Crosstalk between Notch and Wnt Signalling Pathways in Vertebrates

A thesis submitted to The University of Manchester for the Degree of PhD in Developmental Biology in the Faculty of Life Sciences

2012

Ana Hidalgo Sastre
# Table of Contents

Table of Contents ................................................................................................................................. 3
List of Figures ........................................................................................................................................ 5
List of Tables ......................................................................................................................................... 7
Abstract ................................................................................................................................................ 9
Author Contributions ............................................................................................................................. 11
Declaration ........................................................................................................................................... 13
Acknowledgements ............................................................................................................................... 15
List of Abbreviations ............................................................................................................................. 17

Chapter 1: General Introduction ............................................................................................................ 19
  1.1 Development and gene regulatory networks .............................................................................. 21
  1.2 Development, crosstalk and understanding of disease .............................................................. 24
  1.3 Notch signalling, structure and features ................................................................................... 25
    1.3.1 Structure of the Notch receptor .......................................................................................... 25
    1.3.2 Regulation of Notch by post-translational modifications .................................................. 26
    1.3.3 Canonical Notch ligands structure .................................................................................... 28
    1.3.4 Regulation of DSL ligands by post-translational modifications ........................................ 29
    1.3.5 Canonical Notch signalling pathway ................................................................................ 30
    1.3.6 Alternative Notch signalling pathways ............................................................................. 32
    1.3.7 Endocytosis and intracellular trafficking of Notch ligands and receptor ......................... 36
  1.4 Nuts and bolts of Wnt signalling .................................................................................................. 39
    1.4.1 Structure of Wnt ligands and secretion .............................................................................. 39
    1.4.2 Wnt receptors at glance ..................................................................................................... 40
    1.4.3 Canonical Wnt/β-catenin signalling pathway .................................................................... 43
    1.4.4 Non-canonical Wnt signalling pathways .......................................................................... 45
  1.5 Importance of Notch and Wnt signalling pathways in development and disease ................. 46
  1.6 Interactions between Wnt and Notch signalling pathways ....................................................... 55
  1.7 Setting the scene .......................................................................................................................... 58
References ............................................................................................................................................... 60

Chapter 2: Paper 1 ................................................................................................................................ 77
  Abstract .............................................................................................................................................. 78
  Introduction ....................................................................................................................................... 79
  Materials and methods ....................................................................................................................... 82
  Results .............................................................................................................................................. 84
  Discussion ....................................................................................................................................... 96
  References ....................................................................................................................................... 98

Chapter 3: Paper 2 ................................................................................................................................ 105
  Abstract .......................................................................................................................................... 106
  Introduction .................................................................................................................................... 107
  Materials and methods ..................................................................................................................... 110
  Results .......................................................................................................................................... 113
  Discussion ..................................................................................................................................... 129
  References ..................................................................................................................................... 132

Chapter 4: Paper 3 ................................................................................................................................ 141
  Abstract .......................................................................................................................................... 142
  Key words: Notch, Wnt, β-catenin, Hey1, inhibition, Rbpj, transcription-dependent. Introduction ................................................................................................................................. 142
  Introduction .................................................................................................................................... 143
  Materials and methods ..................................................................................................................... 145
  Results .......................................................................................................................................... 149
  Discussion ..................................................................................................................................... 164
List of Figures

Chapter 1

Figure 1.1: Mechanism of interactions between signalling pathways to generate cell 22
Figure 1.2: Structure of Drosophila and mammalian Notch receptor proteins 27
Figure 1.3: Structure of Drosophila and mammalian Notch ligands 29
Figure 1.4: Mammalian Notch signalling pathway 31
Figure 1.5: Possible mechanisms of ligand independent and dependent Notch 35
Figure 1.6: Structure of Frizzled receptor 41
Figure 1.7: Structure of LRP/Arrow receptor 41
Figure 1.8: Structure of Ror2 receptor 42
Figure 1.9: Structure of Ryk receptor 42
Figure 1.10: Canonical Wnt/β-catenin signalling pathway 44
Figure 1.11: Notch-mediated lateral inhibition during sensory organ precursor 47
Figure 1.12: Wingless as a morphogen during wing margin development 48
Figure 1.13: Notch binary cell fate assignment during mecanosensory organ 51
Figure 1.14: Role of Notch and Wnt signalling pathways in intestine cell lineage 53
Figure 1.15: Role of Notch signalling in skin stem cells 55

Chapter 2

Figure 2.1: Xenopus β-catenin stimulates the greatest fold increase in reporter gene 89
Figure 2.2: Identifying a mammalian cell line that robustly responds to Wnt signalling 91
Figure 2.3: Comparing activation of TCFAdTATA reporter gene by stabilising β-catenin 93
Figure 2.4: Characterisation of Notch constructs 95
Supplementary Figure 2.1: Wnt signalling in MEF cells 101
Supplementary Figure 2.2: β-catenin accumulation in CHO-K1 and NIH-3T3 cell lines 102
Supplementary Figure 2.3: β-catenin accumulation in HEK 293T cell line 103

Chapter 3

Figure 3.1: Membrane bound Notch inhibits β-catenin transcriptional activity in 119
Figure 3.2: ΔEGF_N1 functions independently of CBF1/Rbpj 121
Figure 3.3: Notch at the membrane inhibits β-catenin transcriptional activity 123
Figure 3.4: Characterisation of the membrane-restricted C-terminal deletion Notch 125
Figure 3.5: The ANK repeats of Notch are required to inhibit β-catenin transcriptional 127
Figure 3.6: Mouse ΔEGF_N1 inhibits Xenopus β-catenin transcriptional activity 128
Supplementary Figure 3.1: Whole cell lysate from HEK293T 136
Supplementary Figure 3.2: Quantitative analysis of S45Fβ-catenin fluorescence 137
Supplementary Figure 3.3: Quantitative analysis of S45Fβ-catenin fluorescence 138
Supplementary Figure 3.4: Quantitative analysis of S45Fβ-catenin fluorescence 139
Chapter 4

Figure 4.1: NICD inhibits β-catenin transcriptional activity

Figure 4.2: NICD requires its interaction with Rbpj to block β-catenin transcriptional

Figure 4.3: The Notch target genes Hes/Hey can regulate Wnt signalling

Figure 4.4: Hey1 forms a complex with β-catenin and TCF

Figure 4.5: The Hey1 inhibitory crosstalk to β-catenin is conserved in Xenopus

Supplementary Figure 4.1: Quantitative analysis of S45Fβ-catenin fluorescence

Supplementary Figure 4.2: Quantitative analysis of S45Fβ-catenin fluorescence

Supplementary Figure 4.3: NICD activates expression of Hes/Hey family of proteins

Supplementary Figure 4.4: Rbpj-VP16 R218H cannot activate a Rbpj-dependent

Supplementary Figure 4.5: Dominant negative forms of Hes and Hey proteins inhibit

Supplementary Figure 4.6: Notch target gene Hes5 regulates Wnt signalling

Supplementary Figure 4.7: Notch and the target gene Hey limit Wnt signalling

Supplementary Figure 4.8: The RFPHey1 and RFPDNHey1 can still cause a similar

Chapter 5

Figure 5.1: Proposed model of the functional relationship between Notch and Wnt

Figure 5.2: Axis induction in Xenopus
List of Tables

Table 6.1: Antibodies used for western blot and immunofluorescence 204
Table 6.2: Mutagenesis primers 205
Table 6.3: PCR and sequencing primers 206
Table 6.4: Quantitative PCR primers 207
Abstract

The development of complex metazoans depends on the integration of a handful of signalling pathways that eventually modulate precise patterns of gene expression. The fact that just a few pathways are involved in the generation of such complexity in different organisms, suggests that these are highly regulated and conserved processes. The accurate spatio-temporal coordination of the signalling pathways controls the assignation of different cell fates and their patterning into tissues and organs. The source of diversity relies on the different possible interactions between signalling pathways, such as, the combination of signals and the order in which they are received by the cell or crosstalk. Due to their importance in development, abnormal signalling through these pathways has been strongly associated with developmental disorders, cancers and other diseases. The Notch and Wnt signalling pathways are key components of the intricate network that controls gene expression during development, and genetic analysis in Drosophila has highlighted that interactions between these two signalling pathways are important during this process.

This thesis investigates the cross-regulatory interactions between Notch and Wnt signalling pathways in mammals. Using transcriptional reporter assays and biochemical analysis, I have found two molecular mechanisms underlying the inhibitory crosstalk between Notch and β-catenin, the effector of Wnt signalling pathway, in mammalian cells. At the membrane Notch inhibits β-catenin transcriptional activity through Deltex mediated endocytosis of Notch and a component required for β-catenin activation. This is similar to results observed in Drosophila. In the nucleus, I have identified a novel mechanism by which NICD-dependent transcription of Hes/Hey family of transcription factors prevents the activation of Wnt signalling pathway. This mechanism involves the formation of a physical complex between Hey1 and β-catenin/TCF, which allows Hey to block Wnt transcriptional activation. Additionally, I have found that these two mechanisms are conserved across vertebrates.

Together the findings of this thesis improve our understanding of the molecular mechanism underlying the Notch/Wnt crosstalk. In turn, this will give an insight into unravelling how a handful of signalling pathways can generate sufficient diversity in signalling output to specify the hundreds of different cell fates generated to make a mammal. Elucidating these signalling networks will also contribute to our understanding of diseases, both their aetiology, by knowing how changes in one signal can influence another, and their treatment as mimicking points of crosstalk is likely to generate very specific therapeutic agents.
Author Contributions

This thesis is presented as an alternative format that consists of a general introduction chapter, which reviews previous research, followed by three results chapters written as manuscripts, which contain the main findings of my research, then a general discussion chapter containing a general view of the proposed model, perspectives and conclusions and a final supplementary materials and methods chapter. The contribution of others and my contribution to the work presented in the results chapters of this thesis are summarized as follows:

Chapter 2: Choosing the right tools to study the molecular mechanism behind the crosstalk between Notch and Wnt signalling pathways.

AHS and KB designed the experiments. AHS performed the experiments and wrote the paper. KB edited the paper.

Chapter 3: Membrane-restricted Notch inhibits β-catenin transcriptional activity independently of Rbpj signalling.

AHS and KB designed the experiments. AHS performed the experiments and wrote the paper. KB edited the paper.

Chapter 4: Hey1 modulates Wnt signalling by associating with β-catenin and TCF and regulating its transcriptional activity in vertebrates.

AHS and KB designed the experiments. AHS performed the experiments and wrote the paper. KB edited the paper.
Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of The University of Manchester or any other University or Institute of learning.

Copyright Statement

The author of this thesis (including any appendices and/or schedules to this thesis) owns any copyright in it (the “Copyright”) and s/he has given The University of Manchester the right to use such Copyright for any administrative, promotional, educational and/or teaching purposes.

Copies of this thesis, either in full or in extracts, may be made only in accordance with the regulations of the John Rylands University Library of Manchester. Details of these regulations may be obtained from the Librarian. This page must form part of any such copies made.

The ownership of any patents, designs, trademarks and any and all other intellectual property rights except for the Copyright (the “Intellectual Property Rights”) and any reproductions of copyright works, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property Rights and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property Rights and/or Reproductions.

Further information on the conditions under which disclosure, publication and exploitation of this thesis, the Copyright and any Intellectual Property Rights and/or Reproductions described in it may take place is available from the Head of School of (insert name of school) (or the Vice-President) and the Dean of the Faculty of Life Sciences, for Faculty of Life Sciences’ candidates.
Acknowledgements

I would like to thank my supervisor Dr Keith Brennan for all his help and advice over the last three years. I would also like to thank my advisor, Dr Chris Thompson, for providing helpful discussions and suggestions.

In the lab, I am grateful to Olivier Meurette and very specially to Anna Collu for their practical help and the scientific discussions. I am also thankful to the other members of the Brennan Lab, specially Stephanie Jobling and Rebecca Rock, for the Wednesday morning lab discussions. I would also like to thank Dr Karel Dorey for his help with the in vivo work in Xenopus. I am very thankful to Barbara Schellenberg and Tom Pettini for their practical help, and for proofreading earlier drafts of this thesis, as well as to Rocío Ortuño Casanova for help with the final editing. Thanks as well to my colleagues from the Gilmore and Streuli Labs, for making the day to day in the lab enjoyable, in particular Jolanta Hughes.

Outside the lab, I am very grateful to the good friends I have made in Manchester. You have made these last 4 years unforgettable, making me feel like at home, especially with the big lunches on the weekends. Your support, has been essential to keep me motivated during the tough process of writing. In particular, I would like to thank my dearest friend Elena Martín Ávila. Both of us have sailed side by side on the same boat during this PhD ocean and she has never let go. It has been quite a ride, Elena, but we made it to land!

Last but not least and from the very bottom of my heart, I would like to dedicate the final lines of these acknowledgements to my parents and Conrad, for your endless support. Thank you for been there every day, physically or at the other end of the line, to encourage me, to cheer me up in the hard moments, and to celebrate the good ones. Without you I wouldn’t have been able to finish this thesis.

The work presented in this thesis was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and the Wellcome Trust.

To all of you, muchas gracias!
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAM</td>
<td>A disintegrin and A metalloprotease</td>
</tr>
<tr>
<td>ANK</td>
<td>Ankyrin</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>AS-C</td>
<td>Achaete-scute complex</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>CADASIL</td>
<td>Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>Chinese Hamster Ovary cell line sub-clone K1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CK1</td>
<td>Casein kinase 1</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CRD</td>
<td>Cystein-rich domain</td>
</tr>
<tr>
<td>CSL</td>
<td>CBF1, Suppressor of Hairless Su(H) and Lag-1</td>
</tr>
<tr>
<td>DI</td>
<td>Delta</td>
</tr>
<tr>
<td>DII</td>
<td>Delta-like</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNER</td>
<td>Delta/Notch-like EGF-related receptor</td>
</tr>
<tr>
<td>DSL</td>
<td>Delta, Serrate and Lag-2</td>
</tr>
<tr>
<td>D/V</td>
<td>Dorso/Ventral</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>E(spl)C</td>
<td>Enhancer of split complex</td>
</tr>
<tr>
<td>Fzd</td>
<td>Frizzled</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>Hes</td>
<td>Hairy and Enhancer of Split</td>
</tr>
<tr>
<td>Hey</td>
<td>HES-related with YRPW motif</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney 293 cells transformed with large T-antigen</td>
</tr>
<tr>
<td>Jag</td>
<td>Jagged</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>Mib</td>
<td>Mindbomb</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphocyte Enhancer Factor</td>
</tr>
<tr>
<td>LIN</td>
<td>Lin-12-Notch repeat</td>
</tr>
<tr>
<td>LRP</td>
<td>Low density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>Neur</td>
<td>Neuralised</td>
</tr>
<tr>
<td>NEXT</td>
<td>Notch extracellular truncation</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation sequences</td>
</tr>
<tr>
<td>O-Fut</td>
<td>O-fucosyltransferase</td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEST</td>
<td>Proline, glutamine, serine, threonine-rich</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS with Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RAM23</td>
<td>RBPjκ-associated molecule</td>
</tr>
<tr>
<td>Rbpj</td>
<td>Recombination signal binding protein for immunoglobulin kappa J region</td>
</tr>
<tr>
<td>R. luc</td>
<td>Renilla luciferase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>S1</td>
<td>Cleavage site 1</td>
</tr>
<tr>
<td>S2</td>
<td>Cleavage site 2</td>
</tr>
<tr>
<td>S3</td>
<td>Cleavage site 3</td>
</tr>
<tr>
<td>Ser</td>
<td>Serrate</td>
</tr>
<tr>
<td>SOP</td>
<td>Sensory organ precursor</td>
</tr>
<tr>
<td>Su(H)</td>
<td>Suppressor of Hairless</td>
</tr>
<tr>
<td>TACE</td>
<td>Tumour necrosis factor α-converting enzyme</td>
</tr>
<tr>
<td>TAD</td>
<td>Transcriptional activation domain</td>
</tr>
<tr>
<td>T-ALL</td>
<td>T-cell acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>TBS with Tween-20</td>
</tr>
<tr>
<td>TCF</td>
<td>T-cell factor</td>
</tr>
</tbody>
</table>
Chapter 1: General Introduction
In this thesis I have studied the mechanisms underlying the molecular crosstalk between Notch and Wnt signalling pathways. These two signalling pathways are frequently active during development in the same tissues at the same time. However, they often have opposing effects on cell fate decisions. Consequently, there is the potential for the two pathways to conflict with one another during this process. This conflict can, however, be resolved if the two pathways crosstalk with one another, so that one pathway can limit signalling through the other. Prior to my thesis, an inhibitory interaction between Notch and Armadillo/β-catenin had been described in Drosophila that allowed Notch to limit signalling through the Wnt/β-catenin pathway, producing a Notch-ON/Wnt-OFF cell state. In this thesis, I present evidence that there is a similar crosstalk between the two pathways in vertebrates. However, my experiments also reveal that there are two distinct mechanisms mediating this crosstalk and I will speculate why this maybe the case within the general discussion.

Within the general introduction to this thesis you will find: a summary of the Notch and Wnt signalling pathways (ligands, receptors, and intracellular components), a description of the roles the two pathways play in normal metazoan development and human disease, examples where Notch and Wnt signalling can conflict in development, and a summary of the known molecular mechanisms that underpin the crosstalk between the two pathways.
1.1 Development and gene regulatory networks

Only eight major signalling pathways: Hedgehog (Hh), Janus kinase/Signal transducer and activator of transcription (JAK/STAT), Nuclear factor kappa-light-chain-enhancer of activated β-cells (NF-κβ), Notch, Receptor tyrosine kinase (RTK), Transforming growth factor-β (TGF-β), Wnt/Wingless (Wnt/Wg) and nuclear hormone pathways control the development of complex metazoans from a single cell, the fertilized egg, into adults with multiple cell types (for review see: (Barolo and Posakony, 2002; Gerhart, 1999; Pires-daSilva and Sommer, 2003)). It is extraordinary that all metazoan species, with such varied body plans, can evolve from such a small shared signalling framework. This is rather like music, where a simple collection of 8 notes can be combined to generate an almost endless array of melodies. It is not surprising then, that if these signalling pathways represent the “musical tree of life”, when they are “out of tune” they lead to developmental disorders and adult diseases.

In the past decade, developmental research has addressed the question of how specificity and diversity in cell fate can be achieved from this limited number of signalling pathways, yet without a clear answer. Development of a multicellular organism requires the spatio-temporal coordination of: cell proliferation, cell fate specification and organization of these cells into tissues. Here, we shall focus on “cell fate specification” and on its driving force, an intricate mesh of gene regulatory networks (Macneil and Walhout, 2011). These gene regulatory networks are composed of signal transduction networks and transcription factor networks which control initiation, development and coordination of cell fates within cell populations (Muñoz-Descalzo and Martinez Arias, 2012). Initially, signalling pathways were thought to work in a linear fashion (a starter signal, a messenger, and an effector) (Rodbell, 1995). However, with the years it has become more obvious that signalling pathways interact with one another resulting in complex gene regulatory networks (Alon, 2007). Furthermore, in a communication system we cannot forget the concept of “noise”, fluctuations in the messenger system that influence and alter the outcome of signalling events creating variability in a cell population (Chalancon et al., 2012; Hayward et al., 2008; Martinez-Arias and Hayward, 2006).

These eight major signalling pathways interact as a network to produce the incredible array of cell types found in the animal kingdom, mainly, by three mechanisms. (I) The **sequential** activation of a particular set of signalling pathways in different orders within the
same cell can promote different cell fates (Fig 1.1A). (II) The **simultaneous** activation of two or more signalling pathways can generate diversity by activating the expression of a novel set of fate-inducing genes, which require cooperative binding to the promoters of transcription factors activated by the different pathways (Fig 1.1B). (III) Finally, **crosstalk** between signalling pathways active in the same tissue at the same time can occur. Here signalling through one pathway can be inhibited or activated by signalling through a second pathway, changing the output of the first pathway and leading to different responses within the same cell (Fig 1.1C).

This thesis will focus on how the crosstalk interactions between signalling pathways affects cell fate. I will present this in the context of Notch and Wnt signalling pathways due to the increasing evidence that a complex functional relationship exists between both pathways during cell fate assignation.

**Figure 1.1: Mechanism of interactions between signalling pathways to generate cell diversity.**

A. **SEQUENTIAL**

B. **SIMULTANEOUS**

C. **CROSSTALK**

---

Figure 1.1: Mechanism of interactions between signalling pathways to generate cell diversity. A. Sequential diversity can be generated using the same set of signals in different order. B. Simultaneous activation of two signalling pathways can generate diversity by activating a new set of genes that require co-operative binding of transcription factors activated by the two pathways alone. C. Crosstalk diversity can be generated when two pathways are active at the same time and one exerts an effect over the other.
Wnt and Notch signalling pathways are highly conserved through all metazoans playing key roles in embryonic development and adult tissue maintenance [reviewed in: (Andersson et al., 2011; Bolós et al., 2007; Klaus and Birchmeier, 2008)]. Thorough analysis of both pathways, in several organisms and systems, has suggested that both pathways are closely intertwined [reviewed in: (Hayward et al., 2008; Hurlbut et al., 2007; Martinez-Arias et al., 2002; Muñoz-Descalzo and Martinez Arias, 2012)]. Both signalling pathways are often active in the same tissue at the same time, yet, frequently have opposing effects upon cell fate, suggesting the potential for interactions to occur. The first interaction between Wnt and Notch signalling pathways was described in 1994 in the context of the development and patterning of the wing of Drosophila (Couso and Martinez Arias, 1994).

In many cases, Wnt signalling has been shown to induce the expression of Notch ligands and hence activation of Notch signalling, giving an example of sequential interaction between the two pathways in the specification of cell fate (Fig 1.1A). For instance, mammalian epidermis is maintained through stem cells that differentiate into several lineages which constitute the hair follicle, interfollicular epidermis and sebaceous gland. An in vivo study has shown that in the adult epidermis Wnt/β-catenin signalling induces transcription of Jagged1, which then activates Notch to promote differentiation of hair follicle lineages (Estrach et al., 2006). Furthermore, Notch target genes Hes and Hey are up-regulated by β-catenin activation in the epidermis and expressed in the pre-cortex of the hair follicle where the lineage commitment occurs (Ambler and Watt, 2007). Deletion of Jagged1 results in hair follicles differentiating into cysts of interfollicular epidermis. In contrast, activation of Notch expands hair follicles and enlarges the sebaceous glands (Estrach et al., 2006). An earlier in vitro study has also shown that Wnt signalling induces the expression of Notch ligand, Delta1, within interfollicular stem cells. This inhibits Notch signalling within the stem cells through cis-inhibition, while promoting differentiation of neighbouring transit amplifying cells into keratinocytes at the edge of the clusters (Lowell et al., 2000).

Simultaneous activation of Wnt and Notch signalling pathways can also regulate the expression of fate-inducing genes through cooperative binding of promoters via transcription factors activated by the two pathways (Fig 1.1B). For example, a cooperative interaction of this type occurs between suppressor of Hairless (Su(H)) and Pangolin/DTcf on the promoter element that drives vestigial expression at the dorsal ventral boundary of Drosophila wing (Kim et al., 1996; Klein and Arias, 1999).
Examples of crosstalk between pathways have also been described (see Fig 1.1C), such as the association and phosphorylation of Notch1 and Notch2 intracellular domains by glycogen synthase kinase 3β (GSK3β), a component of the Wnt signalling cascade, modulating their ability to signal. However, this phosphorylation has opposing effects, stimulating Notch1 (Foltz et al., 2002) and inhibiting Notch2 (Espinosa et al., 2003). Notch2 phosphorylation by GSK3β inhibits Notch mediated transcription of the Hes1 promoter. This process can be reverted by the addition of Wnt1 which inhibits GSK3β dependent phosphorylation of Notch2, leading to increased activity of the Hes1 promoter (Espinosa et al., 2003). In contrast, inhibition of GSK3β shortens the half-life of Notch1 intracellular domain, so that GSK3β stimulates Notch1 signalling by protecting the intracellular domain from proteosomal degradation (Foltz et al., 2002).

1.2 Development, crosstalk and understanding of disease

The interactions between the developmental signalling pathways also play a key role in the aetiology of many diseases, including cancer (Andersson et al., 2011; Klaus and Birchmeier, 2008). As above, there are clear examples where the sequential and simultaneous activation of and crosstalk between the Notch and Wnt pathways is important.

Sequential activation of the Notch pathway downstream of the Wnt pathway is clearly seen in colorectal cancer. Nearly, all colorectal cancers are initiated by a mutation that activates the Wnt signalling pathway; in more than 85% of cases this is caused by the homozygous loss of APC, the remaining 10-15% of cases carry mutations in AXIN2 or β-catenin (Korinek et al., 1997; Liu et al., 2000; Morin et al., 1997; Rubinfeld et al., 1997). This increase in Wnt/β-catenin signalling triggers the expression of the Notch ligand Jagged1 (Rodilla et al., 2009). This leads to an increase in Notch signalling that stops goblet-cell differentiation and promotes tumour vasculogenesis, and in co-operation with Wnt signalling, induces cell proliferation (Fre et al., 2009; Rodilla et al., 2009).

The Wnt and Notch signalling pathways also co-operate to transform primary human breast epithelial cells (Ayyanan et al., 2006; Collu and Brennan, 2007). As in the colon, Wnt signalling leads to signalling through the Notch pathway, most likely by inducing the expression of Notch ligands. However, the two signalling pathways must co-operate to transform the breast epithelial cells, as activation of Notch alone by expressing an active form of Notch fails
to transform the cells and expression of a dominant negative form of a Notch ligand abrogates Wnt-mediated transformation (Ayyanan et al., 2006).

**Crosstalk** between Notch and Wnt signalling pathways can also be essential for tissue homeostasis after an adult disease. For example, it has been shown that after acute pancreatitis, Notch inhibits Wnt signalling to regulate pancreatic acinar regeneration (Siveke et al., 2008). Reporter assays demonstrated that in the pancreatic acinar tumour cell line 266-6, Notch inhibits β-catenin mediated transcriptional activation through a mechanism that requires Rbpj transcription. This inhibitory crosstalk was partially relieved in the presence of DAPT, a γ-secretase inhibitor that reduces Notch receptor activation.

Therefore, crosstalk points between signalling pathways could be excellent targets for novel therapeutics. Points of crosstalk have been specifically selected during evolution, within the milieu of signalling that occurs inside cells, to allow cross-regulation between signalling pathways without causing a signalling catastrophe. Therefore, by studying and mimicking conserved crosstalk mechanisms, we should be able to generate very precise therapeutic treatments.

### 1.3 Notch signalling, structure and features

The *notch* gene was first discovered in Drosophila in 1917 with the identification of a fly mutant with a haploinsufficient wing notching/nicking phenotype, which gave the gene its name (Metz and Bridges, 1917). The *NOTCH1* gene was identified 74 years later in humans as an oncogene that is disrupted by the t(7;9)(q34;q34.3) chromosomal translocation in T-cell leukaemia, leading to the expression of an activated form of Notch1 (Ellisen et al., 1991). The number of Notch genes varies from organism to organism. For example, in Drosophila, there is only one Notch gene, whereas in *Caenorhabditis elegans*, there are two Notch genes (*lin-12* and *glp-1*) and in humans and mice there are four (*Notch1, 2, 3, and 4*).

#### 1.3.1 Structure of the Notch receptor

The Notch gene encodes a single-pass transmembrane receptor protein that functions as a membrane bound transcription factor. The extracellular region is composed of a signal peptide, a tandem array of epidermal growth factor factor-like (EGF-like) repeats, and three
cysteine-rich repeats known as Lin-12-Notch repeats (LNR). The main characteristic of the extracellular domain are the EGF-like repeats since they are responsible for ligand binding and subsequent activation of the pathway (Nichols et al., 2007a; Rebay et al., 1991). The number of EGF-like repeats varies between species, ranging from 36 in flies and mammals to 11 in *C. elegans*. The LNR repeats prevent premature receptor activation by metalloprotease cleavage (Sanchez-Irizarry et al., 2004). The intracellular region consists of a Rbpj-associated molecule (RAM23) domain, for the binding of members of the CSL (CBF1 or Rbpj, Su(H), and Lag) family of transcription factors (Tamura et al., 1995), seven cdc10/ankyrin (ANK) repeats (Ehebauer et al., 2005) flanked by nuclear localisation sequences (NLS), a transcriptional activation domain (TAD), a polyglutamine sequence (OPA), and a proline, glutamine, serine, threonine-rich (PEST) domain that is involved in protein degradation (Fig 1.2). The main feature of the intracellular domain is the ANK repeats since they are the major site for protein-protein interactions.

