PhD degree in Molecular Medicine (curriculum in Molecular Oncology)
European School of Molecular Medicine (SEMM),
University of Milan and University of Naples “Federico II”
Settore disciplinare: Bio/10

A NOVEL ROLE OF THE ENDOCYTIC ADAPTOR PROTEINS EPS15 AND EPS15L1 IN THE REGULATION OF NOTCH SIGNALING

Caterina Lucano
IFOM, Milan
Matricola n. R09390

Supervisor: Prof. Pier Paolo Di Fiore
IFOM, Milan

Added Supervisor: Dr. Nina Offenhäuser
IFOM, Milan

Anno accademico 2013-2014
# TABLE OF CONTENTS

TABLE OF CONTENTS ......................................................................................................................... 3

LIST OF ABBREVIATIONS .................................................................................................................. 7

FIGURES INDEX ................................................................................................................................. 9

ABSTRACT ........................................................................................................................................ 13

Chapter 1 INTRODUCTION ............................................................................................................. 15

1. Endocytosis .................................................................................................................................. 15
   1.1 Role of endocytosis in the cell ................................................................................................. 15
   1.2 Clathrin-mediated endocytosis ............................................................................................. 17
   1.3 Clathrin-independent endocytosis ....................................................................................... 19
   1.4 Endocytosis and signaling ....................................................................................................... 20

2. The endocytic adaptor proteins Eps15 and Eps15L1 ................................................................. 23
   2.1 Eps15 and Eps15L1: structure and interactors ...................................................................... 23
   2.2 Role of Eps15 and Eps15L1 in CME and CIE ....................................................................... 24
   2.3 Post-translational modifications of Eps15 .......................................................................... 25
   2.4 Other roles of Eps15 and Eps15L1 in the cell ......................................................................... 26

3. Notch signaling pathway .............................................................................................................. 28
   3.1 Importance of studying the Notch signaling pathway: Notch and human diseases .......... 28
   3.2 Notch signaling: an overview of Notch ligands and receptors ............................................ 30
   3.3 Notch signaling: general pathway overview ......................................................................... 32
   3.4 Regulation of Notch receptors in the signal-receiving cell: glycosylation and proteolysis ......................................................................................................................... 34
   3.5 Regulation of Notch receptors in the signal-receiving cell: endocytosis ............................ 37
   3.6 Endocytic regulation of Notch ligands in the signal-sending cell: D. melanogaster .......... 39
   3.7 Endocytic regulation of Notch ligands in the signal-sending cell: mammals ... 42
   3.8 Regulation of Notch ligands in the signal-sending cell: glycosylation and proteolysis .......... 44
   3.9 Transcriptional feedback regulation of Notch receptors and ligands: lateral inhibition ................................................................................................................................. 45
### Chapter 2 MATERIALS AND METHODS

**1. Solutions and Buffers**

1.1 Phosphate-buffered saline (PBS)

1.2 Tris-HCl (1 M)

1.3 Tris-buffered saline (TBS)

1.4 10X SDS-PAGE running buffer

1.5 10X Western transfer buffer

1.6 JS lysis buffer

**2. Reagents**

2.1 DNA constructs

2.2 TaqMAN assays for RT qPCR

2.3 Antibodies

2.4 RNAi Oligonucleotides

**3. Cell lines and cell culture conditions**

3.1 Cell lines and growth conditions

3.2 Harvesting of cells and long-term storage

**4. Cells transfection and generation of a stable clone**

4.1 Transfection of the C2C12 cell line by microporation

4.2 Generation of a stable C2C12 clone expressing the human receptor Notch1

4.3 RNA interference

**5. Molecular biology, biochemical and immunostaining procedures**

5.1 RT qPCR

5.2 Western Blotting

5.3 Immunofluorescence

5.4 FACS analysis

5.5 Dll1 surface levels measurement

**6. Biological and biochemical assays**

6.1 Notch reporter transactivation assay

6.2 Dll1 internalization assay

6.3 Dll1 internalization assay analysis

6.4 Dll1 recycling assay
Chapter 3 RESULTS

1. Characterization of cell lines to study the role of Eps15 and Eps15L1 in Notch signaling regulation

1.1 The OP9-Dll1 and C2C12 model system

1.2 OP9-Dll1 cells and C2C12 cells express different amounts of Notch ligands and receptors

1.3 OP9, OP9-Dll1 and C2C12 cells express comparable levels of Eps15 and Eps15L1

2. Set-up and characterization of the Notch reporter transactivation assay

2.1 Notch reporter activation in C2C12 cells is efficient and reproducible but is not increased by transient transfection of Notch1

2.2 Generation and characterization of a stable C2C12 clonal cell line overexpressing Notch1 receptor

2.3 The γ-secretase inhibitor DAPT is able to abolish Notch transactivation in C2C12-Notch1 cells

3. Eps15 and Eps15L1 do not have a role in the regulation of Notch receptor signaling in the signal-receiving cell

3.1 Validation of the Notch reporter transactivation assay by silencing of the known Notch regulators Numb and dynamin in C2C12-Notch1 cells

3.2 Silencing of Eps15, Eps15L1 or both Eps15/Eps15L1 in C2C12-Notch1 cells does not alter Notch activation

4. Eps15 and Eps15L1 are key regulators of Notch signaling in the signal-sending cell

4.1 Notch activation in the signal-receiving cell is dependent on the presence in the signal-sending cell of Mib1, dynamin and clathrin, and independent of AP2

4.2 Silencing Numb and Numb-like in the signal-sending cell does not reduce Notch transactivation

4.3 Silencing Eps15 and Eps15L1 in the signal-sending cell reduces Notch transactivation

4.4 Silencing Epsin1 and Epsin2 in the signal-sending cell reduces Notch transactivation

4.5 Eps15/Eps15L1 and Epsin1/Epsin2 act in distinct pathways of Notch ligand activation
4.6 Eps15 and Eps15L1 silencing in the signal-sending cell has a similar impact on the activation of all Notch ligands .................................................................................. 103
4.7 Combined Eps15/Eps15L1 KD in both the signal-sending and signal-receiving cell does not further reduce Notch transactivation compared to combined KD in the signal-sending cell alone ........................................................................................................... 106

5. Dll1 internalization is Eps15 and Eps15L1 independent ........................................ 108
   5.1 Dll1 internalization assay setup and characterization ................................................. 108
   5.2 Dll1 internalization assay validation and characterization ....................................... 111
   5.3 Dll1 surface levels in OP9-Dll1 after KD of Eps15 and Eps15L1 are not altered ............................................................................................................................................. 112
   5.4 Loss of Eps15 and Eps15L1 does not affect receptor-mediated or constitutive Dll1 internalization .................................................................................................................................................. 114

6. Investigating the role of Eps15 and Eps15L1 in Dll1 recycling .............................. 116
   6.1 Dll1 recycling to the cell surface after internalization does not occur in OP9-Dll1 cells in suspension ............................................................................................................................................. 116

7. Assessing the role of Eps15 and Eps15L1 in Dll1 membrane localization in OP9-Dll1 cells ........................................................................................................................................................................ 120
   7.1 Dll1 is predominantly located in lipid rafts in OP9-Dll1 cells ............................ 120
   7.2 Eps15 and Eps15L1 silencing in OP9-Dll1 cells does not alter Dll1 localization in lipid rafts ............................................................................................................................................. 122

Chapter 4 DISCUSSION ............................................................................................... 125
   1. Advantages and pitfalls of our in vitro model system .............................................. 126
   2. Eps15 and Eps15L1 regulate Notch signaling in the signal-sending cell ............... 129
   3. Investigating Dll1 endocytosis and recycling ......................................................... 134
   4. Other possible mechanisms through which Eps15 and Eps15L1 could regulate Notch signaling and future directions ................................................................. 136

REFERENCES ............................................................................................................. 141
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANK</td>
<td>Ankyrin repeats</td>
</tr>
<tr>
<td>AP1</td>
<td>Adaptor Protein 1</td>
</tr>
<tr>
<td>AP2</td>
<td>Adaptor Protein 2</td>
</tr>
<tr>
<td>CADASIL</td>
<td>Cerebral autosomal dominant arteriopathy</td>
</tr>
<tr>
<td>CHC</td>
<td>Clathrin heavy chain</td>
</tr>
<tr>
<td>CIE</td>
<td>Clathrin-independent endocytosis</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CME</td>
<td>Clathrin-mediated endocytosis</td>
</tr>
<tr>
<td>Crn7</td>
<td>Coronin 7</td>
</tr>
<tr>
<td>CSL</td>
<td>CBF1/Suppressor of Hairless/Lag-1</td>
</tr>
<tr>
<td>EHB1</td>
<td>Eps15 homology domain containing protein-binding protein 1</td>
</tr>
<tr>
<td>DKD</td>
<td>Double knock down</td>
</tr>
<tr>
<td>DKO</td>
<td>Double knock out</td>
</tr>
<tr>
<td>DSL</td>
<td>Delta/Serrate/lag-2</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EH</td>
<td>Eps15 homology</td>
</tr>
<tr>
<td>ENTH</td>
<td>Epsin N-Terminal Homology</td>
</tr>
<tr>
<td>Eps15</td>
<td>Epidermal growth factor protein substrate 15</td>
</tr>
<tr>
<td>Eps15L1</td>
<td>Epidermal growth factor protein substrate 15-like1</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain</td>
</tr>
<tr>
<td>KD</td>
<td>Knock down</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>Mib</td>
<td>Mindbomb</td>
</tr>
<tr>
<td>NECD</td>
<td>Notch extracellular domain</td>
</tr>
<tr>
<td>Neur</td>
<td>Neuralized</td>
</tr>
<tr>
<td>NEXT</td>
<td>Notch extracellular truncation</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
</tr>
<tr>
<td>NRR</td>
<td>Negative regulatory region</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small-cell lung carcinomas</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>RAM</td>
<td>Rbp-associated molecule</td>
</tr>
<tr>
<td>SOP</td>
<td>sensory organ precursor</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T-acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>T-NHL</td>
<td>T-cell non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>TAD</td>
<td>transcriptional activation domain</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TMICD</td>
<td>Trans-membrane intracellular domain</td>
</tr>
<tr>
<td>UIM</td>
<td>Ubiquitin interacting motif</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
FIGURES INDEX

INTRODUCTION
Figure 1.1 Endocytic routes of entry into the cell. ..........................................................17
Figure 1.3 Receptor signaling regulation by endocytosis. ........................................... 22
Figure 1.4 Modular structure of the endocytic adaptor proteins Eps15 and Eps15L1.….. 24
Figure 1.5 Structure of Notch ligands and receptors.....................................................32
Figure 1.6 Schematic overview of activation of the Notch signaling pathway. .......... 33
Figure 1.7 Schematic overview of the complex regulation of Notch signaling pathway. ...38
Figure 1.8 Morphological analysis of Eps15/Eps15L1 DKO embryos at 9.5 days post-
fertilization and comparison with Notch signaling loss-of-function KO embryos. 48
Figure 1.9 Downregulation of Notch target genes in Eps15/Eps15L1 DKO embryos at 9.5
days post-fertilization ..................................................................................................51

MATERIALS AND METHODS
Figure 2.1 BD FACSCalibur Optical Path Configuration .............................................68
Figure 2.2 Bioluminrescence reactions catalyzed by Firefly and Renilla luciferase. .......70

RESULTS
Figure 3.1 OP9-Dll1 cells overexpress the Notch ligand Dll1 compared to control OP9
cells. ...................................................................................................................................76
Figure 3.2 OP9-Dll1 and C2C12 cells express different amounts of Notch ligands and
receptors. ................................................................................................................................77
Figure 3.3 OP9, OP9-Dll1 and C2C12 cells express comparable levels of Eps15 and
Eps15L1. ................................................................................................................................78
Figure 3.4 Transient transfection of Notch1 in C2C12 cells does not increase Notch
reporter activity over background levels. ........................................................................ 80
Figure 3.5 Uncorrect processing and rapid loss of Notch1 protein upon transient
transfection in C2C12 cells. ...............................................................................................81
Figure 3.6 Generation of a stable C2C12 clone overexpressing Notch1 receptor. ........82
Figure 3.7 OP9-Dll1 cells are able to efficiently transactivate Notch signaling in C2C12
cells stably overexpressing the tagged-Notch1 receptor. .............................................84
Figure 3.8 C2C12 clone 15A and C2C12 WT cells express comparable levels of Eps15 and Eps15L1. 85

Figure 3.9 DAPT abolishes Notch transactivation in C2C12-Notch1 cells. 86

Figure 3.10 Notch activation in the signal-receiving cell is dependent on both Numb and dynamin2. 88

Figure 3.11 Notch activation in signal-receiving cells is independent of both Eps15 and Eps15L1. 90

Figure 3.12 KD of Mib1 in the signal-sending cell reduces Notch activation in the Notch reporter transactivation assay. 93

Figure 3.13 KD of CHC and dynamin2 but not AP2 in the signal-sending cell reduces Notch activation in the signal-receiving cell. 94

Figure 3.14 KD of Numb and Numb-like in the signal-sending cell does not reduce Notch transactivation. 96

Figure 3.15 Eps15 and Eps15L1 silencing in the signal-sending cell reduces Notch transactivation. 98

Figure 3.16 Epsin1 and Epsin2 silencing in the signal-sending cell reduces Notch transactivation. 100

Figure 3.17 Effect of combined Eps15/Eps15L1 and Epsin1/Epsin2 KDs in the signal-sending cell on Notch transactivation. 102

Figure 3.18 Eps15 and Eps15L1 have a similar role in the regulation of all Notch ligands. 104

Figure 3.19 Efficiency of KD in OP9-Dll4, OP9-Jag1 and OP9-Jag2 cells. 105

Figure 3.20 Silencing of Eps15 and Eps15L1 in both the signal-sending and signal-receiving cell does not further reduce Notch transactivation. 107

Figure 3.21 Schematic representation of the Dll1 internalization assay in OP9-Dll1 cells. 109

Figure 3.22 Receptor-mediated and constitutive internalization of Dll1 in OP9-Dll1 cells. 110

Figure 3.23 Effect of Mib1 and CHC silencing on receptor-mediated and constitutive endocytosis of Dll1 in OP9-Dll1 cells. 111

Figure 3.24 Surface levels of Dll1 after KD in OP9-Dll1 cells at steady state. 113

Figure 3.25 Eps15 and Eps15L1 do not have a role in internalization of the Notch ligand Dll1. 115

Figure 3.26 Schematic representation of the Dll1 recycling assay in OP9-Dll1 cells. 117

Figure 3.27 Dll1 recycling to the cell surface after internalization does not occur in OP9-Dll1 cells in suspension. 118
Figure 3.28 Purification of lipid rafts with Brij58 in OP9-Dll1 cells. ........................................121
Figure 3.29 Eps15 and Eps15L1 KD in OP9-Dll1 cells does not alter Dll1 localization in lipid rafts. .................................................................................................................................123

DISCUSSION

Figure 4.1 The gene dosage hypothesis .................................................................................131
ABSTRACT

Eps15 and Eps15L1 are endocytic adaptor proteins involved in both clathrin-mediated and clathrin-independent endocytosis of receptor tyrosine kinases. Eps15/Eps15L1 double knockout mice are embryonic lethal and display a Notch loss-of-function phenotype, accompanied by downregulation of Notch target genes.

The Notch pathway is an evolutionary conserved signal transduction pathway that regulates multiple aspects of the development of multicellular organisms. Notch signaling activation requires direct contact between a signal-sending cell, expressing the ligand, and a signal-receiving cell, expressing the receptor. Both Notch ligands and receptors are transmembrane proteins, tightly regulated by endocytosis and membrane trafficking.

Given these premises, the overall aim of this thesis was to understand whether Eps15 and Eps15L1 have a direct role in the regulation of Notch signaling, and, if so, whether this regulation takes place in the signal-receiving cell or in the signal-sending cell. Our final goal was to understand the molecular mechanisms by which Eps15 and Eps15L1 affect Notch signaling.

To investigate the role of Eps15 and Eps15L1 in Notch signaling, we set-up a co-culture model system composed of the signal-sending cell OP9-Dll1 that overexpresses the ligand Dll1, and the signal-receiving cell C2C12-Notch1 that overexpresses the receptor Notch1.

To assess the role of Eps15 and Eps15L1 in Notch signaling regulation, we developed an in vitro Notch transactivation reporter assay based on our co-culture model system. We observed no reduction in Notch activity after knockdown (KD) of Eps15 or Eps15L1 in the signal-receiving cell, indicating that the two proteins do not have a role in the regulation of Notch receptors. However, when we performed KD of Eps15 and/or Eps15L1 in the signal-sending cell, we observed a 40-50% reduction in Notch activity. This reduction was
observed for all the Notch ligands tested, Dll1, Dll4, Jag1 and Jag2, indicating that Eps15 and Eps15L1 have a general role in the modulation of Notch ligand activity/signaling.

Since Eps15 and Eps15L1 are known to act in combination with Epsin, a fundamental regulator of Notch ligand internalization, we investigated whether removal of Eps15 and Eps15L1 from the signal-sending cell affected the early steps of Dll1 endocytosis. To do so, we set-up a Dll1 internalization assay to measure induced or constitutive Dll1 endocytosis. Using this assay, we observed a strong reduction in Dll1 endocytosis following KD of known endocytic regulators of Notch ligands, Epsin and Mindbomb1. However, we did not detect any alterations in Dll1 internalization after KD of Eps15, Eps15L1 or both Eps15 and Eps15L1, indicating that the two proteins do not participate in the regulation of the early steps of Notch ligand endocytosis, both induced and constitutive.

Eps15 and Eps15L1 have also been implicated in endocytic recycling. Therefore, we asked whether a recycling function of Eps15/Eps15L1 could be involved in Notch ligand regulation. We attempted to assess Dll1 recycling in OP9-Dll1 cells, however, results suggested that Dll1 does not undergo significant recycling in our experimental conditions, therefore a change of strategy will be necessary. We also assessed Dll1 localization in plasma membrane lipid rafts following KD of Eps15 and Eps15L1, but did not score any change in ligand membrane localization.

In conclusion, we showed that Eps15 and Eps15L1 are key regulators of Notch ligands in the signal-sending cell. However, in contrast to their known role as endocytic adaptors, the two proteins do not appear to be involved in the early steps of Dll1 endocytosis. Given that Eps15 and Eps15L1 have been implicated in other pathways and cellular processes, such as recycling, secretion, degradation, cell-matrix adhesion and cell-cell connection, it is possible that Eps15/Eps15L1 might mediate Notch ligand regulation through one of these other pathways. However, the precise molecular mechanisms by which Eps15 and Eps15L1 regulate Dll1 activity remain to be defined, and will be the focus of future studies.
Chapter 1

INTRODUCTION

1. Endocytosis

1.1 Role of endocytosis in the cell

The plasma membrane is the surface through which the cell mediates molecular exchanges and communications with other cells and the external environment. To do this, the cell takes advantage of mechanisms that mobilize large portions of the plasma membrane, namely endocytosis and exocytosis. Endocytosis mediates the internalization of nutrients and macromolecules contained in the extracellular fluid (Doherty & McMahon, 2009). In addition, endocytosis contributes to the production and renewal of cell membranes through the internalization of lipids and membrane proteins. Through the opposed process of exocytosis, the cell is able to bring newly synthesized proteins and lipids to the plasma membrane, and expel waste and toxic substances from the cytoplasm (Conner & Schmid, 2003; Doherty & McMahon, 2009). However, the role of endocytosis is not only limited to nutrient uptake and interactions with the extracellular environment. Increasing evidence indicates that endocytosis and endocytic proteins are also involved in the regulation of diverse cellular processes including: i) signal transduction, through regulating the assembly of signaling platforms at the plasma membrane; ii) cell polarity, through asymmetrical distribution of membranes and signaling molecules; iii) motility, through the continuous endocytic reorganization of the plasma membrane, which is essential for changes in cell shape; iv) cell division; v) transcription (Scita & Di Fiore, 2010; Sigismund et al., 2012). For the latter two processes, although there is evidence of an involvement of endocytic
proteins, this involvement does not seem to be connected to the plasma membrane or the process of endocytosis itself (Scita & Di Fiore, 2010; Sigismund, et al., 2012).

Cells possess several endocytic routes that differ mainly in the characteristics of the transported cargo and the protein machinery involved in cargo uptake. These routes can be divided into two principal categories: phagocytosis and pinocytosis (Figure 1.1). Phagocytosis mediates the uptake of large particles, such as whole microorganisms or cell debris, through the formation of large membrane expansions. The particles are then directed to the lysosome for degradation by lysosomal enzymes (Figure 1.1). Pinocytosis, instead, mediates the uptake of smaller cargos and involves less extensive modifications of the plasma membrane. Pinocytosis is in turn divided into: i) macropinocytosis, which mediates the unspecific uptake of extracellular fluids; ii) clathrin-dependent endocytosis, which mediates entry into the cell via clathrin coated vesicles; iii) clathrin-independent endocytosis, which includes all endocytic routes that do not depend on the coating protein clathrin (Figure 1.1) (Conner & Schmid, 2003; Doherty & McMahon, 2009; Mayor & Pagano, 2007).
Figure 1.1 Endocytic routes of entry into the cell.

Large particles and microorganisms are internalized through phagocytosis, while fluids are brought into the cell via macropinocytosis. Both mechanisms involve actin remodeling of the plasma membrane and the formation of large vesicles. The majority of solutes and molecules are internalized and conveyed to the endosomal compartment through clathrin-coated or caveolin-coated vesicles. Internalization mechanisms independent of both clathrin and caveolin are also present [adapted from: (Mayor & Pagano, 2007)].

1.2 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME), also referred to as receptor-mediated endocytosis, is a highly specific process by which transmembrane receptors present in the plasma membrane, together with their ligands, are internalized into the cell via ‘coated pits’, i.e., vesicles formed by the cytosolic coat protein clathrin. CME is the main endocytic route responsible for the internalization of receptor tyrosine kinases, G protein-coupled receptors and transferrin (TfR) receptors (Conner & Schmid, 2003; Doherty & McMahon, 2009; McMahon & Boucrot, 2011).
Clathrin is organized in trimers, named triskelia; each triskelion is composed of three clathrin heavy chains, with a molecular weight of \( \approx 180 \) kD each, and three light chains, with a molecular weight of \( \approx 35 \) kD each. When triskelia assemble in solution, they create a lattice cage composed of hexagons and pentagons that gives shape to the rounded vesicles (Faini et al., 2013). In vivo, several adaptor proteins help to coordinate clathrin nucleation, vesicle formation and cargo recruitment at specialized sites in the plasma membrane. Clathrin nucleation and subsequent polymerization allows the deformation of the plasma membrane and the establishment of the vesicle neck. Detachment of clathrin-coated vesicles from the plasma membrane requires the activity of the GTPase dynamin. Dynamin forms a helical polymer around the vesicle neck and allows the release of the vesicle in the cytoplasm following GTP hydrolysis. After vesicle release, clathrin detaches from the surface, allowing vesicle fusion with the intracellular compartment of destination (Figure 1.2) (Conner & Schmid, 2003; Doherty & McMahon, 2009; McMahon & Boucrot, 2011).

A great variety of adaptor proteins can also play a role during the formation of clathrin-coated pits, to further optimize the process. Accessory proteins may participate in vesicle formation, by helping with cargo recruitment, in membrane deformation, or they may perform a scaffold function, acting as bridge to connect components participating in the endocytic process (Reider & Wendland, 2011). The adaptor protein 2 (AP2) complex, for example, is the main component of clathrin-coated pits, after clathrin itself. AP2 is able to interact with cargo proteins, lipids, clathrin and other endocytic accessory proteins at the site of internalization. Other endocytic accessory proteins that also participate in CME, for instance, include: i) Intersectin that binds to components of the endocytic machinery, as well as elements of the actin cytoskeleton; ii) epidermal growth factor receptor substrate 15 (Eps15), a scaffold protein that promotes clustering of AP2; iii) Epsin, an Eps15 binding partner that, in addition to its role of anchoring the cargo to clathrin, can directly bind
phosphatidylinositol lipids and help with membrane deformation through the insertion of an aliphatic helix (McMahon & Boucrot, 2011; Reider & Wendland, 2011).

![Figure 1.2 Genesis of a clathrin coated pit](image)

Clathrin triskelia polymerization allows invagination of the plasma membrane and formation of the vesicle neck. Release of the vesicle in the cytoplasm is mediated by dynamin, which forms a helical polymer around the vesicle neck. Clathrin is subsequently released from the vesicle and is recycled for another endocytic cycle [adapted from: (Doherty & McMahon, 2009)].

### 1.3 Clathrin-independent endocytosis

All the endocytic routes that do not require vesicle coating by clathrin are classified as clathrin-independent endocytosis (CIE). Indeed, CIE is experimentally defined by insensitivity to clathrin removal. Moreover, CIE is sensitive to drugs that interfere with cholesterol organization in the membrane and disrupt lipid raft platforms. Consequently, the endocytic pathways that fall under this definition are highly heterogeneous and require different endocytic machinery (Conner & Schmid, 2003; Doherty & McMahon, 2009; Mayor & Pagano, 2007). CIE can be further classified on the basis of dynamin requirement. It was initially thought that dynamin, given its role in vesicle fission and release from the plasma membrane, would be involved in all forms of endocytosis. However, it was later discovered that some clathrin-independent pathways are also dynamin-independent (Damke et al., 1995; Guha et al., 2003; Mayor & Pagano, 2007; Wu et al., 2014).