1.3.2 Regulation of Notch by post-translational modifications

*O*-linked glycosylation is essential for Notch receptor activity in both flies and mammals (Okajima and Irvine, 2002), but a role for *O*-glycosylation has not yet been described in *C. elegans* (Haines and Irvine, 2003). The first sugar modifications are catalysed by *O*-Fut1 (POFUT1 in mammals), an *O*-fucosyltransferase which adds *O*-fucose to the Serine or Threonine residues of the EGF-like repeats (Haines and Irvine, 2003; Okajima and Irvine, 2002). In addition, *O*-Fut also acts as a chaperone to control the folding and transport of Notch from the endoplasmic reticulum to the cell membrane (Okajima et al., 2005). Consequently, *O*-Fut is essential for Notch receptor function. After the addition of the first fucose, other glycosyltransferases can act upon Notch, such as the Fringe family (Lunatic, Radical, and Manic Fringe in mammals and Fringe in Drosophila). Fringe enzymes catalyse the addition of \( \beta 1,3 N \)-acetylglucosamine to the first *O*-fucose moiety and the elongation of the polysaccharide chain (Moloney et al., 2000).

In the Drosophila wing, Fringe, promotes signalling by Delta as the addition of the sugar groups promotes Delta/Notch binding. On the other hand, Serrate/Notch signalling is inhibited as Fringe mediated modification of Notch disrupts the Serrate/Notch interaction (Haines and Irvine, 2003; Kato et al., 2010; Panin et al., 1997). However, in vertebrates due to the presence of several Notch receptors and ligand molecules, as well as three different Fringe homologues, the situation results in greater complexity (Kopan and Ilagan, 2009). The majority
of the data supports that vertebrate Fringe proteins will modify Notch signalling as their homologue does in Drosophila, promoting signalling from Delta and inhibiting Jagged ligand signalling, but it is not clear yet [reviewed in: (Fiuza and Arias, 2007)]. Recently a new O-glucosyltransferase was identified in Drosophila, Rumi (Acar et al., 2008; Fernandez-Valdivia et al., 2011). Rumi is able to transfer glucose to serine residues of the Notch EGF-like repeats with a consensus sequence C1-X-S-X-P-C2, regulating folding and trafficking of Notch to the cell membrane to allow signalling.

Figure 1.2: Structure of Drosophila and mammalian Notch receptor proteins. Drosophila Notch (dD) and mammalian Notch1 to Notch4 receptors. Notch receptors share a similar structure including: a tandem arrangement of Epidermal Growth Factor-like (EGF-like) repeats and three cysteine-rich LIN12-Notch repeats in the extracellular domain, and a Ram23 domain, two nuclear localising sequences (NLS) domains, seven ankyrin repeats (ANK), one transcriptional activation domain (TAD), one OPA domain and one proline, glutamine, serine, threonine-rich (PEST) domain in the intracellular domain. The N3 receptor has less EGF-like repeats and does not contain a TAD domain. The N4 receptor has fewer EGF-like repeats than N3, only contains one NLS, 5 ANK repeats and does not bear a TAD domain.

Interestingly, there is growing evidence to support that NICD is subject to various post-translational modifications including phosphorylation, ubiquitination, hydroxylation, and acetylation which will be briefly described (see review: (Kopan and Ilagan, 2009)). GSK3β binds NICD at the C-terminal of the ANK repeats and phosphorylates at Thr2068 and/or Ser2070, Thr2074, and Thr2093 (Espinosa et al., 2003; Foltz et al., 2002). E3 ubiquitin ligases, such as Numb or Deltex, ubiquitinate NICD (see section 1.2.7) (reviewed in: (Le Bras et al., 2011)). Also, SEL10 and Cdc4 proteins ubiquitinate NICD in the PEST domain (Tsunematsu et al., 2004). Hydroxylation of NICD is performed by the asparagine hydroxylase FIH-1 (factor inhibiting
hypoxia-inducible factor-1) at residues N1945 and N2012 in the ankyrin repeats (Coleman et al., 2007). Very recently, acetylation and deacetylation have been shown to modify Notch half-life (Guarani et al., 2011).

1.3.3 Canonical Notch ligands structure

Classical Notch ligands belong to the DSL (Delta, Serrate, and Lag-2) family of proteins. In Drosophila, there is only one Delta (Dl) and one Serrate ligand. In contrast, in mammals there are three Delta-like ligands: Delta-like 1 (Dll1), Delta-like 3 (Dll3), and Delta-like 4 (Dll4) and two homologues of Serrate: Jagged1 (Jag1) and Jagged2 (Jag2) (Kopan and Ilagan, 2009) (Fig 1.3).

DSL ligands are type 1 transmembrane proteins that have a similar extracellular arrangement. Within the extracellular domain (ECD) all ligands contain an N-terminal (NT) domain, a DSL domain, and a tandem array of EGF-like repeats. Serrate and its homologues also contain a cysteine-rich domain (Fleming, 1998) (Fig 1.3). The DSL domain and the first three EGF-like repeats are required for the binding of DSL ligands to Notch (Parks et al., 2006). Recently, a conserved DOS (Delta and OSM-11-like) motif has also been identified. The position of the DOS motif has been predicted in Drosophila, and vertebrate Notch ligands overlapping the first two EGF-like repeats that interact with EGF-like repeats 11 and 12 of Notch receptor (Fleming, 1998; Komatsu et al., 2008; Parks et al., 2006). Surprisingly, Dll3, Dll4 and C. elegans do not contain the DOS motif (Komatsu et al., 2008). The intracellular regions of Dl, Ser and Lag-2 contain several lysine residues and a C-terminal PDZ domain, which are required for ligand signalling and cytoskeleton interactions, respectively (Pintar et al., 2007).

In addition to the role of Notch activation through cell-cell interactions (trans-interactions), DSL ligands can also inhibit Notch signalling through cis-interactions when both are on the same cell, in a concentration dependant way (de Celis and Bray, 1997; Jacobsen et al., 1998; Klein and Arias, 1998; Sprinzak et al., 2010). Particularly, if the ligand levels exceed those of the receptor, the ligand will prevent signalling within the expressing cell through cis-inhibition, but will signal to neighbour cells.
1.3.4 Regulation of DSL ligands by post-translational modifications

The Notch DSL ligands undergo glycosylation in the same way the Notch receptors do, O- and N-linked glycans are added at conserved sequences within the EGF-repeats. However, only O-fucose and O-glucose have been shown to have an effect on signalling (Panin et al., 2002).

Modification of DSL ligands by RING finger-containing E3 ubiquitin ligases, Neuralized (Neur) and Mind bomb (Mib), regulates ligand signalling and cell-surface expression (Nichols et al., 2007b). Neur and Mib ligases ubiquitinate the intracellular domain of DSL ligands in order to enhance their endocytosis. In the absence of Neur or Mib, ligands accumulate at the surface of the cell but are inactive (Le Borgne et al., 2005). The role of each ligase in ubiquitination of Notch ligands is still controversial. However, a study performed in mammalian cells has suggested that Mib is responsible for DSL ligand endocytosis that activates Notch signalling, whereas Neur functions downstream, to control lysosomal degradation (Song et al., 2006). It is
possible that the different functional roles for Neur and Mib may be due to different ubiquitin states of the DSL ligands (mono vs. poly-ubiquitination).

### 1.3.5 Canonical Notch signalling pathway

Due to the transmembrane nature of the Notch receptor and the DSL ligands, cell-cell contact is required to trigger a signalling event. Notch is present at the cell surface as a heterodimer where the extracellular domain is linked non-covalently by a heterodimerization region to the juxtamembrane, transmembrane and intracellular domains (Blaumueller et al., 1997; Gordon et al., 2007). The heterodimerization process takes place during secretion of the Notch protein in the Golgi apparatus, where a Furin-like convertase processes Notch at the cleavage site 1 (S1) after the recognition sequence RQRR (Kopan et al., 1996; Logeat et al., 1998) (Fig 1.4). The role of the S1 cleavage is not well defined yet. In mammals, the S1 cleavage and the resulting structure were believed to be essential for cell surface expression of the Notch receptor (Blaumueller et al., 1997), and subsequent activation (Nichols et al., 2007a). However, neither Notch2 receptors (Gordon et al., 2009), nor Drosophila Notch (Kidd and Lieber, 2002) seem to require this process. Once on the cell surface, the EGF-like repeats of the Notch receptor can bind with the Delta-like or Jagged ligands that are expressed on the surface of a neighbouring signal-sending cell. This leads to a conformational change in the Notch receptor, exposing an extracellular cleavage site (S2 cleavage site) below the LNR repeats, between Ala1710 and Val1711, 13 amino acids outside the transmembrane domain (Brou et al., 2000; Mumm et al., 2000). Ectodomain shedding at this site is regulated by ADAM/TACE family of metalloproteases, (Gordon et al., 2007; Kovall and Blacklow, 2010), particularly, ADAM10 metalloprotease Kuzbanian (van Tetering et al., 2009).

Upon cleavage, the remaining membrane-tethered Notch, named the Notch extracellular truncation (NEXT), becomes susceptible to two more cleavages (S3 and S4) mediated by γ-secretase (De Strooper et al., 1999; Schroeter et al., 1998) (Fig 1.4); γ-secretase is a multi-subunit complex that comprises presenilin, nicastrin, presenilin enhancer 2 (Pen2), and anterior pharynx-defective 1 (Aph1) proteins (reviewed in: (Jorissen and De Strooper, 2010)). Both S3 and S4 cleavages occur in the middle of the Notch transmembrane domain, close to the cytoplasmic border (Okochi et al., 2002). The S3 cleavage site has been located between Gly1743 and Val1744 (Schroeter et al., 1998), whereas, the S4 cleavage site has been situated in the centre of four alanine residues, between Ala1731 and Ala1732 (Okochi et al., 2002). These cleavages lead to the secretion of a Notch β-peptide (Nβ) to the extracellular
space and the release of the Notch intracellular domain, known as NICD, from the membrane into the cytoplasm. Interestingly, there is variability in both the site and precision of the S3 cleavage, starting from Val1744 to Ser1747, so that different N-terminus NICD fragments can be generated (Tagami et al., 2008). The amino acid at the N-terminus affects stability, fragments with a N-terminal Ser have a shorter half-life than fragments with a N-terminal Val (Bachmair et al., 1986; Gonda et al., 1989), thus, Val seems to be a stabilising residue. Generation of N-terminal Val-NICD occurs more frequently at the plasma membrane, whereas, generation of a N-terminal Ser-NICD is more common on the endosomes (Tagami et al., 2008).

**Figure 1.4: Mammalian Notch signalling pathway.** Notch receptor is synthesised in the ER as a co-linear precursor which is cleaved by a Furin-like convertase at site 1 (S1) within the Golgi. Cleavage results in two non-covalently associated subunits expressed at the cell surface. Notch signalling is triggered by ligand binding to the EGF-like repeats, exposing a second cleavage site (S2) processed by ADAM metalloproteases, generating a Notch intermediate (NEXT). Two more cleavages occur at site 3 and 4 (S3 and S4) of the transmembrane domain by γ-secretases, releasing NICD. NICD migrates to the nucleus and binds to CSL transcription factors displacing co-repressors and recruiting activators, such as Mastermind.
The released NICD can directly translocate to the nucleus without interacting with a cascade of secondary messengers. In the nucleus, NICD interacts with the CSL family of transcription factors (CBF-1, C-promoter binding factor in humans; Rbpj, recombination binding protein for immunoglobulin kappa J region in mice; Su(H), Suppressor of Hairless in Drosophila; and Lag-1, Lin12 and Gip1 in C. elegans). Once the RAM and the ANK domains of NICD are bound to CSL they create a binding groove for Mastermind proteins (MAML) (Nam et al., 2007), which act as co-activators to initiate expression of target genes. The best characterised Notch target genes are the basic helix-loop-helix transcription factors *Hairy* and *Enhancer of Split* (*Hes*) and *Hes related with YRPW motif* (*Hey*) (Fischer and Gessler, 2007; Iso et al., 2003), nevertheless others involved in proliferation, like, c-Myc (Klinakis et al., 2006; Palomero et al., 2006; Weng et al., 2006) and *cyclin D1* (Jeffries et al., 2002; Joshi et al., 2009; Ronchini and Capobianco, 2001), or apoptosis, such as, *p21* (Rangarajan et al., 2001) can also be expressed depending on cell context (Fig 1.4). Interestingly, biochemical studies have shown that ANK-ANK contacts and Mastermind binding promote cooperative dimerization of this Notch transcriptional complex on the Hes1 promoter (Choi et al., 2012; Kovall and Blacklow, 2010; Nam et al., 2007). In the absence of Notch signalling, CSL functions as a transcriptional repressor by binding co-repressors, such as, Hairless in flies, and histone-deacetylase (HDAC), silencing mediator of retinoid and thyroid receptors (SMRT), SKIP, CBF1-interacting co-repressor (CIR), KyoT2, ETO, MTG16 (reviewed in: (Kovall and Blacklow, 2010)). In general, these repressors link CSL to the HDAC machinery of the nucleus.

1.3.6 Alternative Notch signalling pathways

There are numerous examples of Notch dependent biological events that do not require the canonical DSL ligands nor CSL transcription (see review: (Martinez-Arias et al., 2002). Strong evidence is derived from the study of *abruptex* (*Ax*) and *microchaetae defective* (*Mcd*) Notch alleles during neurogenesis in Drosophila (Brennan et al., 1999; Ramain et al., 2001). These mutants generate what appears to be a gain-of-function Notch phenotype, but it is not due to increased lateral inhibition caused by Su(H)-dependent signalling, additionally, these mutant receptors do not require canonical DSL ligands. The phenotype is, instead, dependent on Deltex and regulated by Shaggy (GSK3β in mammals) (Brennan et al., 1999; Ramain et al., 2001). Loss of function of Deltex in the presence of the Ax and Mcd Notch alleles promotes an excess of microchaetae, in contrast, overexpression of Deltex inhibits neurogenesis and flies do not develop microchaetae. Later studies in Drosophila, have
suggested that Deltex activates Notch signalling in a Su(H)-independent manner but that requires endocytic trafficking to late endosomes (Hori et al., 2004). These studies indicate that a CSL-independent Notch activation exists, however, the molecular mechanism of this pathway is still poorly defined. It is not clear yet, whether the Su(H)-independent mechanism in Drosophila is the same as in vertebrates, although, both seem to involve Deltex.

The first evidence of Rbpj-independent signalling in vertebrates was obtained from differentiation experiments with the myogenic cell line C2C12 (Kuroda et al., 1999; Shawber et al., 1996). Incubation of C2C12 cells, expressing a form of Notch1 that cannot be cleaved by Furin, with Jagged1 expressing cells failed to show activation of a Rbpj-dependent reporter gene, but prevented the differentiation of the cells into myotubes. The failure of the cells to differentiate suggests that signalling had been activated downstream of Notch. Since, several reports have described how NICD can interact with transcription factors other than Rbpj to regulate transcription in a Rbpj-independent fashion. For example, NICD can bind with Smad3 and Smad1, effectors of the transforming growth factor-beta TGF-β/BMP signalling pathway, to induce Hes1 expression in C2C12 cells (Blokzijl et al., 2003; Dahlqvist et al., 2003). Moreover, TGF-β signalling has been shown to induce epithelial-to-mesenchymal transition phenotypes, by transcription of Notch target genes Jagged and Hes1 through a Smad3-dependent event (Zavadil et al., 2004).

NICD can also interact with members of other signalling pathways, such as, the transcription factor HIF-1α to generate a non-canonical Notch signal. HIF-1α is involved in the hypoxia response, and under hypoxic conditions it associates with NICD to Notch promoters inducing the transcription of Notch target genes like Hey1 (Chen et al., 2007; Gustafsson et al., 2005). However, NICD can also bind to members of other signalling pathways to modulate their signalling, such as, repressing NF-κB (Wang et al., 2001), or acting as co-activator of LEF1 (Ross and Kadesch, 2001).

Deltex is a cytoplasmic adaptor E3 ubiquitin ligase that regulates Notch endocytosis independently of DSL ligands (Wilkin et al., 2008; Wilkin et al., 2004) by interacting with Notch ankyrin repeats (Diederich et al., 1994; Matsuno et al., 1995; Zweifel et al., 2003) and mono-ubiquitinating them. From analytical ultracentrifugation studies, it seems that ankyrin repeats 3 and 4 are “hot spots” for Deltex binding, but contact with repeat 5 at around Ala2026 is also likely to be important (Allgood and Barrick, 2011). Furthermore, substitution of this conserved Ala2026 with a valine, results in a Deltex loss-of-function phenotype in flies (Diederich et al.,
1994; Matsuno et al., 1995; Ramain et al., 2001). It has been shown that Notch can be activated by Deltex independently of the DSL ligands and Su(H), but in an endocytic-dependent manner (Hori et al., 2004) (Fig 1.5). However, Deltex can also activate NICD signalling through CSL-dependent transcription via the endosomal trafficking pathway (Wilkin et al., 2008; Wilkin et al., 2004) (see more detail in section 1.2.7 and Fig 1.5).

Deltex homologues have also been identified in mammals (Hu et al., 2003; Ordentlich et al., 1998), but there is no apparent homologue in C. elegans. Overexpression of Deltex in vertebrate cells can inhibit the function of basic helix-loop-helix transcription factors, such as E47 and mammalian achaete-scute homologue 1 (Mash1), in much the same way as membrane-tethered forms of Notch that cannot signal through CSL (Ordentlich et al., 1998; Yamamoto et al., 2001). Deltex and NICD forms that cannot signal through CSL are thought to regulate E47 by inhibiting JNK activity (Ordentlich et al., 1998) and Mash1 and sequestering the transcriptional co-activator CBP/p300 (Yamamoto et al., 2001) (Fig 1.5).

Finally, there are also some non-canonical ligands that have been described over the years. The main characteristic of the non-canonical ligands is that they lack the DSL domain required to interact with Notch. These can be divided into 3 main groups: (I) integral membrane bound, (II) glycosyl phosphatidylinositol GPI-linked membrane bound, and (III) secreted proteins. However, it is only members of the membrane bound ligands (I, and II) that have been described to activate Notch signalling in a Rbpj-independent but Deltex-dependent manner. Therefore, for the purpose of this Introduction I shall focus only on these.

Within the membrane bound non-canonical ligands, Delta-like 1 (Dlk-1) was one of the first reported non-canonical ligands (Laborda et al., 1993), which prevents adipogenesis (Wang et al., 2006). Although, Dlk-1 lacks a DSL domain the rest of the protein structure is very similar to the Delta-like canonical ligands. Due to the structure similarities, it has been suggested that Dlk-1 antagonises Notch signalling by competing with the Delta-like canonical ligands, however, the majority of the data suggests that Dlk-1 interacts with Notch in cis, inhibiting Notch signalling (Baladron et al., 2005; Bray et al., 2008). Another non-canonical ligand is Delta/Notch-like EGF-related receptor (DNER), which activates Notch signalling in trans to elicit glial morphological changes (Eiraku et al., 2002). These glial morphological changes have been linked to a CSL-independent Notch signalling caused by Deltex interaction with Notch and β-arrestin protein Kurz, a protein binding partner of Deltex (Mukherjee et al., 2005).
Figure 1.5: Possible mechanisms of ligand independent and dependent Notch signalling. Upon ligand binding, NICD is released to the cytoplasm where it can interact with Deltex (Dx) inhibiting JNK signalling. NICD/Dx complex also migrates to the nucleus, sequestering p300 to inhibit bHLH transcription factors such as E47 or Mash1. NICD can also translocate to the nucleus to activate CSL transcription factors, as in the canonical Notch signalling pathway. Note that Dx can also activate ligand-independent Notch proteolysis by binding full length Notch at the membrane, ubiquitinating it and inducing its dynamin-dependent endocytosis to the Rab5 positive early endosome (EE) and the Rab7 positive late endosome (LE). Dx can also retain Notch within the limiting membrane of the LE, which can then be activated by S3 cleavage. In contrast, Suppressor of Deltex (SuDx) promotes Notch internalization into internal vesicles of the multivesicular body (MVB), promoting its downregulation. From the EE there are a fast (Rab4) and slow (Rab 11) recycling routes. The route to the trans Golgi network is controlled by Rab 9. Note that there may be differences in the form of NICD that is cleaved from different cellular compartments which can affect NICD turnover.
Within the GPI-linked membrane-bound non canonical ligands there are F3/contactin1 and NB3/contactin6, neural cell recognition molecules. These non DSL ligands release NICD through γ-secretase cleavage to induce oligodendrocyte differentiation (Cui et al., 2004; Hu et al., 2003). However, dominant negative forms of Rbpj do not stop F3 and NB3 Notch activation, whereas, Deltex1 mutants do (Hu et al., 2003; Lu et al., 2008). Therefore, instead of a CSL-dependent activation these receptors trigger the Notch/Deltex signalling pathway to promote oligodendrocyte maturation.

### 1.3.7 Endocytosis and intracellular trafficking of Notch ligands and receptor

Endocytosis has been shown to be essential for ligand-dependent Notch signalling and activation, in both signalling-sending and signalling-receiving cells (Seugnet et al., 1997). Early studies in Drosophila embryos found that the ligand Delta was present in endocytic vesicles (Parks et al., 2006; Seugnet et al., 1997). A key signal to promote ligand endocytosis is ubiquitination of intracellular lysine residues by E3 ligases, but ubiquitination can also promote sorting, and/or degradation (Acconcia et al., 2009) (Fig 1.5). Both Neur and Mib ligases ubiquitinate DSL ligands in the signalling-sending cell in Drosophila and vertebrates, which can then be recognised by Epsin, a protein that promotes the curvature of the membrane to allow clathrin-coated invaginations, and endocytosed in a clathrin dependent manner (Windler and Bilder, 2010). Currently, there are two theories that explain how ligand internalization leads to successful ligand activation which, although different, they are not mutually exclusive: (I) the “ligand activation” model, and (II) the “pulling force” hypothesis. The first model is based on a Drosophila study where Epsin mutants did not affect Delta endocytosis, yet, Delta was unable to signal, suggesting that Delta needs to be ubiquitinated, endocytosed, and sorted before becoming active (Wang and Struhl, 2004). Additionally, studies in mammalian cell culture demonstrate that in signalling sending cells Rab11, a GTPase that regulates the recycling endosome, is important for ligand activation; since over expression of dominant negative forms of Rab11 promote recycling defects (Emery et al., 2005). Also, more recent studies show that during oogenesis dynamin-dependent ligand internalization in the signalling-sending germ cell is required (Windler and Bilder, 2010). Others also agree with the theory and propose that lipid rafts are involved in the activation of the ligands (Heuss et al., 2008); since Dll1 localizes to lipid rafts, whereas, ubiquitination defective ligands do not. Lipid rafts may help to cluster the ligands into a microenvironment and/or promote interactions with specific cofactors (Chitnis, 2006; Simons and Toomre, 2000). The second model suggests that the DSL ligands
bind the extracellular domain of Notch, and are then endocytosed. This generates a physical force that causes a conformational change in the Notch protein exposing the S2 cleavage site, which is normally protected by the LNR repeats (Gordon et al., 2007). Following the subsequent cleavage by ADAM proteases, the extracellular domain is trans-endocytosed into the signalling sending cell (Klueg and Muskavitch, 1999; Klueg et al., 1998; Nichols et al., 2007b; Parks et al., 2000). Cell culture studies where secreted ligands are cross-linked or immobilised also support the “pulling force” theory, since the tension or force generated between the ligand and the extracellular domain of the receptor is necessary for Notch activation (Varnum-Finney et al., 2000).

During canonical Notch signalling endocytosis mediated by Dynamin, Rab5, and Avl proteins is required for Notch activation in signal-receiving cells (Lu and Bilder, 2005; Vaccari et al., 2008) (Fig 1.5). However, since the ligand binding and the S2 cleavage happen at the cell surface, to date, the exact step in which Notch S3 cleavage and signalling activation require endocytosis still remains unclear. Some studies support that endocytosis is required for S3 cleavage to happen, as γ-secretase is more active in acidic environments, such as the one found in the limiting membrane of the endosomes and lysosomes (Pasternak et al., 2003), whereas, other studies support that the S3 cleavage can occur without endocytosis (Tagami et al., 2008) (Fig 1.5).

Different observations support the requirement of the S3 cleavage. The first evidence was obtained from Drosophila shibire mutants which exhibited a neurogenic phenotype that resembled a loss-of-function Notch phenotype (Poodry et al., 1973; Seugnet et al., 1997). The shibire gene encodes for the Drosophila homologue of the mammalian Dynamin, a GTPase required for pinching the Clathrin coated vesicles from the membrane during endocytosis (Chen et al., 1991). Later it was shown that γ-secretase has a higher activation in acidic environments like in the endosomes, suggesting that S3 cleavage is more efficient at lower pH. (Pasternak et al., 2003). Furthermore, defects in endosome acidification proteins lead to accumulation of Notch in the endosomes. Additionally, mono-ubiquitination of Notch intracellular domain has been shown to be essential for endocytosis and γ-secretase-mediated activation to occur (Gupta-Rossi et al., 2004). The strongest evidence comes from one study in Drosophila that has used Rab5 and Avl mutants to impair entry to the early endosome (Vaccari et al., 2008). This study has shown that γ-secretase cleavage and signalling activation of Notch are reduced in Rab5 and Avl mutants, since Notch accumulates at the cell surface and there is hardly any NICD production, whereas, cleavage and activation are enhanced in mutants that
increase endosomal retention. In contrast, mammalian cell culture studies show that active γ-secretase complex can be purified from the plasma membrane, where it processes Notch (Chyung et al., 2005). As well as, a study that shows that γ-secretase has variability in both the site and precision of the S3 cleavage, generating a N-terminus Val-NICD more frequently at the plasma membrane, and a N-terminus Ser-NICD on the endosomes (Tagami et al., 2008). The amino acid at the N-terminus affects stability, and seems that Val-NICD (refer to as the original NICD) is the most stable and therefore, activates signalling. These data argues against the idea that endocytosis is essential to promote S3 cleavage, and supports that γ-secretase cleaves Notch at the plasma membrane. Therefore, it is still controversial whether S3 cleavage requires endocytosis or not. However, the controversy may be due to the different model systems used (Drosophila versus mammalian cell culture), and considering the strict dosage dependence of Notch signalling depending on the context, γ-secretase may play a key role promoting cleavage at different locations depending on the environment.

Inactive Notch receptors present in the surface of the cell are constantly endocytosed and recycled back to the membrane (McGill et al., 2009) or degraded in the lysosome (Jehn et al., 2002) (Fig 1.5). A balance between different E3 ubiquitin ligases including Deltex, and two HECT domain E3 ligases: Suppressor of deltex (Su (dx)) and DNedd4, can modulate Notch by altering its sorting through the endosomes (Wilkin et al., 2008; Wilkin et al., 2004). Deltex ubiquitinates and directs full length Notch at the membrane to an endocytic pathway with two possible outcomes: either Notch is sent to the endosomal trafficking pathway and degraded in the lysosome, or NICD is released of the limiting membrane of the multivesicular body by Presenilin-mediated cleavage (Pasternak et al., 2003), activating Notch signalling in a ligand independent manner (Wilkin et al., 2008). Moreover, very recently Deltex has been shown to be required not only for the incorporation of Notch from the membrane into endocytic vesicles, but also for the transport of Notch from the early endosomes to the multivesicular bodies and then to the lysosomes (Yamada et al., 2011).

The Notch antagonist Numb, which has two homologues in mammals: Numb and Numb-like, inhibits Notch signalling by sorting Notch through the late endosomes for degradation (McGill et al., 2009). Numb is an adaptor protein, therefore, it requires cooperation with Nedd4 family of HECT domain E3 ubiquitin ligase Itch (Su(dx) homologue) in order to ubiquitinate Notch. Furthermore, McGill and colleagues 2009, demonstrated that Drosophila Numb mutants that do not bind with Itch, fail to promote Notch degradation. Recently, it has been shown that Notch receptors can be differentially affected by Numb, with
Numb regulating Notch1 signalling but not Notch3 activity during myogenesis (Beres et al., 2011).

1.4 Nuts and bolts of Wnt signalling

The Wnt1 gene was first discovered in 1982 (Nusse and Varmus, 1982) as a proto-oncogene when the integration of the mouse mammary tumour virus (MMTV) in adjacent DNA induced its over expression leading to mammary gland carcinomas; the Wnt1 gene was then called integration 1 (Int1). A few years later, it was discovered that the Drosophila gene Wingless (wg), which was first described in 1973 as mutant phenotype where flies lacked wings but is, probably, best known for its role in segment polarity during embryonic development, encodes the homologue of mammalian Int1 (Rijsewijk et al., 1987; Sharma, 1973). The current term, Wnt, is an amalgam of wg and Int1 (Nusse et al., 1991). To date, there are 19 mammalian homologues of Wnt1, which can be grouped into 12 subfamilies (reviewed in: (van Amerongen and Nusse, 2009)). However, Wnt proteins are found in almost all metazoans, from sea anemones to humans, meaning that the Wnt gene appeared early in evolution (Guder et al., 2006; Logan and Nusse, 2004). Wnts are also highly conserved proteins, as they are essential for the development of multicellular animals (reviewed in: (Clevers, 2006; van Amerongen and Nusse, 2009)). The first observation that Wnt signalling was shared between flies and vertebrates came from the formation of a double body axis in Xenopus after injection of mouse Wnt1 mRNA, ventrally, in the blastomeres of Xenopus embryos, at 4-cell stage (McMahon and Moon, 1989). Furthermore, this provided a fast and relatively simple assay to study the Wnt signalling pathway in vertebrates.

1.4.1 Structure of Wnt ligands and secretion

Wnt ligands are a large family of secreted hydrophobic glycoproteins that are involved in intracellular signalling during metazoan embryonic development and adult tissue homeostasis (Logan and Nusse, 2004). It is now well known that Wnt proteins take part in many processes, such as cell fate decisions, patterning of the embryo, morphogenesis, stem cell pluripotency, diseases such as Alzheimer, and tumourigenesis (for review see: (De Ferrari and Inestrosa, 2000; Reya and Clevers, 2005; van Amerongen and Nusse, 2009)). Little is known about Wnt protein structure 25 years after the cloning of Wnt1 gene, nevertheless, Wnt ligands share several characteristics including: 23 conserved cysteine residues, a N-terminal signal peptide and several potential N-glycosylation and palmitoylation sites.
(Miller, 2002). The highly conserved pattern of cysteine residues suggests that they are involved in the correct folding of the protein through disulfide bonds (Mason et al., 1992).

Following synthesis, Wnt proteins are subjected to glycosylation and lipid modification. These modifications suggest that both processing and secretion of Wnt proteins are highly regulated events. Glycosylation of Wnt proteins might be required for targeting the molecule to the appropriate exocytic route (Tanaka et al., 2002). The lipid modification entails the attachment of two palmitate moieties, one on the first conserved cysteine residue of the Wnt protein (referred to as C77, as found in mouse Wnt3a) (Willert et al., 2003), and another at a Ser209 (referred to as S209, as found in mouse Wnt3a) (Takada et al., 2006). Palmitoylation seems to be essential for Wnt function (Willert et al., 2003), since the addition of the fatty acids causes the insolubility of Wnts, and it may help to anchor Wnts to the plasma membrane by inserting them into the lipid bilayer (Port and Basler, 2010). Porcupine, a membrane bound O-acetyltransferase, is thought to be involved in the lipid modification of Wnt proteins in the endoplasmic reticulum (Kadowaki et al., 1996; Zhai et al., 2004). Interestingly, Porcupine has also been shown to stimulate N-glycosilation by anchoring Wnts to the endoplasmic reticulum, possibly via acetylation (Tanaka et al., 2002).

A gene essential for Wnt secretion was discovered by two different groups in 2006, wntless (wls) (Banziger et al., 2006), also known as evenness interrupted (evi) (Bartscherer et al., 2006). Wntless is a seven-pass transmembrane protein, present in the Golgi where it physically binds with Wnt (Banziger et al., 2006). Wntless is conserved from worms to humans (Banziger et al., 2006; Bartscherer et al., 2006; Fu et al., 2009; Goodman et al., 2006; Kim et al., 2009). The absence of wntless resembles a Wnt loss-of-function phenotype, cells fail to properly secrete and release Wnt accumulating Wnt proteins in the secretory pathway, mainly in the Golgi (Banziger et al., 2006). Furthermore, no other secreted proteins was found to be affected by wntless loss-of-function, therefore, showing high specificity (for a review see: (Port and Basler, 2010)).

1.4.2 Wnt receptors at glance

Wnt signalling is initiated upon ligand binding to the cell surface receptors, which include Frizzled, LRP5/6 (LDL-receptor related protein 5 and 6, homologues of Arrow in Drosophila), ROR and RYK.
Frizzled (Fzd) receptors are proteins that contain seven transmembrane domains with an extracellular cysteine-rich domain (CRD) to which Wnt can bind (Bhanot et al., 1996) (Fig 1.6). There are at least ten different Frizzled genes in vertebrates, four in Drosophila and three in C. elegans (Wang et al., 1996). Frizzled proteins are localised at the cell membrane; however, it has been suggested in Drosophila that Frizzleds are internalised to regulate the extracellular levels of Wnt (Chen et al., 2003; Dubois et al., 2001). After Fzd-mediated endocytosis, endocytosed Wingless is targeted for lysosomal degradation, this regulates the formation of extracellular Wingless morphogen gradients. Frizzleds function in three different Wnt signalling pathways: the canonical Wnt/β-catenin pathway, the planar cell polarity (PCP) pathway and the Wnt/Ca\textsuperscript{2+} pathway.

LRP5 and LRP6, Arrow in Drosophila, are single-pass transmembrane proteins (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). The structure of LRP5/6 consist of a signal peptide, 24 YWTD motifs in groups of six, spaced by four EGF repeats, three low density

**Figure 1.6: Structure of Frizzled receptor.** Frizzled proteins contain 7 transmembrane domains (green) bridged by a variable domain (yellow), an N-terminal cysteine-rich domain (pink) for Wnt binding, and a short cytosolic C-terminal domain (orange) that interacts with Dishevelled upon Wnt binding. Representation of the receptor within the membrane, each colour corresponds to the described domains.

**Figure 1.7: Structure of LRP/Arrow receptor.** Receptors contain a N-terminal signal peptide (yellow), four EGF repeats (E1 to E4, green) separating the YWTD motifs, three LDLR repeats (red) and a cytoplasmic domain (blue).
lipoprotein receptor (LDLR) repeats, a transmembrane domain and a 200 amino acid cytoplasmic domain containing four PPS/TP repeats (Fig 1.7). There is evidence that Wnt can form a ternary signalling complex bridging between Frizzled and LRP5/6 which activates Wnt/β-catenin signalling.

Figure 1.8: Structure of Ror2 receptor. The extracellular domain contains an immunoglobulin domain (blue), a cysteine-rich domain (CRD) (green) and a juxta-membrane Kringle domain (Kr) (yellow). The intracellular domain includes a tyrosine kinase domain (red).

ROR are single-pass conserved RTKs characterised by the presence of intracellular tyrosine kinase domains and by an extracellular Frizzled-like CRD domain (for binding to Wnt), an immunoglobulin domain and a juxta-membrane Kringle domain (Forrester, 2002) (Fig 1.8). Most vertebrate species bear two Ror genes (Ror1 and Ror2) (Forrester, 2002). However, signalling pathways downstream of this receptor are not well understood. Ror2 has been involved in activation of both canonical (Wnt/β-catenin) (Billiard et al., 2005; Winkel et al., 2008) and non canonical (Wnt5a/JNK) (Oishi et al., 2003) Wnt pathways, including the planar cell polarity (PCP) pathway in Xenopus (Schambony and Wedlich, 2007).

Figure 1.9: Structure of Ryk receptor. Ryk receptor is composed by a glycosylated extracellular domain with homology to the Wnt inhibitory factor-1 (WIF-1) domain; a transmembrane domain with tandem cysteine residues; and an intracellular serine-threonine rich domain and a catalytically inactive RTK-like domain.

Ryk family members contain a glycosylated extracellular domain (smaller than those of other RTKs) with significant homology to the N-terminal domain of the Wnt inhibitory factor-1
(WIF-1) (Halford and Stacker, 2001) (Fig 1.9). The WIF-1 motif is required for Wnt binding. Ryk single pass transmembrane domain contains tandem cysteine residues, and the intracellular domain includes a catalytically inactive receptor tyrosine kinase (RTK)-like domain. Currently, the intracellular pathway activated by Wnt/Ryk is unclear with studies suggesting that both the canonical Wnt/β-catenin and the PCP pathways can be activated (Fradkin et al., 2010). However, some Ryk mutant phenotypes resemble a Wnt loss-of-function phenotype, suggesting that in that context Ryk may be signalling through canonical Wnt pathway and vice versa.

### 1.4.3 Canonical Wnt/β-catenin signalling pathway

A canonical Wnt signalling pathway has been identified in vertebrate and invertebrate model systems (Fig1.10) which regulates the cytosolic and nuclear levels of β-catenin (Armadillo in Drosophila).

In the absence of Wnt ligands, the newly synthesized β-catenin that is free in the cytosol is processed by a destruction complex (Fig 1.10A). This destruction complex is formed by the scaffolding proteins Axin and APC (Hart et al., 1998; Kishida et al., 1998), and the kinases GSK3β (Shaggy in Drosophila) (Yost et al., 1996) and Casein kinase 1 (CK1) (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002). The limiting component within this destruction complex is Axin, as it is the least abundant member, but interacts with all the other components. Thus, it has been suggested that the number of destruction complexes present in the cell may be dependent on the levels of Axin (Lee et al., 2003).

Once β-catenin is bound to the destruction complex, it is initially phosphorylated by CK1α, at Ser45 in the mouse protein, to generate a binding site for GSK3β which subsequently phosphorylates three further N-terminal Ser/Thr residues (Ser33, Ser37 and Thr41). Phosphorylated β-catenin interacts with the E3 ubiquitin ligase β-transducing repeat containing protein (β-TrCP) which targets it for proteosomal degradation (Aberle et al., 1997; Latres et al., 1999). In this way the cytoplasmic concentration of β-catenin is kept low. In unstimulated cells, most of the endogenous β-catenin is found bound to E-cadherin, α-catenin and to the cytoskeleton, regulating cell-cell adhesion (Heuberger and Birchmeier, 2010; Peifer et al., 1992). In the nucleus, in the absence of β-catenin, the TCF/LEF family of transcription factors
act as transcriptional repressors (Brannon et al., 1997) by binding to Groucho proteins (Cavallo et al., 1998).

Figure 1.10: Canonical Wnt/β-catenin signalling pathway. A. In the absence of Wnt ligand, β-catenin is recruited by the Axin destruction complex, phosphorylated and targeted for degradation. B. Extracellular Wnt binds to Fz and LRPS/6 on the cell membrane to activate signalling. Subsequently, Dvl inactivates the destruction complex and β-catenin accumulates in the cytosol. This allows translocation of β-catenin to the nucleus where it activates transcription of target genes upon binding to LEF/TCF transcription factors.

In the presence of Wnt ligands, a receptor complex containing Frizzled and LRPS/6 proteins is formed at the plasma membrane (Fig 1.10B). This induces the phosphorylation of the conserved PPS/TP repeats within the intracellular domain of LRPS/6 by GSK3β, priming a second phosphorylation by CK1α on a flanking serine residue. Subsequently, both Dishevelled (Dvl, Dsh in Drosophila) and Axin are recruited to the membrane, with Dvl interacting with the C-terminal tail of the Frizzled protein and Axin with the hyperphosphorylated LRPS/6 forming an intracellular bridging complex (reviewed in: (van Amerongen and Nusse, 2009)). This sequesters the limiting amount of Axin away from the destruction complex allowing cytoplasmic β-catenin/Armadillo to accumulate quickly. Recently, a new mechanism for Wnt
signalling activation/regulation has been proposed. This mechanism involves the sequestration of GSK3β from the cytosol into multivesicular bodies, as well as, the Wnt receptor complex including: phosphorylated LRP6, phosphorylated β-catenin, Dvl, Axin, and APC (Taelman et al., 2010). Upon stabilization, β-catenin translocates and accumulates in the nucleus (Tolwinski and Wieschaus, 2004a; Tolwinski and Wieschaus, 2004b) where it binds with LEF/TCF family of transcription factors (Molenaar et al., 1996; van de Wetering et al., 1997), physically displaces Groucho (Daniels and Weis, 2005), and recruits transcriptional co-activators, like Pygopus or Legless, (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002), leading to the expression of specific target genes, such as Axin2 and c-Myc (He et al., 1998; Jho et al., 2002).

1.4.4 Non-canonical Wnt signalling pathways

Wnt proteins have also been proposed to activate several other downstream signalling pathways. The best characterised of these non-canonical Wnt pathways are: (I) the planar cell polarity pathway, identified in Drosophila (Adler, 1992; Vinson and Adler, 1987), and (II) the Wnt/calcium pathway, first described in vertebrates (Kühl et al., 2000).

Planar cell polarity signalling refers to the mechanism(s) responsible for organising groups of cells within the plane of the epithelium, with a similar orientation, perpendicular to the apical-basal axis of the cell (for a review see: (Wansleeben and Meijlink, 2011; Wu and Mlodzik, 2009)). In Drosophila, planar cell polarity controls the organization of sensory bristles on the cuticle, photoreceptors in the eye, and hairs on the wing (Goodrich and Strutt, 2011). Planar cell polarity has also been described in vertebrates where it regulates development features, such as convergent extension (CE) (Roszko et al., 2009), neural tube closure, inner ear development (Rida and Chen, 2009), hair orientation in mammals and ciliogenesis (for review see: (Wansleeben and Meijlink, 2011)).

The Wnt/calcium pathway appears to be activated by specific combinations of different Wnts and Frizzled receptors (Kohn and Moon, 2005). Upon receptor stimulation, release of intracellular calcium is a rapid event which activates different calcium dependent enzymes and eventually transcriptional activation (reviewed in: (Kestler and Kühl, 2008)). Intracellular calcium release is required for body plan specification; for instance, in Xenopus embryos dominant negative Wnt11 or dominant negative forms of calcium enzymes promote dorsalization (Kuhl et al., 2000). Calcium signalling has also been involved in the development
of myoblasts (Anakwe et al., 2003) and in slow muscle fiber formation in adult muscles (Naya et al., 2000), and in heart development (Kuhl, 2004).

However, these alternative Wnt signalling pathways are not the main focus of this thesis and therefore, they will not be discussed further.

1.5 Importance of Notch and Wnt signalling pathways in development and disease

Wnt and Notch are crucial members of the signalling network that controls gene expression in embryonic development, adult tissue homeostasis, and disease (Andersson et al., 2011; Clevers, 2006). Both pathways are involved in processes, such as control of proliferation, apoptosis, expression of cell fate determination genes, epithelial to mesenchymal transition (Klaus and Birchmeier, 2008). Additionally, Notch and Wnt signalling pathways have a role in stem cell self-renewal and differentiation (Liu et al., 2010; Reya and Clevers, 2005). Brief examples of this will be given below.

Both pathways play a very important role in developmental patterning. One of the best characterized functions of Notch in development is lateral inhibition, a process which generates a spaced pattern whereby a cell adopts a specific fate preventing the neighbour cells from acquiring the same fate (Bray, 1998) (Fig 1.11). This process is well characterised during the development of the peripheral nervous system of Drosophila (reviewed in: (Gomez-Skarmeta et al., 2003; Munoz-Descalzo et al., 2012)).

In Drosophila, proneural clusters are defined over a few days (reviewed in: (Hayward et al., 2008)). Initially, a group of ectodermal cells acquire the potential to become neural as Wnt signalling promotes the spatially localised expression of the proneural genes from the achaete and scute complex (AS-C) (Fig 1.11). AS-C expression is mosaic over several hours within the cluster of cells, here the cells are in a “transition state” of Notch-OFF/Wnt-ON to Wnt-OFF/Notch-ON. Then it resolves such that only one cell increases the levels of AS-C expression to become a sensory organ precursor, which will form a sensory bristle in the thorax of the flies. This sensory organ precursor uses Notch signalling to suppress the neural potential of the surrounding cells through lateral inhibition (Hartenstein and Posakony, 1990). Lateral inhibition requires Notch-dependent Su(H) activation and expression of bHLH genes from the Hairy and enhancer of split complex (E(spl)C). This process continues with a series of
asymmetric cell divisions on the sensory organ precursor (see Fig 1.13), E(spl)C inhibits AS-C expression in the rest of the cells of the proneural cluster which become epidermal again (Fig 1.11). The determining factor in this process is the Notch:Wingless signalling ratio. A high ratio promotes Notch signalling and favours the epidermal fate through lateral inhibition, whereas, a low ratio favours Wingless signalling biasing the cells towards a sensory organ precursor fate (Munoz-Descalzo et al., 2012). Therefore, proneural clusters are maps of probabilities that progress from a transition state to a definitive fate as sensory organ precursor or they revert to epidermal.

One of the best characterized functions of Wnt in development is to act as a morphogen, regulating gene expression in a concentration dependent manner (Fig 1.12). This is a well studied process during wing margin development in Drosophila (Cadigan, 2002). Initially, Notch signalling leads to the formation of the dorso-ventral (DV) boundary of the wing, where the asymmetrically localised Notch ligands Ser (dorsally) and DI (ventrally) activate the expression of wingless in a stripe of two cells of width. Subsequently, interactions
between neighbour cells establish an organizer centre that controls growth and cell fate along the DV axis of the wing. *Wingless* generates a negative feedback loop that restricts its own expression to the dorso-ventral boundary strip. Additionally, as Ser/Dl are Wingless targets, Wingless contributes to increase Ser/Dl activation, which in turn maintain *wingless* expression. Later, Wingless protein, expressed by Notch at the wing margin, forms a concentration gradient that is highest at the wing margin and falls across both the dorsal and the ventral surfaces of the wing. This gradient diffuses along the disc and functions as a morphogen inducing *senseless* and sensory bristle development at high concentrations, *distalless* and distal limb formation at moderate concentrations and *vestigial* and wing blade formation at low concentrations (Neumann and Cohen 1997) (Fig 1.12). Consequently, the Wingless gradient can pattern the developing wing in Drosophila.

![Diagram](image)

**Figure 1.12: Wingless as a morphogen during wing margin development.** During the development of the wing in Drosophila, the spatially localised Notch ligands Ser (dorsal) and Dl (ventral) in the wing disc, activate the expression of Wingless leading to the formation of the dorso-ventral (DV) boundary. Later on, wg protein, expressed by Notch at the wing margin, forms a concentration gradient that diffuses along the disc and functions as a morphogen. Wingless induces expression of senseless at high concentrations (orange), distalless at moderate concentrations (coral) and vestigial at low concentrations (red).

Notch is also involved in **asymmetric cell fate assignation**, which relies on the localisation of a cell fate determinant to one of the two daughter cells. Asymmetric cell division has been well studied during mechanosensory organ development in Drosophila (Fig 1.13).
Chapter 1: General Introduction

Here during division of a sensory organ precursor cell, Numb and Neuralized are asymmetrically localised to the anterior pole of the cell, determining the identity of the daughter cells (pIIa and pIIb) (Le Borgne and Schweisguth, 2003; Rhyu et al., 1994). Consequently, the posterior daughter cells (pIIa) receives Notch signal and differentiates in a different manner to the anterior daughter cell (pIIb) which contains Neuralized and Numb. The presence of Sara-endosomes in the pIIa cell promotes Notch signalling activation in this cell (Coumailleau et al., 2009). Sara-endosomes are PI3P-containing endosomes to which Sara, an adaptor protein characterised by the presence of a FYVE domain, localises to. In the pIIb cell Neur promotes the endocytosis of Delta, which is at the membrane to interact and activate Notch in the pIIa cell (Emery et al., 2005), generating a unidirectional Notch signalling (Le Borgne and Schweisguth, 2003). Additionally, in the pIIb cell Numb promotes endocytosis of Sanpodo, Notch, and Notch/Sanpodo complexes from the membrane (Couturier et al., 2012; Hutterer and Knoblich, 2005). In contrast, in the pIIa cell, where Numb is absent, Sanpodo is present at the membrane positively regulating Notch signalling through an unknown mechanism (Babaoglan et al., 2009). Eventually, the pIIa cell gives rise to a socket and a shaft cell, and the pIIb cell produces a glial and a pIIb cell (Fig 1.13). This pIIb cell divides once more to generate a neuronal cell and a sheath cell (Wang and Chia, 2005) (Fig 1.13). Therefore, in this context, the endocytic pathway is used to restrict and regulate Delta and Notch proteins to achieve precise cell fates via unidirectional Notch signalling.

Wnt signalling can also specify cell fates, for example in neural crest formation (Wu et al., 2003). Wnt signalling stimulates melanophore differentiation in medial crest cells and, therefore, promotes the diversity of neural crest cell fates (Dorsky et al. 1998). In zebrafish, neural crest cells that are near to domains of Wnt expression mainly produce pigment, whereas the cells that are more distant differentiate into neurons and glia (Dorsky et al., 2000). Wnt cell fate specification occurs by the direct expression of nacre, a gene that specifically drives tissue-specific expression of pigments.

Notch is involved in a series of developmental disorders, including cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). CADASIL is an inherited degenerative vascular disease caused by mutations in the Notch3 gene, which leads to aberrant vessel homeostasis and ultimately to strokes (Joutel et al., 1996). CADASIL is one of the most common hereditary cause of stroke and vascular dementia in adults, after spontaneous stroke (Herve and Chabriat, 2010). It is characterised by progressive vascular smooth muscle cell (VSMC) degeneration and by the accumulation of specific granular
osmiophilic deposits. The majority of the cases arise from missense point mutations in the EGF-like repeats of the Notch3 protein, leading to the addition or loss of a cysteine. This mutation favours the accumulation of Notch extracellular domain at the cytoplasmic membrane of VSMCs (Joutel et al., 2000). A recent study has suggested a molecular mechanism for the processing of mutated Notch3 proteins which involves aggregation between the mutated Notch3 proteins and retention in the endoplasmic reticulum. This results in decreased cell growth and increased sensitivity to other stresses (Takahashi et al., 2010).

Finally, another recent study has suggested that transendocytosis, the endocytosis of the Notch extracellular domain into ligand expressing cells, is disturbed in CADASIL patients (Watanabe-Hosomi et al., 2012). This contributes to the pathogenesis of the disease, as degradation of the mutant Notch3 protein is impaired.

Abnormalities in Wnt signalling also lead to developmental disorders, including altered bone mass. In osteoblasts, Wnt activation leads to bone formation. Bone formation is prevented when the Dickkopf proteins bind to LRP5 causing its internalisation. This process blocks Wnt signalling as it removes an essential receptor protein. High-bone-mass syndrome is an autosomal dominant disease that can occur due to a missense mutation in LRP5 (Boyden et al., 2002; Little et al., 2002). A conserved glycine at residue 171 of LRP5 is replaced by a valine preventing binding of LRP5 with Dickkopf (Boyden et al., 2002; Little et al., 2002). As a result, individuals have increased Wnt signalling and, therefore, bone formation (Levasseur et al., 2005). However, a recent study, using microarray and conditional knock down mice, has suggested that LRP5 influences bone development by promoting the secretion of serotonin from gut cells rather than acting on osteoblasts directly (Yadav and Ducy, 2010).

Unregulated Notch signalling has been implicated in several human cancers (for review see: (Koch and Radtke, 2007)). The clearest example is T cell acute lymphoblastic leukaemia (T-ALL), an aggressive form of leukaemia that affects children and teenagers. More than 50% of T-ALL cases carry an activating mutation in the Notch1 gene (Aster et al., 2008; Weng et al., 2004). Also, a small number of cases present a chromosomal translocation that fuses the 3’ portion of Notch1 to the T cell receptor Jβ locus, leading to the expression of a truncated but constitutively active Notch protein (Ellisen et al., 1991). Notch controls leukemic cell growth increasing the expression of proliferative target genes like c-Myc but mainly by upregulating the PI3K-AKT pathway via Hes1 downregulating the expression of PTEN (Palomero et al., 2008; Palomero et al., 2006). In T-ALL cases where there is a mutational loss of PTEN, there is a constitutive activation of AKT and the crosstalk between Notch and PI3K-AKT pathway is
blocked; in this cases, Notch still contributes to leukemic growth via expression of c-Myc (Medyouf et al., 2009; Palomero et al., 2008; Palomero et al., 2006) and insulin-like growth factor 1 receptor (IGF1R) (Medyouf et al., 2011).

One of the most well studied cancers caused by aberrations in the Wnt signalling pathway is colorectal cancer. In more than 85% of spontaneous cases both alleles of the **APC** gene are lost. Also the hereditary syndrome familial adenomatous polyposis (FAP) is caused by

---

Figure 1.13: Notch binary cell fate assignment during mechanosensory organ development in Drosophila. Once the sensory organ precursor has been specified, the following divisions will produce the cell types required for the sensory organ to form. During mitosis of the sensory organ precursor the cell fate determinants Numb and Neuralized (blue) are asymmetrically localised to the anterior pole of the cell. Upon cytokinesis Sara-containing endosomes (red) are sorted to the posterior cell (pall) and Numb and Neuralized are inherited by the anterior cell (pbII). This asymmetric segregation of endocytic components influences Notch signalling activation between the pall and pbII cells. Sequential asymmetric fate choices give rise to the cell types required to make the sensory bristle: socket, shaft, sheath, neuron, and glia.
the inheritance of one defective APC allele (Kinzler et al., 1991; Nishisho et al., 1991). Mutations in APC tumour suppressor gene generally lead to inappropriate stabilisation of β-catenin (Munemitsu et al., 1995; Rubinfeld et al., 1993) which continuously activates genes associated with cell proliferation, such as c-Myc and Cyclin D1, in epithelial cells. These proliferative cells stay in the crypt and do not migrate to the villus leading to colon cancer (van de Wetering et al., 2002). In the remaining 10-15% of colorectal cancers that lack APC mutations, genetic changes in Axin2 and Ctnnb1 have been described (Lammi et al., 2004). Furthermore, Wnt pathway activating mutations are not restricted to intestine cancer, mutations in Axin have also been found in liver cancers, and oncogeneic Ctnnb1 mutations appear in several solid tumours (for review see: (Clevers, 2006; Reya and Clevers, 2005)).

The evolutionary conserved Notch and Wnt signalling pathways play a critical role in the self-renewal, proliferation and differentiation of embryonic and adult stem cells in tissue assembly and maintenance (reviewed in: (Liu et al., 2010; Munoz-DescaIzo et al., 2012; Wend et al., 2010)). Here we shall discuss two examples (intestine and skin) where Notch and Wnt signalling pathways cooperate to promote stem cell self renewal or differentiation. However, both signalling pathways have roles in numerous other tissues, including the haematopoietic system, nervous system, and mammary gland.

The epithelium of the intestine is one of the most rapidly renewing tissues in the adult, taking between 4 to 5 days. This epithelium is divided into villi and crypts of Lieberkühn (Fig 1.14). In mice, a crypt can generate up to 200 cells per day, which is in equilibrium with the loss of the villus tip (Reya and Clevers, 2005). This cell replacement relies on an active stem cell population at the bottom of the crypt that is positive for the expression of the leucine-rich G protein-coupled receptor 5 (Lrg5⁺) (Barker et al., 2007). Interestingly, it was recently discovered that Lrg5+ cells exclusively express the Wnt target gene Achete scute-like 2 (Ascl2) (van der Flier et al., 2009). LRG5⁺ cells generate transient amplifying (TA) cells, which expand rapidly and give rise to all four mature cell types present in the gut: absorptive enterocytes, and secretory goblet cells, enteroendocrine, and paneth cells (Barker et al., 2008) (Fig 1.14). Differentiated cells occupy the villi, except for paneth cells which migrate to the bottom of the crypt with the stem cells (Bjerknnes and Cheng, 1981).
Figure 1.14: Role of Notch and Wnt signalling pathways in intestine cell lineage determination. Schematic representation of the intestine. Stem cells are at the bottom of the crypt, above them there are the TA cells which give rise to 4 different cell types: the secretory Paneth cells, Goblet cells, and Enteroendocrine cells, and the absorptive Enterocyte cells. The TA cells express Dll/Jag Notch ligands activating the expression of Hes1 in their neighbour cells differentiating into an absorptive cell. In contrast, cells expressing the Notch ligands escape Notch activation and differentiate into the secretory lineage. Epithelial turnover is maintained by a morphogen-like gradient of Wnt signalling across the crypt/villus axis, which is stronger at the base of the crypts and fades towards the villi. (Adapted from Heath, 2010).

In the crypts of the intestine, stem cell renewal is maintained by Notch (Fre et al., 2005; Fre et al., 2009) and Wnt (Sansom et al., 2004; van de Wetering et al., 2002) signalling pathways promoting cell proliferation and suppressing differentiation. Within the TA cells, Notch has been shown to have a proliferative role but this is dependent upon concurrent intermediate Wnt signalling (Fre et al., 2009; van Es et al., 2005). This was demonstrated by targeting NICD expression to the intestinal crypts using a Villin-Cre system, which inhibited the differentiation of the secretory cells and stimulated a striking increase of the TA cell population (Fre et al., 2005). It was later found that this proliferative activity of NICD on TA cells was
extinguished in a TCF4 null background indicating that the proliferative effect of Notch signalling was Wnt-dependent (Fre et al., 2009). Additionally, this study also indicated that the proliferative activity of Notch in the intestine promoted adenoma formation in mice carrying an APC mutation. An increase in Notch signalling is also seen in sporadic adenomas due to the Wnt-induced upregulation of the Notch ligand Jagged1 (Rodilla et al., 2009). In contrast, in the crypt to villus transition Notch regulates cell fate decisions of the TA cells controlling the production of secretory versus enterocyte fate (van der Flier and Clevers, 2009; van Es et al., 2005). The Notch-mediated fate assignation between absorptive versus secretory lineages is illustrated in Fig 1.11, and is dependent on the presence or absence of Hes1 Notch target gene. The Hes1 transcriptional repressor targets Math1, such that cells without Math1 differentiate into enterocytes, and cells that express Math1 commit to a secretory fate as they exit the crypt (Jensen et al., 2000; Yang et al., 2001).