The most studied clathrin-independent, dynamin-dependent endocytic route is caveolae-mediated endocytosis. Caveolae are invaginations of the plasma membrane enriched in...
cholesterol and sphingolipids, and are characterized by the presence of caveolin proteins. Cargos internalized through caveolae are mainly proteins, proteins with lipid anchor, lipids and pathogens (Doherty & McMahon, 2009; Mayor & Pagano, 2007). A second clathrin-independent, dynamin-dependent endocytic route employs the GTPase RhoA, and is responsible for internalization of the β chain of the interleukin 2 receptor. Classical examples of clathrin-independent, dynamin-independent endocytosis include internalization mediated by the GTPase CDC24, which is responsible for cholera toxin endocytosis, and endocytosis mediated by the GTPase ARF6, responsible for internalization of MHC class 1 proteins, β1 integrin and E-cadherin (Doherty & McMahon, 2009; Mayor & Pagano, 2007).

1.4 Endocytosis and signaling

Endocytosis is one of the main mechanisms employed by eukaryotic cells to regulate signal transduction. Cells communicate with the extracellular environment, and with each other, through receptors localized on the plasma membrane. Receptors, following ligand-dependent activation, are internalized and conveyed to different endosomal compartments. From there, they can be directed towards lysosomes for degradation, or back to the plasma membrane through a recycling pathway. In this way, endocytosis regulates signal transduction by controlling the number of available receptors on the plasma membrane (Figure 1.3) (Le Roy & Wrana, 2005; Sigismund, et al., 2012; Sorkin & von Zastrow, 2009).

The function of endocytosis, however, is not only limited to signal attenuation, but also encompasses signal modulation: duration, intensity, integration and spatial distribution of signals can all be regulated by endocytosis (Le Roy & Wrana, 2005; Sigismund, et al., 2012; Sorkin & von Zastrow, 2009). A clear example of this type of endocytic regulation of signaling is provided by the epidermal growth factor receptor (EGFR). The EGFR is a
receptor tyrosine kinase involved in cell growth, survival, proliferation and differentiation. EGFR can be internalized through different endocytic routes, depending on the concentration of extracellular ligand. In the presence of low doses of EGF, EGFR is almost exclusively internalized through a clathrin-dependent route, which supports receptor recycling and prolonged signaling (Polo & Di Fiore, 2006; Sigismund et al., 2005). Conversely, at high EGF concentrations, the EGFR can also be internalized through a clathrin-independent pathway, which conveys part of the receptor towards degradation, thus avoiding excessive stimulation of the pathway (Polo & Di Fiore, 2006; Sigismund, et al., 2005).

Another critical factor in signal transduction regulation by endocytosis, is the endosomal compartment. Endosomes can support signals that originate at the plasma membrane or they can generate their own signals, since they provide specific physical conditions, different from those of the plasma membrane (Sigismund, et al., 2012). For instance, the relatively small volume of endosomes can promote the association between ligands and receptors at lower concentrations. Endosomes are also enriched in certain lipids and proteins, which can provide a specialized scaffold environment, able to assemble specific signaling complexes. Moreover, endosomes possess an acidic pH in their lumen, which can favor specific chemical reactions, such as proteolysis of signaling molecules. Finally, endosomes are also connected to the cytoskeleton by microtubules, thus allowing signal transmission over long distances (Scita & Di Fiore, 2010; Sigismund, et al., 2012).
Figure 1.3 Receptor signaling regulation by endocytosis.

Receptors, activated by ligand, are mainly internalized through CME or CIE. Following internalization, receptors are directed to early endosomes, and from there to lysosomes for degradation, or back to the plasma membrane, through either slow or fast recycling pathways [adapted from: (Scita & Di Fiore, 2010)]
2. The endocytic adaptor proteins Eps15 and Eps15L1

2.1 Eps15 and Eps15L1: structure and interactors

The epidermal growth factor protein substrate 15 (Eps15) and the epidermal growth factor protein substrate 15-like1 (Eps15L1) proteins are endocytic adaptors that are involved in the regulation of receptor tyrosine kinase endocytosis, both CME and CIE. Eps15 is a 150 KDa protein that is localized predominantly in the cytosol and was originally identified as a substrate of the EGFR (Fazioli et al., 1993). A subsequent screening of a mouse keratinocyte cDNA library, performed with the Eps15 homology domain (EH domain) as a probe, led to the identification of Eps15L1, a gene highly homologous to Eps15, whose product is approximately a 125 KDa protein (Wong et al., 1995) (Figure 1.4).

Both Eps15 and Eps15L1 possess a modular structure in which different protein-protein interaction domains can be identified (Figure 1.4). Indeed, in the cell, Eps15 and Eps15L1 act as endocytic adaptors, forming bridges between different proteins during the assembly of endocytic complexes in both CME and CIE. The N-terminal portion of the two proteins contains three EH domains that mediate binding to NPF (asparagine-proline-phenylalanine)-containing endocytic proteins, such as Epsin, Numb, Hrb, Synaptojanin and Stonin (Chen et al., 1998; Doria et al., 1999; Haffner et al., 1997; Martina et al., 2001; Polo et al., 2003; Salcini et al., 1997). The central portion of the two proteins is occupied by an α-helical coiled-coil domain that can mediate homo- and hetero-dimerization of Eps15 and Eps15L1, as well as interactions with the endocytic adaptors Hrs and Intersectin (Bean et al., 2000; Sengar et al., 1999). In their C-terminal region, the two proteins contain several DPF (aspartic acid-proline-phenylalanine) tripeptide repeats that compose the AP2 binding site (Benmerah et al., 1996; Fazioli, et al., 1993; Iannolo et al., 1997). This site is immediately followed by a 14 amino acid sequence that mediates binding to adaptor protein 1 (AP1) (Chi et al., 2008; Kent et al., 2002), and two short sequences called UIM...
(Ubiquitin interacting motif) domains that are responsible for binding to ubiquitinated proteins, as well as monoubiquitination of Eps15 and Eps15L1 (Polo et al., 2002).

**Figure 1.4 Modular structure of the endocytic adaptor proteins Eps15 and Eps15L1.**

Schematic representation of the modular structure of Eps15 and Eps15L1. Key domains are represented as follows: EH domains (orange), α-helix coiled-coil domain (green), DPF motifs (blue), AP1 binding site (purple) and UIM domains (red).

### 2.2 Role of Eps15 and Eps15L1 in CME and CIE

There is extensive evidence linking Eps15 and Eps15L1 to CME. Eps15 interacts with the alpha subunit of AP2 (Benmerah et al., 1996; Benmerah et al., 1995; Benmerah et al., 1998; Iannolo et al., 1997), and the Eps15:AP2 complex, in turn, interacts with Epsin (Chen et al., 1998) and the GTPase dynamin, which is responsible for vesicle pinch-off (Majumdar et al., 2006). Moreover, Eps15 colocalizes with active EGFR both at the plasma membrane and in different endocytic compartments (Torrisi et al., 1999).

The importance of Eps15 in CME was experimentally demonstrated by several studies. In particular, overexpression of a mutant Eps15 lacking the second and third EH domains, was shown to disrupt the localization of AP2, clathrin and dynamin at the plasma membrane (Benmerah et al., 1999). Moreover, overexpression of this truncated form of
Eps15, or microinjection of antibodies blocking Eps15 and Eps15L1 functionality, led to defects in clathrin-dependent EGFR internalization (Carbone et al., 1997).

Over the last 10 years, it has also been demonstrated that EGFR stimulated with high doses of ligand is monoubiquitinated and internalized through CIE (Chen & De Camilli, 2005; Sigismund, et al., 2005). This pathway is preferentially used by the cell to downmodulate EGFR signaling, since it directs the receptor towards lysosomal degradation. In vitro, triple knockdown (KD) of Eps15, Eps15L1 and Epsin1 resulted in inhibition of this pathway, demonstrating that, together, the three proteins play an important role in targeting the ubiquitinated receptor to non-clathrin internalization (Sigismund, et al., 2005).

2.3 Post-translational modifications of Eps15

Eps15 is tyrosine phosphorylated by the EGFR and monoubiquitinated after stimulation with EGF (Fazioli, et al., 1993; van Delft et al., 1997). A mutant form of Eps15, in which Tyr850 is mutated into a phenylalanine, retains its protein binding properties, but is no longer phosphorylated by EGFR. This mutant acts as a dominant negative mutant, inhibiting the internalization of EGFR, but not that of the TfR (Confalonieri et al., 2000). This result is an indication that Eps15 and Eps15L1 might be required for ligand-induced internalization of receptors, as in the case of EGFR, but not for constitutive internalization, as in the case of TfR. The result also shows that tyrosine phosphorylation of Eps15 is critical for the correct function of this endocytic adaptor protein.

Ubiquitination is a post-translational modification that is often associated with endocytosis and trafficking (Sigismund, et al., 2012). For instance, the EGFR is ubiquitinated by the E3 ubiquitin ligase Cbl (de Melker et al., 2001); ubiquitination of the EGFR appears to be required for CIE but it is not essential for CME (Huang et al., 2007; Sigismund, et al., 2005). Importantly, however, EGFR interaction with Cbl is required for ubiquitin-independent endocytosis of EGFR (Huang, et al., 2007), suggesting that Cbl-mediated
EGFR internalization may not be related to receptor ubiquitination, but may be related to the recruitment of ubiquitinated endocytic adaptors, containing UIM domains, such as Eps15 (Polo, et al., 2002).

Finally, it has also been demonstrated that Eps15, together with Epsin, is phosphorylated during mitosis. This phosphorylation inhibits the interaction of the two proteins with the endocytic protein AP2, and has been postulated to cause dissociation of the two proteins from clathrin-coated pits, resulting in the mitotic block of endocytosis (Chen et al., 1999).

2.4 Other roles of Eps15 and Eps15L1 in the cell

The high number of Eps15 and Eps15L1 interactors, the role of the two proteins in both CME and CIE, and their post-translational modifications at different stages of the cell cycle and during signaling events, all suggest that Eps15 and Eps15L1 are involved in the regulation of several cellular processes, possibly in a highly dynamic manner.

For example, there are many lines of evidence indicating that the biological function of Eps15 and Eps15L1 is not restricted to the plasma membrane. Indeed, it has been demonstrated that Eps15 is able to interact with AP1, a member of the AP adaptor protein family involved in the formation of clathrin-coated vesicles in the trans-Golgi network secretory pathway (Chi, et al., 2008). Moreover, Eps15 is recruited by ubiquitinated coronin7 (Crn7), an F-actin regulator, and this binding promotes the recruitment of Crn7 to the trans-Golgi network, where it contributes to the formation of tubular carriers (Yuan et al., 2014). Taken together, these two observations strongly indicate a possible role for Eps15 in the regulation of trans-Golgi network trafficking.

In the literature there are also reports of two isoforms of Eps15, produced by alternative splicing, Eps15b and Eps15s. Eps15b, which completely lacks the three EH domains at the N-terminus, colocalizes with the endosomal sorting protein Hrs in specific endosomal microdomains and mediates the sorting of the EGFR towards the degradative pathway.
(Kostaras et al., 2013; Roxrud et al., 2008). Eps15s, which lacks 111 amino acids at its C-terminus, including the two UIM domains, is involved in the recycling of the internalized EGFR back to the cell surface, in a Rab11-dependent manner (Chi et al., 2011). Several alternative splice forms of Eps15L1 have also been reported, however nothing is known about their functional specificity.

Finally, there is evidence to suggest that Eps15 and Eps15L1 are linked to cell-cell adhesion and connections; Eps15 regulates internalization and trafficking of the components of tight junctions (Murakami et al., 2009) and internalization of connexin43, a base component of gap junction channels (Girao et al., 2009). Moreover both Eps15 and Eps15L1 are involved in the regulation of the internalization of integrin β1, through their binding with the endocytic adaptor Dab2 (Teckchandani et al., 2012).
3. Notch signaling pathway

3.1 Importance of studying the Notch signaling pathway: Notch and human diseases

The Notch pathway is a signal transduction pathway that is highly conserved during metazoan evolution. It regulates numerous aspects of development, and, in particular, it can influence self-renewal, proliferation, differentiation and cell fate specification (Bray, 2006; Fortini, 2009; Kopan & Ilagan, 2009).

Given the importance of the cellular processes that are mediated by Notch and the great variety of tissues in which the pathway is active, it is not surprising that alterations in Notch signaling regulation are the cause of several human diseases, including cancer (Louvi & Artavanis-Tsakonas, 2012). In fact, mutations in genes that encode for different Notch signaling pathway proteins, can cause developmental syndromes that affect several organs, such as the heart, liver, skeleton, kidney and vascular system (Gridley, 2003; Louvi & Artavanis-Tsakonas, 2012; Penton et al., 2012; Yavropoulou & Yovos, 2014). For example:

i) the Alagille syndrome, that is caused by mutations in the Notch ligand Jagged1 gene and results in pleiotropic development of organs and tissues (Li et al., 1997; Oda et al., 1997);

ii) Spondylocostal Dysostosis, a disease that results in an anomalous vertebral development and can be caused by mutations in the Notch ligand Dll3 gene (Bulman et al., 2000) or by inactivating mutations in Lunatic Fringe, that encodes a glycosyltransferase that regulates the Notch receptors (Sparrow et al., 2006);

iii) several types of cardiac congenital diseases, such as Tetralogy of Fallot, that can be caused by mutations in the Notch ligand Jagged1 gene (Eldadah et al., 2001);
iv) the adult disorder CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), a vascular disorder that causes transient ischemic attacks or strokes and has been linked with dominant mutations in the Notch receptor *Notch3* gene (Joutel et al., 1996).

Notch signaling also has a key role in inducing commitment and differentiation of the T-cell lineage, while impairing early B-cell development (Thompson & Zuniga-Pflucker, 2011). Consequently, Notch signaling alterations have been described in a wide variety of hematological tumors, including T and B cell leukemia and lymphoma, and myeloid leukemia (Hernandez Tejada et al., 2014). The archetypal hematological malignancy associated with altered Notch signaling is T-acute lymphoblastic leukemia (T-ALL), an aggressive neoplasm, in which the Notch1 receptor is constitutively activated either by chromosomal translocation (1% of the cases) or by point mutations and frame-shift mutations (55-60% of the cases) (Roy et al., 2007). Similarly, ≈50% of T-cell non-Hodgkin lymphoma (T-NHL) cases and 5-15% of chronic lymphocytic leukemia (CLL) cases carry activating Notch1 mutations (Roy, et al., 2007). Moreover, expression of the Notch1 receptor and the ligands Jagged1 and Dll1 are associated with poor prognosis in acute myeloblastic leukemia (Hernandez Tejada, et al., 2014).

Numerous lines of evidence also point to an involvement of altered Notch signaling in solid tumors. Studies in primary human breast cancers have demonstrated that increased expression of the Notch1 receptor and the ligand Jagged1, at the mRNA level, correlates with increased tumor aggressiveness and poor prognosis (Nwabo Kamdje et al., 2014). It has also been demonstrated that increased Notch signaling due to loss of expression of the Notch antagonist Numb, occurs in approximately 50% of breast carcinoma cases (Pece et al., 2004) and in one third of non-small-cell lung carcinomas (NSCLCs) (Westhoff et al., 2009). Moreover, activating Notch mutations have been detected in ~10% of NSCLCs (Westhoff, et al., 2009). Aberrant Notch signaling has also been implicated in the development and progression of prostate cancer (Leong & Gao, 2008), malignant
melanoma (C. S. Muller, 2012), osteosarcoma (McManus et al., 2014), renal epithelial cancers (Sirin & Susztak, 2012), colorectal cancer (Noah & Shroyer, 2013), gliomas and medulloblastomas (Stockhausen et al., 2012).

Given the extensive role of Notch signaling in cancer, several agents targeting and inhibiting Notch signaling are now into early clinical development. In particular, both agents that impair Notch proteolitic activation (such as γ-secretase inhibitors GSIs) and monoclonal antibodies that interfere with Notch ligand/receptor binding are now being tested (Takebe et al., 2014). In this context, gaining an in-depth understanding of the molecular mechanisms regulating Notch activation could potentially evolve in the identification of new successful cancer therapies.

3.2 Notch signaling: an overview of Notch ligands and receptors

Notch signaling activation requires direct contact between a signal-sending cell, expressing the ligand, and a signal-receiving cell, expressing the receptor. Signal transduction is short range, very quick and extremely responsive to modulation and downregulation (Fortini, 2009). Both ligands and receptors are single pass type I transmembrane proteins. *Drosophila melanogaster* possesses one Notch receptor and two Notch ligands, Delta and Serrate. Mammalian cells, on the contrary, possess four paralogous Notch receptors (Notch1-4), three Delta-like ligands (Dll1, Dll3 and Dll4) and two Serrate-like ligands (Jagged1, Jagged2) (Figure 1.5) (Bray, 2006; Fortini, 2009; Kopan & Ilagan, 2009).

The N-terminal portion of Notch receptors contains the Notch extracellular domain (NECD), which is composed of several epidermal growth factor (EGF)-like repeats (Figure 1.5). *D. melanogaster* Notch and the mammalian Notch1-4 receptors differ in the number of EGF-like repeats that they contain (ranging from 29-36). Some of these EGF-like repeats undergo glycosylation as a post-translational modification and mediate interactions with the ligands. In particular, repeats 11-12 mediate *trans* interactions with ligands.
expressed on neighboring cells, while repeats 24-29 are important for inhibitory cis interactions with ligands expressed in the same cell. A negative regulatory region (NRR) is also localized in the NECD, following the EGF repeats (Figure 1.5). This region is composed by three cysteine-rich repeats (LNR) and a heterodimerization domain, and has a key role in preventing the activation of the receptor in the absence of ligand. Three cleavage sites (S1, S2 and S3) are localized in an unstructured loop that protrudes from the NRR domain. The S1 cleavage site is the one that allows the proper maturation of the protein, while the S2 and S3 sites are fundamental for Notch conversion in its active form: i.e., the Notch Intracellular domain (NICD). The NICD is composed of a Rbp-associated molecule (RAM) domain, followed by seven ankyrin repeats (ANK) and a nuclear localization sequence (NLS) (Figure 1.5). The RAM and ANK domains are essential for interactions with transcriptional coactivators, such as Mastermind, in the nucleus. Finally, a transcriptional activation domain (TAD) and a PEST domain (Pro-Glu-Ser-Thr), which acts as a signal peptide for receptor degradation, are localized at the C-terminal portion of the protein (Figure 1.5) (Bray, 2006; Fortini, 2009; Kopan & Ilagan, 2009).

Notch ligands are also transmembrane proteins that contain EGF-like repeats in their extracellular domain. Both the D. melanogaster ligands, Delta and Serrate, and the mammalian ligands, Delta-like (Dll1, Dll3, Dll4) and Serrate-like (Jagged1 and Jagged2), contain a cysteine-rich N-terminal Delta/Serrate/LAG-2 (DSL) domain, which is responsible for their interaction with the receptor (Figure 1.5). These ligands are also called canonical DSL ligands, in contrast to non-canonical ligands that lack the DSL domain. These non-canonical ligands are a group of structurally diverse proteins, whose function is still unclear, but which presumably modulate Notch receptor activity (D'Souza et al., 2010; D'Souza et al., 2008; Kopan & Ilagan, 2009).
3.3 Notch signaling: general pathway overview

The Notch receptor is synthesized in the signal-sending cell as an approx. 300 kDa precursor protein; its N-terminal portion undergoes extensive O-linked and N-linked glycosylation and O-linked fucosylation in the Golgi compartment (Figure 1.6). In the trans-Golgi network, the precursor is cleaved at the S1 cleavage site by a Furin-like convertase. The resulting N-terminal and C-terminal fragments are then reassembled, via a non-covalent bond, to generate the mature heterodimeric form of the receptor, which is finally exported to the plasma membrane (Bray, 2006; Fortini, 2009; Kopan & Ilagan, 2009).

Notch receptor activation occurs at the plasma membrane following binding of the ligand (Figure 1.6). A second cleavage at the S2 site, mediated by the metalloprotease ADAM10/TACE, produces an active form of the receptor (Notch extracellular truncation, NEXT) that is, however, still anchored to the membrane. It is now accepted that endocytosis of the ligand in the signal-sending cell occurs simultaneously with receptor
binding. This generates the force required for a conformational change in the receptor that exposes the S2 cleavage site, making it accessible to the ADAM10/TACE protease. A subsequent cleavage at the S3 site, mediated by the aspartyl protease γ-secretase, releases the Notch intracellular domain (NICD) soluble fragment, which then translocates into the nucleus (Figure 1.6). The γ-secretase-mediated cleavage can occur both at the plasma membrane and in the endosomal compartment. Once in the nucleus, NICD associates with the DNA-binding protein CSL, which in absence of NICD acts as transcriptional repressor. Together, NICD and CSL recruit the co-activator Mastermind and form a transcriptional complex that, upon the recruitment of additional co-activators, is able to activate transcription of downstream target genes (Figure 1.6) (Bray, 2006; Fortini, 2009; Kopan & Ilagan, 2009).

Figure 1.6 Schematic overview of activation of the Notch signaling pathway.

The Notch receptor is produced as a 300 kDa precursor, which undergoes glycosylation, fucosylation and cleavage at the S1 site. The mature receptor is exported to the membrane as a
hetero-oligomer, where it is able to bind ligand expressed in the signal-sending cell. Ligand binding activates signaling via a second and third cleavage of the receptor at the S2 and S3 sites. The active form of the receptor (NICD) is released from the plasma membrane and translocates into the nucleus, where it forms complexes with the transcription activators CSL and Mastermind (Mam) and activates transcription of target genes. Cis-interaction between ligand and receptor result in signaling inhibition [adapted from (Hori, et al., 2013)].

3.4 Regulation of Notch receptors in the signal-receiving cell: glycosylation and proteolysis

Notch signaling activation is tightly regulated both in the signal-sending and in the signal-receiving cell. Both Notch receptors and ligands undergo glycosylation, phosphorylation, ubiquitination, proteolysis and recycling into endosomal compartments (Figure 1.6 and 1.7). The combination of these events is critical for correct Notch signal transduction (Fortini, 2009; Kopan & Ilagan, 2009).

Notch receptors are glycoproteins; many of their EGF repeats undergo both O- and N-glycosylation. The effects of this post-translational modification are numerous and complex, but they all contribute to the regulation of Notch receptors during their synthesis and secretion (Fortini, 2009; Kopan & Ilagan, 2009; Takeuchi & Haltiwanger, 2014). Here, I report some examples of the effects of Notch glycosylation:

1) The *D. melanogaster* O-fucosyltransferase, O-fut1 (Pofut1 in mammals), catalyzes the addition of O-fucose to several EGF-like repeats in the Notch extracellular domain (Okajima & Irvine, 2002). Loss of O-fut1 in flies, or Pofut1 in mice, causes strong Notch-defective phenotypes. (Okajima & Irvine, 2002; Shi & Stanley, 2003). In vivo studies in *D. melanogaster* demonstrated that O-fut1 also possesses a chaperone activity, distinct from its fucosylation activity, which promotes Notch folding and its export to the plasma membrane (Okajima et al., 2008; Okajima et al., 2005). Similarly, in mammals, Pofut1 is required to generate a fully functional Notch receptor, but it is not absolutely required for Notch export to the plasma
membrane or folding, as it can be substituted, to a certain extent, by other enzymes in the cell (Stahl et al., 2008).

2) The D. melanogaster N-acetylglucosaminyItransferase, Fringe (whose mammalian paralogs are Lunatic, Radical and Manic Fringe), catalyzes the addition of N-acetylglucosamine to the O-fucose localized on the twelfth EGF-like repeat of the Notch receptor (Moloney et al., 2000). This modification is essential for the interaction of Notch with its ligand. In vivo evidence suggests that modifications by Fringe can modulate the Notch receptor response to Delta and Serrate ligands (Fleming et al., 1997; Panin et al., 1997; Xu et al., 2007). The mammalian system is more complicated with the presence of three different Fringe enzymes, four Notch receptors and five ligands. Thus, the mechanism by which mammalian Fringe modulates Notch responsiveness to ligands is not yet fully understood, despite the fact that the question has been addressed by numerous studies (Rampal et al., 2005; Shimizu et al., 2001; Yang et al., 2005).

3) A third enzyme involved in Notch glycosylation is the glycosyltransferase Rumi, identified in D. melanogaster. This enzyme catalyzes the addition of O-glucose to specific serine residues in the extracellular domain of the receptor (Acar et al., 2008). In the absence of Rumi activity, Notch is exported to the plasma membrane and is able to bind to the ligand, but is not cleaved at the S2 site. Hence, it has been hypothesized that the absence of Rumi causes the generation of misfolded receptors that cannot be processed by the ADAM10/TACE protease (Acar, et al., 2008). In mammals, three Rumi homologs exist, but only the homolog PoglUt1 shows in vitro enzymatic activity and is able to rescue Notch phenotypes when overexpressed in mutant flies (Takeuchi et al., 2011).