Within the skin, Notch signalling does not promote stem cell self renewal but rather promotes terminal differentiation and growth suppression. The epidermis in vertebrates consists of four layers, basal (innermost), spinous, granular, and cornified (Fig 1.12). Like the intestine, the epidermis is under constant cell replacement, and it is the most important defence barrier of the body against external factors. Therefore, deregulation of skin cell differentiation can result in dehydration, infection, atopic disease, or cancer (Demehri et al., 2009; Zhang et al., 2009). The skin holds two niches of stem cells in the adult, one in the basal layer of the interfollicular epidermis (Blanpain and Fuchs, 2009; Fuchs and Raghavan, 2002), and a second in the bulge of the hair follicle (Blanpain and Fuchs, 2006; Pasolli, 2011). Differentiation and proliferation of adult epidermal stem cells within the interfollicular epidermis is controlled by Notch signalling (Ambler and Maatta, 2009; Massi and Panelos, 2012; Okuyama et al., 2008) (Fig 1.12). Notch determines spinous cell fate and induces terminal differentiation by inducing Ascl2 expression, although, Hes1 expression is required for maintenance of immature spinous cells (Moriyama et al., 2008). Notch also induces expression of the cell cycle regulator p21, directly via NICD-Rbpj complexes binding to p21 promoter (Rangarajan et al., 2001), and indirectly through activation of Calcineurin/NFAT (Mammucari et al., 2005), contributing to the cell cycle arrest of proliferating keratinocytes and initiation of terminal differentiation. However, it should be noted that this Notch mediated induction of p21 has only been described in mice. A homologue of p53 tumour suppressor, p63, is expressed within the basal layer of the skin where it promotes the self renewal of keratinocyte stem cells (Nguyen et al., 2006). Notch also restricts the proliferation of keratinocytes and promotes terminal differentiation by suppressing p63 expression, although, at the same time
p63 inhibits Notch transcription and function. Self-renewal of the skin is believed to depend on the proliferation of the stem cells of the basal layer, formed by a subpopulation of keratinocytes. Wnt/β-catenin signalling is expressed in these keratinocytes contributing to stemness and to the proliferative potential (Zhu and Watt, 1999). Wnt/β-catenin also influences the development of epidermal Langerhans cells, a dendritic cell population in the epidermis of the skin (Becker et al., 2011). However, Notch1 normally represses Wnt signalling directly or indirectly through p21 increase (reviewed in: (Panelos and Massi, 2009)).

These two examples of self-renewing tissues in adult tissues illustrate how Notch and Wnt signalling pathways play complex context-dependent and cell type specific roles in stem cell biology and tissue regeneration. Additionally, this section describing the role of Notch and Wnt signalling pathways during development and disease, clearly pinpoints how intimate is the relationship between the two networks.

1.6 Interactions between Wnt and Notch signalling pathways

Throughout this Introduction I have reviewed how cell fate decisions require the integration of several signalling inputs at the level of transcription and signal transduction. I
have also shown that the Notch and Wnt signalling pathways are extraordinarily intertwined during development and disease in a variety of systems. Here, I will describe what was known about the molecular mechanisms that underpin the crosstalk between these pathways at the start of my thesis. However, it is worth noting that two significant publications appeared in 2011 that describe similar work to the one presented on this thesis (Acosta et al., 2011; Kwon et al., 2011). There are important differences between these publications and our experiments and these are covered in detail within the discussion sections of Chapters 3 and 4.

Different members of the Wnt signalling pathway have been shown to interact with Notch. For instance, a physical interaction between the NH₂-terminal half of Dishevelled and the intracellular domain of Notch (at the C-terminus, distal to the cdc10/ankyrin repeats) has been described in yeast two hybrid systems (Axelrod et al., 1996). Through this interaction, Dishevelled provides an inhibitory crosstalk between the two pathways blocking Notch signalling. Other studies have also seen a similar inhibitory crosstalk between Dishevelled and Notch during patterning of the wing (Ramain et al., 2001) and the eye of Drosophila (Strutt et al., 2002). A recent study in Drosophila embryos and S2 cell culture, has shown that Wingless signalling increases the stability of the interaction between Notch and Dishevelled (Munoz-Descalzo et al., 2010). Furthermore, this study also shows that Wingless increases the endocytosis and trafficking of Notch, particularly, the ligand independent traffic of Notch by promoting the Dishevelled-Notch interaction at the membrane and the subsequent downregulation by degradation (Munoz-Descalzo et al., 2010). Additionally, current work from the Brennan lab, using a combination of cell culture and Xenopus embryo development, has shown that in vertebrates, unlike Drosophila, Dishevelled inhibits Notch signalling by physically associating with CSL transcription factors in the nucleus (Giovanna Collu, unpublished). Therefore, to date two mechanisms by which Dishevelled can inhibit Notch signalling have been described, one in the cytosol and one in the nucleus.

On the other hand, strong genetic interactions between the *notch* and *wingless* genes had been described in Drosophila during wing development, suggesting that Notch could interact with Wingless to inhibit Wingless signalling (Brennan et al., 1997; Couso and Martinez Arias, 1994; Wesley, 1999; Young and Wesley, 1997). Nevertheless, the first indication that Notch protein could negatively regulate Wg/Armadillo signalling in Drosophila directly, came from flies where the visceral mesoderm-specific enhancer of the *Ultrabitorax* (*Ubx*) gene, regulated by Wg/Armadillo signalling, was more active in the absence of Notch (Lawrence et al., 2001). This suggested that Notch can suppress Wg/Armadillo signalling but did not indicate
how. Subsequently, Notch was shown to regulate the function of Armadillo by modulating its amounts and transcriptional activity in Drosophila wing discs. Therefore, the proposed model suggested that Notch downregulates the transcriptional activity of Wnt signalling by physically interacting with Armadillo and degrading it (Hayward et al., 2005). This degradation was later shown to involve an active pool of β-catenin present near the adherent junctions, which has escaped degradation by the destruction complex and can now associate with Notch and traffic through the endosomes with it (Sanders et al., 2009). Reporter experiments in mammalian culture cells suggested that the same may be happening in mammals (Hayward et al., 2005). Additionally, an in vivo study in adult mice has shown that inactivation of Notch1 in the epidermis increases β-catenin abundance and signalling (Nicolas et al., 2003). These authors showed similar results in reporter assays performed in primary keratinocytes, however, did not propose any mechanism for the inhibitory crosstalk over Wnt/β-catenin signalling.

Notch has also been shown to interact with other components of the Wnt pathway in Drosophila, including Axin and APC (Hayward et al., 2006; Munoz-Descalzo et al., 2011). Axin and APC have been shown to negatively modulate the ligand-independent traffic of Notch (Hayward et al., 2006; Munoz-Descalzo et al., 2011). Therefore, in Drosophila, Axin, APC and Notch co-operate in vivo negatively modulating Armadillo, independently of the destruction complex. This work is in agreement with previous observations that Axin can regulate Armadillo, independently of the destruction complex (Tolwinski et al., 2003), since it could be through its role in the endocytosis and trafficking of Notch. Therefore, in Drosophila the ligand independent traffic of Notch results in the degradation of the active Armadillo, inhibiting Wnt signalling activation. This ligand independent traffic of Notch is driven, in part, by three components of the Wnt signalling pathway: Dishevelled, Axin, or APC.

Specific NICD and β-catenin interactions had previously been described in a study that was investigating the inhibitory effect of NICD in osteoblastogenesis. This study shows that NICD overexpression inhibits the response of bone marrow stromal cells (ST-2 cells) to Wnt/β-catenin signalling (Deregowski et al., 2006). From this study a model mechanism for NICD-mediated Wnt inhibition was proposed: following Notch activation, NICD decreases β-catenin levels in the cytoplasm and increases Hes1 transcription. Subsequently, they suggest that Hes1 may associate with Groucho/TCF complex, preventing Groucho displacement by β-catenin and therefore, inhibiting Wnt signalling activation. Nevertheless, no physical association between Hey1 and Groucho or TCF was shown in this study. Thus, the molecular mechanism by which Notch inhibits β-catenin in mammals is still a matter of debate.

57
1.7 Setting the scene

In this thesis, I have investigated the molecular mechanism(s) behind the interaction between Notch and Wnt signalling pathways in vertebrates. In particular, how Notch can influence Wnt/β-catenin signalling. Before proceeding to the results section, I would like to write a few words to explain my personal interest in these two signalling pathways, as well as, to provide a brief overview of the structure of the results presented in this thesis.

Within the general introduction I have presented the reasons why the Notch and Wnt signalling pathways are two of the most important networks during animal development and adult homeostasis. Both work through highly conserved mechanisms to form, shape and maintain tissues. Furthermore, deregulation of these mechanisms leads to many different diseases, including cancer, which nowadays affects 1 in 3 adults. Therefore, knowing how these pathways interact during normal development is an important consideration when designing therapeutics for disease treatment. Thus, an important question that stood out for me in this puzzle was: by which mechanism do these two signalling pathways crosstalk in vertebrates?

Prior to starting my PhD, several points of crosstalk had been described between the Notch and Wnt signalling pathways, including a possible crosstalk mechanism at the level of β-catenin. Though, the underlying molecular mechanisms were, in many cases, poorly understood. Often, genetic work in Drosophila had highlighted the point of crosstalk. However, a lack of appropriate reagents and systems in Drosophila has made it difficult to analyse the molecular mechanism biochemically. In contrast, there are many reagents available for analysis of signalling pathways in mammalian cell culture models. In addition, these systems allow the manipulation of signalling pathways in isolation, without the background hub-bub of signalling that occurs in vivo, making mammalian cell culture systems ideal for unravelling the molecular mechanisms that underlie signalling crosstalk. Therefore, I set out to elucidate the molecular mechanism underpinning these two signalling pathways in a vertebrate context.

First, I needed to find the right system to study crosstalk interactions, i.e.: a reporter assay, a cell line and Notch and Wnt constructs. The results of these experiments are described in Chapter 2. Secondly, once the system was well established, I set out to elucidate the crosstalk. For that I needed to activate Wnt signalling by transfecting cells with plasmids that encode components of the pathway and monitor signalling through the expression of
luciferase-reporter genes and western blotting for β-catenin. Notch signalling was similarly activated by transfecting plasmids encoding components of the pathway. I found that there are two possible mechanisms by which Notch inhibits Wnt signalling at the level of β-catenin, one that happens at the membrane, and one that happens in the nucleus. Therefore, I divided the work into two different chapters (Chapter 3 and Chapter 4). Finally, given the observed inhibitory effect of Notch on Wnt signalling I was keen to explore the role of this mechanism during Xenopus development.

The research performed for my PhD is presented as an alternative format thesis. The next 3 chapters (Chapter 2, 3 and 4) represent a comprehensive collection of the results from my research and are written in the form of a submitted manuscript. The first is a methods manuscript designed to find a system to study crosstalk interactions. Manuscripts 2 and 3 contain the bulk of the experimental work carried out during my PhD, describing the two mechanisms that I found by which Notch inhibits Wnt/β-catenin signalling. Following these, there is a general discussion chapter (Chapter 5) that summarises and discusses all the findings and provides a summary of the future proposed experiments. Each paper contains a brief materials and methods section. Additionally, there is a supplementary materials and methods chapter (Chapter 6) that contains a completed version of all the materials and methods used in the experiments presented in this thesis, as well as, a description of the cloning and mutagenesis constructs that I generated to conduct this work.
References


Liu, W., Dong, X., Mai, M., Seelan, R. S., Taniguchi, K., Krishnadath, K. K., Halling, K. C., Cunningham, J. M., Boardman, L. A., Qian, C. et al. (2000). Mutations in AXIN2 cause...


Chapter 1: General Introduction


Chapter 1: General Introduction


Chapter 1: General Introduction


Chapter 2: Paper 1

Choosing the Right Tools to Study the Molecular Mechanism behind the Crosstalk between Notch and Wnt Signalling Pathways.

Ana Hidalgo Sastre¹ & Keith Brennan¹.

¹Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester, M13 9PT, UK.
Author for correspondence (e-mail: keith.brennan@manchester.ac.uk)

Running title: The right tools to study a molecular crosstalk mechanism.

Author contribution: AHS and KB designed the experiments. AHS performed the experiments and wrote the paper. KB edited the paper.

Note for the reader: The aim of this study was to find a robust experimental approach to investigate the interaction between Notch and Wnt signalling pathways. This short research methods paper describes the identification of a cell line, a reporter gene, and two Notch expression constructs appropriate for the experiments described in Chapters 3 and 4.
Abstract

This manuscript describes the identification of a mammalian cell culture system to investigate the crosstalk between Notch and Wnt signalling pathways. This involved the selection of: (I) a Wnt reporter plasmid that cannot be directly regulated by the transcriptional repressors Hes and Hey which are Notch target genes, (II) a mammalian cell line in which we can activate both pathways reliably and (III) Notch expression constructs that differentiate between Notch transcription-dependent and -independent mechanisms. We found that the TCFAdTATA Wnt reporter plasmid (E-box free), the HEK293T cell line, and a membrane-restricted (ΔEGF_N1) and a nuclear-restricted (NICD) form of Notch were the ideal reagents.

Key words: Wnt reporter assay, HEK293T, Notch, Wnt, crosstalk.
Chapter 2: The Right Tools to Study a Molecular Crosstalk Mechanism

Introduction

The Notch and Wnt signalling pathways are two of the most important in metazoan development helping to pattern and shape the embryo through their control of cell fate assignation (Hayward et al., 2008; Logan and Nusse, 2004; Muñoz-Descalzo and Martinez Arias, 2012). Furthermore, these pathways are also involved in tissue homeostasis in the adult (Andersson et al., 2011; Grigoryan et al., 2008). Therefore, it is not surprising that both pathways must be under tight spatio-temporal control. Furthermore, de-regulation of the pathways leads to developmental disorders and degenerative diseases, such as cancer (Andersson et al., 2011; Klaus and Birchmeier, 2008; Reya and Clevers, 2005). It is currently well known that signalling pathways are not a linear chain of molecular events, but instead, complicated networks of interactions (Alon, 2007; Barolo and Posakony, 2002). Part of the tight control that ensures proper development and tissue maintenance is carried out by crosstalk between the signalling pathways (Alon, 2007). Studies in Drosophila have provided strong evidence for an inhibitory interaction between Notch and Wnt signalling pathways (Sanders et al., 2009). These authors suggest that a membrane-restricted form of Notch limits Wnt signalling at the level of β-catenin by physically interacting with it and targeting it for degradation. In mammals, results suggest that Notch may regulate Wnt signalling similarly in vivo (Nicolas et al., 2003) and in vitro (Deregowski et al., 2006). However, these studies do not address the molecular mechanism that underpins this crosstalk. In addition, in the mammalian cell culture experiments Notch signalling was activated by expressing NICD rather than a membrane-restricted form of Notch. Therefore, it is not clear yet whether the mechanism described in Drosophila is conserved in vertebrates, or how NICD is regulating Wnt signalling.

To unravel the molecular mechanism that underpins the crosstalk between Notch and Wnt signalling pathways in vertebrates, it is essential to choose the best available tools. It would be ideal to have a simple system in which we can activate the two pathways reliably. Additionally, it would be good to be able to monitor signalling through the two pathways both biochemically and genetically, without interference from other signalling inputs. For this reason, we propose to use mammalian cell lines as a model system. In mammalian cell lines we can perform transient transfections of Notch and Wnt constructs to activate the signalling pathways and monitor this signalling with luciferase reporter assays. Moreover, we can use cell fractionation to establish the localisation of pathway components. It is therefore important, before starting this study, to choose the right reporter plasmid, the right cell line and the right constructs to activate the two pathways.
It is essential to choose a right reporter to monitor Wnt signalling that is not regulated by Notch signalling simply due to the presence of Rbpj or Hes/Hey binding sites (Fischer and Gessler, 2007). Dual-luciferase assay is widely used to determine rapidly and accurately the activity of a given promoter (Promega). The widely available Wnt reporter plasmid, TOPflash, contains a firefly luciferase gene expressed upon β-catenin mediated transcriptional activation due to the presence of four TCF/LEF binding sites (Wnt response elements) with the sequence ‘AGATCAAGGGGGA’ (Korinek et al., 1997) (Fig 2.1A). This reporter plasmid also contains a c-fos promoter with two Ephrussi-boxes (E-boxes) with the sequence ‘CANNTG’ (Ephrussi et al., 1985) (Fig 2.1A). The Notch target genes Hes and Hey are bHLH transcriptional repressors that bind to E-boxes (Fischer and Gessler, 2007). Consequently, Notch signalling may regulate the TOPflash reporter gene through Hes/Hey expression. Therefore, an ideal reporter plasmid to study Notch1 modulation of Wnt signalling would either have mutated E-boxes, such as MOPflash, or not contain E-boxes at all, like TCFAdTATA (Fig 2.1A). In fact, the TCFAdTATA reporter plasmid only contains a TATA box, with the sequence ‘GGGGCTATAAAAGGGG’, regulated by four TCF sites (Fig 2.1A).

It is also important to choose a cell line in which signalling of the pathway of study can be clearly reported, in this case Wnt signalling. Many different mammalian cell lines are available. For our analysis, we have chosen a series of easily accessible and widely used cell lines that are straightforward to culture and can easily be transfected with plasmid DNA. These include: CHO-K1, a subclone of the parental Chinese hamster ovary cell line derived by T.T. Puck in Boston in 1958 (Puck et al., 1958); NIH-3T3, a mouse embryonic fibroblast cell line established by George Todaro in Washington in 1969 (Jainchill et al., 1969); HEK293 and HEK293T generated from human embryonic kidney cells by Alex Van der Eb and Frank Graham in the Netherlands in 1970’s (Graham et al., 1977) and wild type mouse embryonic fibroblasts (MEF). These cell lines are also widely used in the biochemical analysis of cell signalling.

In the literature there is controversy regarding Notch and Wnt crosstalk, with authors arguing for a Notch transcription-dependent mechanism (Deregowski et al., 2006; Nicolas et al., 2003), versus authors arguing for a transcription-independent mechanism (Hayward et al., 2005; Sanders et al., 2009). Therefore, it was essential in this study to be able to differentiate between Notch transcription-dependent and -independent crosstalk mechanisms. To generate a membrane-restricted form of Notch, others have generated fusion proteins between the Notch intracellular domain and the extracellular and transmembrane (TM) domains of the receptor tyrosine kinase (RTK) Torso (TNotch) (Hayward et al., 2005) or with the extracellular and TM domain of CD8 (Sanders et al., 2009). In contrast, we have generated ΔEGF_N1 and...
ΔEGF+LNR_N1, two forms of mammalian Notch1 that lack the 36 extracellular EGF-like repeats of the Notch receptor. The ΔEGF+LNR_N1 construct also lacks the LNR domain up to the S1 cleavage site. Deletion of the EGF-like repeats prevents ligand binding (Lawrence et al., 2000; Rebay et al., 1991) and hence the S2 and S3 consecutive cleavages that activate Notch signalling (Mumm et al., 2000). These constructs, however, have the advantage over previously used constructs in that they contain the Notch transmembrane domain. Furthermore, the ΔEGF_N1 construct will also undergo S1 cleavage by Furin (Logeat et al., 1998) and will be present as a heterodimer on the cell surface, like the endogenous full length Notch1 protein (Blaumueller et al., 1997).

Two different constructs are commonly used to activate Notch signalling, N^ΔE (Jarriault et al., 1995; Schroeter et al., 1998) and NICD1 (Coffman et al., 1993; Rebay et al., 1993). We have generated a construct similar to N^ΔE, that we have named ΔN_N1. The original N^ΔE construct starts at amino acid Ile1704, that is 7 amino acids before the S2 cleavage site (which happens at Val1711). In contrast, our ΔN_N1 construct starts at amino acid Val1711, just at the S2 cleavage site. Both N^ΔE and ΔN_N1 constructs are initially targeted to the cell membrane, where they are spontaneously cleaved by γ-secretase, releasing NICD into the cytosol. NICD can translocate to the nucleus and initiate transcriptional activation. The NICD construct is translated in the cytoplasm, once synthesised, it travels to the nucleus to activate transcription.

In summary, we have generated a Wnt reporter that cannot be repressed by Notch target genes Hes and Hey, identified a cell line that can be used to monitor Wnt signalling biochemically and genetically, and generated a panel of plasmids encoding different forms of the Notch receptor. Together, this collection of tools enables us to study the inhibitory crosstalk between Notch and Wnt signalling pathways in a mammalian cell line and elucidate the underlying molecular mechanism.
Materials and methods

This is a general description of the methods used for this manuscript. Further details are given in the supplementary materials and methods on chapter 6.

Cell culture and transfections

CHO-K1 cells were cultured in Ham’s F12 medium supplemented with 10% FBS, 1% non-essential amino acids, 50 µg/ml penicillin and 50 µg/ml streptomycin. NIH-3T3, HEK293T and MEF cells were cultured in DMEM medium supplemented with 10% FBS, 50 µg/ml penicillin and 50 µg/ml streptomycin. HEK293 cells were cultured in EMEM medium supplemented with 10% FBS, 1% non-essential amino acids, 1% Na Pyruvate, 50 µg/ml penicillin and 50 µg/ml streptomycin. Cells were maintained at 37°C and 5% CO₂ in a humidified incubator. Cells were transfected using Lipofectamine and Plus reagent (Invitrogen) or X-Treme transgene 9 transfection reagent (Roche) according to manufacturer’s instructions.

Luciferase assays

Cells were plated at a density of 2 x 10⁵ cells/well of a 24 well plate. Cells were transfected in triplicate with 250 ng DNA cocktail containing the desired expression plasmids, 50 ng of the reporter plasmid (TOPflash, MOPflash, or TCFAdTATA), and 20 ng of pRL-CMV as an internal control. To adjust transfections to a constant final amount of 250 µg of DNA, the cocktails were supplemented with pcDNA3.1(+). The cell culture medium was changed 3 hr after transfection and cells were lysed 48 hr post-transfection using 1 x Passive lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter assay system (Promega) according to manufacturer’s instructions with a MicroLumatPlus plate reader (Berthold Technologies). Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU), compared to empty vector. Statistical analysis was perform using one-way ANOVA and Tukey’s post-hoc tests, for experiments containing more than two samples, or with a Student T-Test for data sets with only two samples.

cDNA constructs

A description of the constructs used in the experiments for this manuscript can be found on the supplementary materials and methods on Chapter 6 section 6.4.

S100/P100 cytosolic fractionation

Cells were washed in 2 x in 3 ml of 1 x TBS (10 mM Tris-HCl pH 7.4 + 140 mM NaCl) + 2 mM CaCl₂, placed on ice and lysed in 1 ml of lysis buffer (1 x TBS + protease inhibitor cocktail
set (Calbiochem Cat# 539131)) + 10 µl of 100 mM PMSF. Subsequently, cells were poured into a cold 2 ml dounce homogenizer and lysed with 30 strokes. Cell lysates were centrifuged at 1500 x g for 5 min at 4°C. The supernatant was poured into a cold Beckman ultracentrifuge tube (Polyallomer 11x60mm) and centrifuged for 90 min at 50000 x g in a TLA 100 rotor in a Beckman Coulter Optima TLX-120 Ultracentrifuge at 4°C. After the spin, the supernatant (100 µl) was mixed with 2 x Laemmli’s buffer (100 µl), boiled for 3 min and stored at -20°C; this represents the cytosol fraction.

**Western blotting**

Total cell lysis and subsequent western blotting were performed as described (Stylianou et al., 2006). For more details on buffers and procedure see supplementary materials and methods on Chapter 6.

**Immunofluorescence**

HEK293T cells were seeded on nitric acid treated cover slips at 4 x 10⁵ cells per well of a 6 well plate. Transfection was performed 24 hr later and cells were fixed for 10 min in 1 x PBS containing 4% formaldehyde 24 hr post transfection. Following 3 x washing with 1 x PBS, coverslips were incubated with rabbit α-myc-Tag primary antibody diluted 1:100 in blocking solution (3% goat serum (Biosera, Sussex, UK), 0.1% Triton-X100, 0.05% NaN₃ in TBS) in a humidified chamber for 1 hr. Cells were subsequently washed and incubated with fluorescence goat α-rabbit Alexa 594 secondary antibody in blocking solution. Coverslips were mounted with VECTASHIELD mounting medium for fluorescence with DAPI H-1200 (Vector Laboratories). Images were captured with a Zeiss LSM 700, AxioObserver flexible confocal microscope (Carl Zeiss MicrolImaging GmbH, Germany) using Zeiss ZEN 2011 software (Carl Zeiss MicrolImaging GmbH, Germany). The confocal software was used to determine the optimal number of Z sections when acquiring 3D optical stacks. Either maximum intensity projections of these 3D stacks or a single z-stack images are shown in the results.
Results

TCFAdTATA Wnt reporter plasmid shows the greatest increase in activity in response to β-catenin expression.

In order to determine which of the three reporter plasmids responds the most strongly to Wnt/β-catenin signalling activation, a constitutively active form of the Wnt transcription factor LEF1, LEF1-VP16 was transfected in HEK293T cells along with the different reporters: TOPflash, MOPflash, or TCFAdTATA (Fig 2.1A). Out of all of them, TOPflash and MOPflash showed the highest fold increase in relative luciferase units when transfected with LEF1-VP16, compared to cells transfected with the same reporter gene and an empty vector (Fig 1.1B). In contrast TCFAdTATA showed the lowest activation of the Wnt reporter for LEF1-VP16. Nevertheless, this activation was clearly significant when compared with the activation caused by co-transfecting an empty vector (Fig 2.1B).

To further investigate which reporter was best to study inhibition of Wnt signalling by Notch, we determined which of the three reporter plasmids responds most strongly to Wnt/β-catenin signalling activation when it is initiated by β-catenin, the key effector of the Wnt signalling pathway (Korinek et al., 1997; Molenaar et al., 1996; Morin et al., 1997). When β-catenin was transfected in HEK293T cells along with TOPflash, MOPflash, or TCFAdTATA we found that all reporter plasmids showed significant activation compared to cells transfected with the same Wnt reporter plasmid and an empty vector (Fig 2.1C). More interestingly, out of all of the Wnt reporters used, TCFAdTATA clearly showed the highest significant activation when Wnt signalling was triggered with β-catenin. From these results and due to the lack of E-boxes in TCFAdTATA, we conclude that TCFAdTATA is the best Wnt/TCF reporter plasmid available to study the inhibitory crosstalk between Notch and Wnt signalling pathways in mammalian cell lines. Therefore, this Wnt reporter plasmid was selected to be used in subsequent experiments.

CHO-K1 and HEK293T cell lines show a robust activation of Wnt signalling.

In order to determine which of the cell lines available responds the most strongly and robustly to Wnt/β-catenin signalling, LEF1-VP16 was transfected in CHO-K1, NIH-3T3, HEK293, HEK293T (Fig 2.2) or MEF (Sup Fig 2.1A) cells along with TCFAdTATA reporter. Results showed that expressing LEF1-VP16 activated the TCFAdTATA reporter gene in all the cell lines, and there were no significant differences between them in the ability of LEF1-VP16 to induce transcription (Fig 2.2A). This result, although interesting, did not shed any light on which cell line would be the most appropriate to use to report Wnt signalling. To further investigate this,
we tested which of the four cell lines reports an equally robust Wnt signalling activation when transfected with expression plasmids encoding different activators of the Wnt pathway, including the Wnt1 ligand, Dishevelled2 (Dvl2) adaptor protein, or β-catenin (Fig 2.B-E and Sup Fig 2.1A). In CHO-K1 cells, Wnt1 and Dvl2 did not show significant activation compared with an empty vector used as a control. Instead, β-catenin activation was significantly higher (Fig 2.2B). In NIH-3T3 cells, none of the Wnt signalling activators stimulated a significant increase in reporter gene activity (Fig 2.2C). Similar results were observed for HEK293 cells (Fig 2.2D). In HEK293T cells, only Wnt1 ligand showed significant activation of Wnt signalling, whereas, Dvl2 and β-catenin did not (Fig 2.2E).

Additionally, whole cell lysates on western blot showed no obvious accumulation of β-catenin when transfected with expression plasmids encoding different Wnt pathway activators, including plasmids encoding β-catenin itself compared to the empty vector (Sup Fig 2.1B and 2.2). This fits with the lack of obvious signalling in NIH 3T3, HEK293 and MEF cells (Fig 2.2C and D and Sup 2.1 A). The failure to observe an accumulation in HEK293T cells could be due to the membrane pool of β-catenin masking changes in the cytoplasmic pool. However, changes in β-catenin were clearly visible in HEK293T cells following S100/P100 cytosolic fractionation (Sup Fig 2.3). Due to the fact that CHO-K1 and HEK293T were the only cell lines that demonstrated a significant activation of a component of the Wnt signalling pathway, we chose to continue our experiments using these two cell lines.

**HEK293T cell line, the perfect model to report Wnt signalling activation.**

β-catenin is the key effector of the Wnt signalling pathway (Korinek et al., 1997; Molenaar et al., 1996; Morin et al., 1997). Therefore, it is important to observe a robust activation of the Wnt reporter in the cell line used in subsequent experiments by transfecting a plasmid encoding β-catenin. In the absence of Wnt ligand, the free cytosolic β-catenin is phosphorylated by a destruction complex formed by the scaffolding proteins, Axin and APC (Hart et al., 1998; Kishida et al., 1998), GSK3β (Yost et al., 1996), and CK1 (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002), and targeted for proteosomal degradation by βTrCP E3 ubiquitin ligase (Aberle et al., 1997; Latres et al., 1999). To avoid results being complicated by this, we decided to work with a form of β-catenin that contains a serine to phenylalanine substitution at amino acid 45, S45Fβ-catenin. This mutation mimics a spontaneous mutation described in colorectal cancer (Morin et al., 1997; Polakis, 1999; Samowitz et al., 1999). This form of β-catenin cannot be phosphorylated by CK1 which normally primes β-catenin for phosphorylation by GSK3β (Amit et al., 2002; Liu et al., 2002); it is the phosphorylation of β-catenin at serine 33 and 37 by GSK3β that leads to its recognition by β-TRCP and, thus, its
targeting to the proteosome (Aberle et al., 1997; Latres et al., 1999). To determine whether CHO-K1 or HEK293T cells respond strongly to Wnt/β-catenin signalling activated at the level of β-catenin, an expression plasmid encoding S45Fβ-catenin was transfected in the two cell lines, along with the TCFAdTATA reporter plasmid. Although, reporter gene activation was observed in both cell lines, HEK293T cells clearly responded most strongly (Fig 2.3A).

We then tested the response of each cell line to the activation of the Wnt/β-catenin signalling pathway at the level of endogenous β-catenin. To do this, we treated both cell lines with LiCl, which inhibits GSK3β (Davies et al., 2000; Klein and Melton, 1996; Stambolic et al., 1996). This leads to the stabilisation of free cytosolic β-catenin which can then translocate to the nucleus and activate transcription. Both CHO-K1 and HEK293T cells were treated overnight with either 20 mM LiCl or 20 mM KCl, as a control. Results clearly showed that HEK293T cells had a much greater capacity to respond to the accumulation of endogenous β-catenin (Fig 2.3B). HEK293T cells were able to robustly report Wnt signalling from both endogenous and ectopic sources. Therefore, these data clearly demonstrated that HEK293T cell line was the appropriate to study crosstalk mechanisms between the Notch and Wnt signalling pathways.

ΔEGF_N1 and NICD, the ideal constructs to differentiate signalling inactive and signalling active functions of Notch.

To study the molecular mechanism underlying the crosstalk between Notch and Wnt signalling pathways it was important to differentiate whether Notch would require transcriptional activation or not. In the lab we had available several Notch constructs that remove different portions of the extracellular domain, but still produce a Notch protein that can localise to the membrane (Fig 2.4A). All of these constructs are expected to prevent interaction with DSL ligands but are expected to interact very differently with γ-secretase. The ΔN_N1 construct, which starts near the S2 cleavage site (Val1711), is expected to mimic the transitory NEXT intermediate in Notch signalling. Therefore, the ΔN_N1 construct is expected to be spontaneously cleaved by γ-secretase. The ΔEGF+LNR_N1 construct starts at the S1 cleavage site (Glu1655) and is not expected to be a substrate for γ-secretase. Therefore, the ΔEGF+LNR_N1 construct is expected to be restricted to the plasma membrane. The ΔEGF_N1 construct only lacks the 36 EGF-like repeats but still contains the LNR repeats and, like ΔEGF+LNR_N1, is not expected to be a substrate for γ-secretase. However, the ΔEGF_N1 construct has the advantage that it will undergo S1 cleavage, like the full length Notch protein, and will be present on the cell surface as a heterodimer (due to the presence of the LNR repeats). We also have an expression plasmid encoding the Notch intracellular domain (NICD) (Fig 2.4A).
The ability of the different Notch constructs to activate Notch signalling was tested with a Notch reporter assay. For this assay different Notch constructs were transfected along with 10xRbpj-luc, a Notch reporter plasmid that contains 10 Rbpj binding sites upstream of the minimal adenovirus AdTATA. As expected, the membrane-bound forms of Notch: ΔEGF_N1 and ΔEGF+LNR_N1 showed no significant activation of the Notch reporter (Fig 2.4B). In contrast, ΔN_N1 that is expected to be a substrate of γ-secretase, as well as NICD, strongly activated the 10xRbpj-luc reporter, like a constitutively active form of the Rbpj transcription factor, Rbpj-VP16 (Fig 2.4B). In addition, expression of the constructs was analysed by western blotting (Fig 2.4C). Results showed that all Notch constructs were expressed at comparable levels when transfected in HEK293T cells (Fig 2.4C). Notice that the ΔEGF_N1 construct appears as a doublet (Fig 2.4C, line 2). The upper band represents the co-linear form of ΔEGF_N1, prior to S1 cleavage. The lower band is the Notch transmembrane (N\textsuperscript{TM}) protein formed by S1 cleavage, which will be present as heterodimer with the truncated extracellular domain of ΔEGF_N1 at the membrane. In contrast, ΔEGF+LNR_N1, which is not expected to undergo S1 cleavage, is present as a single band in the western blot (Fig 2.4C line 5). ΔN_N1 is expected to undergo S2 cleavage, but this removes a short peptide making it difficult to distinguish between the full length and truncated forms of ΔN_N1 and, therefore, it appeared as a single band (Fig 2.4 line 6).

Since the aim of this investigation was to differentiate between a Notch inhibitory mechanism that occurs at the membrane and a mechanism that happens in the cytosol or the nucleus, it was important to choose the Notch constructs that would enable to clearly distinguish between these two possibilities. From the membrane-restricted constructs, ΔEGF_N1 was structurally the most similar to the FLN, yet it was signalling inactive (Fig 2.4 A, B). NICD was chosen over ΔN_N1 as signalling active form of Notch because, in contrast to ΔN_N1, NICD is synthesized within the cytosol and can translocate to the nucleus without passing through the membrane compartment. Finally, we confirmed the localization of the selected constructs by immunofluorescence. As expected, we found that ΔEGF_N1 was largely present at the membrane; the cytosolic staining was punctate and is thus, likely to represent the ΔEGF_N1 protein localised to vesicles within the secretory or endocytic machinery of the cell (Fig 2.4D). In contrast, NICD is exclusively found in the nucleus of the cells (Fig 2.4E). These data strongly suggest that ΔEGF_N1 and NICD are the ideal Notch constructs to differentiate between signalling inactive or signalling active function of Notch that would either remain at the membrane or be restricted to the nucleus, respectively.
Figure 2.1: Xenopus β-catenin stimulates the greatest fold increase in reporter gene activity with TCFAdTATA. A. Diagram showing the structure of the three different Wnt signalling reporter plasmids. B. HEK293T cells were transfected with 10 ng of LEF1-VP16 along with 50 ng of TOPflash, MOPflash, or TCFAdTATA Wnt reporter plasmids and 20 ng of the transfection control reporter pRL-CMV per well. The experiment was performed in triplicate. The ratio of firefly to Renilla luciferase was calculated for each condition. The average fold increase in relative luciferase units (RLU) is shown for HEK293T cells transfected with LEF1-VP16 compared to cells transfected with the empty expression vector, pcDNA3.1(+), 48 hr post transfection. Data are presented as mean fold change (+/- SEM) in RLU (**P<0.001 one-way ANOVA and Tukey’s post-hoc test, N=1). C. HEK293T cells were transfected as before with 100 ng of Xenopus β-catenin (Xβ-catenin). The experiment was performed in triplicate. The average fold increase in RLU 48 hr after transfection is shown for HEK293T cells transfected with Xβ-catenin compared to cells transfected with pcDNA3.1(+). Data are presented as mean fold change (+/- SEM) in RLU (**P<0.001 one-way ANOVA and Tukey’s post-hoc test, N=1).
Chapter 2: The Night Tools to Study a Molecular Crosstalk Mechanism

A. TOPflash

MOPflash

TCF AdTATA

B.

C.

***

Fold change in RLU

TOP Flash  MOP Flash  TCF AdTATA

Empty vector  LEF1-VP16

Fold change in RLU

TOP Flash  MOP Flash  TCF AdTATA

Empty vector  β-catenin
Figure 2.2: Identifying a mammalian cell line that robustly responds to Wnt signalling. A. Cell lines were transfected with 12.5 ng of LEF1-VP16 was transfected in each of the cell lines: CHO-K1, NIH-3T3, HEK293, and HEK293T cells along with 50 ng of TCFAdTATA and 20 ng of pRL-CMV, per well. The experiment was performed in triplicate. The average fold increase in relative luciferase units (RLU) 48 hr after transfection is shown for cells transfected with LEF1-VP16 compared to cells transfected with empty vector (pcDNA3.1(+)). Data are presented as mean fold change (+/- SEM) in RLU (NS $P>0.05$ one-way ANOVA and Tukey’s post-hoc test, $N>3$). B-E. The different cell lines were transfected with several components of the Wnt pathway, including 2.5 ng of Wnt1, 125 ng of Dvl2, 25 ng of $\beta$-catenin, 50 ng of TCFAdTATA and 20 ng of pRL-CMV, per well. The average fold increase in relative luciferase units (RLU) 48 hr after transfection is shown for cells transfected with the different Wnt components compared to cells transfected with empty vector. Data are presented as mean fold change (+/- SEM) in RLU (NS $P>0.05$; *$P<0.05$; ***$P<0.001$ one-way ANOVA and Tukey’s post-hoc test, $N\geq3$). B. Graph showing activation of the TCFAdTATA reporter gene in CHO-K1 cells. The reporter was significantly activated by transfection with $\beta$-catenin. C & D. Graph showing activation of the TCFAdTATA reporter gene in NIH-3T3 and HEK293 cells, respectively. The reporter was not activated by transfection with the different Wnt pathway components. E. Graph showing activation of the TCFAdTATA reporter gene in HEK293T cells. The reporter was significantly activated by transfection with Wnt1.
Chapter 2: The Night Tools to Study a Molecular Crosstalk Mechanism

A.

![Graph A]

- Choice K1
- NIH-3T3
- HEK293
- HEK293T

- Empty vector
- LEF1-VP16

B.

![Graph B]

- CHO-K1
- Empty vector
- Wnt1
- Dvl2
- β-catenin

C.

![Graph C]

- NIH-3T3
- Empty vector
- Wnt1
- Dvl2
- β-catenin

D.

![Graph D]

- HEK 293
- Empty vector
- Wnt1
- Dvl2
- β-catenin

E.

![Graph E]

- HEK 293T
- Empty vector
- Wnt1
- Dvl2
- β-catenin
Figure 2.3: Comparing activation of TCFAdTATA reporter gene by stabilising β-catenin in CHO-K1 and HEK293T cells. A. CHO-K1 and HEK293T cells were transfected, as before, with 12.5 ng of S45Fβ-catenin, 50 ng TCFAdTATA and 20 ng pRL-CMV, per well. The average fold increase in RLU 48 hr after transfection is shown for cells transfected with S45Fβ-catenin compared to cells transfected with pcDNA3.1(+). Data are presented as mean fold change (+/- SEM) in RLU (**P<0.01 Student T-test, N=1). TCFAdTATA activation was stronger in HEK293T cells compared to CHO-K1 cells. B & C. Cells were transfected with an empty vector, 50 ng of TCFAdTATA and 20 ng of pRL-CMV per well. 24 hr after transfection cells were treated over night with 20 mM LiCl to inhibit GSK3β. 20 mM KCl were used as control. Cells were lysed 48 hr post transfection. The LiCl treatment significantly increased Wnt activity compared to KCl treatment (**P< 0.001 Student T-test, N=2).
Chapter 2: The Night Tools to Study a Molecular Crosstalk Mechanism

A. 

B. 

C. 

93
**Figure 2.4: Characterization of Notch constructs.** A. Diagram of the different Notch constructs used. All constructs contain a myc tag within the intracellular domain before the OPA and PEST domains. Those carrying the IgG k-chain signal peptide also contain a His/myc tag at the N-terminus, just after the signal peptide. B. Identifying signalling deficient and competent Notch molecules. Several different Notch plasmids were transfected (25 ng) into HEK293T cells in the presence of 50 ng of the Notch reporter plasmid p10xRbpj-luc and 20 ng of the transfection control plasmid pRL-CMV. Notice that ΔEGF_N1 and ΔEGF+LNR_N1 did not activate Notch signalling. In contrast, ΔN_N1 and NICD activated Notch signalling to a similar extent as Rbpj-VP16. Experiments were performed in triplicate and cells were lysed 48 hr post transfection. Data are presented as mean fold change (+/- SEM) in RLU (NS P >0.05; ***P< 0.001 one-way ANOVA and Tukey’s post-hoc test, N≥3). C. Western blot showing expression of the different Notch constructs used in this study. Expressed protein was detected by probing the western blot with an antibody that recognises the myc epitope tag found within all the proteins. Note that a myc antibody was used instead of a NICD one to be able to identify only the transfected constructs. Renilla luciferase is shown as a loading control. The position of molecular weight markers (in KDa) is shown. D & E. Immunofluorescence analysis of ΔEGF_N1 and NICD constructs. Cells were transfected with 25 ng of ΔEGF_N1 or NICD and fixed 24 hr post transfection. Expressed protein was detected by staining with a primary antibody against the myc epitope tag (α-myc-Tag Rabbit, Cell Signaling) and a secondary antibody Goat α-Rabbit Alexa 594 (Molecular probes). Scale bar is 51 µm. For ΔEGF_N1 a single z-stack through the middle of the cell is show. For NICD a maximum projection of the z-stack is show.
Discussion

We found that TCFAdTATA was the best Wnt reporter plasmid, HEK293T the best mammalian cell line and ΔEGF_N1 and NICD the best Notch constructs to study the molecular mechanism behind the crosstalk between Notch and Wnt signalling pathways.

Luciferase reporter assays were developed in HEK293T cells to establish a Wnt reporter plasmid to look at the interaction between Notch and Wnt signalling pathways. Previous experiments showing that Notch modulates Wnt signalling (Deregowski et al., 2006; Hayward et al., 2005; Jin et al., 2009) have used the TOPflash reporter plasmid to monitor Wnt signalling. Consequently, it is not clear from these experiments whether the results observed are a direct effect of Notch on Wnt signalling or an indirect effect through the expression of the Notch target genes Hes and Hey, which would bind to the E-boxes within the c-fos promoter of TOPflash (Fischer and Gessler, 2007). Accordingly, we have developed two plasmids that lack E-boxes: MOPflash in which the E-boxes within the c-fos promoter are mutated, and TCFAdTATA which is a minimal adenoviral TATA box regulated by four TCF sites and lacks any E-boxes (Fig 2.1A). We chose TCFAdTATA for our ongoing work as it showed the greatest fold increase in activity in response to Wnt/β-catenin signalling (Fig 2.1C).

We initially screened five different mammalian cell lines: CHO-K1, NIH-3T3, MEF, HEK293 and HEK293T (hamster, mouse and human), to establish which one would be most suitable for our experiments. We chose these cell lines because they are easy to culture and to transfect with plasmid DNA. In addition, CHO-K1 cells have been shown to process full length Notch properly and to have low Notch signalling (Giovanna Collu and KB, unpublished results), whilst knock down experiments should be straightforward in NIH-3T3, MEF, HEK293, and HEK293T cells using pre-designed mouse and human siRNA sequences. Furthermore, HEK293T cells express SV40 large T-antigen, a helicase that recognises the SV40 origin present in the backbone of several plasmids, such as pcDNA3.1, pcDNA6, and pSecTag, causing replication of the plasmid outside S phase (DuBridge et al., 1987). This leads to episomal amplification of the transfected plasmid.

We assayed the response of the cell lines to Wnt signalling by measuring β-catenin accumulation and the expression of a transfected reporter gene (Fig 2.2 and Sup Fig 2.1, 2.2). The accumulation of β-catenin was only readily visible in CHO-K1, NIH-3T3, and MEF cells from whole cell lysates (Sup Fig 2.1B, 2.2). The high levels of β-catenin associated with the membrane in HEK293T cells made it impossible to visualise changes in the cytosolic pool of
β-catenin in whole cell lysates, compared to the other cell lines (Sup Fig 2.3A). However, changes in cytosolic β-catenin were clearly visible in HEK293T cells following S100/P100 fractionation by ultracentrifugation (Sup Fig 2.3B). On the other hand, reporter gene assays were only reliable in CHO-K1 and HEK293T cells (Fig 2.2 and 2.3). In fact, activation of the Wnt reporter genes was minimal in NIH-3T3, HEK293, and MEF cells (Fig 2.2 and Sup Fig 2.1A) except following transfection with a plasmid encoding LEF1-VP16, an activated form of LEF1 (Fig 2.2 and Sup 2.1A). HEK293T cells demonstrated a significantly more robust response than CHO-K1 cells when transfected with a plasmid encoding for a stabilised form of β-catenin (Fig 2.3A), or following LiCl treatment (Fig 2.3B). We assumed that the failure to activate a Wnt reporter gene in the NIH-3T3 and MEF cell lines is due to the absence or limitation of a factor(s) downstream of β-catenin that is/are required for transcription. However, we have not investigated this further. Subsequent experiments have been conducted with HEK293T cells.

Finally, we tested four different mammalian Notch1 constructs: ΔEGF_N1, ΔEGF+LNR_N1, ΔN_N1 and NICD, to establish which ones would be most suitable for our experiments. On the one hand, we needed a membrane-restricted form of Notch that would not activate signalling. Therefore, we chose ΔEGF_N1 because it is bound and restricted to the membrane, undergoes S1 cleavage similar to FLN, it is not detected in the nucleus of the cells, and it does not activate Notch signalling (Fig 4B,D). On the other hand, we needed a signalling active form of Notch that would not be bound to the membrane. For that reason we chose NICD (Fig 4B,E). These two constructs are clearly different and would allow us to distinguish between signalling-dependent and -independent crosstalk mechanisms between the Notch and Wnt pathways.
References


Supplementary Figure 2.1: Wnt signalling in MEF cells. A. Wnt signalling activation in MEF cells was tested by transfecting different components of the Wnt pathway (2.5 ng of Wnt1, 125 ng of Dvl2, 25 ng of β-catenin, and 12.5 ng of LEF1-VP16, 50 ng of TCFAdTATA and 20 ng of pRL-CMV per well). The average fold increase in relative luciferase units (RLU) 48 hr after transfection is shown for cells transfected with the different Wnt components compared to cells transfected with empty vector. Data are presented as mean fold change (+/− SEM) in RLU (NS $P>0.05$; *$P<0.05$; ***$P<0.001$ one-way ANOVA and Tukey’s post-hoc test, N≥3). B. Western blot showing the levels of β-catenin in whole cell lysates taken from MEF cells transfected with expression plasmids encoding different components of the Wnt pathway. Note that the antibody used to detect total β-catenin also recognises the expressed V5 tagged β-catenin. Renilla luciferase is shown as a loading control. The position of molecular weight markers (in KDa) is shown.
Supplementary Figure 2.2: β-catenin accumulation in CHO-K1 and NIH-3T3 cell lines after Wnt signalling activation. A and B. Western blot of whole cell lysates showing levels of β-catenin in whole cell lysates taken from cells transfected with expression plasmids encoding different components of the Wnt pathway (Wnt1, Dvl2, and β-catenin). The position of molecular weight markers (in KDa) is shown. Note that the antibody used to detect total β-catenin also recognises the expressed V5 tagged β-catenin. A. Tubulin is shown as a loading control. B. GSK3β is shown as a loading control.
A. HEK293T

<table>
<thead>
<tr>
<th>Component</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-catenin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dvl2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Wnt1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Empty vector</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B. HEK293T

<table>
<thead>
<tr>
<th>Component</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>S45Fβ-catenin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Empty vector</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Supplementary Figure 2.3: β-catenin accumulation in HEK 293T cell line. A. Western blot showing lysates taken from HEK293T cells transfected with different components of the Wnt pathway (Wnt1, Dvl2, and β-catenin). The position of molecular weight markers (in KDa) is shown. GSK3β was used as a loading control. B. Western blot showing levels of β-catenin from a cytosolic fractionation of lysates taken from HEK293T cells transfected with S45Fβ-catenin. Note the accumulation of β-catenin in the cytosolic fraction compared with empty vector (compare lane 1 and 2). Note that this is only the cytosol fraction and does not include any β-catenin from the membrane whereas the whole cell lysate does (see panel A on this figure).
Chapter 3: Paper 2

Membrane-restricted Notch Inhibits β-catenin Transcriptional Activity Independently of Rbpj Signalling.

Ana Hidalgo Sastre¹ & Keith Brennan¹.

¹Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester, M13 9PT, UK.
Author for correspondence (e-mail: keith.brennan@manchester.ac.uk)

Running title: Membrane-restricted Notch inhibits β-catenin activity.

Author contributions: AHS and KB designed the experiments. AHS performed the experiments and wrote the paper. KB edited the paper.

Note for the reader: Once a robust system to study the crosstalk between Notch and Wnt signalling pathways was set up (Chapter 2), I investigated whether Notch inhibition of Wnt signalling at the level of β-catenin was conserved from invertebrates to vertebrates. The experiments performed are presented in the format of a research paper which could be sent for publication to The Journal of Cell Science.
Abstract

Notch and Wnt signalling pathways are two of the key eight signalling networks that shape and model animals during development. Their interaction generates the great diversity of cell types that we can find in metazoans, more than 200 different cells in human bodies. Notch and Wnt pathways are often active at the same time within a tissue, however, they typically have opposite effects upon cell fate decisions, for instance in stem cell self-renewal versus lineage commitment. Therefore, there is great potential for an inhibitory crosstalk to occur. It has been suggested in Drosophila that a full length Notch at the membrane can inhibit Wnt signalling by targeting the amount and activity of Armadillo (β-catenin, in vertebrates). Though, the mechanism that underpins this inhibitory role of Notch on Wnt signalling is not well understood in vertebrates. Here we show a mechanism by which membrane-restricted Notch1 modulates the transcriptional activity of β-catenin in mammalian cells, independently of DSL ligands or CSL transcription factors. Moreover, we demonstrate that Notch1 requires its cdc10/ANK intracellular domain to antagonise β-catenin activity. Thus, it is likely that this membrane mechanism works through the Deltex-mediated Notch trafficking pathway. Finally, our data suggests that this inhibitory crosstalk mechanism is conserved from invertebrates to vertebrates. We believe this mechanism contributes to promote robust cell-fate decisions during development.

Key words: Notch, Wnt, membrane, inhibition, β-catenin, CSL-independent, vertebrates.
Introduction

In mammals there are 4 different Notch genes (Notch 1 to 4) which have different spatio-temporal expression patterns and play distinct roles in cell fate decisions during development (Andersson et al., 2011; Artavanis-Tsakonas et al., 1999). Notch proteins are single pass transmembrane receptors present on the surface of the cells as heterodimers (Blaumuller et al., 1997). Their extracellular domain is linked by a non-covalent Ca\(^{2+}\)-dependent bond to the Notch transmembrane (N\(^{TM}\)) molecule, which comprises extracellular juxtamembrane, transmembrane and intracellular domains (Blaumuller et al., 1997). The extracellular domain of Notch contains 36 epidermal growth factor-like (EGF) repeats required for ligand binding (Rebay et al., 1991) and three cysteine-rich domains (LNR) that function to limit signalling in the absence of ligand (Sanchez-Irizarry et al., 2004). Upon binding with its ligand, Delta-like or Jagged in mammals, Notch extracellular domain is pulled (Nichols et al., 2007) generating an open conformation that is accessible to ADAM/TACE metalloproteases (Gordon et al., 2007; Kovall and Blacklow, 2010). These proteases cleave Notch at the S2 cleavage site to generate a short-lived intermediate, NEXT (Notch extracellular truncation) that is a substrate for \(\gamma\)-secretase (De Strooper et al., 1999; Schroeter et al., 1998). Cleavage by \(\gamma\)-secretase at the S3 cleavage site releases the Notch intracellular domain (NICD) from the membrane. NICD is the signalling effector of the Notch pathway. Its main structural characteristics are a group of seven cdc10/Ankryn (ANK) repeats that are involved in a variety of molecular interactions (Ehebauer et al., 2005) and a RAM23 domain for binding with members of the CSL family of transcription factors (CBF1 in humans, Suppressor of Hairless (Su(H)) in Drosophila, Lag-2 in C.elegans, and Rbpj in mice) (Tamura et al., 1995). Once NICD translocates to the nucleus, it interacts with CSL transcription factors and the Mastermind co-activator (Nam et al., 2007) to activate the expression of specific target genes, such as, members of the Hes and Hey family.

There is evidence to support that Notch can also signal in a CSL-independent manner in vertebrates (Kuroda et al., 1999; Martinez-Arias et al., 2002; Shawber et al., 1996) and Drosophila (Brennan et al., 1999b; Ramain et al., 2001). In fact, numerous experiments from Drosophila indicate that Notch can modulate Wnt signalling in a Su(H)-independent manner by targeting Armadillo (Hayward et al., 2006; Hayward et al., 2005; Lawrence et al., 2001; Ramain et al., 2001; Sanders et al., 2009). The strongest evidence is derived from the study of Abruptex (Ax) and Microchaetae defective (Mcd), Notch alleles, in Drosophila where flies carrying these mutations display a loss of bristles phenotype due to Notch antagonism of Wingless signalling.
(Brennan et al., 1999b; Ramain et al., 2001). Furthermore, this loss of bristles phenotype can be rescued activating downstream Wingless signalling through the loss of Shaggy function, but not the loss of Su(H). The latter result clearly demonstrates that the loss of bristles is not due to excessive signalling during lateral inhibition. Other studies have also shown that loss of Notch function can bypass the requirement for Wingless signalling to initiate gene expression in certain developmental situations (Brennan et al., 1999a; Lawrence et al., 2001). This suggests that Notch can downregulate Wnt signalling independently of Su(H). Consistent with this, eliminating Notch1 expression in the skin of mice leads to tumours with high levels of Wnt signalling (Nicolas et al., 2003).

Mechanistically, the relationship between the pathways is less well understood. In Drosophila, functional interactions between Notch and several components of the Wnt signalling pathway have been described, including Dishevelled (Axelrod et al., 1996; Munoz-Descalzo et al., 2010; Ramain et al., 2001) (Giovanna Collu, unpublished), Axin (Hayward et al., 2006; Munoz-Descalzo et al., 2011), APC (Munoz-Descalzo et al., 2011), GSK3β (Espinosa et al., 2003; Foltz et al., 2002) and β-catenin (Hayward et al., 2005; Sanders et al., 2009). Recently, Notch/β-catenin interactions have also been detailed in vertebrates (Acosta et al., 2011; Kwon et al., 2011). From this work it is unclear whether Notch must be present at the membrane or in the nucleus to effect the crosstalk on β-catenin, or whether it is mediated by Notch itself or downstream effectors of the pathway. Consequently, it is not clear whether the same molecular mechanism is conserved across species.

It is well established that the key parameter of the Wnt signalling pathway is the stability and localisation of a soluble pool of β-catenin (Gottardi and Gumbiner, 2001; Logan and Nusse, 2004; MacDonald et al., 2009; Reya and Clevers, 2005). In the absence of Wnt ligand the free cytosolic β-catenin interacts with a destruction complex, formed by CK1, APC, Axin and GSK3β, which phosphorylates β-catenin and targets it for degradation via the proteosome (Aberle et al., 1997). The Wnt ligand interacts with Frizzled and LRP receptors at the surface of the membrane priming intracellularly the recruitment of the cytoplasmic adaptor protein Dishevelled, as well as, the rest of the destruction complex to the membrane. This allows the accumulation of a hypophosphorylated form of β-catenin in the cytosol (Tolwinski and Wieschaus, 2004). This activated form of β-catenin can now enter the nucleus and bind members of the TCF/LEF family of transcription factors to activate expression of target genes such as c-Myc and Axin2 (Logan and Nusse, 2004).
Here we examine whether a membrane-restricted form of Notch can limit Wnt signalling in a mammalian system and the mechanism that underpins this crosstalk. We demonstrate that in the presence of a membrane-restricted Notch1 receptor, β-catenin transcriptional activity is reduced. Since the membrane-restricted form of Notch cannot activate the expression of downstream target genes, these results reveal an effect of Notch on Wnt signalling that is independent of both DSL ligands and CSL transcription factors function. This mechanism seems to require Deltex-mediated endocytosis of Notch. Our results provide a mechanism for the interaction between Notch and Wnt signalling pathways that has implications for the cell fates during development and perhaps the formation of tumours.
Materials and methods

This is a general description of the methods used for this manuscript. Further details are given in the supplementary materials and methods on Chapter 6.

Cell culture and transfections

Human embryonic kidney cells that stably express the large T antigen of simian virus 40 (HEK293T) were obtained from Dr Anthony Brown (Weill Medical College, Cornell University, New York, USA) and from Dr Valerie Kouskoff (Paterson Institute for Cancer Research, Manchester, UK). HEK293T cells were cultured in DMEM medium supplemented with 10% FBS and 50 µg/ml penicillin and 50 µg/ml streptomycin. Cells were maintained at 37°C and 5% CO₂ in a humidified incubator. Cells were transfected using Lipofectamine and PLUS reagent (Invitrogen) or X-Treme transgene 9 transfection reagent (Roche), according to manufacturers' instructions.