Notch maturation and activation is tightly regulated by a series of proteolytic cleavages that occur in proximity of the trans-membrane domain of the receptor (van Tetering & Vooijs, 2011). The first cleavage is performed by the protease Furin-like convertase that
cleaves the Notch precursor at the S1 cleavage site, generating two fragments that are then reunited by a calcium-dependent, non-covalent bond to form the mature heterodimeric receptor (Blaumueller et al., 1997; Logeat et al., 1998). The heterodimeric receptor is then exported to the plasma membrane (Figure 1.6 and 1.7).

The second Notch cleavage occurs at the S2 site and is mediated by the metalloprotease ADAM10/TACE (Brou et al., 2000; Mumm et al., 2000; Parks et al., 2000). This event follows binding of the receptor to the ligand, and promotes the removal of the extracellular domain of Notch, generating a form of the receptor called NEXT, which is still anchored to the plasma membrane (Figure 1.6 and 1.7) (Nichols et al., 2007). The S2 cleavage site is situated in the NRR region of the receptor. This region has the role of preventing the proteolytic event in the absence of ligand. The S2 site, in fact, is exposed to the protease only after a conformational change of the receptor induced by ligand binding (Gordon et al., 2007; Sanchez-Irizarry et al., 2004).

The NEXT fragment undergoes a third proteolytic cleavage at the S3 site that is embedded in the trans-membrane domain. This third cleavage, which corresponds to the last step in the proteolytic regulation of Notch signaling, is mediated by the γ-secretase protease complex and generates the soluble active Notch fragment, NICD (Figure 1.6 and 1.7) (De Strooper et al., 1999; Schroeter et al., 1998; Struhl & Greenwald, 1999). All these proteolytic steps are tightly controlled in order to ensure that only properly processed receptors, correctly activated by the ligand, have signaling activity (Bray, 2006; Fortini, 2009; Kopan & Ilagan, 2009).
3.5 Regulation of Notch receptors in the signal-receiving cell: endocytosis

Endocytosis and membrane trafficking have also been known for a long time as essential regulators of Notch activity (Baron, 2012; Fortini & Bilder, 2009; Kandachar & Roegiers, 2012). The first observation indicating an endocytic requirement for Notch activation both in the signal-sending and in the signal-receiving cell, comes from the analysis of the *shibire* mutant in *D. melanogaster*. *Shibire*, the *Drosophila* homolog of dynamin, is a protein required for the release of endocytic vesicles from the plasma membrane. *Shibire* mutant cells were shown to be defective both in sending and in receiving Notch signals (Seugnet et al., 1997). A further requirement for endocytosis and endocytic regulatory proteins in the signal-receiving cell was demonstrated in the *Drosophila* sensory organ precursor (SOP) cell. This cell generates two daughter cells, the pIIa and pIIb cells (Hartenstein & Posakony, 1989); pIIa cells require Notch activation for fate specification, while overexpression of Notch in the pIIb cells causes a switch into a pIIa cell (Hartenstein & Posakony, 1990).

During SOP cell mitosis, the endocytic adaptor protein Numb is asymmetrically segregated into the pIIb cell; there it binds Notch and interacts with other endocytic adaptor proteins, and acts as a negative regulator of the Notch receptor (Berdnik et al., 2002; Tang et al., 2005). The mechanism through which Numb negatively regulates Notch is still unknown, however, two hypotheses have been proposed. The first hypothesis proposes that Numb inhibits Notch by promoting its endocytosis, therefore, its removal from the plasma membrane and subsequent degradation (McGill & McGlade, 2003). The second hypothesis instead proposes that Numb alters Notch intracellular trafficking, and prevents newly synthesized receptor to reach specific plasma membrane micro-domains (Couturier et al., 2012). Moreover, Numb might also modulate Notch activity in an indirect fashion, by sequestering the positive Notch regulator Sanpodo (Hutterer & Knoblich, 2005; O'Connor-Giles & Skeath, 2003). AP1, a complex of endocytic adaptor proteins that localizes to the trans-Golgi network and recycling endosomes, is also able to suppress Notch signaling
activation in the pIIb cells, probably by impairing accumulation of Notch or Sanpodo at the apical membrane of the cell (Benhra et al., 2011).

Ubiquitination of Notch also plays an important role in the regulation of Notch trafficking (Fortini, 2009). A number of E3 ubiquitin ligases, such as Nedd4 and Cbl, have been shown to target Notch for degradation (Gupta-Rossi et al., 2004; Jehn et al., 2002; Sakata et al., 2004; M. B. Wilkin et al., 2004), while the E3 ligase Deltex acts as a positive regulator of Notch influencing its endosomal partitioning (M. Wilkin et al., 2008).

Of note, non-activated Notch is also internalized, subjected to endosomal trafficking, and either recycled or degraded. This constitutive endocytosis of Notch is exploited by the cell to modulate receptor levels at the plasma membrane (Figure 1.7) (Fortini, 2009; Kopan & Ilagan, 2009).

![Figure 1.7 Schematic overview of the complex regulation of Notch signaling pathway.](image)

The figure shows a schematic representation of a signal-sending cell (blue) that expresses the Notch ligands Delta and Jagged, and a signal-receiving cell (green) that expresses the receptor Notch. The main points of Notch receptor and ligand regulation are marked by numbers. (1)
Receptor maturation: the Notch receptor, before export to the plasma membrane, undergoes several steps of maturation, including glycosylation of the EGF repeats in the extracellular domain and a proteolytic cleavage at the S1 site, followed by reassembly of the two protein fragments. (2) Endosomal sorting: numerous endocytic proteins regulate the export of the receptor to the plasma membrane or its entry into the endosomal compartment. From here the receptor can be directed towards the degradative pathway, resulting in downregulation of the signal, or towards a recycling pathway that recycles receptor back to the plasma membrane. (3) Ligand maturation: the two ubiquitin E3 ligases Neuralized (Neur) and Mindbomb (Mib) and the endocytic protein Epsin promote the internalization of inactive DSL ligands, directing them towards a recycling pathway during which they are activated. (4) Regulated proteolysis: ligand-dependent Notch activation is controlled by a series of tightly controlled proteolytic cleavages that generate the fragment NEXT and later the soluble NICD fragment that translocates into the nucleus. (5) Transcriptional switch and target genes expression: in the nucleus NICD binds the transcriptional activators CSL and Mastermind (Mam); together they form a ternary complex capable of activating Notch target genes transcription. (6) Signal downregulation: once generated, the NICD fragment, can only be downregulated by degradation, which mediates the disassembly of the NICD/CSL/Mam complex [adapted from (Ilagan & Kopan, 2007)].

3.6 Endocytic regulation of Notch ligands in the signal-sending cell: *D. melanogaster*

The regulation of Notch ligands in the signal-sending cell is still a poorly defined process that remains under investigation today. It is known that endocytosis and endosomal trafficking play a crucial role in the activation and signaling ability of Notch ligands (D'Souza, et al., 2008; Musse et al., 2012). The majority of data regarding this aspect of Notch regulation has been obtained in *D. melanogaster* and only subsequently confirmed in mammalian models. As already reported in the previous section, the first data demonstrating a critical role for endocytosis in the regulation of Notch signaling, came from the analysis of the dynamin *shibire* mutant in *D. melanogaster* (Seugnet, et al., 1997). The novel observation, however, was that not only this requirement was true for the signal-receiving cell, but also for the signal-sending. Dynamin-dependent Delta endocytosis in the signal-sending cell was in fact necessary to produce an active NICD in the signal-receiving cell (Parks, et al., 2000). It was subsequently demonstrated that endocytosis of Notch ligands is promoted by monoubiquitination of their cytoplasmatic tails. The enzymes
responsible for this monoubiquitination are the ubiquitin E3 ligases Neuralized (Neur) and Mindbomb (Mib) (Itoh et al., 2003; Lai et al., 2001; Lai et al., 2005; Wang & Struhl, 2005). Moreover, the endocytic adaptor protein liquid facet (homolog of mammalian Epsin) was also found to be strictly required for ubiquitin-dependent ligand internalization and Notch signaling (Wang & Struhl, 2004; Wang & Struhl, 2005).

As previously described, Epsin is an endocytic adaptor that participates in CME and CIE together with Eps15 and Eps15L1 (Chen, et al., 1998; Sigismund, et al., 2005). Likewise Eps15 and Eps15L1, Epsin possesses two UIMs that mediate interactions with ubiquitinated proteins, and is itself subject to ubiquitination (Polo, et al., 2002). Moreover, it has been shown that the presence of an Epsin N-Terminal Homology (ENTH) domain localized at the N-terminal of Epsin, can induce membrane curvature (Chen, et al., 1998; Ford et al., 2002). Epsin also possesses two clathrin-binding domains (Drake et al., 2000), a DPW (Asp-Pro-Trp)-rich region that binds the endocytic adaptor AP2, and three NPF motifs that bind endocytic proteins containing an EH domain, such as Eps15 and Eps15L1 (Rosenthal et al., 1999). A recent structure-function analysis performed in D. melanogaster, demonstrated that one of the two UIM domains and the ENTH domain of Epsin are essential for Notch signaling activation (Xie et al., 2012). However, the clathrin-binding domain seems to be dispensable, indicating that in the D. melanogaster female germline Notch ligand is endocytosed via a CIE route (Xie, et al., 2012). However, CME involvement in Notch ligand activation in D. melanogaster remains at present controversial, with different observations suggesting that clathrin requirement in Notch ligand endocytosis might be context dependent (Eun et al., 2008; Hagedorn et al., 2006; Windler & Bilder, 2010).

To explain the requirement for ligand endocytosis in Notch signaling, two non-mutually exclusive models have been proposed. The first model, called the ‘recycling model’, proposes that the newly synthesized ligand exported to the plasma membrane is not competent to signal; the ligand needs to be internalized and recycled back to the plasma
membrane in order to be active. During this process, it has been proposed that the ligand undergoes an unknown post-translational modification or a conformational change (D'Souza, et al., 2008; Kandachar & Roegiers, 2012; Le Borgne, 2006; Musse, et al., 2012). The major supporting data for this model comes from studies in the D. melanogaster SOP cell. In this cell, it has been demonstrated that the GTPase Rab11 that is active in recycling endosomes, and Sec15, a component of the exocist complex, are necessary for Delta recycling and Notch activation (Emery et al., 2005; Jafar-Nejad et al., 2005). Moreover, in the same cell it has been shown that the Eps15 homology domain containing protein-binding protein 1 (dEHBP1) interacts with Rab11 and Sec15 to assist in the exocytosis and recycling of Delta (Giagtzoglou et al., 2012).

Another variant of the ‘recycling model’ proposes that recycling is needed to relocate the ligand to a specific lipid-enriched microdomain of the plasma membrane, which might favor clustering of the ligand. This model is supported by two different studies. The first study proposes that the phosphoinositide-binding motif of Neuralized mediates endocytosis of Delta after its ubiquitination, suggesting that Delta might need to be located in a specific lipid microdomain environment to be activated (Skwarek et al., 2007). The second study suggests that changes in the glycosphingolipid composition of the membrane can rescue the Delta and Serrate signaling and trafficking defects obtained after Mindbomb inhibition (Hamel et al., 2010).

The second model, called the ‘pulling forces model’ proposes that, after binding to Notch, ligand, endocytosis in the signal-sending cell is required to exert a mechanical force on the receptor in the signal-receiving cell. This force would be able to induce a conformational change in the receptor that allows unmasking of the S2 metalloprotease cleavage site, and subsequent activation of the pathway (D'Souza, et al., 2008; Kandachar & Roegiers, 2012; Le Borgne, 2006; Musse, et al., 2012). This second model is mainly supported by the fact that, although in SOP cells, Rab11 and Sec15 are required to activate Notch signaling, neither Rab11 nor Rab5 are necessary for Notch ligand to activate Notch in the D.
melanogaster germline or in the developing eye, excluding a role for recycling requirement in this context (Banks et al., 2011; Windler & Bilder, 2010). Major support for this model also comes from studies performed in mammalian cells that will be discussed in the next section.

3.7 Endocytic regulation of Notch ligands in the signal-sending cell: mammals

The role of the mammalian orthologs of the D. melanogaster genes, Neuralized, Mindbomb and Liquid facet, has also been explored. Genetic studies in knockout (KO) mice have helped to clarify the role of these proteins in vivo. In particular, it has been shown that Neuralized1 and Neuralized2 deletion in mice, contrary to expectations based on D. melanogaster data, does not lead to a lethality phenotype, since the mice are healthy and display few Notch related phenotypes (Koo et al., 2007; Ruan et al., 2001; Vollrath et al., 2001). The same is true for the Mindbomb2 KO mice (Koo, et al., 2007). In contrast, Mindbomb1 KO mice are embryonic lethal and conditional inactivation of Mindbomb1 in different tissues, such as the endothelium, skin epithelium, cerebellum and apical ectodermal ridge, revealed phenotypes characteristic of Notch loss-of-function mutants (i.e., Dll4, Jag1 and Jag2 mutant mice) (Koo et al., 2005; Koo, et al., 2007). These data suggest that in mammals Mindbomb1 is the key ubiquitin E3 ligase involved in the regulation of Notch ligands. This role of Mindbomb1 was confirmed by reciprocal bone marrow transplantation experiments that demonstrated that Mindbomb1 in the thymic and splenic microenvironments is essential for Notch dependent T and Marginal Zone B cell development, respectively (Song et al., 2008). Moreover, silencing of Mindbomb1 in the OP9-Dll1 cell line, a stromal murine cell line that overexpresses the ligand Dll1, abolished T cell development in vitro and inhibited Dll1 endocytosis (Song, et al., 2008).

A double KO mouse for the two murine Epsins (Epsin1 and Epsin2) has also been generated (Chen et al., 2009). While the single KO mice for these two proteins are viable
and show no phenotypic defects, the Epn1/Epn2 double KO (DKO) mouse is embryonic lethal and displays phenotypes that recapitulate several defects caused by Notch loss-of-function (Chen, et al., 2009). Moreover, a recent in vitro study characterizing the endocytic and cellular components necessary for Dll1 ligand activation, confirmed a fundamental role of Epsin1 and Epsin2, together with clathrin (but not AP2 and caveolin1) in the regulation of Dll1 activity and its endocytosis (Meloty-Kapella et al., 2012). Using a cell-bead optical tweezer system, the authors also demonstrated that the primary role for Notch ligand endocytosis is not recycling, but rather to generate mechanical forces needed for the conformational change and subsequent activation of the receptor, thus supporting the “pulling force” model (Meloty-Kapella, et al., 2012; Shergill et al., 2012).

Nevertheless, the requirement for Notch ligand recycling in mammalian cells has not been disproved and seems to be at least context dependent, especially in polarized cells. There are in fact data that report that Neuralized is able to promote Dll1 recycling from the basolateral to the apical membrane of polarized cells (Benhra et al., 2010). Biochemical proof of the requirement of ligand recycling in mammalian cells has been obtained using a mutant Dll1 protein lacking the 17 lysine residues in its intracellular domain (Heuss et al., 2008). This mutant protein is not ubiquitinated and undergoes endocytosis, but is not able to be recycled to the plasma membrane or to bind the Notch1 receptor efficiently. Moreover, a chimeric protein obtained fusing the extracellular domain of the ligand Dll1 and the intracellular domain of the ligand Dll3, which contains no lysine residues, is internalized, recycled to the plasma membrane and is able to interact with Notch1, but is not able to promote signal transduction (Heuss, et al., 2008). A lysine residue (K613) has also been identified in the cytoplasmic tail of Dll1 as a critical residue for Notch signaling activation; mutation of Dll1-K613 to R generated a ligand that was not able to physically interact with Notch1 receptor or to activate Notch signaling, although its endocytic recycling and stability were unaltered (Zhang et al., 2011). However, localization of the mutant to lipid raft microdomains was enhanced (Zhang, et al., 2011). It has also been
demonstrated that mammalian Dll1 contains a glycosphingolipid binding domain and is associated with specific lipidic microdomains of the plasma membrane, suggesting that ligand trafficking and activity can be modulated by their localization in a lipid environment (Heuss et al., 2013). Further studies aiming to elucidate the role of internalization and recycling in Notch ligand regulation have been performed on the Notch ligand Dll4. In particular, it has been shown that Dll4 is also able to undergo internalization and recycling, and to interact with Mindbomb1 (Shah et al., 2012). However, Dll4 showed a different localization in the plasma membrane domains compared to Dll1, with Dll4 being excluded from lipid rafts. Taken together, these results indicate that in mammalian cells the two Notch ligand activation models, “recycling/relocalization” and “pulling forces”, are likely to be not mutually exclusive, and, at least in some contexts, the two events could take place consecutively.

3.8 Regulation of Notch ligands in the signal-sending cell: glycosylation and proteolysis

Notch ligands, as Notch receptors, also appear to be regulated by glycosylation and proteolysis. At present, the role of glycosylation in Notch ligand regulation is mainly unexplored; both *D. melanogaster* Notch ligands, Delta and Serrate, as well as their mammalian counterparts, Dll1 and Jagged1, contain several consensus sequences for O-fucosylation. It has been demonstrated that both *D. melanogaster* and mammalian ligands are substrates of O-fut1/Pofut1 and that the added O-fucose can be elongated by Fringe (Panin et al., 2002). However, a very recent paper argues against a substantial regulation of Notch ligands activity by this modification, since a Dll1 protein with mutated O-fucosylation sites was able to reach the cell surface; likewise wild type Dll1 expressed in cells lacking Pofut1, was also able to reach the cell surface and to activate Notch (J. Muller et al., 2014).
Notch ligands can undergo two proteolytic cleavages in the proximity of their transmembrane domain. These two cleavages are mediated by ADAM proteases and by γ-secretase. Although it is clear that ligand proteolysis affects Notch signaling by decreasing ligand levels and availability at the plasma membrane, there is still controversy over whether the products of the proteolytic events have an intrinsic activity (D'Souza, et al., 2008). Mammalian DSL ligands can be cleaved by numerous ADAMs: ADAM9, ADAM10, ADAM12, ADAM17; however, only the ADAM10 and ADAM17 homologues are responsible for cleavage of the *D. melanogaster* ligands. These proteases cleave Notch ligands at multiple sites and the product of this cleavage is a portion of the ligand still anchored to the plasma membrane (TMICD) (Zolkiewska, 2008). In mammalian cells, the TMICD can undergo a second cleavage by γ-secretase (Ikeuchi & Sisodia, 2003; Six et al., 2003). Moreover, there is evidence that the product of this second cleavage (ICD) translocates into the nucleus and activates the transcription of various reporter genes (Hiratochi et al., 2007; Kolev et al., 2005; LaVoie & Selkoe, 2003). Together, these observations have provided the basis for the speculation that the ICD could mediate reverse signaling in the signal-sending cell. However, two recent studies in mice overexpressing various versions of Dll1-ICD and in endothelial cells overexpressing Jagged1-ICD, Dll-ICD and Dll4-ICD, indicate that this proposed bi-directional ligand signaling might not have much relevance in physiological contexts (Liebler et al., 2012; Redeker et al., 2013).

### 3.9 Transcriptional feedback regulation of Notch receptors and ligands: lateral inhibition

A last aspect that needs to be discussed in the context of Notch regulation, both in the signal-sending and the signal-receiving cell, is the concept of “lateral inhibition”. This concept refers to a transcriptional feedback mechanism that explains how Notch signaling
can lead to different cell fates in two initially identical progenitor cells, as in the case of neuroblast differentiation in the neurogenic region of the *D. melanogaster* embryo (Bray, 2006; Fortini, 2009). In this example, two initially equivalent interacting cells express comparable levels of Notch receptor and DSL ligands, and are at the same time both a signal-sending and signal-receiving cell. Notch ligands, in addition to being able to activate Notch receptors in *trans* on the neighboring cell, can negatively modulate Notch receptors in the same cell in a *cis* fashion, both at the plasma membrane and during the secretory pathway. When a stochastic difference in ligand and receptor levels arises in the two initially identical cells, the cell with a stronger Notch signaling will overcome the *cis*-inhibition of Notch by the ligand, resulting in upregulation of receptor expression. In contrast, in the cell with the weaker Notch signaling, receptor activity is downmodulated and ligand expression increases. In this way, a stable feedback amplification loop is established and the two cells adopt a signal-receiving and a signal-sending cell fate, respectively (Bray, 2006; Fortini, 2009).

This mechanism has also been investigated in mammalian cells through a quantitative time-lapse microscopy approach that allowed the authors to determine that while the Notch response to Delta in *trans* is graded, its response to Delta in *cis* is sharp and occurs at a fixed threshold (Sprinzak et al., 2010). These results contribute to our understanding of “lateral inhibition” in a multicellular organism, explaining how this mechanism can amplify small initial differences between neighboring cells.
4. Eps15/Eps15L1 and Notch signaling regulation

4.1 Eps15, Eps15L1 and Eps15/Eps15L1 knockout mice

Genetically engineered mice are an effective model system to study the in vivo function of a protein. KO mouse models, in which a specific protein has been inactivated (or knocked out) by disrupting its gene of origin, are particularly useful in studying the biological roles of proteins. The loss of the protein of interest can cause changes in the mouse phenotype at different levels: it can cause lethality, alteration of developmental processes, behavioral problems and other biochemical or physiological alterations.

To obtain insights into the physiological function of Eps15 and Eps15L1 in mammals, KO mice for Eps15, Eps15L1 and Eps15/Eps15L1 combined (i.e., the double KO, DKO) were generated in our laboratory, prior to the beginning of this thesis project. The Eps15 KO mice are viable, healthy and fertile and do not show any obvious phenotype beside a defect in B cell lymphopoiesis (Pozzi et al., 2012). In contrast, the Eps15L1 KO mice are perinatal lethal due to a defect in the nervous system that impairs feeding (Alberici et al., manuscript in preparation). Eps15/Eps15L1 DKO mice, however, are embryonic lethal. The mice die at midgestation, around 10.5 days after fertilization. Morphological analysis of 9.5-day-old Eps15/Eps15L1 DKO embryos revealed numerous developmental defects, such as cardiovascular defects and absence of limb buds. Moreover, more severely affected embryos showed an open neural tube, absence of turning and lack of caudal somites (Figure 1.8A).
Figure 1.8 Morphological analysis of Eps15/Eps15L1 DKO embryos at 9.5 days post-fertilization and comparison with Notch signaling loss-of-function KO embryos.

A. Photographs displaying the phenotype of Eps15-KO and Eps15/Eps15L1-DKO littermates at 9.5 days post-fertilization. The Eps15-KO embryo displays a wild type phenotype. Arrows indicate the midbrain/hindbrain boundary (Bd), the limb bud (Lb) and the heart (Ht). The asterisk in the middle picture indicates the absence of limb bud. All the photographs are at the same magnification.

B. Photographs displaying the midgestation embryo phenotype of the Tie2Cre/Notch1-KO [adapted from (Limbourg et al., 2005)], Dll4-KO [adapted from (Duarte et al., 2004)], Numb-KO [adapted from (Zilian et al., 2001)], Epsin1/Epsin2 KO [adapted from (Chen, et al., 2009)] and Mib1-KO [adapted from (Koo, et al., 2005)].
4.2 Eps15 and Eps15L1: hypothesis on Notch signaling regulation

Most of the alterations identified in the Eps15/Eps15L1 DKO mice phenocopy various characteristics of Notch signaling loss-of-function mice, such as the Notch1 KO (Limbourg, et al., 2005; Swiatek et al., 1994), Notch1/Notch4 DKO (Krebs et al., 2000), Dll1 KO (Hrabe de Angelis et al., 1997), Dll4 KO (Duarte, et al., 2004), Jagged1 KO (Xue et al., 1999), Numb KO (Zilian, et al., 2001), Epsin1/Epsin2 DKO (Chen, et al., 2009) and Mib1 KO (Koo, et al., 2005) (Table 1 and Figure 1.8B).

To understand if Notch signaling, together with other signaling pathways, was indeed deregulated in the absence of Eps15 and Eps15L1, a microarray analysis was performed on 9.5-day-old embryos, comparing 3 sets of Eps15 KO, Eps15L1 KO and Eps15/Eps15L1 DKO littermates. The majority of the genes found altered in the DKO mice recapitulated the morphological changes in the embryos; in particular, genes involved in cardiac and neural development, in hematopoiesis and in metabolism were deregulated (Table 2). Numerous genes were also found altered in the signaling category; however, no single pathway was prominently affected. Major signaling pathways, such as Smad, β-catenin and Hedgehog, were not deregulated. However, the Notch target genes Hes1, Hes5 and NRAP were downregulated in DKO embryos. To confirm these findings, a QPCR analysis of 9.5-day-old embryos was performed, using Epsin1/Epsin2 DKO embryos as a control for alterations in Notch signaling target genes (Figure 1.9). Notch target genes Hes1, Hes5 and NRAP were confirmed as downregulated in the Eps15/Eps15L1 DKO, to the same extent as in Epsin1/Epsin2 DKO mice (Figure 1.9A). In contrast, the β-catenin target genes Axin2 and Lef1, the TGF-β signaling proteins Smad6 and Smad7, and the Hedgehog target gene Gli1, were shown to be unaltered in Eps15/Eps15L1 DKO mice, confirming the microarray data (Figure 1.9B-D).

Taken together, these observations suggest that loss of both Eps15 and Eps15L1 correlates with a Notch loss-of-function phenotype. Considering the fact that these proteins are also
known to interact with two major Notch signaling regulators, Numb and Epsin (Chen, et al., 1998; Salcini, et al., 1997), we hypothesized that Eps15 and Eps15L1 could also act as Notch regulators, either in the signal-sending or in the signal-receiving cell.