Luciferase assays

HEK293T cells were plated at a density of 2 x 10⁵ cells/well of a 24 well plate. After 24 hr cells were transfected in triplicate with a total of 250 ng DNA cocktail containing the desired DNA plasmids and 50 ng of the Wnt reporter plasmid TCFAdTATA. As an internal control 20 ng of pRL-CMV plasmid was used. To maintain transfections with a constant amount of total DNA, the plasmid pcDNA3.1(+) was used. The cell culture medium was changed 3 hr after transfection when Lipofectamine and PLUS reagent (Invitrogen) were used. Cells were lysed 48 hr post-transfection using 1 x Passive Lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter assay system (Promega), according to manufacturer’s instructions, with a MicroLumatPlus plate reader (Berthold Technologies). Data are presented as mean fold change (+/−SEM) in relative luciferase units (RLU), compared to β-catenin. Statistical analysis was perform using one-way ANOVA and Tukey’s post-hoc tests for more than two samples or with a Student T-test for data with two samples.

cDNA constructs

A description of the constructs used in the experiments for this manuscript can be found on the supplementary materials and methods on Chapter 6 section 6.4.

Notice that ΔEGF_N1 lacks the extracellular 36 EGF-like repeats of the Notch receptor, and therefore, is unable to interact with DSL ligands. However, it contains the 3 Lin-12/Notch repeats (LNR) that form a regulatory domain to prevent access of ADAM/TACE proteases to
the S2 cleavage site (Gordon et al., 2007) and the generation of the NEXT (Notch extracellular truncation) signalling intermediate (Mumm et al., 2000).

S45Fβ-catenin is a stable form of the β-catenin protein with a serine 45 to phenylalanine (S45F) mutation expected to prevent its phosphorylation by CK1 (Amit et al., 2002; Liu et al., 2002; Yanagawa et al., 2002). Phosphorylation of serine 45 allows GSK3β to bind β-catenin and phosphorylate serine and threonine residues at amino acids 33, 37 and 41 (Yost et al., 1996). Once phosphorylated on these amino acids, β-catenin is recognised by E3 ubiquitin ligase β-TrCP targeting β-catenin for proteosomal degradation (Aberle et al., 1997). Consequently, the S45F mutations should prevent the phosphorylation of β-catenin by GSK3β and therefore, its degradation.

**S100/P100 cytosolic fractionation**

Cells were washed twice in 3 ml of 1 x TBS (10 mM Tris-HCl pH 7.4 + 140 mM NaCl + 2 mM CaCl₂), placed on ice and lysed in 1 ml of lysis buffer (1 x TBS + freshly added protease inhibitor cocktail set I (Calbiochem)) + 10 µl of 100 mM PMSF. Subsequently, cells were poured into a cold 2 ml dounce homogenizer and sheared with 30 strokes. Cell lysates were centrifuged at 1500 x g for 5 min at 4°C. The supernatant was centrifuged for 90 min at 100000 x g in a TLA 110 rotor in a Beckman Coulter Optima TLX-120 Ultracentrifuge at 4°C. After the spin, 100 µl of the supernatant, containing the cytosolic fraction, were mixed with 100 µl of 2 x Laemmli buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol), boiled for 3 min and stored at -20°C. The pellet, containing the membrane fraction, was washed twice in 1 x TBS, re suspended in 1 ml of 2 x Laemmli buffer, boiled for 3 min and kept at -80°C.

**Western blotting**

Total cell lysis and subsequent western blotting were performed as described (Stylianou et al., 2006). For more details see supplementary materials and methods, Chapter 6.

**Immunofluorescence**

HEK293T cells were seeded on nitric acid treated coverslips at 4 x 10⁵ cells/well of a 6 well plate. Transfection was performed 24 hr later, as described above. Cells were fixed 24 hr post transfection for 10 min in 1 x PBS containing 4% formaldehyde. Following three washes with 1 x PBS, coverslips were incubated either with α-myc-Tag rabbit primary antibody (Cell signaling) for Notch constructs, or with mouse α-V5 Alexa 488 conjugated antibody (AbD SeroTec) to detect S45Fβ-catenin, diluted 1:100 in blocking solution (3% goat serum (Biosera, Sussex, UK), 0.1% Triton-X100, and 0.05% NaN₃ in TBS) in a humidified chamber for 1 hr.
Subsequently, cells were washed and coverslips for Notch constructs were incubated with fluorescence goat α-rabbit Alexa 594 secondary antibody in blocking solution. Coverslips were mounted with VECTASHIELD mounting medium for fluorescence with DAPI H-1200 (Vector Laboratories). Images were captured with a Zeiss LSM 700, AxioObserver flexible confocal microscope (Carl Zeiss MicroImaging GmbH, Germany), using the Zeiss ZEN 2011 software (Carl Zeiss MicroImaging GmbH, Germany). The confocal software was used to determine the optimal number of Z sections when acquiring 3D optical stacks. Either maximum intensity projections of these 3D stacks or single z-section images are shown in the results.

**Quantitative Image analysis**

The fluorescence intensity of nuclear S45Fβ-catenin was determined with image J software (open source software, National Institute of Health, USA) by measuring the integrated fluorescence intensity of the nucleus and cytoplasm of cells transfected with different plasmids. The integrated density of each cell was measured at the middle plane of a z series. The maximum nuclear to cytosol ratio was set to 0.75 (as this was the maximum intensity observed when S45Fβ-catenin was transfected alone). Negative nuclear to cytosol ratios (ie: when nuclear corrected total fluorescence was negative) were set to zero. At least 25 cells were measured per condition. For ratio measurements, the following formulas were used:

**Whole cell signal** = Integrated density (sum of the intensity of the pixels for one cell)

**Nuclear signal** = Integrated density of the nucleus of a cell

**Cytosol fluorescence** = whole cell signal – nuclear signal

**Corrected total fluorescence (CTF)** = Integrated density – (Area of selected cell or nucleus x Mean fluorescence of background readings).

**Nuclear to cytosol ratio** = Nuclear CTF / Cytosol CTF
Results

Notch at the membrane modulates the transcriptional activity of β-catenin.

Previous work has shown that Notch can inhibit Wnt signalling (Deregowski et al., 2006; Hayward et al., 2005; Nicolas et al., 2003; Sanders et al., 2009). However, it is unclear whether this crosstalk is mediated by a membrane-restricted form of Notch or through the transcription of downstream target genes. To analyse whether a membrane-restricted form of Notch can inhibit Wnt signalling, we have generated a form of Notch1, ΔEGF_N1, that is membrane-restricted (Fig 3.1D) and unable to signal (Fig 3.2A-C). On the other hand, ΔEGF_N1 does undergo S1 cleavage in the Golgi and therefore, is present at the cell surface as a heterodimer, like the full length Notch protein (Fig 3.2C lane 2).

Transfection of Wnt1, S45Fβ-catenin (a stabilised form of β-catenin) or LEF1-VP16 (a constitutively active form of the LEF1 transcription factor), activated Wnt signalling as expected (Fig 3.1A, B). Wnt signalling was monitored by co-transfecting the cells with the TCFAdTATA reporter plasmid (Fig 2.1), which contains a minimal adenoviral TATA box regulated by four TCF/LEF binding sites (Chapter 2). Co-expression of ΔEGF_N1 significantly reduced Wnt signalling activation by both Wnt1, and S45Fβ-catenin (Fig 3.1A, B). In contrast, LEF1-VP16 activity did not seem to be affected by the presence of this membrane-restricted form of Notch1 (Fig 3.1A). These results indicate that a membrane-restricted form of Notch can inhibit Wnt signalling and that it appears to do so at the level of β-catenin.

To find out whether the inhibition S45Fβ-catenin transcriptional activity by ΔEGF_N1 was due to protein degradation or not, a cytosolic fractionation for S45Fβ-catenin, in the absence or presence of ΔEGF_N1, was performed. Expression of S45Fβ-catenin in HEK293T cells elevated the overall levels of endogenous β-catenin (lower band of doublet, Fig 3.1C lane 2) and showed accumulation of S45Fβ-catenin (Upper band of doublet, Fig 3.1C compare lane 1 and 2) in the cytosol. Interestingly, both S45F and endogenous β-catenin protein levels did not change when ΔEGF_N1 was present in the cytosol (Fig 3.1C compare lanes 2 and 3). Similar results were obtained with whole cell lysates either for β-catenin or S45Fβ-catenin (Sup Fig 3.1 A, B). This suggests that, although, Notch can inhibit Wnt signalling at the level of β-catenin, the protein still accumulates in the cytosol. This result demonstrates that a membrane-restricted Notch inhibits the transcriptional activity of β-catenin without clearly degrading the protein.
To investigate whether membrane-restricted Notch inhibits β-catenin transcriptional activity by altering its localisation, we analysed the localisation of S45Fβ-catenin in the presence or absence of ΔEGF_N1 by immunofluorescence. In the absence of Notch, S45Fβ-catenin was generally distributed throughout the cell (Fig 3.1E). Additionally, S45Fβ-catenin presented a bright punctuate pattern at the membrane which resemble vesicles. To obtain an indication of the amount of β-catenin present in the nucleus and to account for variations in transfection, we performed a quantitative analysis looking at nuclear levels relative to whole cell levels (Sup Fig 3.2A). ΔEGF_N1 was mainly localised at the membrane, with some present in vesicle-like structures within the cytoplasm but none in the nucleus, either alone or when co-transfected with S45Fβ-catenin (Fig 3.1D,F). In the presence of ΔEGF_N1 S45Fβ-catenin had a similar distribution to that observed when it was transfected alone (Fig 3.1G). However, there were less bright vesicle-like punctate at the membrane (compare Fig 3.1E with 3.1G), and the quantitative analysis, which takes into account transfection differences, suggested a slight shift towards higher levels of S45Fβ-catenin in the nucleus (Sup Fig 3.2B).

These data demonstrates membrane-restricted Notch inhibits β-catenin transcriptional activity without altering β-catenin normal expression or localisation to the nucleus.

**Inhibition of β-catenin signalling by Notch happens at the membrane independently of Rbpj.**

ΔEGF_N1 is likely to inhibit Wnt signalling in a CSL-independent fashion as it is membrane-restricted, and CSL transcription factors are predominantly nuclear. However, to confirm that the effects of ΔEGF_N1 are CSL-independent we generated a point mutation, W1758A, in ΔEGF_N1 that will eliminate Notch ability to bind CSL. As controls, we generated two other point mutations, V1744G and A2026V (Fig 3.2A). During wild type canonical Notch signalling S3 cleavage mediated by γ-secretase occurs between Gly 1743 and Val 1744 (De Strooper et al., 1999; Schroeter et al., 1998). The V1744G mutation, alters the precision of the γ-secretase cleavage upon ligand binding, resulting in cleavages at different positions within the transmembrane domain generating much less stable forms of NICD (Tagami et al., 2008). Given that ΔEGF_N1 does not undergo S3 cleavage, the V1744G mutation is not expected to alter its function. The A2026V mutation has been described before in Drosophila (Nsu42c). Nsu42c is a point mutation in the 5th ANK repeat that, although, it does not affect significantly Notch interactions with Deltex, it results in phenotypes that resemble Deltex loss-of-function (Diederich et al., 1994; Matsuno et al., 1997; Ramain et al., 2001; Zweifel et al., 2003), suggesting that it alters the ability of Deltex to regulate the movement of Notch through the endocytic pathway (Hori et al., 2004; Matsuno et al., 1998; Wilkin et al., 2008).
The ability of the different Notch mutant constructs to activate CSL signalling was tested with a Rbpj-dependent reporter plasmid, p10xRbpj-luc. As expected, membrane bound ΔEGF-N1 was poorly cleaved by γ-secretase and had no transcriptional activity (Fig 3.2B). Likewise, we confirmed that ΔEGF-N1 W1758A, ΔEGF-N1 V1744G, and ΔEGF-N1 A2026V, exhibited negligible levels of Notch/Rbpj-dependent luciferase activity (Fig 3.2B). All constructs were expressed to similar levels as shown by western blot analysis (Fig 3.2C). Localisation was confirmed by immunofluorescence. ΔEGF-N1 was localised predominately at the membrane and in some vesicle-like structures in the cytoplasm (Fig 3.1D). This could be due to cytosolic synthesis, degradation and recycling of the molecule. ΔEGF-N1 W1758A localisation pattern resembled that of ΔEGF-N1, being predominately at the membrane and in some vesicle-like structures in the cytoplasm (Fig 3.2D). In contrast, ΔEGF-N1 V1744G was mainly at the membrane and it could not be detected in the cytosol, leaving a big gap between cell membrane and nuclear envelop (Fig 3.2E). Although, present at the membrane, ΔEGF-N1 A2026V, appeared mainly localised in some vesicle-like pattern in the cytoplasm (Fig 3.2F). None of the membrane-restricted Notch constructs were present in the nucleus of the cells.

To test whether modulation of β-catenin signalling by ΔEGF_N1 requires its ability to interact with CSL transcription factors, ΔEGF-N1 and the point mutants were co-transfected with S45Fβ-catenin and the Wnt reporter (TCFAdTATA). Results showed that there was no difference between the ability of ΔEGF-N1 or ΔEGF-N1 W1758A to inhibit Wnt signalling (Fig 3.3A). However, ΔEGF-N1 V1744G was more potent (Fig 3.3A), whilst ΔEGF-N1 A2026V had no effect on the ability of S45Fβ-catenin to activate transcription. To further analyse this mechanism, localisation of β-catenin was compared in the presence or absence of ΔEGF-N1 point mutants. When S45Fβ-catenin was co-expressed with ΔEGF-N1 W1758A, it still localised to the nucleus of many cells and we observed a reduction of the bright vesicle-like dots at the membrane (Fig 3.3C). In the presence of ΔEGF-N1 V1744G, S45Fβ-catenin appeared in nucleus and cytoplasm but also defining an obvious membrane for the majority of the cells. However, no bright vesicle-like punctate were observed at the membrane, in comparison to when S45Fβ-catenin was transfected alone (Fig 3.3E). In addition, the quantitative analysis suggested that the frequency of intensities shifted towards the middle rather than to lower ones for both ΔEGF-N1 W1758A and ΔEGF-N1 V1744G (Sup Fig 3.3A,B). Finally, in the presence of ΔEGF-N1 A2026V, S45Fβ-catenin distribution and intensity resembled that of S45Fβ-catenin transfected alone (Fig 3.3G and Sup Fig 3.3C). Additionally, the bright vesicle-like punctuate pattern was again visible at the membrane of cells (Fig 3.3G). The Notch point mutants did not change their
localisation in the presence of S45Fβ-catenin (Fig 3.3 B, D, F). These results are in agreement with the luciferase reporter assay for these constructs (Fig 3.3A).

These observations clearly show that the inhibition of Wnt signalling by ΔEGF-N1 is not reliant on its ability to interact with Rbpj. However, we were surprised to see that ΔEGF-N1 V1744G was more potent than ΔEGF-N1 or the ΔEGF-N1 W1758A mutant inhibiting β-catenin transcriptional activity. Interestingly, despite causing an inhibition of Wnt signalling ΔEGF-N1 W1758A and ΔEGF-N1 V1744G point mutants did not change the localisation of S45Fβ-catenin to the nucleus.

**Notch requires the cdc10/ANK domain to inhibit β-catenin activity.**

Next we mapped the domains of Notch required for inhibition of β-catenin transcriptional activity. To do this we deleted different domains of the membrane bound Notch construct from the C-terminus. ΔEGF-N1 Δ425 lacks the TAD, OPA, and PEST domains, ΔEGF-N1 Δ671 also lacks cdc10/ANK repeats, whilst ΔEGF-N1 Δ764 lacks nearly all of the intracellular domain except for 15 amino acids (Fig 3.4A). Notch/Rbpj-dependent signalling of these truncated constructs was tested in a Notch reporter assay. As expected we found that they were signalling deficient (Fig 3.4B). As shown by western blot, all truncated membrane bound Notch constructs are expressed to similar levels (Fig 3.4C) and they localise to the membrane, as expected (Fig 3.4D-F).

We observed that Notch mutants lacking the cdc10/ANK domain had no effect on S45Fβ-catenin transcriptional activity (Fig 3.5A). This is in agreement with our previous result where we had shown that the point mutant for the ANK domain that alters the interaction with Deltex, ΔEGF-N1 A2026V, had no effect on the ability of β-catenin to activate signalling (Fig 3.3A,G).

Localisation of β-catenin in the absence or presence of ΔEGF-N1 Δ425, Δ671, and Δ764 was verified by immunofluorescence (Fig 3.5B-G). When S45Fβ-catenin was co-transfected with ΔEGF-N1 Δ425 the protein was present in both the cytosol and the nucleus, some of it also appeared at the membrane in the form of bright vesicle-like punctate (Fig 3.5C). Quantitative analysis suggested a shift towards higher levels of S45Fβ-catenin in the nucleus (compare Sup Fig 3.4A with Sup Fig 3.2A). In the presence of ΔEGF-N1 Δ671 and ΔEGF-N1 Δ764, S45Fβ-catenin distribution resembled the pattern of S45Fβ-catenin transfected alone, present throughout the cell and with many bright vesicle-like dots at the membrane (Fig 3.5E,G and SupFig 3.4B,C). None of the Notch deletion constructs changed their localisation
when co-transfected with S45Fβ-catenin compared to when they were transfected alone (compare Fig 3.4D-F with Fig 3.5B,D,F).

This results indicate that Notch inhibition of β-catenin transcriptional activity requires the cdc10/ANK domain present in the intracellular domain of Notch, and possibly binding to Deltex protein.

**Membrane-restricted Notch inhibition of β-catenin is conserved across species.**

Finally, we wanted to address whether this Notch inhibitory crosstalk mechanism was conserved across vertebrate species, therefore, we chose to work with Xenopus constructs. When Xenopus β-catenin (Xβ-catenin) was co-transfected with ΔEGF_N1, interestingly, we found that this murine membrane-restricted form of Notch inhibited Xβ-catenin ability to activate Wnt signalling (Fig 3.6). Therefore, this observation suggests that this mechanism is conserved across species.
Figure 3.1: Membrane bound Notch inhibits β-catenin transcriptional activity in vertebrate cells. A. Relative Wnt/β-catenin luciferase activity of HEK293T cells transfected with different components of Wnt signalling pathway (2.5 ng of Wnt1 and 10 ng of LEF1-VP16, per well) alone or in the presence of 25 ng, per well, of the signalling inactive form of Notch, ΔEGF_N1. To monitor Wnt signalling 50 ng of TCFAdTATA Wnt reporter plasmid and 20 ng of the control reporter plasmid pRL-CMV were also transfected. The experiment was performed in triplicate and cells lysed 48 hr post transfection. Data are presented as mean fold change (+/- SEM) in RLU (*P<0.05; **P<0.001 one-way ANOVA and Tukey’s post-hoc test, N=1). B. Wnt signalling was induced by transfecting HEK293T cells as before with S45F β-catenin. The presence of the membrane-restricted Notch, ΔEGF_N1, significantly reduced the levels of ectopic Wnt signalling activated by β-catenin. Data are presented as mean fold change (+/- SEM) in RLU (*P<0.05; **P<0.001 one-way ANOVA and Tukey’s post-hoc test, N=3). C. Cytosolic fractionation showing by western blot expression of β-catenin. Notice that in the presence of S45Fβ-catenin there is an accumulation of both ectopic S45Fβ-catenin and endogenous β-catenin levels in comparison with the empty vector (compare lane 1 and 2). Expression of ΔEGF_N1 does not alter β-catenin protein levels (compare lane 2 and 3). D-G. Effects of ΔEGF_N1 on the stability of S45F β-catenin by confocal microscopy analysis. DAPI (blue) was used to stain the nuclei. Red represents ΔEGF_N1 and green is S45Fβ-catenin. Cells were transfected and stained as described in the materials and methods. D. Localisation of ΔEGF_N1. E. Localisation of S45Fβ-catenin. F & G. Localisation of S45Fβ-catenin and ΔEGF_N1 when co-transfected. Notice the localisation of S45Fβ-catenin is not significantly altered by the presence of ΔEGF_N1. Quantitative analysis of S45Fβ-catenin fluorescence in the nucleus versus the cytosol is shown in Sup Fig 3.2.
Chapter 3: Membrane-restricted Notch Inhibits β-catenin Activity

A. Bar graph showing the effect of Notch inhibition on β-catenin activity. Ncat = Nanog, Lcat = LDLR.

B. Bar graph showing the effect of Notch inhibition on β-catenin activity. S5 and S2 are conditions tested.

C. Western blot analysis showing the expression of β-catenin under different conditions. S5 and S2 are conditions tested.

D. Fluorescence images showing the localization of ΔEGF-N1 and DAPI in cells.

E. Fluorescence images showing the localization of S45F-β-catenin and DAPI in cells.

F. Fluorescence images showing the localization of ΔEGF-N1 + S45F-β-catenin and DAPI in cells.

G. Fluorescence images showing the localization of ΔEGF-N1 + S45F-β-catenin and DAPI in cells.
Chapter 3: Membrane-restricted Notch Inhibits β-catenin Activity

**Figure 3.2: ΔEGF_N1 functions independently of CBF1/Rbpj.** A. Diagram of the Notch point mutants used. All constructs contain a myc tag within the intracellular domain before the OPA and PEST domains (red hexagon in C-terminus). Those carrying the IgG k-chain signal peptide (Blue hexagon in N-terminus) also contain a His/myc tag at the N-terminus just after the signal peptide (green hexagon). Big asterisks represent the mutations. B. Identifying signalling deficient and competent Notch molecules. Relative Notch/Rbpj luciferase activity of HEK293T cells transfected, as before (Chapter 2), with 25 ng of ΔEGF_N1, the point mutants ΔEGF_N1 W1758A, ΔEGF_N1 V17744G, and ΔEGF_N1 A2026V, or 12.5 ng of Rbpj-VP16, per well. Signalling was monitored using 50 ng of a Notch reporter plasmid 10xRbpj-luc and 20 ng of pRL-CMV were used as a transfection control. ΔEGF_N1 and the point mutants are rarely cleaved from the membrane and do not activate the CBF1 reporter. Experiments were performed in triplicate and cells were lysed 48h post transfection. Data are presented as mean fold change (+/- SEM) in RLU (NS P>0.05; ***P<0.001 one-way ANOVA and Tukey’s post-hoc test, N=3). C. Western blot showing protein expression of ΔEGF_N1 and the point mutants ΔEGF_N1 W1758A, ΔEGF_N1 V17744G, and ΔEGF_N1 A2026V. Proteins are expressed to similar levels. Expressed protein was detected by probing the western blot with an antibody that recognises the myc epitope tag found within all the proteins (α-myc clone 4A6, Upstate). Renilla luciferase is shown as a loading control. The position of molecular weight markers (in KDa) is shown. D-F. Localisation of membrane-restricted Notch constructs by immunofluorescence and confocal microscopy analysis. Cells were transfected with 25 ng of ΔEGF_N1 W1758A, ΔEGF_N1 V17744G, and ΔEGF_N1 A2026V and fixed 24h post transfection. Expressed protein was detected by staining with a primary antibody against the myc epitope tag (α-myc-Tag Rabbit, Cell Signaling) and a secondary antibody Goat α-Rabbit Alexa 594 (Molecular probes). Scale bar is 51 µm. A single z-section through the middle of the cell is show.
Chapter 3: Membrane-restricted Notch Inhibits β-catenin Activity

A.

B.

C.

D.

E.

F.
Figure 3.3: Notch at the membrane inhibits β-catenin transcriptional activity independently of CBF1/Rbpj transcription. A. Relative Wnt/β-catenin luciferase activity of HEK293T cells transfected with S45Fβ-catenin alone or in the presence of ΔEGF_N1 or the point mutants ΔEGF_N1 W1758A, ΔEGF_N1 V17744G, and ΔEGF_N1 A2026V. Cells were transfected as before. Signalling was monitored using 50 ng of the Wnt reporter plasmid TCFAdTATA and 20 ng of pRL-CMV were used as a transfection control. Experiments were performed in triplicate and cells were lysed 48h post transfection. Data are presented as mean fold change (+/- SEM) in RLU (NS $P>0.05$; **$P<0.01$; ***$P<0.001$ one-way ANOVA and Tukey’s post-hoc test, N ≥ 3). B-G. Confocal microscopy analysis of S45Fβ-catenin localisation in the presence of ΔEGF_N1 W1758A, ΔEGF_N1 V17744G, and ΔEGF_N1 A2026V. Cells were transfected with 25 ng of S45Fβ-catenin and either 25 ng of ΔEGF_N1 or the point mutants and fixed 24h post transfection. Expressed protein was detected by staining with a primary antibody against the myc epitope tag (α-myc-Tag Rabbit, Cell Signaling) and a secondary antibody Goat α-Rabbit Alexa 594 (Molecular probes) for Notch constructs, or with a V5-Tag: Alexa 488 (AbDSeroTec) primary conjugated antibody for β-catenin. Scale bar is 51 µm for Notch images and 100 µm for β-catenin images. A single z-section through the middle of the cell is show. Quantitative analysis of S45Fβ-catenin fluorescence in the nucleus versus the cytosol is shown in Sup Fig 3.3.
Chapter 3: Membrane-restricted Notch Inhibits $\beta$-catenin Activity

A.

B.

C.

D.

E.

F.

G.
Figure 3.4: Characterisation of the membrane-restricted C-terminal deletion Notch constructs. A. Diagram of the Notch deletion constructs used. All constructs carry the IgG k-chain signal peptide (Blue hexagon N-terminus) and contain a His/myc tag at the N-terminus just after the signal peptide (green hexagon). B. Identifying signalling competency of the Notch molecules. HEK 293T cells were transfected, as before, with 25 ng of ΔEGF_N1, ΔEGF_N1 Δ671, ΔEGF_N1 Δ764 or NICD, or 62.5 ng of ΔEGF_N1 Δ425, per well. Signalling was monitored using 50 ng of a Notch reporter plasmid 10xRbpj-luc and 20 ng of pRL-CMV were used as a transfection control. ΔEGF_N1 and the deletion mutants are rarely cleaved from the membrane and do not activate the CBF1 reporter. Experiments were performed in triplicate and cells were lysed 48h post transfection. Data are presented as mean fold change (+/- SEM) in RLU (NS P>0.05; ***P<0.001 one-way ANOVA and Tukey’s post-hoc test, N=2) C. Western blot showing protein expression of ΔEGF_N1 and the deletion constructs ΔEGF_N1 Δ425, ΔEGF-N1 Δ671, and ΔEGF-N1 Δ764. Proteins are expresses to similar levels. Expressed protein was detected by probing the western blot with an antibody that recognises the myc epitope tag found within all the proteins (α-myc clone 4A6, Upstate). Renilla luciferase is shown as loading control. The position of molecular weight markers (in KDa) is shown. D-F. Localisation of membrane-restricted ΔEGF_N1 Δ425, ΔEGF_N1 Δ671, and ΔEGF_N1 Δ764 Notch deletion constructs by immunofluorescence and confocal microscopy analysis. Cells were transfected with 62.5 ng of Δ425 or 25 ng of the deletion constructs Δ671 and Δ764, per well, and fixed 24h post transfection. Expressed protein was detected by staining with a primary antibody against the myc epitope tag (α-myc-Tag Rabbit, Cell Signaling) and a secondary antibody Goat α-Rabbit Alexa 594 (Molecular probes). Scale bar is 51 μm. A single z-section through the middle of the cell is show.
Chapter 3: Membrane-restricted Notch Inhibits β-catenin Activity

A. Diagram showing αEF_N1 and αEF_N1 Δ425, αEF_N1 Δ671, and αEF_N1 Δ764

B. Graph showing fold change in RLU

C. Table showing expression levels

D. Images of αEF_N1 Δ425, DAPI, and Merged

E. Images of αEF_N1 Δ671, DAPI, and Merged

F. Images of αEF_N1 Δ764, DAPI, and Merged
Figure 3.5: The ANK repeats of Notch are required to inhibit β-catenin transcriptional activity.

A. Relative Wnt/β-catenin luciferase activity of HEK293T cells transfected with 25 ng of S45Fβ-catenin alone or in the presence of 62.5 ng of ΔEGF_N1Δ425, or 25 ng of ΔEGF_N1Δ671 or ΔEGF_N1Δ764, per well. Signalling was monitored using 50 ng of the Wnt reporter plasmid TCFAdTATA and 20 ng of pRL-CMV were used as a transfection control. Experiments were performed in triplicate and cells were lysed 48h post transfection. Data are presented as mean fold change (+/- SEM) in RLU (NS P>0.05; *P<0.05; one-way ANOVA and Tukey’s post-hoc test, N≥3).

B-G. Localisation of S45Fβ-catenin in the presence of ΔEGF_N1Δ425, ΔEGF_N1Δ671, and ΔEGF_N1Δ764 by confocal microscopy analysis. Cells were transfected as above and fixed 24h post transfection. Notch proteins were detected by staining with a primary antibody against the myc epitope tag (α-myc-Tag Rabbit, Cell Signaling) and a secondary antibody Goat α-Rabbit Alexa 594 (Molecular probes). S45Fβ-catenin was detected with a V5-Tag: Alexa 488 (AbDSeroTec) conjugated primary antibody. Scale bar is 51 µm for Notch images and 100 µm for β-catenin images. A single z-section through the middle of the cell is show. Quantitative analysis of S45Fβ-catenin fluorescence in the nucleus versus the cytosol is shown in Sup Fig 3.4.
Chapter 3: Membrane-restricted Notch Inhibits β-catenin Activity

A.