**Table 1.** Summary of the main phenotypic effects displayed by embryos knocked out for the principal Notch signaling components.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Receptor/ligand/other</th>
<th>KO lethality</th>
<th>Cardiovascular defects</th>
<th>Placental and yolk sac defects</th>
<th>Defects in nervous system</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notch1</td>
<td>Receptor</td>
<td>&lt; 11.5 dpc</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>(Swiatek, et al., 1994)</td>
</tr>
<tr>
<td>Tie2Cre/Notch1</td>
<td>Receptor</td>
<td>&lt; 11.5 dpc</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>(Limbourg, et al., 2005)</td>
</tr>
<tr>
<td>Notch1/Notch4</td>
<td>Receptor</td>
<td>&lt; 11.5 dpc</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>(Krebs, et al., 2000)</td>
</tr>
<tr>
<td>Dll1</td>
<td>Ligand</td>
<td>&lt; 11.5 dpc</td>
<td>YES</td>
<td>/</td>
<td>YES</td>
<td>(Hrabe de Angelis, et al., 1997)</td>
</tr>
<tr>
<td>Dll4</td>
<td>Ligand</td>
<td>&lt; 11.5 dpc</td>
<td>YES</td>
<td>YES</td>
<td>/</td>
<td>(Duarte, et al., 2004)</td>
</tr>
<tr>
<td>Jag1</td>
<td>Ligand</td>
<td>&lt; 11.5 dpc</td>
<td>YES</td>
<td>/</td>
<td>/</td>
<td>(Xue, et al., 1999)</td>
</tr>
<tr>
<td>Numb</td>
<td>Endocytic adaptor</td>
<td>&lt; 11.5 dpc</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>(Zilian, et al., 2001)</td>
</tr>
<tr>
<td>Epsin1/2</td>
<td>Endocytic adaptors</td>
<td>&lt; 11.5 dpc</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>(Chen, et al., 2009)</td>
</tr>
<tr>
<td>Mib1</td>
<td>E3 ligase</td>
<td>&lt; 11.5 dpc</td>
<td>YES</td>
<td>YES</td>
<td>/</td>
<td>(Koo, et al., 2005)</td>
</tr>
<tr>
<td>Eps15/Eps15L1</td>
<td>Endocytic adaptors</td>
<td>&lt; 11.5 dpc</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>(unpublished)</td>
</tr>
</tbody>
</table>
Table 2. Summary of the major gene categories found altered in the microarray analysis performed on 9.5-day-old Eps15/Eps15L1 KO embryos.

<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>UP</th>
<th>DOWN</th>
</tr>
</thead>
<tbody>
<tr>
<td>cardiac</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>neural</td>
<td>8</td>
<td>49</td>
</tr>
<tr>
<td>hematopoiesis</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>adhesion</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>cytoskeleton</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>metabolism</td>
<td>31</td>
<td>20</td>
</tr>
<tr>
<td>trafficking</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>signaling</td>
<td>19</td>
<td>27</td>
</tr>
<tr>
<td>transcription</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>RIKEN</td>
<td>7</td>
<td>22</td>
</tr>
<tr>
<td>other</td>
<td>16</td>
<td>15</td>
</tr>
</tbody>
</table>

Figure 1.9 Downregulation of Notch target genes in Eps15/Eps15L1 DKO embryos at 9.5 days post-fertilization.

A. Bar graph depicting the relative expression levels of Hes1 (white bars), Hes5 (grey bars) and NRAP (black bars) Notch target genes in wild-type (WT), Eps15/Eps15L1-DKO (DKO), Eps15L1-KO (15L1), Eps15-KO (15) and Epsin1/Epsin2-DKO (E1/2) 9.5-day-old embryos, as determined by QPCR.
B. Bar graph depicting the relative expression levels of axin2 (white bars) and lef1 (black bars) Wnt/β-catenin target genes in wild-type (WT), Eps15/Eps15L1-DKO (DKO), Eps15L1-KO (L1KO) and Eps15-KO (15KO) 9.5-day-old embryos as determined by QPCR.

C. Bar graph depicting the relative expression levels of smad7 (white bars) and smad6 (black bars) in wild-type (WT), Eps15/Eps15L1-DKO (DKO), Eps15L1-KO (L1KO) and Eps15-KO (15KO) 9.5-day-old embryos as determined by QPCR.

D. Bar graph depicting the relative expression levels of the Hedgehog target gene Gli (black bars) in wild-type (WT), Eps15/Eps15L1-DKO (DKO), Eps15L1-KO (L1KO) and Eps15-KO (15KO) 9.5-day-old embryos, as determined by QPCR.

A-D. All values were normalized on GAPDH and on the relative expression levels of WT embryos. At least 3 independent embryos were analyzed for each gene, except for Epsin1/Epsin2-DKO (n=1). Results are expressed as mean ± SD.
5. Aims

A number of observations point to a role for Eps15 and Eps15L1 in the endocytic regulation of Notch signaling:

i) Morphological analysis of the Eps15/Eps15L1 DKO mice suggests that ablation of the two proteins causes a putative Notch loss-of-function phenotype, since Eps15/Eps15L1 embryos closely resemble those of KO mice for different components of the Notch pathway; moreover the Notch target genes Hes1, Hes5 and NRAP are downregulated in Eps15/Eps15L1 DKO embryos.

ii) Eps15 and Eps15L1 physically interact with Numb and Epsin, key regulators of Notch receptor and ligand activity, respectively.

iii) Eps15 and Eps15L1 act in complex with Epsin1 to modulate EGFR endocytosis.

iv) Both Eps15 and Eps15L1 possess UIM domains, one of the two essential domains needed for Epsin-dependent activation of Notch ligands.

Despite these observations, there is no direct evidence demonstrating the involvement of Eps15 and Eps15L1 in the endocytic regulation of Notch.

Thus, the overall aim of this project is to characterize the involvement of Eps15 and Eps15L1 in Notch signaling regulation. Specifically, we will first assess whether Eps15 and Eps15L1 are able to directly affect Notch signaling (Results Chapter 1-4). To achieve this, it is necessary to set-up a suitable model system that allows measurement of trans activation of Notch (Results chapter 1-2). Subsequently, we will investigate whether Eps15 and Eps15L1 modulate Notch signaling by regulating the activity of the receptor in the signal-receiving cell or the activity of the ligand in the signal-sending cell (Result Chapters 3 and 4). Finally, to define the molecular mechanisms underlying the putative regulation of Notch signaling by Eps15 and Eps15L1, we will assess if this occurs through modulation of the receptor/ligand endocytosis/recycling (Results Chapters 5-7).
Chapter 2

MATERIALS AND METHODS

1. Solutions and Buffers

1.1 Phosphate-buffered saline (PBS)

NaCl 137 mM
KCl 2.7 mM
Na₂HPO₄ 10 mM
KH₂PO₄ 2 mM

8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ were dissolved in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl and the volume was brought to 1 litre with distilled H₂O.

1.2 Tris-HCl (1 M)

121.1 g of Tris base were dissolved in 800 ml distilled H₂O. The pH was adjusted to 7.4, 7.6 or 8.0 with HCl, and distilled H₂O was added to bring the volume up to 1 litre.

1.3 Tris-buffered saline (TBS)

NaCl 137 mM
KCl 2.7 mM
Tris HCl pH 7.4 25 mM

8 g of NaCl, 0.2 g of KCl, and 3 g of Tris base were dissolved in 800 ml of distilled H₂O. The pH was adjusted to 7.4 with HCl and distilled H₂O was added to bring the volume up
to 1 litre.

1.4 10X SDS-PAGE running buffer

Glycine 192 mM
Tris HCl, pH 8.3 250 mM
SDS 1%

1.5 10X Western transfer buffer

Glycine 192 mM
Tris HCl, pH 8.3 250 mM

For 1X western transfer buffer, the 10X stock was diluted 1:10 with ddH2O and 20% v/v ethanol.

1.6 JS lysis buffer

HEPES, pH 7.4 50 mM
NaCl 150 mM
Glycerol 10%
Triton X-100 1%
MgCl2 1.5 mM
EGTA 5 mM

200X Protease inhibitor cocktail (Calbiochem, 539131), sodium pyrophosphate pH 7.5 20mM, sodium fluoride 250 mM, PMSF 2 mM, and sodium orthovanadate 10 mM, final concentrations, were added to the buffer just before use.
2. Reagents

2.1 DNA constructs

The pBabe plasmid expressing human Notch1 HA-tagged at the N-terminal and FLAG-tagged at the C-terminal (pBABE-HAN1FLAG) was a gift from Maddalena Donzelli (IEO, Milano). The pCS2 plasmid expressing murine Notch1 Intracellular domain (pCS2-ICV-6MT) was generated by Schroeter and colleagues (Schroeter, et al., 1998). The firefly luciferase Notch reporter construct pGa981-6 was generated by Kurooka and colleagues (Kurooka et al., 1998). The renilla luciferase pRL-TK vector has been purchased from Promega.

2.2 TaqMAN assays for RT qPCR

The TaqMAN assays for RT qPCR (Applied Biosystems) were:

- Notch1: mm00435245_m1
- Notch2: mm00803077_m1
- Notch3: mm00435270_m1
- Notch4: mm00440525_m1
- Dll1: mm00432841_m1
- Dll3: mm00432854_m1
- Dll4: mm00444619_m1
- Jagged1: mm00496902_m1
- Jagged2: mm00439935_m1
- Numb: mm00477927_m1
- Numb-like: mm00477931_m1
2.3 Antibodies

The following antibodies were used:

**Immunofluorescence**: armenian hamster monoclonal anti-Dll1 HMD1-3 Biolegend (1:500).

**Western blot**: mouse monoclonal anti Eps15 3T (Poupon et al., 2002) (1:3), rabbit polyclonal anti Eps15L1 home made raised against full-length mouse Eps15L1 fused to GST (1:2000), mouse monoclonal anti Epsin1 ZZ3 home made raised against aminoacids 249-401 of human Epsin1 (1:100) (a gift from Simona Polo), rabbit polyclonal anti Epsin2 (a gift from Pietro de Camilli) (1:500), mouse monoclonal anti Vinculin Sigma (1:10000), mouse monoclonal anti HA 16B12 Covance (1:1000), mouse monoclonal anti FLAG Sigma (1:10000), rabbit polyclonal anti Mib1 M5948 Sigma (1:1000), mouse monoclonal anti AP2 610501 BD Bioscience (1:1000), mouse monoclonal anti Dynamin1/2 Hudy-1 EDM Millipore (1:2000), rabbit monoclonal anti Clathrin heavy chain D3C6 Cell signaling (1:1000), mouse monoclonal anti- NUMB Ab21 (Colaluca et al., 2008) raised against amino acids 537–551 of human NUMB (1:250), rabbit monoclonal anti Caveolin-1 D46G3 Cell Signaling (1:1000), mouse monoclonal anti β-Tubulin AA2 Sigma (1:10000), rabbit monoclonal anti EGFR 4267 Cell Signaling (1:1000), rabbit monoclonal anti Dll1 2588 Cell Signaling (1:2000).

**Cytofluorimetry**: armenian hamster monoclonal anti Dll1 HMD1-3 Alexa Fluor 647 Biolegend (1:250), goat polyclonal anti human IgG Alexa Fluor 647 Invitrogen (1:200)
### 2.4 RNAi Oligonucleotides

**Table 3. RNAi Oligos sequences and final concentrations**

<table>
<thead>
<tr>
<th>Targeted protein/oligo name</th>
<th>Oligo sequence 5’-3’ (guide)</th>
<th>Oligo type/company</th>
<th>Final concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control_CTR</td>
<td>AGACGAACAGUCACCGAC</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Negative Control_CTR 2</td>
<td>AGCGAACACGTACCCGAC</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Negative Control_CTR 3</td>
<td>AGACGAACAGUCACCGACCCCC</td>
<td>IBONI (Riboxx)</td>
<td>5 nM</td>
</tr>
<tr>
<td>Numb_01</td>
<td>UUCUUAUCUUGUCUGCCACCCCC</td>
<td>IBONI (Riboxx)</td>
<td>5 nM</td>
</tr>
<tr>
<td>Numb_02</td>
<td>UAAACGUCUUUCUUUCUCCCCC</td>
<td>IBONI (Riboxx)</td>
<td>5 nM</td>
</tr>
<tr>
<td>Numb-like_02</td>
<td>AGGUAAACAGAGUCUCCACCCCC</td>
<td>IBONI (Riboxx)</td>
<td>5 nM</td>
</tr>
<tr>
<td>Numb-like_03</td>
<td>ACUCUGCCGUAACUUGUCCCCCC</td>
<td>IBONI (Riboxx)</td>
<td>5 nM</td>
</tr>
<tr>
<td>dynamin 2</td>
<td>UGAACUGCAGGAUAGUCCCCC</td>
<td>IBONI (Riboxx)</td>
<td>5 nM</td>
</tr>
<tr>
<td>Eps15_51</td>
<td>CCCUUAACUGAAUGAUCAUUUCA</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Eps15_52</td>
<td>CCCAGGCAUAGUAGUCCCCAAGAA</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Eps15_53</td>
<td>AGAUAGAGUUCAAAGGGAGAGUAAU</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Eps15L1_54</td>
<td>GCAAAGUUGUAGGCAUCUUUGAAA</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Eps15L1_55</td>
<td>GAAAACCUUCUACGAGCUACUUU</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Eps15L1_56</td>
<td>GGCAUCAGGAGAGUUCACUGUGUU</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Mindbomb1</td>
<td>GGUGCUAAGCUAGACAUUCAGGAUA</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>AP2</td>
<td>UCAAGCGCAUGGCAUGCCCCC</td>
<td>IBONI (Riboxx)</td>
<td>5 nM</td>
</tr>
<tr>
<td>Clathrin Heavy Chain</td>
<td>GGAAGCAAUUCAUCAAGACCCC</td>
<td>IBONI (Riboxx)</td>
<td>20 nM</td>
</tr>
<tr>
<td>Epsin1_48</td>
<td>GACUGGCUCUGAGGCUGUACCAA</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Epsin1_49</td>
<td>GCAAGAACUGGAGGACUCGCAACAA</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Epsin1_50</td>
<td>AGUUUGAGGCGGAGGAGGACGAGUA</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Epsin2_66</td>
<td>GAGCUCUUCUACGUAUUCACAGUA</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Epsin2_67</td>
<td>CGGAGAUCUAGGCAUGGUGGUUGAA</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
<tr>
<td>Epsin2_68</td>
<td>CCCACCUUCUACCCAGCUACUCAG</td>
<td>Stealth (Invitrogen)</td>
<td>10 nM</td>
</tr>
</tbody>
</table>
3. Cell lines and cell culture conditions

3.1 Cell lines and growth conditions

The stromal murine cell line OP9 (Kodama et al., 1994) was obtained by ATCC. The stromal murine cell line OP9 constitutively overexpressing the Notch ligand Dll1 (OP9-Dll1) was generated in the Zunica-Pflucker laboratory (Schmitt & Zuniga-Pflucker, 2002). The OP9-Dll4, OP9-Jagged1 and OP9-Jagged2 cell lines (Van de Walle et al., 2011) were a gift from Tom Taghon (Ghent University Hospital). All the OP9 and OP9-derived cell lines were maintained in Minimum Essential Medium with GlutaMAX™ (MEM GlutaMAX™, Gibco) supplemented with 20% fetal bovine serum (FBS, Hyclone), 1% Non Essential Aminoacids (NEAA) 100x (EuroClone) and 1% Sodium Pyruvate 100 mM (EuroClone).

The C2C12 cell line, obtained by ATCC, is a subclone of the murin myoblast cell line established by Yaffe and Saxel (Yaffe & Saxel, 1977), and it was maintained in Dulbecco's Modified Eagle Medium with GlutaMAX™ (DMEM GlutaMAX™, Gibco) supplemented with 10% FBS (Hyclone).

All cells were kept cultured in 10 cm cell culture dishes under sterile conditions in a humidified incubator at 37 °C and 5% CO₂. Culturing media was changed every 2/3 days; cells were harvested at 80-90% confluency and replated at a ratio of 1:3-1:6 for OP9 and OP9-derived cell lines, and 1:5-1:10 for C2C12 and C2C12 derived cell lines.

3.2 Harvesting of cells and long-term storage

Harvesting of the cells was performed as follows: cells were washed twice with sterile Phosphate Buffer Saline (PBS) solution without Calcium and Magnesium, and subsequently incubated with trypsin/EDTA solution (0,05% trypsin, 0,02% EDTA in PBS 1x, EuroClone) at 37°C. After the detachment of the cells, trypsin was inhibited by addition of FBS containing growth medium. Trypsin/EDTA was removed by
centrifugation, cells were diluted to the desired concentration in growth medium and replated.

For long-term storage, cells were frozen and stored in liquid nitrogen. Cells were harvested by trypsinization and 1 million cells was resuspended in 1 ml of freezing solution, composed by 90% FBS (Hyclone) and 10% dimethyl-sulphoxide (DMSO), and placed in cryovials (Nalgene). Vials were placed immediately at -80°C in a cribox, and after overnight storage transferred to liquid nitrogen.

4. Cells transfection and generation of a stable clone

4.1 Transfection of the C2C12 cell line by microporation

Microporation is a transfection technique that uses a gold-plated pipette tip as an electroporation device. Electroporation is an effective technique for transfection in which an electric field is applied to increase the permeability of the cell membrane, inducing the formation of micro-pores. The pores, or "electropores", allow the passage of molecules (such as nucleic acids), ions and water from one side to the other of the plasma membrane. After electroporation, cells are able to spontaneously close the pores and continue growing. The pore formation is a very rapid event that takes about 1 second, while pore closure takes a few minutes. Amplitude of the electric pulses, their duration, their number and the interval between pulses are parameters that can be varied to optimize the protocol, until a threshold is reached, beyond which the diameter of pores increases and the total area of the pores is too large to allow repair, creating an irreversible damage to the cell. During electroporation the applied electric field causes the passage of a current flow through the cell, and this may cause considerable heating and subsequent cell death. Microporation, in contrast to conventional electroporation, allows the generation of a more uniform electric
field with minimal heat production, metal ion dissolution, pH variation and oxide formation.

Microporation of C2C12 cells was carried out using the Neon transfection system microporation kit (MPK10096, Invitrogen), specific for mammalian cells, in accordance with the manufacturer instructions. C2C12 transfection efficiency with microporation technique reaches 98%, in contrast to 40% efficiency obtained by cationic lipid transfection.

C2C12 cells were detached from the plate and $1\times10^6$ cells were resuspended in 100 µl of resuspension buffer R (MPK10096, Invitrogen). DNA was then added to the cells according to these concentrations:

- 40 ng/µl of pBABE-HAN1FLAG
- 40 ng/µl of pCS2-ICV-6MT
- 8 ng/µl Firefly luciferase Notch reporter pGa981-6
- 0.4 ng/µl Renilla luciferase pRL-TK

Upon the addition of 4 ml Electrolytic buffer E (MPK10096, Invitrogen), the microporation tube was inserted into the pipette station to form the microporation unit. The cell/DNA mixture was uploaded into the microporation gold-tip. The microporation parameters, previously optimized for the best transfection efficiency, were set as this:

- Pulse voltage: 1100 V
- Pulse width: 30 ms
- Pulse number: 2

Following microporation, samples were transferred to pre-heated culture medium and plated according to the experimental conditions. Cells were allowed to recover overnight and the following day the culture medium was changed, or the experiment performed.
4.2 Generation of a stable C2C12 clone expressing the human receptor Notch1

To obtain a C2C12 cell line stably expressing the receptor Notch1, a total of 1x10⁶ C2C12 cells were transfected with 4 µg of pBABE-HAN1FLAG plasmid via microporation, as previously described. Twenty-four hours after transfection, cells were detached from the plate and diluted to 4 different concentrations (1:5, 1:10, 1:15 and 1:20) to avoid that they reach confluency before selection. Forty-eight hours after transfection we began selection of pBABE-HAN1FLAG expressing cells, adding 1.5 µg/ml Puromycin to the growth medium, according to the concentration previously established during the killing curve. A killing curve is a dose-response experiment where the cells are subjected to increasing amounts of antibiotic to determine the minimum antibiotic concentration needed to kill all the cells over the course of one week. After 7 days of selection, about 100 Puromycin resistant clones were present on the transfected plates, while in the control non-transfected plate, all cells were dead. To obtain subclones derived from a single cell, 40 clones were trypsinized, picked from the plates with the aid of a pipette tip, counted and resuspended at a concentration of 1 cell in 100 µl. 100 µl of cell suspension were then plated in each well of a 96 well plate. Each clone was plated 12 times. The next day, 100 µl of fresh medium were added to each well and then the medium was changed every 2 days for 10-15 days. Clones that survived expansion were trypsinized and expanded at first in 48 well plate, then 24 well plate, 6 well plate and finally into a 10 cm cell culture dish. An aliquot of the survived clones was analyzed by western blot for the presence of the Notch1 protein, while the remaining cells were frozen in liquid nitrogen. Based on the results of the western blot, cells that expressed the Notch1 receptor were thawed and subsequently tested in a Notch reporter transactivation assay for their ability to signal.
4.3 RNA interference

To perform RNA interference (RNAi) in C2C12-derived and OP9-derived cells we transfected the cells with RNA oligonucleotides (oligos) through the use of artificial lipid vesicles, called liposomes (Lipofectamine RNAi Max, Invitrogen). Lipofectamine RNAi Max is a cationic lipid, optimized for RNA molecules transfection. Cationic lipids form an aggregate of vesicles around the DNA or RNA molecule, establishing electrostatic bonds with the negative charges of the nucleic acid itself. Cationic liposomes are then able to interact with negative charges present on the plasma membrane and facilitate the entry of the nucleic acid into the cell.

To perform RNAi, on day 1 cells were detached from the plate, counted and resuspended in fresh medium at a concentration of 0.5x10^5 cells/ml. Two ml of cell suspension (for a total of 1x10^5 cells) were transferred in a sterile 15 ml falcon. 500 µl of transfection mix were then added dropwise to the cell suspension, and the content of the falcon was plated in 1 well of a 6 well plate.

Transfection mix was prepared as follows:

- **mix 1**: 5 µl of Lipofectamine RNAi MAX (Invitrogen) were added to 250 µl of Opti-MEM fresh medium (Gibco)
- **mix 2**: Oligos at the desired final concentration (concentrations and sequences are specified in Materials and methods section 2.4, Table 3) were added to 250 µl of Opti-MEM fresh medium (Gibco)
- **Transfection mix**: mix1 was added to mix2 and incubated 20 minutes at room temperature before use

RNA interfered cells were then transferred in a humidified incubator at 37°C and 5% CO₂ for 24 hours. On day 2 medium was changed and transfection was repeated as described before on adherent cells. On day 3 medium was changed again or cells were detached, counted and plated at the desired concentration depending on the experimental condition; cells were used to perform the experiment on day 4.
5. Molecular biology, biochemical and immunostaining procedures

5.1 RT qPCR

Quantitative real time polymerase chain reaction (RT qPCR) is a technique used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount) of one or more specific sequences in a nucleic acid sample. TaqMan® chemistry uses a fluorogenic probe to enable the detection of a specific PCR product as it accumulates during PCR cycles.

Total RNA was isolated from $1 \times 10^6$ or $1 \times 10^5$ plated cells using RNeasy Mini kit (Quiagen) in accordance with the manufacturer’s instructions. Reverse transcription was performed with SuperScript VILO cDNA Synthesis Kit (Invitrogen) starting from 1 µg RNA. RT qPCR was performed using TaqMan chemistry (Invitrogen) employing a 7500 Real-Time PCR System instrument (Applied Biosystems), according to the manufacturer’s instructions. Probes were purchased from Applied Biosystems as previously described in Reagents section. The results were normalized on the expression of the housekeeping gene GAPDH. The 2-delta delta ct method was employed to analyze the relative changes in gene expression (Livak & Schmittgen, 2001).

5.2 Western Blotting

To obtain a proteic lysate, cells were placed on ice, washed twice with cold PBS and lysed in JS buffer directly in the cell culture plates using a cell-scraper. Lysates were allowed to incubate for 15 minutes and then clarified by centrifugation at 16,000 rpm for 15 minutes at 4°C in a 5415 R Eppendorf centrifuge. Protein concentration in the samples was measured using the BCA Protein Assay Reagent KIT (Pierce), using as standard bovine serum albumin (BSA), following manufacturer’s instructions. Desired amounts of proteins were loaded onto precast polyacrylamide gels for electrophoresis (Biorad) together with a prestained protein molecular weight marker (Biorad). Gels were run at 200V in Running
proteins were transferred in Western transfer tanks (Biorad) onto nitrocellulose (Schleicher and Schuell) using Western Transfer buffer at 100V for 1 hour. Membranes were stained with Ponceau (Sigma) to determine the efficiency of protein transfer. Membranes were blocked 1 hour in 5% low fat milk in TBS 1% Triton X-100 (TTBS) (for incubation with polyclonal antibodies) or in 5% BSA in TTBS (for incubation with monoclonal antibodies). After blocking, filters were incubated with the primary antibody, diluted in the same solution as blocking, in for 1 hour at room temperature, or overnight at 4°C, followed by three washes of 10 minutes each in TTBS and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted in TTBS for 1 hour. After incubation with the secondary antibody, the filter was washed 3 times in TTBS as previously described and the bound secondary antibody was revealed using either the SuperSignal West Pico Chemiluminescent Substrate (Pierce) or the SuperSignal West Femto Chemiluminescent Substrate (Pierce), and the Chemidoc system (Biorad).

5.3 Immunofluorescence

Cells were detached, counted and plated on 13 mm glass coverslips (coated with 15 μg/ml Poly-D-lysine at 37°C for 30 minutes) at a density of 1x10⁴ cells/coverslip. 24 hours later, cells were fixed at 4°C in 4% paraformaldehyde (in 1X PBS) for 10 minutes, washed with PBS and permeabilized and blocked in a solution of 0.05% Triton X-100, 1% BSA and 10% serum in 1X PBS for 1 hour at room temperature. Cells were then incubated overnight at 4°C in a humidified chamber with the primary antibody diluted in 0.05% Triton X-100, 1% BSA in TBS. The following morning cells were washed 3 times with 0.05% Triton X-100, 1% BSA in TBS and incubated for 1 hour with fluorescently labeled secondary antibodies (Amersham) diluted in TBS. After 3 washes with TBS, nuclei were DAPI-stained for 5 minutes and cells washed again 2 times with H₂O. Coverslips were immediately mounted with mowiol and examined under a wide-field fluorescence 86-microscope (Olympus) with
the help of the associated software MetaMorph (Molecula Devices). Images were further processed with the ImageJ software.

5.4 FACS analysis

Flow cytometry assay is a technique for counting, examining and sorting cells suspended in a stream of fluid. It allows simultaneous multi-parametric analysis of the physical and/or chemical characteristics of single cells flowing through a fluidic, optical and electronic detection apparatus. A beam of laser light of a determined single wavelength is directed onto a hydro-dynamically focused stream of fluid. Each suspended cell pass through a small nozzle, one at time, and scatters the light in a certain way; moreover fluorescent chemicals found in the cell or attached to the cell membrane can be excited into emitting light at a higher wavelength than the light source. The detectors pick up this combination of scattered and fluorescent light and, by analyzing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive information about the physical and chemical structure of each individual cell. FSC (forward scatter) correlates with the cell size and SSC (side scatter) depends on the inner density and granularity of the cell (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness).

The FACS-Calibur flow cytometer (BD Biosciences), used in this work, employs a dual-laser technology, an air-cooled argon laser and a red diode laser, that allows flexibility and sensitivity for multicolor analysis (Figure 2.1). The 488 nm excitation laser allows the detection of green (FL1: FITC, Alexa Fluor 488), yellow (FL2: PE, PI) and red fluorochromes (FL3: PE-Cy5, PerCP, PE-Cy7), while the 635 nm excitation laser allows the detection of other red fluorochromes, such as APC and Alexa Fluor 647 (FL4) (Figure 2.1).

Data generated by flow-cytometers were analyzed using FlowJo software. Data were
plotted in a single dimension, to produce a histogram, and mean fluorescence of the histogram was calculated and compared between samples.

Figure 2.1 BD FACSCalibur Optical Path Configuration

Schematic representation of lasers and fluorescence detectors of the FACS-Calibur flow cytometer (BD Biosciences) [adapted from BD Biosciences website].

5.5 Dll1 surface levels measurement

To measure Dll1 surface levels, OP9-Dll1 cells (KD or not) were detached from the plate and counted. 1×10^5 cells were resuspended in 50 µl of cold Opti-MEM (Gibco) containing 10% BSA, and placed in a 1,5 ml eppendorf on ice. Cells were stained 1 hour at 4°C by adding to the cell suspension 50 µl of Opti-MEM (Gibco) containing anti Dll1 HMD1-3 Alexa Fluor 647 Biolegend, for a final concentration of 2 µg/ml in 100 µl final volume. After staining cells were washed twice in cold PBS, resuspended in cold PBS containing 2% BSA, and immediatedly analyzed using FACS-Calibur flow cytometer (BD Biosciences).
6. Biological and biochemical assays

6.1 Notch reporter transactivation assay

Reporter gene technology is often used to monitor cellular events associated with signal transduction and gene expression. The term ‘reporter gene’ is used to define a gene with a phenotype easily distinguishable from endogenous proteins, such as ability to generate luminescence. The reporter unit is composed by a promoter followed by the reporter gene; the promoter binds transcription factors that are activated in response to a signalling cascade. Activation of the promoter allows expression of the reporter gene, which in turn provides information on the activity of the signal transduction pathway.

To monitor activation of Notch signalling pathway, we chose as reporter unit the plasmid pGA981-6, encoding the reporter gene Firefly luciferase, an enzyme that catalyzes the oxidation of a substrate with subsequent luminescence emittance (figure 2.2). Upstream of the reporter gene are located 6 binding sites for the transcription factor CSL followed by the minimal promoter of the β-globin. To normalize reporter gene activity on transfection efficiency we cotransfected, together with the reporter gene, the plasmid pRL-TK, encoding for the constitutively expressed Renilla luciferase, another enzyme belonging to the luciferase family (Figure 2.2).

To perform the Notch reporter transactivation assay, 1x10^6 C2C12 or C2C12-Notch1 cells (KD or not) were transfected via microporation, as previously described, with the Firefly luciferase construct and the Renilla luciferase construct. 1x10^4 transfected cells were plated in each well of a 96-well plate. For each experimental condition, 8 technical replicas were performed. In order to be able to process more samples in parallel, we were able to downscale the initial co-culture procedure (originally performed in a 24 well plate) into a 96 well plate assay.

24 hours after transfection, 1x10^4 OP9 or OP9-Dll1, OP9-Dll4, OP9-Jagged1 and OP9-Jagged2 cells (KD or not) were plated on top of C2C12-Notch1 cells. After 24 hours
coculture, cells were lysed and luciferase activity was detected with Dual-Luciferase Reporter Assay System KIT (Promega) according to the manufacturer’s instructions. When specified, 1 µg/ml DAPT (D5942 Sigma) was added to C2C12-Notch1 cells 16 hours prior to coculture; treatment was maintained until the end of the experiment.

Figure 2.2 Bioluminescence reactions catalyzed by Firefly and Renilla luciferase.

Firefly luciferase is a 614 kD monomeric protein that is not posttranslational modified. For this reason it is able to function as a reporter immediately after translation. Firefly luciferase emits photons through the oxidation of the beetle luciferin substrate through a reaction that requires ATP, Mg$^{2+}$ and O$_2$. Renilla luciferase is a 36 kD monomeric protein which also does not need posttranslational modifications to perform its enzymatic activity. The luminescence reaction catalyzed by Renilla luciferase employs O$_2$ to oxidize the substrate Coelenterazine.

6.2 Dll1 internalization assay

The Dll1 endocytosis assay that we set up in OP9-Dll1 cells allowed us to follow the internalization of the ligand in time, at the early steps of the endocytic process. The assay permits to follow both receptor mediated Dll1 endocytosis (following stimulation with a preclustered recombinant N2Fc protein that should mimic the physiological conditions of Notch extracellular domain endocytosis) and constitutive endocytosis (through the use of a fluorochrome conjugated antibody anti-Dll1).

Subconfluent OP9-Dll1 cells were detached from the plate by mild trypsinization (0,025% trypsin), resuspended at 5x10$^4$ cells /50 µl of pre warmed Opti-MEM containing 5% FBS
and transferred into a well of a 2 ml volume V-bottom Corning 96 well storage system plate (Sigma). Cells were then allowed to equilibrate at 37°C for 30 minutes. Internalization was performed at 37°C for 2, 4 and 6 minutes by adding 50 µl of 2x internalization mix to the cell suspension.

2x internalization mix consisted of:

- 2 µg/ml Notch2-Fc/chimera (N2Fc) (N9161 Sigma) preclustered for 15 minutes at 4°C with 20 µg/ml anti human IgG Alexa Fluor 647 (A-21445 Invitrogen) in Opti-MEM

OR

- 4 µg/ml anti Dll1 Alexa Fluor 647 (HMD1-3, Biolegend) in Opti-MEM

Internalization was stopped by transferring cells on ice and washing them twice with cold PBS. Surface stain was removed by treatment with 0.5 µg/ml Pronase (P6911 Sigma) in PBS, 30 minutes at 4°C. The Pronase reaction was blocked by the addition of 500 µl Opti-MEM containing 5% FBS. Samples were then washed in cold PBS and resuspended in 400 µl of PBS 2% BSA and immediately analyzed with BD FACSCalibur flow cytometer.

### 6.3 Dll1 internalization assay analysis

For each experimental condition of the Dll1 endocytosis assay, two different samples were analyzed: one sample not treated with pronase, which represents the total fluorescence, given by the surface bound Dll1 and the internalized Dll1 (TOTAL), and one sample treated with pronase, which represents the internalized fluorescence, since the Dll1 on the membrane had been depleted by pronase treatment (INTERNALIZED). After analysis with the flowJo software (version 9.4), the quote of Dll1 bound to the membrane at each experimental timepoint (BOUND) is calculated subtracting the INTERNALIZED fluorescence from the TOTAL fluorescence. The ratio between INTERNALIZED and BOUND fluorescence was determined for each time point. This data was used to obtain the
internalization curve (x-axis: time, y-axis: INTERNALIZED/BOUND). Internalization rate constants (Ke) were extrapolated from the internalization curves (at early time points: 2, 4 and 6 min) and correspond to the slope of the best-fitting curve. All the analysis and statistics, following extrapolation of the fluorescence values by flowJo, were performed with the Excel software.

6.4 Dll1 recycling assay

As a first approach to measure Dll1 recycling, we tried to modify the internalization assay previously described. OP9-Dll1 cells were detached and 5x10⁴ cells were resuspended in 50 µl of pre warmed Opti-MEM containing 5% FBS + 50 µl of 2x internalization mix. Internalization was performed at 16°C for 60 minutes. Internalization mix 2x was prepared as follows:

- 2 µg/ml N2Fc preclustered for 15 minutes at 4°C with 20 µg/ml anti human IgG not conjugated, in Opti-MEM

OR

- 4 µg/ml anti Dll1 not conjugated in Opti-MEM

OR

- Opti-MEM

Treatment with 0.5 µg/ml Pronase (P6911 Sigma) in PBS, 30 minutes at 4°C was employed to remove surface stain. Pronase reaction was blocked by the addition of Opti-MEM containing 5% FBS. Samples were then washed in cold PBS, resuspended in Opti-MEM containing 5% FBS and released at 37°C for 2, 5, 10 and 30 minutes. Recycling was stopped by transferring cells on ice. Membrane staining was performed 1h at 4°C with 2 µg/ml anti Dll1 Alexa Fluor 647. Samples were analyzed for Dll1 surface staining with BD FACSCalibur flow cytometer. 1 µg/ml cycloheximide (Sigma) was added to OP9-Dll1 cells 3 hours prior to detachment and maintained until the end of the experiment.
6.5 Lipid rafts extraction in sucrose gradient

Lipid microdomain isolation in OP9-Dll1 cells was performed according to (Heuss, et al., 2008) and (Shah, et al., 2012) with modifications. Eighty % confluent 10 cm plates of OP9-Dll1 (KD or not) were transferred on ice, washed twice with cold PBS and lysed in a total volume of 400 µl/plate of buffer A (20 mM Tris, pH 7.5, 150 mM NaCl) containing 30 µl/ml of protease inhibitor cocktail (Calbiochem) and 1% in weight of Brij58 (Sigma). Cells were removed from the plate with the help of a cell scraper and quantified using the BCA Protein Assay Reagent KIT (Pierce), following manufacturer’s instructions.

In the meantime the tubes containing the 5%-30% continuous sucrose gradient were produced using the Biocomp Gradient Master:

- Prepare a 5% and a 30% sucrose solution
- Level the magnetic platform with a leveller
- Place the tube (Polypropylene, Thinwall, 5.0 mL, 13 x 51 mm, Beckman Coulter) in the holder and mark the middle level
- Add 3 ml of the 5% sucrose solution to the tube
- With the help of a 2.5 ml syringe and a long needle, add ≈ 2 ml of the 30% sucrose solution underneath the 5% layer until the separation front comes to the marked line
- Put on the cap and remove excess liquid
- Put the holder containing the tubes into the platform and run the 5%-30% program

200 µg of lysates were loaded on the top of the gradient. The gradient was then subjected to centrifugation for 16 hours at 4°C at 37000 rpm in a swing out 55ti Rotor (Beckman Coulter). Thirteen fractions of 400 µl each were collected from the top of the gradient. 30 µl of each fraction were separated by SDS page, transferred to a nitrocellulose membrane and probed for specific markers (Caveolin1, Tubulin and EGFR) and for the presence of Dll1.
Chapter 3

RESULTS

1. Characterization of cell lines to study the role of Eps15 and Eps15L1 in Notch signaling regulation

1.1 The OP9-Dll1 and C2C12 model system

To study the biology of Notch ligands and receptors in vitro it was necessary to employ a co-culture cell model system in which a signal-sending cell, expressing a Notch ligand, and a signal-receiving cell, expressing a Notch receptor, come into close contact.

As a model for the signal-sending cell, we used the bone marrow, stromal, murine cell line OP9, overexpressing the Notch ligand Dll1 (OP9-Dll1). The OP9-Dll1 cell line was generated in 2002 by Schmitt and Zuniga-Pflucker as tool to study the role of Dll1 in thymocyte development in vitro (Schmitt & Zuniga-Pflucker, 2002). Since then, this cell line has been used in numerous studies to investigate Notch ligand regulation and signaling, since it allows the technical development of biological and biochemical in vitro assays (de Pooter & Zuniga-Pflucker, 2007; Gravano & Manilay, 2010; Heuss, et al., 2008; Heuss, et al., 2013; Holmes & Zuniga-Pflucker, 2009; Mohtashami et al., 2010; Shah, et al., 2012; Song, et al., 2008).

Before starting to set-up the co-culture model system, we wanted to confirm Dll1 expression in the OP9-Dll1 cells. To do so, we performed an immunofluorescence (IF) analysis of Dll1. As expected, OP9-Dll1 cells overexpressed the ligand Dll1 compared to the control OP9 cells (Figure 3.1).

As model for the signal-receiving cell, we used the murine, myoblast cell line C2C12, a subclone of the myoblast cell line established by Yaffe and Saxel (Yaffe & Saxel, 1977),
that has been reported to expresses the Notch2 receptor (Ono et al., 2007). Since an antibody against the Notch2 receptor was not available, we decided to verify the levels of Notch2 transcript in C2C12 cells by QPCR analysis. The levels of Notch2 mRNA in C2C12 cells were found to be increased 2-fold compared to those of the housekeeping gene GAPDH (Figure 3.2).

**Figure 3.1 OP9-Dll1 cells overexpress the Notch ligand Dll1 compared to control OP9 cells.**

Immunofluorescence photographs of control OP9 cells (A) compared to OP9-Dll1 cells (B). Cells (1.5 x 10⁴) were plated on glass coverslips 24 hours before fixation. The primary antibody, anti-Dll1, was detected with a secondary anti-hamster Alexa-Fluor 568 antibody. Scale bar corresponds to 10 µm. Photographs are representative of three repeats.

**1.2 OP9-Dll1 cells and C2C12 cells express different amounts of Notch ligands and receptors**

Most cell lines express both Notch ligands and receptors; this is something we wanted to avoid in our model system, since cis interactions between ligand and receptor in the same cell could lead to misinterpretation of our data. Therefore, we decided to assess by QPCR analysis the mRNA levels of all Notch ligands and receptors in OP9-Dll1 and C2C12 cells. This was done to ensure that OP9-Dll1, as signal-sending cells, expressed mainly Notch ligands, and C2C12, as signal-receiving cells, expressed mainly Notch receptors. Using TaqMan technology (Applied Biosystems), we assessed the mRNA levels of all Notch
ligands and receptors in both OP9-Dll1 and C2C12 cells, normalizing to the housekeeping gene GAPDH. As expected, we found that in OP9-Dll1 cells, the Dll1 transcript was expressed at 50-fold higher levels than GAPDH, while in C2C12 cells it was virtually absent. Dll3 and Dll4 transcripts were undetectable in both cell lines. In contrast, Jag1, but not Jag2, was detectable at significant levels in OP9-Dll1 cells, while in C2C12 cells these ligands were barely detectable. Considering Notch receptors, we observed that Notch1 and Notch4 transcripts were absent or barely detectable both in OP9-Dll1 cells and in C2C12 cells. Small amounts of Notch2 and Notch3 transcript were detected in OP9-Dll1 cells, while in C2C12 cells, we observed levels of endogenous Notch2 transcript 2-fold higher than the housekeeping gene GAPDH (Figure 3.2).

**Figure 3.2** OP9-Dll1 and C2C12 cells express different amounts of Notch ligands and receptors.

Bar graph depicting the relative mRNA levels of all the Notch ligands (Dll1, Dll3, Dll4, Jag1, Jag2) and receptors (Notch1, Notch2, Notch3, Notch4) in OP9-Dll1 (gray) and C2C12 (black) cell lines as determined by QPCR. Values were normalized to GAPDH. The y-axis has been compressed for clarity. Values are expressed as the mean ± SD. Two independent experiments were performed.
1.3 OP9, OP9-Dll1 and C2C12 cells express comparable levels of Eps15 and Eps15L1

After we verified the suitability of our model system, we decided to assess by WB analysis, the levels Eps15 and Eps15L1 expression in the two cell lines. This was done to understand if the model system could be employed to study the roles of Eps15 and Eps15L1 in Notch signaling by downregulation of the proteins or by overexpression.

We found that Eps15 and Eps15L1 proteins were expressed at comparable levels in OP9, OP9-Dll1 and C2C12 cells (Figure 3.3A and B).

![WB analysis of Eps15 and Eps15L1 expression in OP9, OP9-Dll1 and C2C12 cells](image)

Figure 3.3 OP9, OP9-Dll1 and C2C12 cells express comparable levels of Eps15 and Eps15L1.

WB analysis of Eps15 and Eps15L1 expression in OP9 (A), OP9-Dll1 (A) and C2C12 (B) cells. Positive controls for western blotting were adult mouse brain extract (Mouse brain) and human HeLa cells (HeLa). For each sample, 10 µg of total protein lysate were resolved by SDS-Page and blotted onto a nitrocellulose membrane. All samples were loaded on the same gel. Specific antibodies, as indicated on the right, were used to probe the membrane. Black arrows indicate two bands, at 150 kDa and 125 kDa, for Eps15 and three bands, at 130 kDa, 110 kDa and 70 kDa, for Eps15L1. These molecular weights differ from those predicted from the amino acid sequence of Eps15 and Eps15L1, which are respectively 98 KDa and 99 KDa. For Eps15, the higher band represents the primary product of mRNA translation, while the lower band is possibly a degradation product or the result of alternative splicing/incomplete mRNA translation. For Eps15L1, the higher band represents the primary product of mRNA translation, while the minor bands are the result of alternative splicing. Additional bands, not marked by arrows, are nonspecific bands (Coda et al., 1998; Polo, et al., 2002).
2. Set-up and characterization of the Notch reporter transactivation assay

2.1 Notch reporter activation in C2C12 cells is efficient and reproducible but is not increased by transient transfection of Notch1

After having characterized the OP9-Dll1/C2C12 co-culture cell model system, we set-up a Notch transactivation, luciferase reporter assay to act as a readout of Notch activity in the signal-receiving C2C12 cells. C2C12 cells were transiently co-transfected with a Notch-responsive, firefly, luciferase reporter construct that measures Notch signaling activation, and a control reporter construct that constitutively expresses renilla luciferase, as an internal control for transfection efficiency (C2C12-Luc). When these C2C12-Luc cells are co-cultivated with the OP9-Dll1 signal-sending cells, the Notch receptor (in C2C12-Luc) is activated by the Dll1 ligand (in OP9-Dll1) and the NICD is released. The NICD then translocates to the nucleus and activates transcription of the Notch luciferase reporter gene.

To test the efficiency and reproducibility of the reporter assay, we co-cultivated, for 24 hours, OP9 control cells that do not express Dll1 ligand with: i) C2C12-Luc cells, to determine the background noise in our model system, or with ii) C2C12-Luc cells that had been transiently transfected with NICD to bypass ligand-dependent Notch activation. This experiment allowed us to assess the theoretical signal-to-noise ratio of the luciferase reporter assay. From our data, we could observe a clear and reproducible activation of the luciferase reporter gene in the C2C12-Luc cells transfected with NICD (Figure 3.4, gray bars).

We then proceeded to co-cultivate C2C12-Luc cells with OP9-Dll1 cells, to measure ligand-dependent activation of endogenous Notch receptor. We observed a modest, but statistically significant activation of endogenous Notch (approximately 2.5-fold; Figure 3.4, compare ‘NT’ bars). Since we require a model system that would allow us to observe a reduction in Notch activation (as would be expected upon KD of Eps15 or Eps15L1), this
level of activation was not sufficient for our experiments. To obtain a higher level of Notch activation, we thought to increase receptor levels by transiently overexpressing Notch1 in C2C12 cells 24 hours prior to co-culture. For this purpose, we used a pBABE-HAN1FLAG construct encoding full-length human Notch1 receptor, tagged with a C-terminal FLAG-tag and an N-terminal HA-tag. However, we did not score any difference between this and the previous co-culture condition, indicating that the ectopically expressed tagged-Notch1 receptor was either not functional or not expressed correctly (Figure 3.4, compare black bars).

![Figure 3.4](image)

**Figure 3.4 Transient transfection of Notch1 in C2C12 cells does not increase Notch reporter activity over background levels.**

C2C12 cells were transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct (C2C12-Luc). At the same time, C2C12-Luc cells were further transiently transfected with an NICD construct (NICD), with a Notch1 full-length construct (Notch1) or with no construct (NT). 24 hours after transfection, C2C12-Luc cells were co-cultured for 24 hours with OP9 (gray bars) or OP9-Dll1 (black bars) cells, before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. Statistical significance was determined using the Student’s T-test: **= p < 0.01, *** = p < 0.001. Values are expressed as the mean ± SEM. Three independent experiments were performed for the NICD condition. Six independent experiments were performed for the Notch1 condition.
To understand why the ectopically expressed Notch1 receptor was not active in the luciferase reporter assay, we performed a WB analysis of C2C12-Luc cells at 24, 48 and 72 hours after transfection with the pBABE-HAN1FLAG construct. The pBABE-HAN1FLAG construct encodes a Notch1 receptor HA-tagged at the N-terminal, and FLAG-tagged at the C-terminal. The full-length precursor of the receptor (300 kDa) is detectable by WD either with an anti-HA antibody or with an anti-FLAG antibody. After processing of the receptor, the N-terminal domain (180 kDa) is detectable with the anti-HA antibody, while the C-terminal fragment (120 kDa) is detectable with the anti-FLAG antibody. Following Notch activation it would also be possible to detect the NICD fragment (80 kDa) with the anti-FLAG antibody.

Using the anti-HA antibody, we detected a smeared band of approx. 150 – 200 kD at 24 hours after transfection, but not at 48 and 72 hours (Figure 3.5). This data suggested that the transiently transfected Notch1 is not correctly processed and that the expression of the protein decays after 24 hours post-transfection, indicating that the protein is not expressed during the timeframe of the co-culture assay.

![WB analysis of C2C12-Luc cells](image)

**Figure 3.5 Uncorrect processing and rapid loss of Notch1 protein upon transient transfection in C2C12 cells.**

WB analysis of C2C12-Luc cells not transfected (C2C12) or transiently transfected with the HA-Notch1-FLAG construct at 24 (24h), 48 (48h) and 72 (72h) hours post-transfection. For each sample, 10 µg of total lysate were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control.
2.2 Generation and characterization of a stable C2C12 clonal cell line overexpressing Notch1 receptor

Since expression of the transiently transfected Notch1 receptor in C2C12 cells was not maintained for sufficient time to allow efficient transactivation of the reporter, we decided to generate a stable cell line that constitutively expressed the receptor. We transfected C2C12 cells with the pBABE-HAN1FLAG construct; 48 hours after transfection we selected the cells with 1.5 mg/ml of puromycin. After 7 days of selection, we picked and expanded 40 single clones. Nine clones survived clonal expansion and were assessed for tagged-Notch1 expression by WB analysis with an anti-HA and an anti-FLAG antibody. Four clones (1A, 1B, 2A, 15A) stably expressed the correctly processed HA-Notch1-FLAG, with the expected approximate MW of ≈ 300 KDa for the full-length form (Figure 3.6, black arrow), and ≈ 180 KDa and ≈ 120 KDa respectively for the N- and C-terminal portions (Figure 3.6, asterisks). One clone (11A) expressed HA-Notch1-FLAG that was not processed correctly, possibly due to degradation or partial translation. The remaining clones did not express HA-Notch1-FLAG (Figure 3.6).

Figure 3.6 Generation of a stable C2C12 clone overexpressing Notch1 receptor.

WB analysis of C2C12-Notch1 clones stably transfected with the HA-Notch1-FLAG construct (1A, 1B, 2A, 11A, 11B, 15A, 15B, 16A, 16B). C2C12 cells transfected with an empty vector (EV) were used as a control. For each sample, 10 µg of total protein lysate were separated by SDS-PAGE.
and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control. The black arrow indicates the full-length form of the receptor, the asterisks indicate the N- and C-terminal portions respectively.