B.

C.

D.

E.

F.

G.
Figure 3.6: Mouse ΔEGF_N1 inhibits Xenopus β-catenin transcriptional activity. Relative Wnt/β-catenin signalling shown by a luciferase reporter assay in HEK293T cells transfected with 25 ng of S45Fβ-catenin alone or in the presence of 25 ng of ΔEGF_N1 per well. Signalling was monitored using 50 ng of the Wnt reporter plasmid TCFA-ΔTATA and 20 ng of pRL-CMV were used as a transfection control. Experiments were performed in triplicate and cells were lysed 48h post transfection. Data are presented as mean fold change (+/- SEM) in RLU (***P<0.001 one-way ANOVA and Tukey’s post-hoc test, N=2).
Chapter 3: Membrane-restricted Notch Inhibits β-catenin Activity

Discussion

In this study we elucidate a molecular mechanism by which the Notch1 receptor regulates Wnt signalling. We have demonstrated that a signalling deficient, membrane-restricted form of Notch1 can reduced S45Fβ-catenin transcriptional activity by about 20% (Fig 3.1B & 3.6). This inhibition of Wnt signalling by ΔEGF_N1 is independent of its interaction with CSL/Rbpj in the nucleus (Fig 3.3A) or DSL ligands at the membrane. Interstingly, we found that these membrane restricted forms do not alter the stability of β-catenin (Fig 3.1B,C), or its localisation (Fig 3.1G, Sup Fig 3.2B). Furthermore, we found that Notch requires the cdc10/ANK repeats present on the intracellular domain to inhibit β-catenin transcriptional activity (Fig 3.5A). Therefore, these data lead us to propose the following model mechanism: membrane-restricted Notch, which is continuously endocytosed and either recycled back to the membrane or trafficked to be degraded in the endosomes by Deltex, uses its ANK repeats to bind a component required for the activation of β-catenin and sequester it or possibly degrade it. By these means, in the presence of Notch, although, β-catenin can still translocate to the nucleus it is not able to activate the transcription of target genes. Finally, our data suggest that this mechanism is conserved across species (Fig 3.6).

It has been shown that signalling inactive forms of Drosophila Notch that are restricted to the membrane, like Torso Notch (TNotch) and CeN, can inhibit Wnt signalling in Drosophila (Hayward et al., 2005). These chimeras contain the Notch intracellular domain fused to the extracellular and transmembrane domains of the Torso receptor tyrosine kinase or CD8, respectively. Thus, these proteins cannot interact with DSL ligands and lack the S2 and S3 cleavage sites. Therefore, these proteins are not expected to stimulate transcription of CSL-dependent targets. These proteins have both been shown to inhibit Wingless/Armadillo signalling in the developing wing disc. For CeN, it has been shown that the inhibition of Wingless/Armadillo signalling is through the recruitment of Armadillo to the apical side of the cell, followed by its subsequent endocytosis and degradation (Sanders et al., 2009). Interestingly, a stabilised form of Armadillo (Armadillo\textsuperscript{10}) accumulates in the wing discs in the absence of Notch, suggesting that Notch helps to maintain low levels of Armadillo in the cell (Sanders et al., 2009). We observe similar results in our mammalian system, in which the membrane-restricted forms of Notch, that cannot interact with CSL, negatively regulate β-catenin transcriptional activity, but they do not change the localisation of stable S45Fβ-catenin to the nucleus (Fig 3.3). However, this suggests that the regulation of Wnt signalling by a membrane-restricted form of Notch is conserved between species.
In a very recent study carried out in mouse embryonic stem cells and colorectal cell lines, Kwon and colleagues (2011), have also shown that Notch1 inhibits β-catenin transcriptional activity. However, unlike our study, they have used N^ΔE constructs that undergo spontaneous γ-secretase mediated cleavage (Jarriault et al., 1995; Schroeter et al., 1998). In addition, they suggest that Notch1 degrades active β-catenin by physically associating with it, via the RAM domain and that Notch-induced degradation of β-catenin requires Numb. Numb is an adaptor protein that antagonises Notch signalling by recruiting the E3 ubiquitin ligase Itch to ubiquitinate Notch and, thus, promote its trafficking through the endosomal system, leading its degradation (McGill et al., 2009). In contrast, we demonstrated no obvious change in the levels and localisation of β-catenin (Fig 3.1C, 3.1G), although, there is a marked reduction of signalling (Fig 3.1B). In addition, our experiments indicate that the ANK domain is important (Fig 3.3A, 3.5A). Firstly, the A2026V mutation within the 5th ANK repeat abolishes the ability of ΔEGF_N1 to inhibit Wnt/β-catenin signalling (Fig 3.3A). Secondly, the inhibitory function of ΔEGF_N1 was also lost when the ANK domain was deleted in ΔEGF_N1 Δ671 and ΔEGF_N1 Δ764 (Fig 3.5A). Furthermore, these results point to a role of Deltex in the process, particularly the A2026V mutation. This mutation mimics the N^Glu2c mutation described in Drosophila (Diederich et al., 1994; Matsuno et al., 1997; Zweifel et al., 2003). This mutation is known to alter the binding between Notch and Deltex, leading to Deltex-like mutant phenotypes in Drosophila. The marked difference in our results, and those of Kwon and colleagues (2011), most likely stems from the Notch constructs used, ours are membrane-restricted whilst theirs will undergo spontaneous γ-secretase mediated cleavage leading to the nuclear localisation of NICD. It is interesting to note that we found that a mutant NICD protein that cannot interact with Rbpj cannot inhibit β-catenin signalling (see Chapter 4), much like the N^ΔE construct lacking the RAM domain. However, the mechanism by which NICD proteins inhibit Wnt/β-catenin signalling is distinct (see Chapter 4; (Deregowski et al., 2006)).

In our mammalian system we have shown that in the presence of Notch, although, β-catenin localises to the nucleus its transcriptional activity was reduced by at least a 20%. Given that the Notch receptor at the membrane is continuously endocytosed by Deltex, for trafficking or recycling (Matsuno et al., 1998; Wilkin et al., 2008), and that our Notch mutants with altered Deltex binding (A2026V, Δ671 and Δ764) do not have an effect on Wnt/β-catenin transcriptional activity, it is possible that Notch at the membrane sequesters a component required for β-catenin activation and, by these means, stops Wnt signalling transcription. For example, Dishevelled, generally a cytoplasmic protein that releases β-catenin from the destruction complex, that has also been shown to be imported to the nucleus and form a
complex with β-catenin and TCF to stabilise and regulate Wnt signalling transcription (Gan et al., 2008; Itoh et al., 2005). The Wnt transcription factor TCF could be another possibility, since recently it has been shown in sea urchin that in the presence of Notch, TCF is exported from the nucleus (Sethi et al., 2012). Alternatively, ΔEGF_N1 could sequester LRP5/6 whose own endocytosis is important for Wnt/β-catenin signalling (Kikuchi and Yamamoto, 2007; Yamamoto et al., 2006; Yamamoto et al., 2008).

The dual role of Notch receptor as an activator of the Notch signalling pathway and an inhibitor of β-catenin transcriptional activity highlights how Notch signalling pathway can regulate cell fate decisions in which Notch and Wnt have opposing effects. For instance, the temporal transition from a state of Notch-ON/Wnt-OFF to Notch-OFF/Wnt-ON is often critical for a cell to progress from precursor to terminally differentiated (Hayward et al., 2008; Munoz-Descalzo et al., 2012; Muñoz-Descalzo and Martínez Arias, 2012). A clear example of this requirement can be seen during the initial neurogenesis (Gomez-Skarmeta et al., 2003) and myogenesis in Drosophila (Brennan et al., 1999a; Rusconi and Corbin, 1998). Clusters of precursor cells are defined through spatial and temporal specific interactions between Notch and Wnt signalling pathways. Notch initially suppresses the development of the proneural clusters or muscle progenitor clusters through a modest inhibition of Wingless signalling (see Chapter 5, section 5.2.2). However, this repression does not require ligand binding or the activation of Su(H)-dependent transcription. Instead, it requires Deltex function (Brennan and Gardner, 2002; Ramain et al., 2001). In the absence of Notch, this initial repression is lost and development proceeds precociously (Brennan et al., 1999a; Brennan et al., 1999b). This scenario is very similar to the mechanism we describe here, where a membrane-restricted form of Notch can reduced Wnt signalling within vertebrate cells.

In summary, we have shown that in vertebrates Notch regulates the Wnt signalling by inhibiting the transcriptional activity of β-catenin, possibly through Deltex-mediated endocytosis of Notch complexed with a component required for β-catenin activation. Our results are in agreement with previous studies in Drosophila (Hayward et al., 2005; Sanders et al., 2009). Therefore, this new mechanism of Notch is highly conserved from invertebrates to vertebrates, and highlights a function for Notch in establishing a threshold for Wnt signalling by regulating the transcriptional activity of β-catenin. This function will play crucial roles in patterning and cell fate decisions during the development of organisms and tissue homeostasis. This function may also be important in malignant situations contributing to the understanding of Notch as a tumour suppressor (Deregowski et al., 2006; Nicolas et al., 2003) as loss of Notch will lead to an increase in Wnt signalling.
Chapter 3: Membrane-restricted Notch Inhibits β-catenin Activity

References


Chapter 3: Membrane-restricted Notch Inhibits β-catenin Activity


Supplementary Figure 3.1: Whole cell lysate from HEK293T. A. Western blot of whole cell lysates showing expression of β-catenin alone or in the presence of ΔEGF_N1. B. Western blot of whole cell lysates showing expression of S45Fβ-catenin alone or in the presence of ΔEGF_N1. A & B. HEK293T cells were transfected with 250 ng of empty vector (EV) as a control, and either 25 ng of β-catenin (A) or 25 ng of S45Fβ-catenin (B) alone, or with 25 ng of ΔEGF_N1. Expressed protein was detected by probing with an antibody that recognises total β-catenin. Renilla luciferase is shown as a loading control. The position of molecular weight markers (in KDa) is shown.
Supplementary Figure 3.2: Quantitative analysis of S45Fβ-catenin fluorescence intensity in the nucleus versus the cytosol. A. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells transfected with S45Fβ-catenin alone (as shown in Fig 3.1F). B. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells co-transfected with S45Fβ-catenin and ΔEGF-N1 (as shown in Fig 3.1H). Note that in the presence of ΔEGF_N1 the spectrum shifts towards the blue colours, meaning that the intensity of β-catenin increases in the nucleus.
Supplementary Figure 3.3: Quantitative analysis of S45Fβ-catenin fluorescence intensity in the nucleus versus the cytosol in the presence of ΔEGF_N1 point mutants. A. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells transfected with S45Fβ-catenin in the presence of ΔEGF_N1 W1758A (as shown in Fig 3.3C). B. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells co-transfected with S45Fβ-catenin and ΔEGF-N1 V1744G (as shown in Fig 3.3E). C. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells transfected with both S45Fβ-catenin and ΔEGF_N1 A2026V (as shown in Fig 3.3G).
Supplementary Figure 3.4: Quantitative analysis of S45Fβ-catenin fluorescence intensity in the nucleus versus the cytosol in the presence of ΔEGF_N1 deletion mutants. A. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells transfected with S45Fβ-catenin in the presence of ΔEGF_N1 Δ425 (as shown in Fig 3.5C). B. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells co-transfected with S45Fβ-catenin and ΔEGF-N1 Δ671 (as shown in Fig 3.5E). C. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells transfected with S45Fβ-catenin along with ΔEGF_N1 Δ764 (as shown in Fig 3.5G).
Chapter 4: Paper 3

Hey1 Modulates Wnt Signalling by Associating with β-catenin and TCF and Regulating its Transcriptional Activity in Vertebrates.

Ana Hidalgo Sastre¹ & Keith Brennan¹.

¹Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester, M13 9PT, UK.
Author for correspondence (e-mail: keith.brennan@manchester.ac.uk)

Running title: Hey1 inhibits Wnt signalling by associating with β-catenin and TCF.

Author contribution: AHS and KB designed the experiments. AHS performed the experiments and wrote the paper. KB edited the paper.

Note for the reader: Previously, we demonstrated that a membrane-restricted Notch1 modulates Wnt/β-catenin signalling by reducing β-catenin transcriptional activity in vertebrate cells (Chapter 3). In this chapter the possibility that the inhibitory crosstalk between Notch and Wnt signalling pathways could also require Rbpj-dependent transcription was studied. The experiments performed are presented in the format of a research paper which could be sent for publication in Molecular and Cellular Biology.
Abstract

Throughout development and during adult tissue homeostasis, cells are exposed to continuous cycles of self-renewal and differentiation. These processes are controlled by an intricate network of eight signalling pathways. Notch and Wnt are key members of this network that generates and maintains diversity. Often they have opposing effects on cell fate decisions and experiments in Drosophila clearly indicate a crosstalk between the pathways. Here we explore the mechanism of this interaction in vertebrate cells and find that it is mediated in part by NICD-dependent transcription. We demonstrate that NICD interaction with Rbpj and subsequent transcription of the Notch target genes Hes5 and Hey1 regulates Wnt/β-catenin transcriptional activity. Particularly, Hey1 assembles into a physical complex with β-catenin and TCF in the nucleus to inhibit Wnt signalling. Furthermore, our results suggest that Hey1/β-catenin inhibitory crosstalk mechanism is conserved across vertebrates. Thus, we identify a dual function for NICD as an inhibitor of Wnt signalling and an activator of the Notch pathway. The inhibition of Wnt/β-catenin signalling by NICD-dependent transcription sharpens the distinction between opposing Notch/Wnt responses allowing the robust differentiation of cell fates.

Key words: Notch, Wnt, β-catenin, Hey1, inhibition, Rbpj, transcription-dependent.
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF

Introduction

Wnts are secreted glycoproteins that initiate cellular responses by binding a receptor complex formed by Frizzled and LRP proteins at the cell surface. This causes the recruitment to the membrane of Dishevelled and a complex of proteins formed by Axin, APC, GSK3β, and CK1. In the absence of Wnt, this complex phosphorylates cytoplasmic β-catenin and targets it for proteosomal degradation (Logan and Nusse, 2004; van Amerongen and Nusse, 2009). Consequently, Wnt signalling can govern the concentration and activity of this pool of free cytoplasmic β-catenin, as well as, the localisation, since β-catenin can enter the nucleus once it is stabilised (Tolwinski and Wieschaus, 2004). Within the nucleus, β-catenin binds with TCF/LEF family of transcription factors (Molenaar et al., 1996; van de Wetering et al., 1997) and recruits transcriptional co-activators including, Pygopus and Bcl9 (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002) to modulate gene expression.

Notch proteins are single-pass transmembrane receptors which upon activation act as transcription factors triggering cell fate decisions during development (Artavanis-Tsakonas et al., 1999). The main characteristic of the extracellular domain of Notch receptors is a tandem array of 36 EGF-like repeats. The DSL (Delta, Serrate, Lag2) family of ligands binds Notch through this array of EGF-like repeats (Nichols et al., 2007; Rebay et al., 1991). This generates tension within the extracellular domain promoting a conformational change that exposes a proteolytic cleavage site for the ADAM10 and TACE metalloproteases, near to the transmembrane domain (reviewed in: (Kovall and Blacklow, 2010)). Cleavage by these metalloproteases generates a short lived intermediate known as NEXT (Notch extracellular truncation) that is rapidly cleaved, within the transmembrane domain, by γ-secretase (De Strooper et al., 1999; Schroeter et al., 1998). This second cleavage event releases Notch intracellular domain (NICD) which translocates to the nucleus where it binds to members of the CSL (CBF1, Su(H), Lag1) family of transcription factors to initiate transcriptional activation (Andersson et al., 2011; Kopan and Ilagan, 2009). The main feature of the intracellular domain is an array of seven ANK (ankyrin) repeats that mediates protein interactions with CSL transcription factors and other proteins (Ehebauer et al., 2005; Nam et al., 2007).

Interestingly, Notch and Wnt signalling pathways are often active at the same time to regulate the development and maintenance of a particular tissue (reviewed in: (Muñoz-Descalzo et al., 2012; Muñoz-Descalzo and Martinez Arias, 2012)). However, they frequently have opposing effects, specifying different cell fates (Hayward et al., 2008). Therefore, it is not surprising that signalling through the two pathways is under strict spatio-temporal control to
prevent a conflict between them during cell fate specification. Part of this tight control happens through interactions between the pathways themselves: either when the transcription factors at the base of the two signalling pathways converge onto the promoters of shared target genes, for instance, in Drosophila Suppressor of Hairless (Su(H)) and Pangolin/DTcf converge on the promoter that drives the expression of vestigial, a gene implicated in the development and patterning of the wing (Kim et al., 1996; Klein and Arias, 1999); or when both pathways mutually and sequentially activate each other, for example, during vertebrate somitogenesis and wing patterning in flies Wnt signalling triggers the up-regulation of Notch ligands activating Notch in neighbouring cells, Notch/CSL activity leads to the expression of Wnt genes (Galceran et al., 2004; Micchelli et al., 1997). Recently, a third mode of interaction between Notch and Wnt signalling pathways has emerged, uncovered from studies in Drosophila and mammalian stem and cancer cells where Notch can modulate the amount and transcriptional activity of Armadillo/β-catenin (Chapter 3 on this thesis and (Hayward et al., 2005; Kwon et al., 2011; Sanders et al., 2009). This work has led to a proposed model in which Notch down regulates Wnt signalling by associating with a pool of Armadillo/β-catenin present at the membrane and degrades it through endosomal trafficking. The proposed model also indicates that this inhibition of Wnt signalling is not reliant on the transcriptional activity of Notch. In contrast, several other studies have suggested that the transcriptional function of Notch is required for the inhibition of the Wnt/β-catenin pathway (Acosta et al., 2011; Deregowski et al., 2006; Hayward et al., 2005; Nicolas et al., 2003).

Although, the latter studies have not provided a detailed molecular mechanism for an interaction between NICD or its transcriptional targets and β-catenin. On the other hand, this direct antagonism between Notch and Wnt signalling ensures that a clear distinction is made between Notch-ON/Wnt-OFF states and vice versa, allowing the robust differentiation of cell fates.

In this study we investigate the interaction between Notch and Wnt signalling pathways in vertebrates. We show that NICD can inhibit Wnt/β-catenin signalling through Rbpj-dependent activation of Hes/Hey family of target genes. Furthermore, we show that Hey1 inhibits β-catenin by assembling into a physical complex with β-catenin and TCF, in the nucleus of the cells. We also demonstrate that this mechanism is conserved across vertebrates. Together, these data allows to propose the first molecular mechanism for Notch modulation of Wnt signalling pathway in vertebrates involving Rbpj-dependent transcription. This mechanism may be relevant during vertebrate development and, perhaps, in cancerous situations.
Materials and methods

A general description of the methods used for this manuscript is detailed here. Further information can be found in the supplementary materials and methods on Chapter 6.

Cell culture and transfections

Human embryonic kidney cells expressing the large T antigen, HEK293T, were a kind gift from Dr Anthony Brown (Weill Medical College, Cornell University, New York, USA) and from Dr Valerie Kouskoff (Paterson Institute for Cancer Research, Manchester, UK). These cells were cultured in DMEM medium supplemented with 10% FBS and 50 μg/ml penicillin and 50 μg/ml streptomycin. HEK293T cells were maintained at 37°C and 5% CO₂ in a humidified incubator. Cells were transfected using Lipofectamine and PLUS reagent (Invitrogen) or X-Treme transgene 9 transfection reagent (Roche), according to manufacturer’s instructions.

Luciferase assays

HEK293T cells were seeded at a density of 2 x 10⁵ cells per well of a 24 well plate. After 24 hr cells were transfected in triplicate. A DNA cocktail containing the desired plasmids, 50 ng of TCFAdTATA, a Wnt reporter plasmid generated in the Brennan lab, and 20 ng of pRL-CMV plasmid, used as internal control, was prepared. The plasmid pcDNA3.1(+) was used to ensure all transfections contained 250 ng of DNA. The cell culture medium was changed 3 hr after transfection when Lipofectamine and Plus reagent (Invitrogen) were used. Cells were lysed 48 hr post-transfection using 1 x Passive Lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using the Dual Luciferase Reporter assay system (Promega), according to manufacturer’s instructions, with a MicroLumatPlus plate reader (Berthold Technologies). Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU), compared to the positive control, S45Fβ-catenin in most cases. Statistical analysis was perform using one-way ANOVA and Tukey’s post-hoc tests.

cDNA constructs

A description of the constructs used in the experiments for this manuscript, as well as, the primers used for mutagenesis and sequencing can be found on the supplementary materials and methods on Chapter 6 section 6.4.
S100/P100 cytosolic fractionation

To perform a cytosolic fractionation, 48 hr post transfection cells were washed twice in 3 ml of 1 x TBS (10 mM Tris-HCl pH 7.4 + 140 mM NaCl) + 2 mM CaCl₂, placed on ice and lysed in 1 ml of lysis buffer (1 x TBS + protease inhibitor cocktail set I (Calbiochem) + 10 µl of 100 mM PMSF. Subsequently, cells were poured into a cold 2 ml dounce homogenizer and sheared with 30 strokes. Cell lysates were centrifuged at 1500 x g for 5 min at 4°C. The supernatant was centrifuged for 90 min at 100000 x g in a TLA 110 rotor in a Beckman Coulter Optima TLX-120 Ultracentrifuge at 4°C. After the spin, 100 µl of the supernatant, representing the cytosolic fraction, were mixed with 100 µl of 2 x Laemmli’s buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue and 20% glycerol), boiled for 3 min and stored at -20°C. The pellet, representing the membrane fraction, was washed twice in 1 x TBS, resuspended in 1 ml of 2 x Laemmli’s buffer, boiled for 3 min and kept at -80°C.

Nuclear Immunoprecipitation (IP)

RFP-Trap_A beads (Chromo Tek GmbH, Martinsried, Germany) were used according to the manufacturer’s instructions. Briefly, cells were washed in 1 x PBS and lysed in 500 µl IP lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 1 x protease inhibitor cocktail set (Calbiochem Cat# 539131) freshly added). The lysate was centrifuged at 21,000 x g for 10 min at 4°C. 50 µl of the supernatant were retained as total cell lysate sample. The pellet was re-suspended in 500 µl of nuclear lysis buffer (10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 0.5% NP-40, 1 x protease inhibitor cocktail set (Calbiochem Cat# 539131) freshly added) and incubated for 10 min at 4°C. Lyophilised DNase I and NaCl were added to a final concentration of 1µg/µl and 150 mM, respectively. The sample was incubated for 30 min at 4°C. The lysate was centrifuged at 20,000 x g for 10 min at 4°C, 50 µl of supernatant were taken as nuclear input sample. 450 µl were mixed with 20 µl of beads slurry previously equilibrated in 500 µl of lysis buffer. The mixture was incubated with gentle end-over-end mixing for 2 hr at 4°C. Beads were separated by centrifugation at 2700 x g for 4 min at 4°C. Subsequently, 50 µl of supernatant were retained as non-bound sample. The pellet containing the bound fraction was washed once in lysis buffer and twice in wash buffer (10 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.5 mM EDTA), re-suspended in 2 x Laemmli buffer, and heated at 60°C for 10 min.

Western blotting

Total cell lysis and subsequent western blotting were performed as described (Stylianou et al., 2006). For more details see supplementary materials and methods, Chapter 6.
Immunofluorescence

HEK293T cells were seeded on nitric acid treated coverslips at 4 x 10^5 cells per well of a 6 well plate. Transfection was performed after 24 hr, as described above. Cells were fixed 24 hr post transfection for 10 min in 1 x PBS containing 4% formaldehyde. Following three washes with 1 x PBS, coverslips were incubated either with anti-myc-Tag rabbit primary antibody (Cell signaling) for Notch constructs, or with mouse anti-V5 Alexa 488 conjugated antibody (AbD SeroTec) to detect S45Fβ-catenin, diluted 1:100 in blocking solution (3% goat serum (Biosera, Sussex, UK), 0.1% Triton-X100, and 0.05% NaNO₃ in TBS) in a humidified chamber for 1 hr. Subsequently, cells were washed and coverslips for Notch constructs were incubated with fluorescence goat anti-rabbit Alexa 594 secondary antibody in blocking solution. Coverslips were mounted with VECTASHIELD mounting medium for fluorescence with DAPI H-1200 (Vector Laboratories). Images were captured with a Zeiss LSM 700, AxioObserver flexible confocal microscope (Carl Zeiss MicroImaging GmbH, Germany) using the Zeiss ZEN 2011 software (Carl Zeiss MicroImaging GmbH, Germany). The confocal software was used to determine the optimal number of Z sections when acquiring 3D optical stacks. Either maximum intensity projections of these 3D stacks or single z-section images are shown in the results.

Quantitative Image analysis

The fluorescence intensity of nuclear S45Fβ-catenin was determined with image J software (open source software, National Institute of Health, USA) by measuring the integrated fluorescence intensity of the nucleus and cytoplasm of cells transfected with different plasmids. The integrated density of each cell was measured at the middle plane of a z series. The maximum nuclear to cytosol ratio was set to 0.75 (as this was the maximum intensity observed when S45Fβ-catenin was transfected alone). Negative nuclear to cytosol ratios (ie: when nuclear corrected total fluorescence was negative) were set to zero. At least 25 cells were measured per condition. For ratio measurements, the following formulas were used:

Whole cell signal = Integrated density (sum of the intensity of the pixels for one cell)

Nuclear signal = Integrated density of the nucleus of a cell

Cytosol fluorescence = whole cell signal – nuclear signal

Corrected total fluorescence (CTF) = Integrated density – (Area of selected cell or nucleus x Mean fluorescence of background readings).

Nuclear to cytosol ratio = Nuclear CTF / Cytosol CTF
Quantitative PCR

Total RNA was extracted from cells using peqGOLD TriFast solution (peqlab Biotechnologie GmbH) according to manufacturer’s instructions. A total of 2 μg of RNA were reverse transcribed to cDNA in a 20 μl reaction using the High Capacity RNA-to-cDNA Master Mix kit (Applied Biosystems), following manufacturer’s instructions. The resultant cDNA were used as template for the quantitative PCR. Quantitative PCR was performed in triplicate, in a total volume of 20 μl, using the primers listed on Chapter 6, Table 6.4, and Fast SYBR Green PCR Master Mix (Applied Biosystems), according to manufacturer’s instructions. The relative amounts of the PCR products were analysed using the comparative RQ method and using PPIA gene as an internal normalization control.
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF

Results

NICD inhibits Wnt signalling activity at the level of β-catenin.

We recently demonstrated that a membrane-restricted Notch1 protein antagonizes Wnt/β-catenin signalling by reducing β-catenin transcriptional activity in vertebrate cells (Chapter 3). Here, we address whether the inhibitory crosstalk between Notch and Wnt signalling could also require Rbpj-dependent transcription.

Previously, Hayward and colleagues (2005), have shown that mouse Notch1 can modulate Wnt signalling activity in HEK293T cells. In that study, researchers tested the ability of a form of Notch1, ΔN-N1, in which all the extracellular domain had been removed except for 13 amino acids, to inhibit β-catenin. ΔN-N1 was readily cleaved by γ-secretase and strongly activated a Rbpj-dependent reporter. Researchers showed that ΔN-N1 suppresses β-catenin activity. However, it is not clear from these experiments whether ΔN-N1 inhibits Wnt/β-catenin signalling due to its initial targeting to the plasma membrane or due to its ability to activate Rbpj-dependent transcription. To further study this mechanism, we activated Notch signalling in HEK293T cells by transfecting the Notch intracellular domain (NICD) and members of the Wnt signalling pathway including Wnt1, Dvl2, or a constitutively active form of the Wnt transcription factor LEF1, LEF1-VP16. Wnt signalling was monitored using TCFAdTATA reporter plasmid (see Chapter 2). Co-expression of NICD with Wnt1 ligand or Dvl2 inhibited Wnt signalling (Fig 4.1A). This result confirms that a form of Notch which can activate Rbpj-dependent transcription but that is not membrane targeted can modulate Wnt signalling. On the other hand, NICD was unable to decrease LEF1-VP16 induced transcription (Fig 4.1A). This was in agreement with previous results (Ross and Kadesch, 2001). These data clearly place a point of crosstalk below Wnt ligand and Dvl at the membrane and above LEF1-VP16 within the nucleus. To localize the point of crosstalk more accurately, we examined whether NICD can inhibit the function of β-catenin itself. To avoid degradation of the expressed β-catenin by the destruction complex, we used a stabilised form of β-catenin, S45Fβ-catenin, with a point mutation changing Serine 45 to Phenylalanine (S45F), to prevent its phosphorylation by CK1 and therefore, subsequent phosphorylation by GSK3β. NICD expression inhibited S45Fβ-catenin transcriptional activity, significantly (Fig 4.1B).