We then chose three clones with different levels of tagged-Notch1 expression (clone 1A, 2A and 15A) and tested them in the Notch transactivation reporter assay. Of these, clone 15A, which displayed the highest level of expression, showed the best induction of the Notch reporter upon co-culture with OP9-Dll1 cells (Figure 3.7). The level of Notch activity in clone 15A was approx. half of the maximum activation achieved by transfection of NICD.

After validating the efficiency of clone 15A in the co-culture assay, we wanted to verify that the expression levels of Eps15 and Eps15L1 proteins did not change following clonal expansion of the cells. We performed a WB analysis of total cell lysates and found that Eps15 and Eps15L1 proteins were expressed at similar levels in clone 15A cells and in the wild-type (WT) C2C12 cells (Figure 3.8).

Based on these results, we concluded that clone 15A was a suitable model to study the role of Eps15 and Eps15L1 in Notch signaling regulation. For sake of clarity, clone 15A is hereafter referred to as C2C12-Notch1.
Figure 3.7 OP9-Dll1 cells are able to efficiently transactivate Notch signaling in C2C12 cells stably overexpressing the tagged-Notch1 receptor.

C2C12 cells were transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct. C2C12 cells were then further transiently transfected with an NICD construct (C2C12 NICD), with no construct (NT) or had been stably transfected with a tagged-Notch1 full-length construct (C2C12 1A, C2C12 2A, C2C12 15A). 24 hours after reporter transfection, transfected C2C12 cells were co-cultured for 24 hours with OP9 (gray bars) or OP9-Dll1 (black bars) cells, before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. Values are expressed as the mean ± SEM. Independent experiments were performed as follows: C2C12 NICD n = 6; C2C12 1A n = 1, C2C12 2A n = 1, C2C12 15A n = 3.
Figure 3.8 C2C12 clone 15A and C2C12 WT cells express comparable levels of Eps15 and Eps15L1.

WB analysis of Eps15 and Eps15L1 expression in C2C12-Notch1 clone 15A (15A) and C2C12 WT cells (C2C12). For each sample, 10 µg of total protein lysate were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control.

2.3 The γ-secretase inhibitor DAPT is able to abolish Notch transactivation in C2C12-Notch1 cells

To further verify that the induction of the luciferase reporter in C2C12-Notch1 cells following co-culture with OP9-Dll1 was due to ligand-induced activation of the receptor, we treated cells with 1 μM of γ-secretase inhibitor DAPT, before (16 hours), and during co-culture (24 hours). Co-culture of OP9-Dll1 and C2C12-Notch1 cells, as previously shown, induced a significant increase in relative luciferase activity compared to co-culture with OP9 cells (Figure 3.9). This increase was significantly reduced after treatment with DAPT, confirming the specificity of the co-culture system.
Figure 3.9 DAPT abolishes Notch transactivation in C2C12-Notch1 cells.

C2C12-Notch1 cells were transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct. 24 hours after reporter transfection, transfected C2C12-Notch1 cells were co-cultured for 24 hours with OP9 or OP9-Dll1 cells in the presence or absence of the γ-secretase inhibitor DAPT. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line indicates 100% of the luciferase activity, while the red line indicates 50% of the signal, to help visualize changes in luciferase activity. Values are expressed as the mean ± SEM. Three independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test: ** p < 0.01, *** p < 0.001.
3. **Eps15 and Eps15L1 do not have a role in the regulation of Notch receptor signaling in the signal-receiving cell**

3.1 Validation of the Notch reporter transactivation assay by silencing of the known Notch regulators Numb and dynamin in C2C12-Notch1 cells

Having set-up the Notch transactivation reporter assay, we next assessed whether KD of the known Notch regulators, Numb and dynamin, had the expected effects on Notch activity in our reporter assay. Numb is a negative regulator of Notch activity (McGill et al., 2009; McGill & McGlade, 2003; Pece, et al., 2004), therefore silencing Numb should result in increased Notch activity. Dynamin, on the other hand, is a GTPase responsible for endocytic vesicles pinching from the membrane, and is necessary for Notch signaling in both the signal-sending and the signal-receiving cell (Seugnet, et al., 1997). Silencing dynamin, therefore, should result in decreased Notch activity. Mammalian genome contains three dynamin genes: dynamin1, that is selectively expressed in neurons, dynamin2, that is ubiquitously expressed, and dynamin3, that is predominantly expressed in the brain and testis (Ferguson & De Camilli, 2012).

We silenced Numb and dynamin2 in C2C12-Notch1 cells by lipofectamine transfection of specific siRNA oligos. As controls, we used a mock transfected sample containing only the lipofectamine tranfection reagent, and three different negative control siRNA oligos. Cells were then co-cultured with OP9-Dll1 cells to activate Notch, or with OP9 cells as a negative control. We found that, in the mock transfected sample, the addition of lipofectamine alone to C2C12-Notch1 cells resulted in a 2-fold increase in Notch activation compared with untreated cells (Figure 3.10A). This was possibly due to a change in composition of the plasma membrane, resulting in a basal activation of Notch signaling. Transfection with three different control siRNA oligos, however, did not cause any further changes in Notch activation compared with the mock transfected cells (Figure 3.10A). As expected, Notch reporter activity was increased upon Numb KD and was
significantly downregulated upon dynamin2 KD (Figure 3.10A). The efficient downregulation of Numb and dynamin2 protein levels following KD was confirmed by WB analysis (Figure 3.10B).

Thus we can conclude that the Notch reporter transactivation assay is a suitable assay to investigate the contribution of endocytic players to Notch activation in the signal-receiving cell.

**Figure 3.10** Notch activation in the signal-receiving cell is dependent on both Numb and dynamin2.

A. Numb or dynamin2 (Dyn2) expression was silenced in C2C12-Notch1 cells by lipofectamine transfection of specific siRNAs. Lipofectamine alone (Mock) or three different control siRNA oligos (CTR, CTR2, CTR3) were used as controls for KD specificity. 24 hours after the second KD round, cells were transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct. 24 hours after reporter transfection, transfected C2C12-Notch1
cells were co-cultured for 24 hours with OP9 or OP9-Dll1 cells, before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line indicates 100% of the CTR luciferase activity, while the red line indicates 50% of the signal, to help visualize changes in luciferase activity. Values are expressed as the mean ± SEM. Four independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test: * p < 0.05, *** p < 0.001.

B. Efficiency of Numb and dynamin2 (Dyn2) KD was assessed by WB analysis of the same samples shown in A. At the end of the co-culture, samples were lysed and proteins were extracted. Proteins were then separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control. Blot is representative of four repeats.

3.2 Silencing of Eps15, Eps15L1 or both Eps15/Eps15L1 in C2C12-Notch1 cells does not alter Notch activation

After validating the reporter assay, we evaluated the effects of silencing Eps15 and Eps15L1 proteins on Notch activation in the signal-receiving cell. We observed no effects on Notch reporter activity following KD of Eps15 and Eps15L1, either singly or combined (Figure 3.11A). We confirmed these data by repeating the assay with three different siRNA oligonucleotides for each protein, to exclude off-target effects. Similar results were obtained with all three siRNAs tested, and data were pooled in Figure 3.11A. We also confirmed the efficient downregulation of Eps15 and Eps15L1 protein levels after KD by WB analysis (Figure 3.11B).

Based on these data, we can conclude that Eps15 and Eps15L1 do not have a role in the regulation of Notch receptor activity in the signal-receiving cell.
Figure 3.11 Notch activation in signal-receiving cells is independent of both Eps15 and Eps15L1.

A. Eps15 (15), Eps15L1 (L1) or both Eps15/Eps15L1 (15/L1) were silenced in C2C12-Notch1 cells by lipofectamine transfection of specific siRNAs. Lipofectamine alone (Mock) and the negative control siRNA oligo (CTR) were used as controls for knockdown (KD) specificity. 24 hours after the second KD round, cells were then transiently transfected with the Notch-reporter firefly luciferase construct and a renilla luciferase control construct. 24 hours after reporter transfection, transfected C2C12-Notch1 cells were co-cultured for 24 hours with OP9 or OP9-Dll1 cells before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line indicates 100% of the CTR luciferase activity, while the red line indicates 50% of the signal, to help visualize changes in luciferase activity. Values are expressed as the mean ± SEM. Five independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test.
B. Efficiency of Eps15 (15), Eps15L1 (L1) and Eps15/Eps15L1 KDs was assessed by WB analysis of the same samples shown in A. At the end of the co-culture, samples were lysed and proteins were extracted. Proteins were then separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control. Blot is representative of five repeats.
4. Eps15 and Eps15L1 are key regulators of Notch signaling in the signal-sending cell

4.1 Notch activation in the signal-receiving cell is dependent on the presence in the signal-sending cell of Mib1, dynamin and clathrin, and independent of AP2

Since removal of Eps15 and Eps15L1 in the signal-receiving cell did not affect Notch activation, we next tested whether their removal in the signal-sending cell could do so. First, we verified that KD of known Notch ligand regulators in the signal-sending cell affected Notch activation in the signal-receiving cell, as assessed by our transactivation reporter assay.

Initially, we verified in our system the involvement of the E3 ubiquitin ligase Mib1, a key regulator of Notch ligand activation and internalization in both *D. melanogaster* and mammalian cells (Itoh, et al., 2003; Koo, et al., 2005; Song, et al., 2008). As expected, KD of Mib1 in OP9-Dll1 cells completely abolished Notch transactivation in the signal-receiving C2C12-Notch1 cells (Figure 3.12A). Efficient downregulation of Mib1 protein levels after KD was confirmed by WB analysis (Figure 3.12B).

We next evaluated the roles of the endocytic proteins clathrin, dynamin2 and AP2 in our mammalian model system. Signaling by Delta, in *D. melanogaster*, can occur both by CME and CIE (Windler & Bilder, 2010), while in a mammalian context Dll1 signaling has been recently shown to be clathrin dependent (Meloty-Kapella, et al., 2012). As previously reported, dynamin is needed for Notch signaling both in the signal-sending and in the signal-receiving cell (Seugnet, et al., 1997). Conversely, recent evidence proposes that AP2 is dispensable for Dll1 activity (Meloty-Kapella, et al., 2012).

When we silenced the three proteins in OP9-Dll1 cells and performed the Notch reporter transactivation assay, we observed that Notch reporter activity was not inhibited upon AP2 KD, but was significantly impaired upon KD of dynamin2 or clathrin heavy chain (CHC)
in OP9-Dll1 cells (Figure 3.13A). Efficient downregulation of AP2, dynamin2 and CHC protein levels after KD was confirmed by WB analysis (Figure 3.13B). Therefore our data are a confirmation that Dll1 endocytic activation might be mediated by a dynamin-dependent, clathrin-dependent AP2-independent pathway. Moreover we can conclude that the Notch reporter transactivation assay is also a suitable assay to investigate the contribution of endocytic players to Notch ligands activation in the signal-sending cell.

**Figure 3.12** KD of Mib1 in the signal-sending cell reduces Notch activation in the Notch reporter transactivation assay.

A. Mindbomb1 (Mib1) expression was silenced in OP9-Dll1 cells by lipofectamine transfection of specific siRNAs. Lipofectamine alone (Mock) or a negative control siRNA oligo (CTR) were used as controls for KD specificity. 48 hours after the second KD round, cells were co-cultured for 24 hours with C2C12-Notch1 cells, transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct, before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line indicates 100% of the CTR luciferase activity, while
the red line indicates 50% of the signal, to help visualize changes in luciferase activity. Values are expressed as the mean ± SEM. Three independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test: *** p < 0.001.

**B.** Efficiency of Mindbomb1 (Mib1) KD was assessed by WB analysis of the same samples shown in A. At the end of the co-culture, samples were lysed and proteins were extracted. Proteins were then separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control. Blot is representative of three repeats.

**Figure 3.13 KD of CHC and dynamin2 but not AP2 in the signal-sending cell reduces Notch activation in the signal-receiving cell.**

**A.** AP2, dynamin2 (Dyn2) or clathrin heavy chain (CHC) expression was silenced in OP9-Dll1 cells by lipofectamine transfection of specific siRNAs. Lipofectamine alone (Mock) or a negative control siRNA oligo (CTR) were used as controls for KD specificity. 48 hours after the second KD round, cells were co-cultured for 24 hours with C2C12-Notch1 cells, transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct, before
measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line indicates 100% of the CTR luciferase activity, while the red line indicates 50% of the signal, to help visualize changes in luciferase activity. Values are expressed as the mean ± SEM. Three independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test: *** p < 0.001.

B. Efficiency of AP2, dynamin2 (Dyn2) or clathrin heavy chain (CHC) KD was assessed by WB analysis of the same samples shown in A. At the end of the co-culture, samples were lysed and proteins were extracted. Proteins were then separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control. Blot is representative of three repeats.

4.2 Silencing Numb and Numb-like in the signal-sending cell does not reduce Notch transactivation

As already described, Numb is a negative regulator of Notch activity in the signal-receiving cell, both in *D. melanogaster* and in mammals (Couturier, et al., 2012; Hutterer & Knoblich, 2005; McGill, et al., 2009; McGill & McGlade, 2003; Pece, et al., 2004; Tang, et al., 2005). In the genesis of the Drosophila sensory organ, the precursor cell divides asymmetrically, generating two daughter cells with different fates, the pIIa and pIIb. This is due to asymmetric partitioning of Numb and its role as a negative regulator of the Notch receptor. However, endocytosis of Delta is also involved in pIIa/pIIb specification (Polo & Di Fiore, 2006). Considering these premises, we asked if Numb could also regulate the activity of the Notch ligands in the signal-sending cell.

To address this question we silenced Numb and its homolog Numb-like in OP9-Dll1 cells, by lipofectamine transfection of specific siRNA oligos. We then co-cultured the cells with C2C12-Notch1 cells and performed the Notch reporter transactivation assay. As a positive control we used OP9-Dll1 cells in which Mindbomb1 KD had been performed (Figure 3.14A).

We observed that Notch reporter activity was not inhibited following KD of Numb and Numb-like, either singly or combined (Figure 3.14A). Since an antibody against Numb-
like was not available, we confirmed the efficient downregulation of the mRNA levels after KD by QPCR analysis (Figure 3.14B-D).

Based on these data we can conclude that neither Numb nor Numb-like have a role in the regulation of Notch ligands activity in the signal-sending cell.

**Figure 3.14 KD of Numb and Numb-like in the signal-sending cell does not reduce Notch transactivation**

A. Numb, Numb-like (N-L), combined Numb+Numb-like (Numb/N-L) or Mindbomb1 (Mib1) expression was silenced in OP9-Dll1 cells by lipofectamine transfection of specific siRNAs. A negative control siRNA oligo (CTR) was used as controls for KD specificity. 48 hours after the second KD round, cells were co-cultured for 24 hours with C2C12-Notch1 cells, transiently
transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct, before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line indicates 100% of the CTR luciferase activity, while the red line indicates 50% of the signal, to help visualize changes in luciferase activity. Values are expressed as the mean ± SEM. Four independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test: *** p < 0.001.

**B-D.** Efficiency of Numb (B), Numb-like (N-L) (C), combined Numb+Numb-like (Numb/N-L) (B-C) or Mindbomb1 (Mib1) (D) KD, was assessed by QPCR analysis of the same samples shown in A. At the end of the co-culture, samples were lysed and total mRNA was extracted. The bar graph depicts the relative mRNA levels of the KD samples normalized on the CTR sample. Values are expressed as the mean ± SEM. Four independent experiments were performed.

### 4.3 Silencing Eps15 and Eps15L1 in the signal-sending cell reduces Notch transactivation

After verifying that the Notch reporter assay is able to detect alterations in the signal-sending cell that affect Notch activation in the signal-receiving cell, we evaluated the effects of silencing Eps15 and Eps15L1 in OP9-Dll1 cells. To exclude siRNA off-target effects, we used three different siRNA oligonucleotides for each protein silenced: siRNAs 51, 52 and 53 for Eps15 and siRNAs 54, 55 and 56 for Eps15L1. We observed an approx. 50% reduction of Notch reporter activity after KD of Eps15 with all the three oligonucleotides tested and an approx. 40% reduction of Notch reporter activity after KD of Eps15L1 (Figure 3.15A). Western blot analysis confirmed the efficient silencing of Eps15 and Eps15L1 proteins, with all siRNA oligonucleotides used (Figure 3.15B). We subsequently repeated the co-culture assay with one single siRNA oligonucleotide for each protein silenced (52 for Eps15 and 54 for Eps15L1), and obtained consistent results (Figure 3.17A).

We also performed combined Eps15/Eps15L1 in OP9-Dll1 cells, and we observed that the combined KD did not further reduce the levels of Notch transactivation obtained by the KD of the single proteins (Figure 3.17A).
In conclusion these results indicate that Eps15 and Eps15L1 are necessary for the regulation of the Notch ligand Dll1 activity in the signal-sending cell. The results also suggest that the two proteins are not functionally redundant, but they both appear to have a critical function in Notch ligand regulation.

Figure 3.15  Eps15 and Eps15L1 silencing in the signal-sending cell reduces Notch transactivation.

A. KD of Eps15 (51, 52, 53) and Eps15L1 (54, 55, 56) was performed in OP9-Dll1 cells by lipofectamine transfection of specific siRNAs. Lipofectamine alone (Mock) or a negative control siRNA oligo (CTR) were used as controls for KD specificity. 48 hours after the second KD round, cells were co-cultured for 24 hours with C2C12-Notch1 cells, transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct, before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line indicates 100% of the CTR luciferase activity, while the red line indicates 50% of the signal, to help visualize
changes in luciferase activity. Values are expressed as the mean ± SD and are the result of a single experiment.

B. Efficiency of Eps15 and Eps15L1 KD was assessed by WB analysis of the same samples shown in A. At the end of the co-culture, samples were lysed and proteins were extracted. Proteins were then separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control.

4.4 Silencing Epsin1 and Epsin2 in the signal-sending cell reduces Notch transactivation

Epsin1 and Epsin2 are known to be key regulators of mammalian Dll1 (Meloty-Kapella, et al., 2012). Epsins are endocytic adaptor proteins that participate in CME and CIE together with Eps15 and Eps15L1 (Chen, et al., 1998; Sigismund, et al., 2005). Epsin1 and Epsin2 have been proposed, together with clathrin, to have a fundamental role in the regulation of Dll1 activation by endocytosis (Meloty-Kapella, et al., 2012).

To investigate the involvement of Epsin1 and Epsin2 in activation of Notch signaling, we silenced their expression in the signal-sending OP9-Dll1 cells. Again, we used three different siRNA oligonucleotides (siRNAs 48, 49 and 50 for Epsin1 and siRNAs 67, 68 and 69 for Epsin2) for each protein to guard against off-target effects. We observed that KD of Epsin1 and Epsin2 in OP9-Dll1 cells impaired Notch transactivation by about approx. 50% and 40%, respectively (Figure 3.16A). WB analysis confirmed the efficient KD of both proteins with all siRNA oligonucleotides used (Figure 3.16B). We subsequently repeated the Notch reporter transactivation assay silencing Epsin1 and Epsin2 with one single siRNA oligonucleotide for each protein (48 for Epsin1 and 69 for Epsin2), and obtained consistent results (Figure 3.17A).

As we did for Eps15/Eps15L1, we also performed combined Epsin1/Epsin2 KD in OP9-Dll1 cells. Interestingly, similarly to what we observed for Eps15/Eps15L1, we could not score a further reduction in Notch reporter activity after combined Epsin1/Epsin2 KD compared to the single KDs (Figure 3.17A).
Figure 3.16 Epsin1 and Epsin2 silencing in the signal-sending cell reduces Notch transactivation.

A. KD of Epsin1 (48, 49, 50) and Epsin2 (66, 67, 68) was performed in OP9-Dll1 cells by lipofectamine transfection of specific siRNAs. Lipofectamine alone (Mock) or a negative control siRNA oligo (CTR) were used as controls for KD specificity. 48 hours after the second KD round, cells were co-cultured for 24 hours with C2C12-Notch1 cells, transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct, before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line indicates 100% of the CTR luciferase activity, while the red line indicates 50% of the signal, to help visualize changes in luciferase activity. Values are expressed as the mean ± SD and are the result of a single experiment.

B. Efficiency of Epsin1 and Epsin2 KD was assessed by WB analysis of the same samples shown in A. At the end of the co-culture, samples were lysed and proteins were extracted. Proteins were then separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control.
4.5 Eps15/Eps15L1 and Epsin1/Epsin2 act in distinct pathways of Notch ligand activation

Eps15/Eps15L1, together with Epsin1, regulate CIE of EGFR (Sigismund, et al., 2005). To understand if, similarly, a combined action of Eps15/Eps15L1 together with Epsin1/Epsin2 might regulate Dll1 activity, and to determine whether these two protein families are acting in the same or distinct pathways leading to Notch ligand activation, we decided to perform the quadruple KD of Eps15/Eps15L1/Epsin1/Epsin2 in OP9-Dll1. Indeed, after the quadruple KD in OP9-Dll1 cells, we observed a complete block of Notch transactivation that was similar to the block observed following Mib1 KD (Figure 3.17A). Moreover, KD of any given combination of Eps15 or Eps15L1 with Epsin1 or Epsin2 resulted in an almost complete loss of Notch transactivation (Figure 3.17A). WB analysis confirmed the efficient KD of all proteins (Figure 3.17B).

Together, these data suggest that the Eps15 and Epsin protein families likely act in two distinct, non-redundant, pathways leading to activation of the Notch ligand Dll1.
Figure 3.17 Effect of combined Eps15/Eps15L1 and Epsin1/Epsin2 KDs in the signal-sending cell on Notch transactivation.

A. Eps15 (15), Eps15L1 (L1), Eps15/Eps15L1 (15/L1), Epsin1 (E1), Epsin2 (E2), Epsin1/Epsin2 (E1/E2), Eps15/Epsin1 (15/E1), Eps15/Epsin2 (15/E2), Eps15L1/Epsin1 (L1/E1), Eps15L1/Epsin2 (L1/E2), Eps15/Eps15L1/Epsin1/Epsin2 (Qdr) or Mindbomb1 (Mib1) expression was silenced in OP9-Dll1 cells by lipofectamine transfection of specific siRNAs. Lipofectamine alone (Mock) or a negative control siRNA oligo (CTR) were used as controls for KD specificity. 48 hours after the second KD round, cells were co-cultured for 24 hours with C2C12-Notch1 cells, transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct, before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line
indicates 100% of the CTR luciferase activity, while the red line indicates 50% of the signal, to help visualize changes in luciferase activity. Values are expressed as the mean ± SEM. At least three independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student's T-test: *** p < 0.001.

B. Efficiency of KD was assessed by WB analysis of the same samples shown in A. At the end of the co-culture, samples were lysed and proteins were extracted. Proteins were then separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control.

4.6 Eps15 and Eps15L1 silencing in the signal-sending cell has a similar impact on the activation of all Notch ligands

The partial effect observed on Dll1 activity after combined KD of Eps15/Eps15L1 in our in vitro model system, does not explain the strong Notch-like phenotype displayed by Eps15/Eps15L1-DKO embryos (see Introduction Section 4.1-4.2). We therefore decided to perform a Notch transactivation assay to screen the other three Notch ligands, Dll4, Jag1 and Jag2, to understand the contribution of Eps15/Eps15L1 to their activation. We obtained OP9 cells stably overexpressing Dll4, Jag1 or Jag2 (Van de Walle, et al., 2011). Eps15/Eps15L1 were silenced in these cells using siRNA oligo 51 and 52 for Eps15, and 54 and 56 for Eps15L1, either singly or together. Data obtained with both siRNA oligos were consistent, and therefore pooled into figure 3.18.

In all samples, we observed a similar reduction in Notch reporter activity as observed after KD of Eps15/Eps15L1 in OP9-Dll1 cells. Single KD of Eps15 or Eps15L1 inhibited Notch activity by approx. 50% and the double KD did not significantly increase the effect. Mib1 KD was performed in each cell line as a positive control (Figure 3.18A-C). We confirmed the efficient downregulation of the target proteins, following KD, by WB analysis (Figure 3.19).

These data indicate that the mechanism of action of Eps15/Eps15L1 on Notch ligands regulation is likely conserved on all Notch ligands.
Figure 3.18 Eps15 and Eps15L1 have a similar role in the regulation of all Notch ligands.

A-C. Eps15 (15), Eps15L1 (L1), Eps15/Eps15L1 (15/L1) and Mindbomb1 (Mib1) expression was silenced in OP9-Dll4 (A), OP9-Jag1 (B) or OP9-Jag2 (C) cells by lipofectamine transfection of specific siRNAs. Lipofectamine alone (Mock) or a negative control siRNA oligo (CTR) were used as controls for KD specificity. 48 hours after the second KD round, cells were co-cultured for 24 hours with C2C12-Notch1 cells, transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct, before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line indicates 100% of the CTR luciferase activity, while the red line indicates 50% of the signal, to help visualize changes in luciferase activity. Values are expressed as the mean ± SEM. Four independent experiments were performed. Statistical
significance between KD and CTR samples was calculated using the Student’s T-test: \( * = p < 0.05, \) 
\( ** = p < 0.01, \) \( *** = p < 0.001. \)

**Figure 3.19 Efficiency of KD in OP9-Dll4, OP9-Jag1 and OP9-Jag2 cells.**

**A-D.** Efficiency of Eps15 and/or Eps15L1 (A-C), and Mib1 (D only) KD in OP9-Dll4 (A, D), OP9-Jag1 (B, D) and OP9-Jag2 (C, D) cells was assessed by WB analysis of the same samples shown in Figure 3.18. At the end of the co-culture, samples were lysed and proteins were extracted. Proteins were then separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Specific antibodies, as indicated on the right, were used to probe the membrane. Vinculin was used as a loading control. Blot is representative of four repeats.
4.7 Combined Eps15/Eps15L1 KD in both the signal-sending and signal-receiving cell does not further reduce Notch transactivation compared to combined KD in the signal-sending cell alone

We next asked whether silencing of Eps15 and Eps15L1 at the same time in both the signal-sending and signal-receiving cell could unmask a further downregulation of Notch signaling and mimic the severe phenotype of the Eps15/Eps15L1 DKO mice in vitro. To address this question, we performed a Notch reporter transactivation assay after combined KD of Eps15/Eps15L1 in both OP9-Dll1 and C2C12-Notch1 cells and observed a similar reduction in Notch reporter activity as observed in OP9-Dll1 Eps15/Eps15L1 double KD cells alone (Figure 3.20). Thus, Notch activity was only partially inhibited and the combined KD in both signal-sending and signal-receiving cells did not significantly increase this effect.

This result is consistent with our data showing no effect of Eps15/Eps15L1 KD in the signal-receiving cells and supports the notion that Eps15/Eps15L1 are important regulators of Notch ligand activation. However, we could not replicate in vitro a complete Notch loss of function phenotype due to the lack of Eps15/Eps15L1, indicating that even if the two endocytic adaptor proteins play an important role in the modulation of Notch ligand activation, probably impairment of other signaling pathways is involved in the severe lethality phenotype of the mice (see Introduction Section 4.1-4.2).
Figure 3.20 Silencing of Eps15 and Eps15L1 in both the signal-sending and signal-receiving cell does not further reduce Notch transactivation.

A. Eps15/Eps15L1 (15/L1) expression was silenced in OP9-Dll1 cells and C2C12-Notch1 cells by lipofectamine transfection of specific siRNAs. A negative control siRNA oligo (CTR) was used as controls for KD specificity. 48 hours after the second KD round, OP9-Dll1 cells were co-cultured for 24 hours with KD C2C12-Notch1 cells, transiently transfected with the Notch-reporter firefly luciferase construct and the renilla luciferase control construct, before measuring luciferase activity. The bar graph depicts the relative luciferase activity of the Notch-reporter firefly luciferase normalized to the activity of renilla luciferase. The blue line indicates 100% of the CTR luciferase activity, while the red line indicates 50% of the signal, to help visualize changes in luciferase activity. Values are expressed as the mean ± SEM. Four independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test: ** = p < 0.01, *** = p < 0.001.
5. Dll1 internalization is Eps15 and Eps15L1 independent

5.1 Dll1 internalization assay setup and characterization

In the previous chapter, we demonstrated that, in the signal-sending cell, both Eps15 and Eps15L1 are required to achieve efficient transactivation of Notch in the signal-receiving cell.

Endocytosis is strictly required in the signal-sending cell to generate a ligand that is capable of activating the Notch receptor in the signal-receiving cell (Parks, et al., 2000; Seugnet, et al., 1997). Two non-mutually exclusive hypotheses have been formulated to explain the requirement for ligand endocytosis in Notch signaling:

i) Notch ligands need to undergo endocytosis and recycling to be correctly processed and activated;

ii) the endocytic pulling forces that are generated following ligand-receptor binding, generate a conformational change in the receptor, that promotes receptors activation.

Since it is known that Epsins are key regulators of Notch ligand endocytosis (Meloty-Kapella, et al., 2012; Wang & Struhl, 2004; Wang & Struhl, 2005), and since Eps15 and Eps15L1 are known to act in combination with Epsin1 to regulate EGFR CIE (Sigismund, et al., 2005), we asked whether Eps15 and Eps15L1 could also have a role in the regulation of Notch ligands internalization.

To test this hypothesis, we first needed to set-up a Dll1 endocytosis assay in OP9-Dll1 cells. Evidences from D. melanogaster literature indicate that Notch ligands can undergo two different types of endocytosis: i) constitutive endocytosis, that is independent of receptor binding, and ii) receptor-stimulated endocytosis, that occurs upon binding to the receptor. To follow constitutive Dll1 internalization, we used an anti-Dll1 antibody conjugated to an Alexa 647 fluorochrome. We chose this system because we reasoned that an antibody that binds to surface Dll1 would be internalized as the ligand undergoes constitutive endocytosis. To measure receptor-stimulated endocytosis of Dll1, we took
advantage of a Notch2-Fc recombinant protein that we preclustered with a secondary antibody conjugated to an Alexa 647 fluorochrome. Again, we reasoned that the preclustered Notch-Fc protein would bind to the Dll1 ligand, mimicking the interaction between ligand and receptor and allowing, in turn, clusterization of Dll1 in the plasma membrane.

With these tools, we set-up a cytofluorimetry-based, quantitative, Dll1 internalization assay. OP9-Dll1 cells were detached from the plate by mild trypsinization, to avoid ligand loss. Anti-Dll1 antibody (to follow constitutive internalization) or preclustered Notch-Fc (to follow receptor-mediated internalization), were added to the cell suspension at 37°C. Dll1 internalization was performed at 37°C. At different time-points (2, 4 and 6 minutes) internalization was stopped on ice, and samples were treated or not with pronase, to remove all Dll1 present on the membrane and therefore to determine respectively the internalized quote of Dll1 and the total amount of Dll1 (internalized+bound). Samples were quantified by cytofluorimetry analysis. The bound quote of Dll1 was then calculated subtracting the internalized Dll1 value from the total Dll1 value (Figure 3.21).

**Figure 3.21 Schematic representation of the Dll1 internalization assay in OP9-Dll1 cells.**

The cartoon schematically depicts the main passages of the Dll1 internalization assay protocol. OP9-Dll1 cells in suspension were treated either with a preclustered N2Fc recombinant protein or with an antibody anti Dll1. Internalization was performed at 37°C; at different timepoints (2, 4 and 6 minutes) internalization was stopped and samples were treated (Internalized) or not (Total) with pronase. Samples were quantified by cytofluorimetry analysis.
By plotting the internalized/bound ratio against time (as a $y = mx$ equation), we were able to calculate the endocytic rate constant ($K_e$), which is a cellular constant that defines the probability of a receptor to be internalized in 1 minute at 37°C (Wiley & Cunningham, 1982). The endocytic rate constant ($K_e$) was extrapolated from the internalization curves and corresponds to the slope ($m$) of the best-fitting curve.

As expected, we observed that the $K_e$ for constitutive internalization ofDll1 (0.0385) is lower than the $K_e$ for receptor-mediated internalization ofDll1 (0.1195) in OP9-Dll1 cells (Figure 3.22), indicating that constitutive internalization is a slower process than receptor-mediated internalization. Interestingly, the $K_e$ for receptor-mediated internalization ofDll1 is in line with the published value of the ligand induced $K_e$ of EGF (0.185), following binding with EGF at physiological concentrations (i.e. 1ng/ml), measured in murine embryonic fibroblasts NIH 3T3 (Sorkin & Duex, 2010).

Taken together these observations suggest that the Dll1 internalization assay could approximately mimic the physiological conditions at which endocytosis of a membrane bound protein occurs.

![Graph showing receptor-mediated and constitutive internalization of Dll1 in OP9-Dll1 cells.](image)

**Figure 3.22** Receptor-mediated and constitutive internalization of Dll1 in OP9-Dll1 cells.

Line graph comparing the ratio of internalized/surface-bound Dll1 after receptor-mediated internalization of Dll1 (black diamonds) and constitutive internalization of Dll1 (white squares) in...
OP9-Dll1 cells at 2, 4 and 6 minutes of internalization at 37°C. Three independent experiments were performed, a representative one is shown here. The endocytic rate constant $K_e$ (slope) is shown next to each line.

5.2 Dll1 internalization assay validation and characterization

To validate the two Dll1 internalization assays, we decided to assess the effects of silencing Mib1 or CHC on both receptor-mediated and constitutive internalization of Dll1. We observed that receptor-mediated internalization of Dll1 was almost completely abolished after Mib1 KD and significantly inhibited after CHC KD (Figure 3.23A), consistent with a model in which binding of Notch to Dll1 leads to Dll1 ubiquitination and subsequent internalization (Itoh, et al., 2003; Lai, et al., 2005; Song, et al., 2008). In contrast, constitutive endocytosis of Dll1 was not affected by CHC KD and was reduced by 50% by Mib1 KD (Figure 3.23B). This result suggests that constitutive internalization of Dll1 is mediated by a CIE pathway, but may still be dependent on ubiquitination of the ligand.

**Figure 3.23** Effect of Mib1 and CHC silencing on receptor-mediated and constitutive endocytosis of Dll1 in OP9-Dll1 cells.

A-B. Mindbomb1 (Mib1), or clathrin heavy chain (CHC) expression was silenced in OP9-Dll1 cells by lipofectamine transfection of specific siRNAs. A negative control siRNA oligo (CTR) was used as control for KD specificity. 48 hours after the second KD round, cells were detached from
the plate and the cytofluorimetry-based internalization assay was performed as described in methods.

A. The bar graph depicts the endocytic rate constants for receptor-stimulated internalization of Dll1. The red line indicates 100% of the signal, while the blue line indicates 50% of the signal, to help visualize changes. Values are expressed as the mean ± SEM. Four independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test: ** = p < 0.01, *** = p < 0.001.

B. The bar graph depicts the endocytic rate constants for constitutive internalization of Dll1. The red line indicates 100% of the signal, while the blue line indicates 50% of the signal, to help visualize changes. Values are expressed as the mean ± SEM. Four independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test: * = p < 0.05.

5.3 Dll1 surface levels in OP9-Dll1 after KD of Eps15 and Eps15L1 are not altered

Internalization is considered a “first-order kinetics” process. Therefore, the rate of internalization depends on the concentration of receptor-ligand complexes at the cell surface (Sorkin & Duex, 2010). In the case of Dll1 internalization, the endocytic rate constant Ke would be dependent on the amount of Dll1 protein at the membrane at any given time point. Therefore, to compare changes in the Ke following different treatments, it is necessary to assess that the levels of Dll1 protein at the membrane are comparable in all the samples.

To take this variable into account, before measuring the effects of Eps15/Eps15L1 KD on Dll1 internalization, we measured surface levels of Dll1 in steady state OP9-Dll1 cells after KD of Eps15 and Eps15L1. We also evaluated the effects of Mib1, clathrin and Epsin1 KD, to control for previous and future experiments.

To measure Dll1 surface levels, we stained OP9-Dll1 cells in suspension, with an antibody anti Dll1 conjugated to an Alexa 647 fluorochrome. We then quantified the samples by cytofluorimetry analysis.

We observed that Eps15, Eps15L1 and the combined Eps15/Eps15L1 KD did not alter Dll1 surface levels in steady state OP9-Dll1 cells (Figure 3.24). Similarly, CHC KD had no
effect, while Epsin1 KD and Mindbomb1 KD resulted in a small but significant increase in Dll1 at the cell surface (Figure 3.24). Combining Eps15 with Epsin1 KD did not show any change in Dll1 surface levels compared to Epsin1 KD alone (Figure 3.24).

Taken together these observations suggest that it will be possible to compare Ke rates for all the samples, taking in consideration the differences observed in the Mindbomb1 and Epsin1 samples.

Since the endocytic rate constant depends on the internalized/bound ratio, an increase in Dll1 levels at the membrane should result in an increased bound value, therefore a decreased Ke. On the other hand, a decrease in the internalization rate of a membrane bound protein should lead to increased surface levels of the same protein, consistent with an endocytic block. In the case of the results shown above for Mindbomb1 KD, however, the reduction that we observe in the Ke value can be only partially ascribed to the increased Dll1 levels, since amount of reduction in the Ke is not mirrored by the same amount of increase in Dll1 levels.

![Graph showing surface levels of Dll1 after KD in OP9-Dll1 cells at steady state](image)

**Figure 3.24 Surface levels of Dll1 after KD in OP9-Dll1 cells at steady state**

Eps15 (15), Eps15L1 (L1), Eps15/Eps15L1 (15/L1), Epsin1 (E1), Mindbomb1 (Mib1), clathrin heavy chain (CHC) and Eps15/Epsin1 (15/E1) expression was silenced in OP9-Dll1 cells by lipofectamine transfection of specific siRNAs. A negative control siRNA oligo (CTR) was used as control for KD specificity. 48 hours after the second KD round, cells were detached from the plate.
and Dll1 surface levels were measured by cytofluorimetry analysis. The bar graph depicts the mean fluorescence corresponding to Dll1 protein levels at the membrane at steady state. Values are expressed as the mean ± SEM. Four independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test: * = p < 0.05.

5.4 Loss of Eps15 and Eps15L1 does not affect receptor-mediated or constitutive Dll1 internalization

After validating the internalization assays and controlling the effects of silencing of proteins of interest on surface levels of Dll1, we proceeded to evaluate the effect of Eps15/Eps15L1 KD on Dll1 receptor-mediated and constitutive internalization. We performed both single and combined KDs of Eps15 and Eps15L1 and observed that none of these KDs affected Dll1 internalization, either receptor-stimulated or constitutive (Figures 3.25A and B). However, when we performed KD of Epsin1, we scored a significant inhibition of receptor-stimulated internalization of Dll1, but not of constitutive internalization (Figures 3.25A and B). A small decrease in the Ke rate following Epsin1 KD was expected, according to the measured increase in Dll1 surface levels. However, as in the case of Mindomb1, the reduction that we observe in the Ke value of stimulated Dll1 internalization (50%) can be only partially ascribed to the increased Dll1 levels. Taken together, these results are in line with published data showing that Epsin1 is a fundamental player in Notch ligand internalization (Meloty-Kapella, et al., 2012). When we then combined Eps15/Epsin1 KD, we observed the same effect as Epsin1 alone (Figure 3.25A). In conclusion these findings indicate that Eps15 and Eps15L1 are not involved in the early steps of Dll1 internalization, either alone or in conjunction with the Epsins.
Figure 3.25 Eps15 and Eps15L1 do not have a role in internalization of the Notch ligand Dll1.

**A-B.** Eps15 (15), Eps15L1 (L1), Eps15/Eps15L1 (15/L1), Epsin1 (E1), and Eps15/Epsin1 (15/E1) expression was silenced in OP9-Dll1 cells by lipofectamine transfection of specific siRNAs. A negative control siRNA oligo (CTR) was used as control for KD specificity. 48 hours after the second KD round, cells were detached from the plate and the cytofluorimetry-based internalization assay was performed as described in methods.

**A.** The bar graph depicts the endocytic rate constants for receptor-stimulated internalization of Dll1. The red line indicates 100% of the signal, while the blue line indicates 50% of the signal, to help visualize changes. Values are expressed as the mean ± SEM. Four independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test: *** = p < 0.001.

**B.** The bar graph depicts the endocytic rate constants for constitutive internalization of Dll1. The red line indicates 100% of the signal, while the blue line indicates 50% of the signal, to help visualize changes. Values are expressed as the mean ± SEM. Four independent experiments were performed. Statistical significance between KD and CTR samples was calculated using the Student’s T-test.
6. Investigating the role of Eps15 and Eps15L1 in Dll1 recycling

6.1 Dll1 recycling to the cell surface after internalization does not occur in OP9-Dll1 cells in suspension

It is well accepted that Notch ligands, at least in some contexts, need to go through a recycling process in order to be activated. The precise role of this recycling step is still unknown; some speculate that the ligand needs to be recycled in order to go through an unknown post-translational modification, while others speculate that recycling is needed to relocalize the ligand to specific membrane compartments, known as lipid rafts (Hamel, et al., 2010; Heuss, et al., 2008; Heuss, et al., 2013; Wang & Struhl, 2004; Windler & Bilder, 2010).

Since Eps15 has previously been ascribed a role in EGFR recycling (Chi, et al., 2011), we wanted to test whether Eps15 and Eps15L1 might also be involved in Dll1 recycling. As a first approach, we decided to analyze Dll1 recycling from a quantitative point-of-view, employing a fluorescence-based recycling assay. To set-up this recycling assay, we adapted the Dll1 internalization assays described in Results Section 5 to measure the amount of internalized Dll1 ligand that is recycled to the cell surface. OP9-Dll1 cells were detached from the plate by mild trypsinization. Dll1 internalization was performed, following the addition of preclustered NFκ to the cells, at 16°C for 1 hour. At this temperature, recycling is impaired (Ren et al., 1998), resulting in accumulation of ligand in the cytoplasm. Internalization was stopped on ice, and all samples were treated with pronase, to remove all Dll1 present on the membrane. Cells were shifted to 37°C for up to 30 minutes to stimulate Dll1 recycling. Treatment with cyclohexamide was used to inhibit de novo protein synthesis thereby avoiding export of newly synthesized proteins to the plasma membrane. The amount of recycled Dll1 was then determined by surface staining at 4°C with an anti Dll1 Alexa647; the recycled Dll1 quote was the quantified by cytofluorimetric analysis (Figure 3.26).
The cartoon schematically depicts the main passages of the Dll1 recycling assay protocol. Following receptor-mediated internalization at 16°C for 1 hour (to impair recycling and accumulate the protein in the cytoplasm), OP9-Dll1 cells in suspension were treated with pronase to deplete the quote of Dll1 still on the membrane. OP9-Dll1 cells were afterwards released at 37°C to allow recycling. Samples were then stained at 4°C with an anti Dll1 Alexa 647, and the recycled Dll1 quote was quantified by cytofluorimetric analysis.

To set-up the assay, we initially monitored the rate of receptor-dependent Dll1 internalization at 16°C. OP9-Dll1 cells were incubated with preclustered NFc at 16°C for up to 4 hours. At various time points, the amount of membrane-bound vs. internalized Dll1 was measured as previously described in section 5.1. We observed a steady decrease in surface Dll1, accompanied by an increase in internalized Dll1, over time, with approximately 80% of Dll1 protein internalized by 4 hours (Figure 3.27A).

Having verified an efficient internalization of Dll1 at 16°C, we then measured Dll1 recycling. After stimulating Dll1 internalization at 16°C for 1 hour, cells were shifted to 37°C for up to 30 minutes, and the amount of recycled Dll1 determined, both in the presence and absence of cyclohexamide. We observed that, after 30 minutes, only 6% of the internalized Dll1 was recycled back to the cell surface (Figure 3.27B). This result contrasts with published data which shows that Dll1 recycling in OP9-Dll1 cells is a rapid process, with almost all internalized Dll1 being recycled back to the plasma membrane after 15 – 30 minutes incubation at 37°C (Heuss, et al., 2008).
To exclude that the Notch-Fc complexes prevented detection of the recycledDll1, we also performed a recycling assay following Dll1 constitutive internalization. However, we observed no recycling when we performed Dll1 internalization either with the anti-Dll1 antibody or without any antibody.

Taken together, these results suggest that Dll1 recycling may not occur in OP9-Dll1 cells in suspension. Therefore, to assess the role of Eps15/Eps15L1 in Dll1 recycling a change of strategy is necessary. In particular we will try to perform a biotinilation-based assay on adherent OP9-Dll1 cells, as described by (Heuss, et al., 2008).

![Diagram A](image1.png)

**Figure 3.27** Dll1 recycling to the cell surface after internalization does not occur in OP9-Dll1 cells in suspension.

**A. Efficiency of receptor mediated Dll1 internalization at 16°C.** OP9-Dll1 cells in suspension were incubated with preclustered N2Fc at 16°C for 30, 60, 120, 180 and 240 minutes. The amount of internalized (white circle) vs surface-bound (black square) Dll1 was quantified by
cytofluorimetric analysis. Two independent experiments were performed: a representative one is shown here.

**B. Dll1 recycling to the cell surface after receptor-mediated internalization.** OP9-Dll1 cells in suspension were incubated with preclustered N2Fc at 16°C for 1 hour. After treatment with pronase, cells were incubated for 2, 5, 10 and 30 minutes at 37°C, to allow Dll1 recycling. The graph shows the amount of recycled Dll1 in the presence (white rhombus) or absence (black triangle) of cyclohexamide, as quantified by cytofluorimetric analysis. Two independent experiments were performed: a representative one is shown here.
7. Assessing the role of Eps15 and Eps15L1 in Dll1 membrane localization in OP9-Dll1 cells

7.1 Dll1 is predominantly located in lipid rafts in OP9-Dll1 cells

In addition to the quantitative approach to assess Dll1 recycling, we also assessed recycling from a qualitative point-of-view, looking at localization of Dll1 in plasma membrane lipid rafts.

To determine whether Eps15/Eps15L1 have a role in directing Dll1 to lipid microdomains, we first set-up the experimental conditions for identifying the location of proteins in different membrane fractions. We decided to perform a biochemical purification of detergent-resistant membrane (i.e., lipid rafts) in a sucrose gradient according to (Heuss, et al., 2008) and (Shah, et al., 2012) with modifications. We lysed OP9-Dll1 cells in a lysis buffer containing Brij58 detergent. We then fractionated 200 µg of cell lysates by ultracentrifugation in a 5 – 30% continuous sucrose gradient. The distribution of Dll1 in the different fractions was then analyzed by WB. We observed, in two independent experiments, that Dll1 was enriched in fractions 7 and 8 (Figure 3.28). As a marker for lipid raft localization, we used caveolin1, which was enriched in fractions 7, 8, 9 and 10. We also assayed our fractions for EGFR, a receptor known to be localized in lipid rafts (Abulrob et al., 2004; Mineo et al., 1999; Yamabhai & Anderson, 2002), and found that it was enriched in the same fractions as Dll1 (Figure 3.28). As a negative control for lipid rafts, we used tubulin, which has been employed as a marker for cytoplasmic fractions. Tubulin was detected in the first 6 fractions of the gradient. Together, these results confirm a lipid raft localization of Dll1 in OP9-Dll1 cells and indicate that our purification protocol is a suitable and reliable method for determining Dll1 localization in the plasma membrane.
A-B. WB analysis of two independent biochemical purifications of detergent resistant membrane in a 5 – 30% continuous sucrose gradient. OP9-Dll1 cells were lysed in a Brij58 containing lysis buffer. 200 µg of total protein lysate were loaded on the gradient and ultracentrifugated for 16 hours at 4°C at 37000 rpm in a swing out 55ti rotor. At the end of centrifugation, 13 fractions were collected (each fraction with a total volume of 400 µl) and then 30 µl of each fraction were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. 2 µg (WCE 1/100) and 4 µg (WCE 1/50) of whole cell extract (WCE) were loaded on gel as positive controls. Specific antibodies, as indicated on the right, were used to probe the membrane.

Figure 3.28 Purification of lipid rafts with Brij58 in OP9-Dll1 cells.
7.2 Eps15 and Eps15L1 silencing in OP9-Dll1 cells does not alter Dll1 localization in lipid rafts

Next, we assessed the effect of silencing of Eps15 and Eps15L1 in OP9-Dll1 on the localization of Dll1 in lipid rafts. We observed that in the control, Dll1 was enriched in fractions 7 and 8, while caveolin1 was enriched in fractions 7, 8 and 9 (Figure 3.29A), as observed in untreated cells (Figure 3.28A-B). This distribution did not change upon Eps15 or Eps15L1 single KD, nor upon Eps15/Eps15L1 double KD (Figures 3.29B-D). The distribution of caveolin, tubulin and the EGFR were also unchanged (Figures 3.29B-D). Together, these results suggest that Eps15/L1 are not necessary for the localization of Dll1 into lipid microdomains.
Figure 3.29 Eps15 and Eps15L1 KD in OP9-Dll1 cells does not alter Dll1 localization in lipid rafts.

A-D. WB analysis of OP9-Dll1 cell lysates after a biochemical purification of detergent-resistant membrane in a 5 – 30% continuous sucrose gradient. siRNA oligo mediated knockdown of Eps15
(B), Eps15L1 (C), Eps15/Eps15L1 (D), was performed in OP9-Dll1 prior to the experiment. A negative control oligo CTR (A) was used as a control for KD specificity. For each condition, 200 µg of total protein lysate, extracted in a Brij58 containing lysis buffer, were loaded on the sucrose gradient and ultracentrifugated for 16 hours at 4°C at 37000 rpm in a swing out 55ti rotor. At the end of centrifugation, 13 fractions were collected (each fraction with a total volume of 400 µl) and then 30 µl of each fraction was separated by SDS-PAGE and blotted onto a nitrocellulose membrane. 2 µg (WCE 1/100) and 4 µg (WCE 1/50) of whole cell extract (WCE) were loaded on gel as positive controls. Specific antibodies, as indicated on the right, were used to probe the membranes. Blots are representative of two independent experiments.
Chapter 4

DISCUSSION

In this work, we show that the endocytic adaptor proteins Eps15 and Eps15L1 play a critical role in the regulation of Notch signaling, acting at the level of the signal-sending cell. Several observations had previously suggested a role for Eps15 and Eps15L1 in the endocytic regulation of Notch signaling, such as their interaction with the endocytic adaptors, and Notch regulators, Epsin and Numb (Chen, et al., 1998; Salcini, et al., 1997). However, the first data directly linking the two proteins to Notch regulation came from the analysis of Eps15/Eps15L1 DKO mice (see Introduction Section 4.1). Morphological analysis of Eps15/Eps15L1 DKO embryos revealed severe developmental defects, similar to those observed in several Notch loss-of-function KO mice, such as Notch1-KO (Limbourg, et al., 2005; Swiatek, et al., 1994), Notch1/Notch4-DKO (Krebs, et al., 2000), Dll11-KO (Hrabe de Angelis, et al., 1997) and Epsin1/Epsin2-DKO (Chen, et al., 2009). Indeed, a defect in Notch signaling in Eps15/Eps15L1 DKO mice was confirmed by gene expression microarray analysis of 9.5-day-old DKO embryos, which revealed that the Notch target genes Hes1, Hes5 and NRAP were downregulated (see Introduction Section 4.2).

Employing an *in vitro* co-culture assay consisting of C2C12-Notch1 signal-receiving cells in co-culture with OP9-Dll1 signal-sending cells, we have now obtained direct evidence of an Eps15 and Eps15L1 involvement in Notch signaling regulation. We found that Eps15 and Eps15L1 do not act as regulators of the Notch1 receptors, but instead participate in the regulation of the Notch ligands, Dll1, Dll4, Jagged1 and Jagged2. In contrast to the known role of Eps15 and Eps15L1 as endocytic adaptor proteins involved in CME and CIE of the EGFR (Benmerah, et al., 1998; Carbone, et al., 1997; Sgismund, et al., 2005), the
regulatory role that the two proteins exert on Notch ligands does not occur during the early steps of Dll1 endocytosis. Our data further suggest that Eps15 and Eps15L1 do not act together with the endocytic proteins Epsin1 and Epsin2 in regulating Notch ligand signaling, but rather through a distinct mechanism, possibly export to the plasma membrane or recycling.

1. Advantages and pitfalls of our in vitro model system

To be able to obtain direct proof of Eps15 and Eps15L1 regulation of Notch signaling, we needed to set-up an in vitro model system of Notch transactivation. In vitro models present both advantages and disadvantages compared to their in vivo counterparts. In vivo models are complex and recapitulate the biology of an entire organism, with its flexibility and adaptability to environmental changes and stimuli. In vitro systems are in contrast reductionist models, which, on the one hand, is an advantage, as they allow the analysis of specific pathways in the simplest system possible, and, on the other hand, a disadvantage, as they reduce the complexity of the organism to a single condition, which might not be the most representative one.

In the case of Notch signaling, in particular, it is essential to work with a model system composed of a signal-sending cell and a signal-receiving cell, since both ligands and receptors are transmembrane proteins that need to undergo endocytosis and processing to become fully activated (Baron, 2012; Bray, 2006; Fortini, 2009; Kopan & Ilagan, 2009). For this reason, treating signal-receiving cells with a soluble extracellular domain of the ligand to activate Notch receptor, as has already been described (Hicks et al., 2002), does not recapitulate physiological Notch activation.

However, a difficulty often encountered when working with a Notch signaling co-culture model system, is to be able to dissect effects in the signal-sending cell from effects in the signal-receiving cell. In vivo, the distinction between signal-sending and signal-receiving is
even more unclear, since very few cell populations express only Notch ligands or Notch receptors, as in the case of marginal zone B cells (Saito et al., 2003; Sheng et al., 2008; Song, et al., 2008; Tanigaki et al., 2002). The same is true also for in vitro systems, since the majority of cell lines express both Notch ligand and Notch receptors. Thus, in setting up a co-culture model system, it is essential to determine the expression profiles of cells before deciding in favor of particular cell models. The inability to dissect signal-sending effects from signal-receiving effects can lead to misinterpretation of the data since the mechanism of cis-inhibition that Notch ligands operate over Notch receptors works as a molecular switch that can make cells shift between a state of signal-sending and signal-receiving (Miller et al., 2009; Sprinzak, et al., 2010).

Another critique that can be directed to Notch in vitro model systems is that they are composed of only one or two cell types. In contrast, in the organism, Notch signaling is active in numerous cellular compartments and can be differentially regulated according to the tissue specificity of the compartment. This gives rise to different biological outcomes of Notch signaling, such as proliferation, lineage commitment or terminal differentiation, in different cell types (Perdigoto & Bardin, 2013).

When screening the literature to find a good model to recapitulate Notch signaling in vitro, we looked for a physiologically relevant system that would not only allow us to perform biochemical assays, but could also be used to perform biological assays, such as differentiation assays. Given the above considerations, we chose a co-culture system between two different cell lines: OP9-Dll1 as the signal-sending cell that expresses the Notch ligand Dll1, and C2C12 as the signal-receiving cell that expresses Notch receptors. The OP9-Dll1 model system was generated in 2002 by Schmitt and Zuniga-Pflucker; the expression of the Notch ligand Dll1 in the OP9 stromal cell line allowed, through an in vitro culture system, the generation of mature T cells from fetal liver stem cells (de Pooter & Zuniga-Pflucker, 2007; Holmes & Zuniga-Pflucker, 2009; Schmitt & Zuniga-Pflucker, 2002). C2C12 is a mouse skeletal muscle cell line, subclone of the murine myoblast cell
line established by Yaffe and Saxel (Yaffe & Saxel, 1977), that is able to differentiate and form myotubes in vitro (Bains et al., 1984; Lawson & Purslow, 2000). Moreover, it has been demonstrated that ligand-induced Notch signaling can inhibit C2C12 myoblast differentiation (Nofziger et al., 1999; Shawber et al., 1996).

Although the OP9-Dll1 and C2C12 cell lines are widely employed by scientists, we could not find data in the literature describing the expression of Notch ligands and receptors in these cells. Therefore, before proceeding with the co-culture assay, we verified, by Taqman technology, the mRNA profile of all Notch ligands and receptors in these cells. We confirmed that OP9-Dll1 cells express mainly Notch ligands with negligible amounts of Notch receptor transcript. In contrast, C2C12 cells do not express Notch ligand transcripts, but express detectable levels of endogenous Notch2 receptor mRNA. However, the level of Notch2 transcript was insufficient to produce an optimal signal upon activation. Notch activation was also not improved when we transiently transfected C2C12 cells with a construct encoding the Notch1 receptor. To overcome this difficulty, we decided to generate a stable cell line expressing the human receptor Notch1 and from this population we selected several clones. This was done to avoid working with a mass population expressing different levels of Notch receptor, therefore preventing the introduction of further variability to the assay. After testing the clones, we chose the one that yielded the best signal to noise ratio after ligand activation. In doing so, we accepted both advantages and disadvantages of working with a clonal cell line. In a clonal cell line, all cells derive from the same parental cell, meaning that all cells in the population will behave similarly when subjected to a stimulus. However, with a clonal cell line, we lose the intrinsic variability of a cell population and we risk amplifying casual mutations that could have been generated in the parental cell during the subcloning and amplification process.

To validate our model system we performed several controls:
i) treatment with γ-secretase inhibitor DAPT confirmed that the signal detected with the Notch reporter transactivation assay can be indeed ascribed to Notch activation and not to a different pathway;  

ii) silencing of the Notch regulators Numb and dynamin in C2C12 cells confirmed respectively increased activation of the reporter gene, consistent with a removal of inhibition of the Notch receptor, and downregulation of the reporter gene, consistent with a block in Notch receptor endocytosis and consequent S2 cleavage;  

iii) downregulation of the Notch ligand regulator Mindbomb1 in OP9-Dll1 cells completely inhibited activation of the reporter, consistent with a block in ubiquitin-mediated internalization of the ligand Dll1.  

Taken together, these observations on the behavior of the main players involved in Notch signaling regulation are an indication that the co-culture system we chose appears to be an appropriate model system to study Notch activation in vitro.

2. Eps15 and Eps15L1 regulate Notch signaling in the signal-sending cell  

When we reasoned about a possible role of Eps15 and Eps15L1 in the regulation of Notch signaling, we hypothesized different mechanisms of actions. First, it is known that Eps15 and Eps15L1 interact with both Numb and Epsin, two fundamental regulators of Notch signaling in the signal-receiving and signal-sending cell, respectively. Consequently, it was possible that Eps15 and Eps15L1 could carry out their role in combination with these Notch regulators, either modulating the activity of Notch receptors through their interaction with Numb or modulating internalization of the Notch ligands through interaction with Epsin1. Another possibility was that Eps15 and Eps15L1 might regulate Notch signaling via a novel mechanism that is not related to their interactors, Numb and Epsin.
To assess if indeed Eps15 and Eps15L1 do have a direct role in the regulation of Notch-signaling and to dissect if this possible regulation occurs in the signal-sending or in the signal-receiving cell, we performed the Notch reporter transactivation assay in our C2C12/OP9-DLL1 co-culture model system.

We could not detect any role of Eps15 and Eps15L1 in the regulation of Notch receptors in the signal-receiving cell. In contrast, we detected alterations of reporter signal when we depleted Eps15 and Eps15L1 in the signal-sending cell. In particular, Notch signaling was downregulated by 40 – 50% after Eps15 or Eps15L1 KD in the signal-sending cell. Moreover, we were able for the first time to rule out, in a mammalian context, a role of the endocytic adaptor Numb in the regulation of Notch ligand in the signal-sending cell. Thus, we could exclude the possibility that Eps15 and Eps15L1 are acting in combination with Numb in the regulation of Notch ligand.

Interestingly, simultaneous removal of both Eps15 and Eps15L1 in the signal-sending cell did not further reduce Notch signaling compared to removal of the single proteins. This result suggests that Eps15 and Eps15L1 are both critical components of the same pathway regulating Notch ligands. It is possible to speculate that the two proteins may have a redundant role in the regulation of Notch ligand and that a certain amount of total Eps15 family proteins, irrespective of which, is required to carry out the correct function (Figure 4.1). This hypothesis, namely “gene dosage hypothesis”, has already been suggested in the case of Epsin1 and Epsin2 in the regulation of Notch ligands (Meloty-Kapella, et al., 2012). Indeed, when we performed KD of Epsin 1 and 2 in our model system, we observed the same behavior that we observed following Eps15 and Eps15L1 KD: i.e. the double KD was similar to the single KDs, with no further decrease of transactivation signaling. To verify this gene dosage theory in our model system, it would be necessary to rescue Eps15L1 KD with overexpression of Eps15, or vice versa, to understand if a certain quantity of the Eps15 protein, no matter if it is Eps15 or Eps15L1, is needed to obtain an optimal Notch signaling (Figure 4.1).
Figure 4.1 The gene dosage hypothesis

Cartoon depicting the gene dosage hypothesis applied to Eps15 and Eps15L1 in the regulation of Notch ligand signaling. The presence of Eps15 and Eps15L1 in the signal-sending cell gives rise to a fully functional Notch ligand, able to activate Notch signaling. Removal of either Eps15 or Eps15L1, or of both proteins, completely inactivates the pathway, giving rise to a semifunctional Dll1. Overexpression of Eps15 (or vice versa) gives rise to the minimal levels of protein required for Dll1 activation, therefore rescuing Dll1 activation.

When we subsequently deleted Eps15 and Eps15L1 in combination with Epsin1 and Epsin2, we scored a complete inhibition of Notch signaling. We can explain this with two different hypotheses.

i) The first hypothesis that we formulated is that Eps15 and Eps15L1 might have the same role as Epsin1 and Epsin2 in the regulation of Notch ligands, mediating their internalization, possibly in two parallel pathways. Consequently if abolishing the Eps15/Eps15L1-mediated route leads to a 50% reduction of the signal, and abolishing the Epsin1/Epsin2-mediated route leads again to a 50% reduction of the signal, it could explain why, when all of them are depleted from the cell, Notch signaling is completely
abrogated. Of course, this hypothesis only holds true if removal of one protein of the family completely destroys the pathway that the family is mediating. However, our results from the DLL1 internalization assay (discussed in section 3) disproved this hypothesis, showing that indeed Eps15 and Eps15L1 do not have a role in the regulation of Notch ligands endocytosis.

ii) The second hypothesis that we formulated to explain these results, is that Eps15 and Eps15L1 could act by regulating a completely different process in ligand activation from Epsin1 and Epsin2, i.e. not internalization. Consequently, when both processes, internalization mediated by Epsin1/2 and the unknown process mediated by eps15/L1, are impaired by the loss of the four proteins, Notch ligands lose all ability to signal. Further evidences about the regulatory process that might be mediated by Eps15 and Eps15L1 will be detailed in section 3 and section 4.

Since in D. melanogaster the two ligands Delta and Serrate appear to be regulated by different mechanisms (Wang & Struhl, 2004; Wang & Struhl, 2005), we were interested to explore whether Eps15 and Eps15L1 are able to regulate all mammalian Notch ligands. To do this, we tested, in our in vitro assay, not only the ligand DLL1, but also the ligands DLL4, Jagged1 and Jagged2. The impairment of Notch signaling after depletion of Eps15 and Eps15L1 in the signal-sending cell was similar for all ligands tested, indicating that the mechanism of action of the two endocytic proteins is conserved for all Notch ligands.

Subsequently, we investigated if contemporary loss of Eps15 and Eps15L1 in both the signal-sending and the signal-receiving cell could uncover a further downregulation of Notch signaling, due to a cross-talk between the two cells. However, our KD experiments in both the signal-sending and the signal-receiving cell ruled out that possibility, further confirming that Eps15 and Eps15L1 do not indeed have any role in the regulation of the Notch receptors.

At this point, it became clear that the data that we obtained in our in vitro study are partially incongruent with our in vivo model system of Eps15/Eps15L1 DKO mice. In fact,
the Eps15/Eps15L1 DKO mouse has a stronger phenotype compared to that of the Eps15 and Eps15L1 single KO mice. In contrast, in the in vitro model system, removal of the single proteins had the same effect as removal of both.

To explain this partial incongruence, two different hypotheses come to mind:

i) First, it is possible that the partial additivity observed in vivo might be due to a higher flexibility of the organism (compared to an in vitro system) that, in the case of a single KO, is able to compensate for the loss of either Eps15 or Eps15L1, possibly through the upregulation and recruitment of other endocytic players. These compensatory mechanisms may come into play in the animal over time. Therefore they are not present when acute silencing of Eps15 and Eps15L1 is performed in our in vitro model system, where we uncover the loss-of-function phenotype already by removing one of the two players.

ii) Second, it is possible that the phenotype of the Eps15/Eps15L1 DKO mice is the result of alterations of different signaling pathways, not just the Notch pathway. Alterations in these pathways may not have been detected by the microarray analysis, because they might not be due to transcriptional alterations, but to altered internalization/trafficking of receptors, such as the EGFR. In this case, the KO mice would have only a partial Notch phenotype. In support of this hypothesis, the extent of downregulation of Notch target genes in the Eps15/Eps15L1 DKO mice amounted to a 50% reduction, comparable with the data obtained with our in vitro transactivation assay after silencing of either Eps15 or Eps15L1. Moreover, the extent of Notch target gene downregulation was similar in both the Epsin1/2 and Eps15/Eps15L1 DKO embryos, and Epsin1/2 and Eps15/L1 KD had comparable effects in our Notch reporter transactivation assay. Therefore it might be safe to speculate that additional Notch-independent mechanisms may contribute to the phenotype of both Epsin1/2 and Eps15/Eps15L1 DKO mice.
3. Investigating Dll1 endocytosis and recycling

To test whether Eps15 and Eps15L1 have a role in modulating Notch ligand endocytosis, we decided to set-up a quantitative, FACS-based, assay, to assess both receptor-stimulated and constitutive endocytosis of Dll1.

We simulated receptor-induced endocytosis using a recombinant NotchFc protein, preclustered with a secondary antibody, in order to mimic receptor clusters on the signal-receiving cell. We followed constitutive endocytosis by treating the cells with a fluorochrome-conjugated antibody, anti-Dll1 (Heuss, et al., 2008; Le Borgne et al., 2005; Meloty-Kapella, et al., 2012; Shah, et al., 2012). Dll1 receptor-mediated internalization stimulated with soluble NotchFc receptor might not recapitulate all aspects of Dll1 endocytosis mediated by membrane-bound receptor. However, the validation of our assay set-up, performing KD of endocytic proteins that are known to regulate the Notch ligands endocytosis both in mammals and in D. melanogaster, would suggest that with our in vitro assay we mimic as close as possible the in vivo physiological condition.

Based on results from our Dll1 internalization assays, we could reject the hypothesis that Eps15 and Eps15L1 participate in the regulation of Notch ligand early endocytic events. Moreover, we excluded that Eps15 and Eps15L1 act in combination with their interactor Epsin1 in this regulatory process, as the combined Eps15/Epsin1 KD did not further reduce Dll1 endocytic rate, compared to Epsin1 alone. Thus, it appears that Eps15 and Eps15L1 act at a different step in the ligand activation process that is not connected to early steps of internalization.

Having concluded that Eps15/L1 are unlikely to be involved in internalization of Dll1, we decided to proceed by assessing whether Eps15 and Eps15L1 regulate Dll1 recycling. Dll1 recycling can be investigated either: i) quantitatively, by measuring the amount of Dll1 present at the plasma membrane; or ii) qualitatively, by assessing the localization of Dll1 in specific plasma membrane microdomains.
We tried to first assess the recycling quantitatively, using a modified version of the internalization assay that we had previously set-up. However, we observed thatDll1 recycling did not occur in our model system under our experimental conditions. This could be due to the fact that recycling was assessed on cells in suspension, and it is possible instead that recycling requires anchorage to the substrate and polarization of the cell. It has long been known in fact that anchorage-dependent cells (i.e. cells that need to be attached to a substrate in order to grow and proliferate) undergo a block in transcription and cell cycle when kept in suspension (Assoian & Zhu, 1997; Reddig & Juliano, 2005; Schulze et al., 1996), and it might be tempting to speculate that also recycling might undergo a similar block. For this reason, we have decided to use a biotinylation-based recycling assay, performed on adherent cells (Heuss, et al., 2008). Membrane proteins will be biotinylated, internalized and recycled to the plasma membrane. After cell lysis, pulldown of biotinylated proteins will be performed, and recycled Dll1 will be detected via western blot analysis. This assay, however, measures recycling only after constitutive internalization of Dll1. We are particularly interested in measuring recycling following Dll1 binding to Notch, since, from data on EGFR regulation, it appears likely that Eps15 and Eps15L1 act in a context of “ligand-induced” trafficking. Indeed, Eps15 and Eps15L1 mediated internalization of EGFR occurs after the receptor binds EGF, and receptor internal trafficking is determined by ligand concentration (Sorkin & Goh, 2008; Tomas et al., 2014). Moreover, Eps15 and Eps15L1 both possess two UIM, and since it is known that Dll1 is ubiquitinated, it is possible to speculate that Eps15/Eps15L1 regulation over Dll1 might be ubiquitin-mediated, and thus tightly regulated. In order to check these hypotheses, we will test whether it is feasible to perform the biotinylation-based recycling assay following Notch2Fc stimulated internalization of the ligand.

To assess Dll1 recycling qualitatively, we decided to perform a lipid raft extraction after KD of Eps15 and Eps15L1 in the signal-sending cell. This experiment was highly reproducible and consistent with published data (Heuss, et al., 2008; Heuss, et al., 2013;
Moreover, when we tested EGFR localization as a control, we found it was localized within the lipid raft enriched fractions, as described in the literature (Abulrob, et al., 2004; Mineo, et al., 1999; Yamabhai & Anderson, 2002). However, we did not observe any changes in Dll1 localization at the membrane after removal of Eps15 and Eps15L1. This result suggests that the two proteins do not have a role in the export of Notch ligands to lipid rafts.

4. Other possible mechanisms through which Eps15 and Eps15L1 could regulate Notch signaling and future directions

When considering what other mechanisms involved in Dll1 activation could be regulated by Eps15 and Eps15L1, different hypothesis come to mind.

First, we can consider hypotheses based on the roles already proposed in the literature for Eps15. For example, it might be possible that Eps15 and Eps15L1 are involved in Notch ligand degradation, since they are already known to participate in the regulation EGFR degradative pathway (Kostaras, et al., 2013; Roxrud, et al., 2008). However, it seems improbable that Eps15/Eps15L1 mediate degradation of Notch ligands, since when we analyzed Dll1 levels at the membrane after Eps15 and Eps15L1 KD, we did not score any changes. Nevertheless, it might still be possible that Dll1 levels at the membrane are more tightly regulated than intracellular levels, meaning that the cell might employ compensatory pathways to keep Dll1 levels at the membrane as constant as possible. To assess this point, it will be necessary to investigate total levels of Dll1 in OP9-Dll1 cells after depletion of Eps15 and Eps15L1. If a change in total levels is confirmed, we could perform a Dll1 degradation assay to further investigate the involvement of Eps15/L1 in this process.

Another possibility is that Eps15 and Eps15L1 could act as regulators of the Dll1 biosynthetic pathway, in particular controlling its trafficking through the Golgi apparatus,
as they already do for EGFR and Coronin7 (Chi, et al., 2008; Yuan, et al., 2014). If Eps15/L1 were to have a regulatory role in the Dll1 secretory pathway, then depletion of the two proteins should give rise to a delay in the Dll1 export. However, we observed that steady state levels of Dll1 at the plasma membrane do not change after Eps15 and Eps15L1 KD, suggesting that this scenario is unlikely. Alternatively, we could speculate that an incorrect trafficking through the Golgi could lead to an incorrect glycosylation of Dll1, resulting in a less active ligand. To test the first hypothesis, we could perform an imaging assay that allows us to monitor the Dll1 biosynthesis over time, whereas, for the second hypothesis we could perform a mass spectrometry analysis to assess glycosylation.

A more unbiased screen that we could perform to understand the molecular mechanism of Eps15 and Eps15L1 in the regulation of Notch ligands, is a structure/function analysis to rescue the phenotype obtained in the Notch reporter transactivation assay using different Eps15 mutants. In particular, we could employ:

i) the Eps15 point mutant L883A that abolishes both binding to ubiquitin and the ability of Eps15 to be ubiquitinated (Woelk et al., 2006); Notch ligands are in fact known to be ubiquitinated and both their internalization and their degradation is ubiquitin-mediated;

ii) the Eps15 mutant Δ14aa, that lacks the 14 amino acids responsible for AP1 binding (Chi, et al., 2008); AP1 is involved in the formation of clathrin-coated vesicles in the trans-Golgi network secretory pathway, therefore the Δ14aa mutant could give us an indication about the role of Eps15 and Eps15L1 in the export of Dll1 to the plasma membrane;

iii) the two Eps15 isoforms, Eps15b or Eps15s, which lack the EH or the UIM domains, respectively (Chi, et al., 2011); Eps15b is known to mediate the sorting of the EGFR towards the degradative pathway, while Eps15s is involved in the recycling of the internalized EGFR back to the cell surface.
Finally, we cannot exclude the possibility that the effect of Eps15 and Eps15L1 on Notch signaling might be of a more indirect nature. There are in fact different several factors that could have an indirect effect on signaling, such as cell morphology, cell adhesion to the substrate, cell polarity, cell proliferation or cell-cell interaction. In the case of Eps15 and Eps15L1, it is known that they regulate trafficking of occludin, a component of tight junctions (Murakami, et al., 2009). Tight junctions are epithelial cell-cell junctions located between the apical and basolateral portions of the plasma membrane within cells. They function as a barrier to regulate the paracellular passages of molecules; moreover they limit the movement of lipids and proteins between the apical and the basolateral portions of the membrane and more recently have been implicated in the control of cell proliferation and gene expression (Gonzalez-Mariscal et al., 2008). Eps15 and Eps15L1, moreover, have been shown to regulate the internalization of connexin43, a component of gap junctions (Girao, et al., 2009). Gap junctions are intercellular membrane channels composed by connexin monomers; they regulate the aqueous exchanges between the cytoplasm of contiguous cells and in doing so they allow the exchange of ions, nucleotides, small molecules and second messengers (Saez & Leybaert, 2014). Several studies also suggest that connexin43 hemichannels contribute to the regulation of signaling induced by mechanical stress in bone tissues (Buo & Stains, 2014). Finally Eps15 has also been shown to regulate internalization of integrin $\beta 1$, through its binding with the endocytic adaptor Dab2 (Teckchandani, et al., 2012). Integrins are transmembrane receptors that act as bridges for cell-cell and cell-extracellular matrix interactions. Integrins also regulate intracellular signaling pathways and cooperate to promote cell growth, cell survival, and cell proliferation. Moreover integrins have been reported to directly regulate Notch signaling (Estrach et al., 2011; Gomez-Lamarca et al., 2014; Stenzel et al., 2011).

Taken together, all these observations suggest that the role of Eps15 and Eps15L1 in the cell is complex and multifaceted, raising the possibility that they do not directly regulate
Notch signaling, but could modulate a cellular process that has as a secondary effect on the Notch pathway.
REFERENCES


LaVoie, M. J., & Selkoe, D. J. (2003). The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. The Journal of biological chemistry, 278(36), 34427-34437.


HOPS and AP-3 complex genes are required for a Deltex-regulated activation of notch in the endosomal trafficking pathway. Developmental cell, 15(5), 762-772.