To determine whether NICD inhibition of S45Fβ-catenin transcriptional activity was due to protein degradation or not, we performed cytosolic fractionation and western blot analysis for S45Fβ-catenin protein levels in the absence or presence of NICD. As expected,
expression of S45Fβ-catenin led to its accumulation in HEK293T cells (upper band of the doublet, Fig 4.1C). Additionally, we observed an accumulation of endogenous β-catenin (lower band of the doublet, Fig 4.1C), presumably, due to sequestration of the destruction complex by the stabilised S45Fβ-catenin (Maher et al., 2010). Interestingly, both S45Fβ-catenin and endogenous β-catenin protein levels did not vary in the presence of NICD (compare lanes 2 and 3 in Fig 4.1C). These results suggest that β-catenin protein is not degraded in the presence of NICD but it cannot activate Wnt signalling.

Next, we asked whether NICD altered the localisation of β-catenin. To answer this, we used immunofluorescence. As expected, NICD localised exclusively to the nucleus (Fig 4.1D) and S45Fβ-catenin was widely distributed throughout the cell, localising to the nucleus, the cytosol and the membrane (Fig 4.1E). At the membrane, S45Fβ-catenin delineated the cell boundaries and accumulated in bright punctate that resemble vesicles (Fig 4.1E). To obtain an indication of the amount of S45Fβ-catenin present in the nucleus and to account for transfection variations, we performed a quantitative analysis looking at nuclear levels relative to whole cell levels (Sup Fig 4.1A). When NICD was co-expressed with S45Fβ-catenin, both NICD and S45Fβ-catenin localisation were unaltered (Fig 4.1F&G). However, S45Fβ-catenin failed to accumulate in the bright punctate pattern at the membrane and the quantitative analysis suggested a slight shift towards higher levels of S45Fβ-catenin in the nucleus (Sup Fig 4.1B). These results indicate that NICD inhibited S45Fβ-catenin transcriptional activity and increased slightly S45Fβ-catenin localisation to the nucleus.

**NICD requires its interaction with Rbpj to inhibit β-catenin activity.**

To address whether the ability of NICD to inhibit β-catenin function was dependent upon its ability to interact with Rbpj and induce Rbpj-dependent transcription, we generated two NICD point mutants, NICD W1758A and NICD A2026V (Fig 5.2A). The W1758A mutation is within the RAM domain and disrupts the interaction with Rbpj (Kato et al., 1997; Lubman et al., 2007; Tamura et al., 1995; Wilson and Kovall, 2006). The A2026V mutation is within the 5th ANK repeat. Mutation of the equivalent residue in the Drosophila Notch protein (N\textsuperscript{Sud2c}) slightly increases the interaction with Deltex, an E3 ubiquitin ligase that regulates the endocytosis of Notch at the membrane, and results in a Deltex loss-of-function phenotype (Diederich et al., 1994; Hori et al., 2004; Matsuno et al., 1997; Zweifel et al., 2003). Given that NICD is a nuclear protein, the A2026V mutation should not affect its function. This point mutant was used as a control. The function of NICD W1758A and NICD A2026V was confirmed with a Notch reporter gene assay (Fig 4.2B). As expected, the W1758A mutation completely abrogated NICD function.
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF

(Fig 4.2B). In contrast, the A2026V point mutation did not alter the ability of NICD to activate transcription (Fig 4.2B). Western blot analysis confirmed that the expression levels of NICD A2026V were comparable to that of the wildtype NICD construct (Fig 4.2C). Similar results were observed with the NICD W1758A point mutant (Giovanna Collu, unpublished). Interestingly, immunofluorescence revealed that the NICD W1758A protein was found within the nucleus and the cytosol (Fig 4.2D), whilst NICD A2026V was exclusively nuclear, resembling the wildtype NICD protein (Fig 4.2E).

To study whether these mutations altered the ability of NICD to inhibit β-catenin function, we expressed the mutant forms of NICD with S45Fβ-catenin, and monitored β-catenin function with the TCFAdTATA reporter plasmid. Expressing NICD W1758A had no significant effect on the ability of β-catenin to activate transcription (Fig 4.2F). In contrast, wildtype NICD and NICD A2026V inhibited β-catenin signalling comparably (Fig 4.2G). Next we investigated whether these mutant NICD proteins altered the localisation of S45Fβ-catenin to modulate its transcriptional activity. Immunofluorescence and quantitative analysis showed that the localisation of NICD W1758A and S45Fβ-catenin was not altered when they were co-expressed (compare Fig 4.2H with Fig 4.2D and Fig 4.2I with Fig 4.1E and see Sup Fig 4.2A). On the other hand, when NICD A2026V and S45Fβ-catenin where expressed together, NICD A2026V localisation appeared to shift slightly to the cytosol (Fig 4.2J), whilst S45Fβ-catenin localisation and quantitative analysis resembled the pattern seen when it was co-expressed with wildtype NICD (compare Fig 4.1G and Fig 4.2K, see Sup Fig 4.2B). These data clearly indicate that NICD has to interact with Rbpj to inhibit Wnt/β-catenin signalling and that to do so NICD does not change S45Fβ-catenin localisation to the nucleus.

The Notch target genes Hey1 and Hey1 can modulate Wnt/β-catenin transcriptional activity

To determine whether the requirement for the interaction between NICD and Rbpj to inhibit Wnt/β-catenin signalling reflects an induction of downstream target genes, we first examined whether an activated form of Rbpj can inhibit β-catenin function. For these experiments, we used a fusion between Rbpj and the transcriptional activation domain of VP16 that can induce a similar array of the Hes/Hey target genes as NICD (Fig 4.3A and Sup Fig 4.3). Like NICD, co-expression of Rbpj-VP16 with β-catenin clearly reduced the ability of β-catenin to activate the TCFAdTATA reporter gene (Fig 4.3B). As our quantitative PCR analysis indicated that both NICD and Rbpj-VP16 induced expression of Hes5 and Hey1 (Fig 4.3A and Sup Fig 4.3), we examined whether these proteins could also inhibit Wnt/β-catenin signalling. Interestingly, both Hes5 and Hey1 were able to inhibit S45Fβ-catenin induced transcription,
although Hey1 was more potent (Fig 4.3B). It should, however, be noted that the TCFAdTATA reporter gene lacks any recognisable E-boxes. Therefore, the repression of the Wnt reporter by Hes5 and Hey1 cannot be explained, simply, by these transcriptional repressors binding to the reporter gene.

To confirm that Rbpj-VP16 was inhibiting Wnt/β-catenin signalling by transcription, we generated a mutant form of Rbpj that cannot bind DNA, by mutating Arginine 218 to Histidine (Chung et al., 1994; Kovall and Hendrickson, 2004; Nam et al., 2003; Wilson and Kovall, 2006). As expected, Rbpj-VP16 R218H was unable to activate the p10xRbpj-luc reporter gene (Sup Fig 4.4). In addition, Rbpj-VP16 R218H was unable to modify S45Fβ-catenin function (Fig 4.3C). We also generated dominant negative (DN) forms of Hey1 and Hes5 proteins, to co-express with Rbpj-VP16 and disrupt the function of the endogenous Hes and Hey proteins induced by the transfection of the Rbpj protein. For Hey1, we did this by mutating the residues that contact the DNA within the DNA binding domain to Alanines, namely Glutamate 58, Lysine 59, and Arginine 62 (Strom et al., 1997). For Hes5 we used the same approach, mutating Glutamate 25, Lysine 26, and Arginine 29 to Alanines. These dominant negative proteins cannot bind DNA but can still dimerise with endogenous Hey and Hes proteins and disrupt their function (Sup Fig 4.5A&B), although, the effects are not so potent when mixing members of the family (Sup Fig 4.5C&D). We initially examined whether the dominant negative Hey1 and Hes5 proteins disrupted Wnt/β-catenin signalling on their own, by expressing them with S45Fβ-catenin. Results showed that S45Fβ-catenin signalling was not affected by DNHey1 (Fig 4.3C). However, DNHes5 still inhibited S45Fβ-catenin function slightly (Sup Fig 4.6A). We then expressed increasing amounts of DNHey1 with Rbpj-VP16 to determine whether this altered the ability of Rbpj-VP16 to disrupt S45Fβ-catenin function. The DNHey1 protein abrogated the effects of Rbpj-VP16 on S45Fβ-catenin in a dose dependent manner (Fig 4.3D). Similar effects were observed with the DNHes5 protein (Sup Fig 4.6B). From these experiments we concluded that the induction of Rbpj-dependent transcription, particularly, of the Hes and Hey family of transcriptional repressors, is required for the inhibition Wnt signalling by Rbpj-VP16.

**Hey1 forms a physical complex with β-catenin and TCF to inhibit Wnt signalling.**

Given the function of Hes and Hey family of proteins as transcriptional repressors, we initially examined whether their expression reduced the levels of Wnt pathway components downstream of β-catenin. We focused on the transcriptional co-activators Pygopus 1, Pygopus 2 and Bcl9 2, as co-expression of Hey1 with TCF4 and β-catenin did not alter the ability of Hey1 to inhibit β-catenin function (Sup Fig 4.7). The TCF4 was provided in this
experiment from an exogenous source that is not regulated by Hey1. This result suggested that Hey1-mediated repression of a TCF family member is not the mechanism by which Hey1 limits Wnt signalling. Therefore, we analysed Pygopus 1, Pygopus 2 and Bcl9 2 by quantitative PCR in sorted HEK293T cells expressing RFP-Hey1, RFP-DNHey1, or RFP. The RFP expressing cells were used as a control for changes in expression due to transfection. We also confirmed that the RFP fusion did not alter the function of Hey1 or DNHey1 (Sup Fig 4.8). We found no significant differences between the mRNA levels of the Wnt co-activators in the RFP-Hey1 or RFP-DNHey1 expressing cells (Fig 4.4A).

An alternative possibility was that Hey1 could physically interact with either β-catenin or members of the TCF family to prevent the formation of a transcriptional complex. To test this hypothesis, we transfected a full length TCF4 protein fused with N-terminal half a Venus GFP (TCFV1) and a full length β-catenin protein fused with the C-terminal half of Venus GFP, (V2β-catenin). Venus GFP will only refold to form a functional fluorescent protein if the TCF4 and β-catenin proteins interact (Fig 4.4B). We found that in the presence of RFP-Hey1, TCFV1 and V2β-catenin still associated to form a functional Venus GFP (Fig 4.4C). Interestingly, we also found that Hey1 co-localised with TCFV1 and V2β-catenin, suggesting that the proteins can aggregate into a large complex (Fig 4.4C). We confirmed the presence of this complex in a co-immunoprecipitation assay, since by using a RFP-trap we were able to isolate TCF4 and S45Fβ-catenin from RFP-Hey1 expressing HEK293T cells and not RFP expressing cells (Fig 4.4D).

These data strongly suggests that Hey1 physically binds TCF4 and S45Fβ-catenin to inhibit their transcriptional activity and therefore, Wnt signalling activation.

**Hey1/β-catenin crosstalk is conserved in Xenopus.**

To test whether Hey1 effects over Wnt signalling are restricted to mammals or whether they are conserved across vertebrates, we tested the ability of *Xenopus tropicalis* Hey1 (XtHey1) to modulate β-catenin in HEK293T cells. Like S45Fβ-catenin, expression of Xenopus β-catenin (Xβ-catenin) resulted in a robust and significant activation of the TCFAdTATA reporter (Fig 4.5A). Co-transfection with XtHey1 resulted in a decrease in reporter activity (Fig 4.5A). In contrast, co-transfection with XtDNHey1 did not alter Xβ-catenin function (Fig 4.5A). Additionally, we found that co-transfection of murine NICD, Rbpj-VP16, Hes5 and human Hey1 reduced Xβ-catenin function (Fig 4.5B) to similar levels as to when murine S45Fβ-catenin was used (4.1B and 4.3B). Similarly, XtHey1 but not XtDNHey1 inhibited the murine S45Fβ-catenin protein (Fig 4.5C). Together these results indicate that the inhibition of Wnt/β-catenin signalling by the NICD, Rbpj and Hey1 axis is conserved across vertebrates.
Figure 4.1: NICD inhibits β-catenin transcriptional activity. Notch intracellular domain limits Wnt signalling at the level of β-catenin. A & B. HEK293T cells were transfected with a Wnt reporter plasmid (TCFAdTATA) and a Renilla luciferase plasmid (pRL-CMV) as a control. Wnt signalling was activated by transfecting Wnt pathway components as indicated including: 2.5 ng of Wnt1, 125 ng of Dvl2, 25 ng of S45Fβ-catenin and 12.5 ng of LEF1-VP16. Notch signalling was activated by transfecting 25 ng of NICD, per well. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU). A. Wnt signalling was activated by expressing Wnt1, Dvl2, and LEF1-VP16. NICD significantly inhibited signalling activated by Wnt1 and Dvl2 (***P<0.001; NSP>0.05, one-way ANOVA and Tukey’s post-hoc test, N≥3). B. Wnt signalling was activated by expressing S45Fβ-catenin. NICD significantly inhibited signalling activated by β-catenin (***P<0.001, one-way ANOVA and Tukey’s post-hoc test, N≥3). C. Cytosolic fractionation of cells expressing S45Fβ-catenin and NICD as indicated. Expressed protein was detected in western blot by probing with an antibody that recognises total β-catenin (α-β-catenin, Bd Transduction lab). Tubulin is shown as a loading control. The position of molecular weight markers (in KDa) is shown. Notice that in the presence of S45F β-catenin an accumulation of both the ectopically expressed S45Fβ-catenin and endogenous β-catenin (compare lane 1 and 2) was observed. Expression of NICD does not alter β-catenin protein levels (compare lane 2 and 3). D-G. Analysis of NICD and S45Fβ-catenin localisation by confocal microscopy. DAPI (blue) was used to stain the nuclei. Scale bar 51 µm for Notch images and 100 µm for β-catenin images. The NICD and S45Fβ-catenin proteins were detected by immunostaining with Myc-Tag primary antibody and Alexa 594 secondary antibody, and with V5 conjugated Alexa 488 antibody, respectively. D. NICD-myc was present in the nucleus of cells. E. S45Fβ-catenin-V5 was found at the plasma membrane, and within the cytoplasm and nucleus. Bright punctate of S45Fβ-catenin were also found delimiting the plasma membrane. F & G. Co-expression of S45Fβ-catenin with NICD. Notice the disappearance of the bright vesicle-like punctate at the membrane. E & G. Quantitative analysis of S45Fβ-catenin fluorescence in the nucleus versus the cytosol is shown in Sup Fig 4.1A and B, respectively.
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF

A. 

B. 

C. 

D. 

E. 

F. 

G.
Figure 4.2: NICD requires its interaction with Rbpj to block β-catenin transcriptional activity.

A. Diagram of the Notch molecules used. The asterisk (*) represents a mutation. B. NICD and the point mutant NICD A2026V significantly activate the Rbpj reporter, 10xRbpj-luc. In contrast, NICD W1758A does not activate Notch reporter signalling. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (**P<0.01; NSP>0.05, one-way ANOVA and Tukey’s post-hoc test, N≥3) C. Western blot showing comparable expression of NICD and NICD A2026V. Renilla luciferase was used as a loading control. The position of molecular weight markers (in KDa) is shown. D-E. Analysis of the localisation of the NICD point mutants by confocal microscopy. DAPI (blue) was used to stain the nuclei. Scale bar is 51 µm. The NICD W1758A mutant was found both within the cytoplasm and the nucleus. F & G. Wnt reporter assays for cells transfected with 25 ng of S45Fβ-catenin in the absence or presence of 25 ng of NICD, NICD W1758A or NICD A2026V. NICD and NICD A2026V can inhibit S45Fβ-catenin function comparably. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (**P<0.01; ***P<0.001; NSP>0.05, one-way ANOVA and Tukey’s post-hoc test, N≥3). H-K. Analysis of S45Fβ-catenin localisation in the presence of NICD W1758A and NICD A2026V by confocal microscopy. Cells where transfected and fixed as described before. Subsequently, cells were immunostained with c-Myc primary antibody and Alexa 594 secondary antibody and with V5 conjugated Alexa 488 antibody as in Fig 1 D-G. DAPI (blue) was used to stain the nuclei. Scale bar is 100 µm. S45Fβ-catenin localisation is not altered by the co-expression of NICD W1758A. I & K. Quantitative data of S45Fβ-catenin fluorescence in the nucleus versus the cytosol is shown in Sup Fig 4.2 A and B, respectively.
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF

A.

B.

C.

D.

E.

F.

G.

H.

I.

J.

K.
**Figure 4.3: The Notch target genes Hes/Hey can regulate Wnt signalling.**

A. Expression of the Notch target genes *Hes1, Hes5, Hey1, Hey2* and *HeyL* was determined by qRT-PCR in HEK293T after transfection with empty vector or 25 ng of Rbpj-VP16, per 6 cm dish. *Hes5* and *Hey1* were the two most upregulated genes.

B-D. Relative β-catenin/TCF luciferase activity of HEK293T cells transfected with 25 ng of S45Fβ-catenin alone or in the presence of different Notch pathway components as indicated. Transfections were performed in triplicate. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (**P<0.001; NSP>0.05, one-way ANOVA and Tukey’s post-hoc test, N≥3). 

B. HEK293T cells were transfected with 25 ng of S45Fβ-catenin alone or in the presence of 12.5 ng of Rbpj-VP16, 12.5 ng of Hes5, and 12.5 ng of Hey1. All Notch pathway components inhibited S45Fβ-catenin.

C. HEK293T cells were transfected with 25 ng of S45Fβ-catenin alone or in the presence of 12.5 ng of Rbpj-VP16 R218H or 12.5 ng of DNHey1. Abolishing the ability of Rbpj-VP16 or Hey1 to bind DNA disrupts their ability to inhibit S45Fβ-catenin.

D. HEK293T cells were transfected with 25 ng of S45Fβ-catenin alone or in the presence of 12.5 ng of Rbpj-VP16 or 12.5 ng of Rbpj-VP16 with increasing amounts of DNHey1 (6.25 ng, 12.5 ng and 18.75 ng). DNHey1 abolishes the ability of Rbpj-VP16 to inhibit S45Fβ-catenin function in a dose dependent manner.
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF
Figure 4.4: Hey1 forms a complex with β-catenin and TCF. A. Expression of the β-catenin/TCF transcriptional co-activators Pygopus 1, Pygopus 2 and Bcl9-2 was determined by qRT-PCR in HEK293T transiently transfected with 1600 ng of RFP-Hey1 or 1600 ng RFP-DNHey1 per 10 cm dish. Cells transfected with 1600 ng of RFP only were used as control. Cells were FACS sorted 24 hr after transfection and mRNA was extracted from RFP positive cells. qRTPCR was performed as described using the required primers. B & C. Confocal images of HEK293T cells transfected with 25 ng of TCFV1 and 25 ng of V2β-catenin either alone. (B) or in the presence of 25 ng of RFPHey1 per 6 cm dish. (C). RFPHey1 did not alter the association between TCFV1 and V2β-catenin. DAPI (blue) was used to stain the nuclei. Scale bar 51 µm. D. Cells transfected with 25 ng of epitope-tagged TCF4-myc, 50 ng of S45Fβ-catenin-V5 and either 25 ng of RFPHey1 or 25 ng of RFP, as indicated per 6 cm dish, were subjected to immunoprecipitation (IP) using RFP-trapA beads. The IP samples were analysed by western blotting, alongside total nuclear lysate with an antibodies against RFP, V5 and myc epitope tags. Co-immunoprecipitation of TCF4 and S45Fβ-catenin was detected. The position of molecular markers (in KDa) is shown.
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF

A.

![Bar chart with RQ values for different samples: RFP, RFP-Hey1, RFP-DNHey1.](chart)

B.

![Images of cells stained for TCFv1 v2β3-cat, DAPI, and merged images.](image)

C.

![Images of cells stained for TCFv1 v2β3-cat, DAPI, and merged images.](image)

D.

<table>
<thead>
<tr>
<th>RFP-Trap</th>
<th>N LYSATE</th>
<th>IP</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCF4-myc</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S45Fβ-cat-V5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RFP-Hey1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RFP</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

![Western blot images showing protein bands for α-Myc, α-V5, and α-RFP.](blot)
**Figure 4.5: The Hey1 inhibitory crosstalk to β-catenin is conserved in Xenopus.** A-C. Relative β-catenin/TCF luciferase activity of HEK293T cells transfected with 25 ng of Xenopus β-catenin or 25 ng of murine S45Fβ-catenin alone, per well, or in the presence of different Notch signalling constructs. Data are presented as mean fold change (+/−SEM) in relative luciferase units (RLU) (***P<0.001; NS P>0.05, one-way ANOVA and Tukey’s post-hoc test, N≥3). A. HEK293T cells were transfected with 12.5 ng of XtHey1 or 12.5 ng of XtDNHey1, per well. XtHey1, but not XtDNHey1, inhibited Xβ-catenin transcriptional activity. B. Cells were transfected with 25 ng of mNICD, 12.5 ng of Rbpj-VP16, 12.5 ng of mHes5, or 12.5 ng of hHey1, per well. mNICD1, mRbpj-VP16, mHes5, and hHey1 inhibit Xβ-catenin signalling. C. XtHey1, but not XTDNHey1, inhibits murine S45Fβ-catenin function.
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF

A.

```
<table>
<thead>
<tr>
<th>Condition</th>
<th>Fold change in RLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xβ-cat</td>
<td>1.2</td>
</tr>
<tr>
<td>Xβ-cat + XHey1</td>
<td>0.02</td>
</tr>
<tr>
<td>Xβ-cat + XDNHey1</td>
<td>1.2</td>
</tr>
</tbody>
</table>
```

B.

```
<table>
<thead>
<tr>
<th>Condition</th>
<th>Fold change in RLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xβ-cat</td>
<td>1.2</td>
</tr>
<tr>
<td>Xβ-cat + mNICTD</td>
<td>0.02</td>
</tr>
<tr>
<td>Xβ-cat + Rbβ1-VP16</td>
<td>1.2</td>
</tr>
<tr>
<td>Xβ-cat + mHes5</td>
<td>1.2</td>
</tr>
<tr>
<td>Xβ-cat + hHey1</td>
<td>0.02</td>
</tr>
</tbody>
</table>
```

C.

```
<table>
<thead>
<tr>
<th>Condition</th>
<th>Fold change in RLU</th>
</tr>
</thead>
<tbody>
<tr>
<td>S45Fβ-cat</td>
<td>1.2</td>
</tr>
<tr>
<td>S45Fβ-cat + XHey1</td>
<td>0.02</td>
</tr>
<tr>
<td>S45Fβ-cat + XDNHey1</td>
<td>1.2</td>
</tr>
</tbody>
</table>
```
Discussion

Here we show that in HEK293T cells Notch signalling behaves as an inhibitor of the Wnt signalling pathway. In our experiments NICD, an active form of Notch restricted to the nucleus of the cells (Fig 4.1D), dramatically reduces the ability of a stabilised form of β-catenin (S45Fβ-catenin) to activate Wnt/TCF transcription (Fig 4.1B). To do so NICD requires its transcriptional activity. We demonstrated that NICD W1758A, a NICD point mutant that cannot bind to Rbpj transcription factor and, therefore, is transcriptionally inactive (Fig 4.2B), did not repress β-catenin activation as shown by a reporter assay (Fig 4.2F). Furthermore, NICD W1758A did not modify the distribution pattern, although, it increased slightly the nuclear localisation of β-catenin in comparison to when β-catenin was transfected alone (compare Fig 4.1E and 4.2I and Sup Fig 4.1A and 4.2A). In agreement with this, a point mutant A2026V within the 5th ANK repeat that does not alter the transcriptional activity of NICD (Diederich et al., 1994; Hori et al., 2004; Matsuno et al., 1998; Zweifel et al., 2003), behaved like the wildtype NICD construct. NICD A2026V decreased β-catenin transcriptional activity (Fig 4.2G), did not affect β-catenin localisation to the nucleus (Fig 4.2K) and modified the distribution pattern similarly to NICD (Sup Fig 4.1B and 4.2B). Additionally, we found that downstream members of the Notch signalling pathway including Rbpj, and Hes and Hey target genes, drastically reduced the transcriptional activity of S45Fβ-catenin (Fig 4.3B). Interestingly, when the DNA binding function of these proteins was abolished the inhibitory effect over β-catenin activity disappeared (Fig 4.3C&D). Surprisingly, Hey1 was not working through its transcriptional repressor function and did not alter expression levels of the Wnt co-activators Pygopus or Bcl9 (Fig 4.4A), or the association between β-catenin and TCF in the nucleus (Fig 4.4C). Instead, we found that Hey1 regulates Wnt signalling by forming a physical complex with β-catenin/TCF in the nucleus (Fig 4.4D). This Hey/β-catenin/TCF complex could potentially block the binding of Wnt transcriptional co-activators, stopping Wnt transcriptional activation. Finally, our results suggest that this mechanism is conserved in Xenopus (Fig 4.5A-C).

Previous experiments have suggested that a nuclear form of Notch can regulate Wnt/β-catenin signalling (Deregowski et al., 2006; Hayward et al., 2005; Nicolas et al., 2003). For example, Hayward and colleagues (2005), have shown that the ΔN-N1 form of Notch, which undergoes spontaneous γ-secretase mediated cleavage to produced NICD, can inhibit Wnt/β-catenin. However, it was not clear from these experiments whether the initial targeting of the ΔN-N1 construct to the plasma membrane was responsible for the inhibition of Wnt/β-catenin signalling, given the ability of membrane restricted forms of Notch to inhibit Wnt signalling (Sanders et al., 2009). Similar result have also been reported very recently by
Kwon and colleagues (2011), using the N\textsuperscript{AE} construct, which like ΔN-N1 is targeted to the plasma membrane before undergoing spontaneous γ-secretase mediated cleavage (Jarriault et al., 1995; Schroeter et al., 1998). Clearer data has come from work looking at osteoblastogenesis in ST-2 cells by expressing NICD (Deregowski et al., 2006). In this case, NICD is shown to inhibit Wnt/β-catenin signalling using the TOPflash reporter gene and a number of endogenous target genes. Unfortunately, the c-fos promoter within the TOPflash reporter gene contains two E-boxes to which the Hes and Hey proteins can bind. Consequently, it is not clear from these results whether NICD is inhibiting Wnt/β-catenin signalling through a complex between TCF, Hes1 and Groucho, as the authors suggest, or simply through the binding of the Hes/Hey transcriptional repressors to the TOPflash reporter gene. Similarly, it is not clear whether the endogenous target genes contain E-boxes. However, our data supports the observations of all of these studies and demonstrates that a nuclear form of Notch can inhibit Wnt/β-catenin signalling by inducing the expression of Hes and Hey genes which once translated into proteins form a complex with β-catenin and TCF.

More recently, an \textit{in vivo} study has shown that in Xenopus development NICD destabilises β-catenin independently of GSK3β (Acosta et al., 2011). Through this mechanism, NICD is able to inhibit Wnt/β-catenin signalling suppressing both the formation of the primary body axis, whose development is dependent on β-catenin-induced transcription, and a secondary body axis generated by ectopically expressing Wnt or β-catenin on the ventral side of the embryo. In our results, we did not see a similar destabilisation of β-catenin, and if anything we observed its accumulation in the nucleus (Sup Fig 4.1B). Also we found that expressing Hey1 did not disrupt the association of TCF and β-catenin in the nucleus (Fig 4.4B&C). It will be interesting to see whether NICD alters the interaction between TCF and β-catenin. Especially, given the recent suggestion that Notch signalling induces the export of TCF from the nucleus during endomesoderm segregation in the sea urchin (Rottinger et al., 2006; Sethi et al., 2012). It is also important to note that the mechanism described here is distinct from the inhibition of Wnt signalling by a membrane-restricted form of Notch, which is both ligand and CSL-independent (Chapter 3, (Hayward et al., 2005; Kwon et al., 2011; Sanders et al., 2009).

Notch/Wnt interactions are essential for the temporal and spatial specification of many different cell fates during development and tissue homeostasis. This function of Notch as a modulator of Wnt signalling is likely to play an important role in these process. For example, Wnt/Notch interactions are important for correctly positioning Wnt expression at the boundary between rhombomeres in the vertebrate hindbrain (Amoyel et al., 2005; Cheng et al.,...
2004; Riley et al., 2004) and at the dorso ventral boundary of the wing primordium of Drosophila (de Celis et al., 1996; Klein and Arias, 1998; Micchelli and Blair, 1999). In these two examples ligand dependent Notch signalling is activated in a broad stripe of cells either side of the boundary. As a result, the boundary cells express Wnt and Wingless proteins, respectively. This generates a concentration gradient of Wnt/Wg ligand that controls growth and differentiation of the adjacent tissue. However, this gradient needs to be maintained for differentiation to be successful, and relies on interactions between the Wnt/Wg signalling and the Notch pathway (Cheng et al., 2004; Diaz-Benjumea and Cohen, 1995; Rulifson et al., 1996). Within the developing wing, Wingless signalling induces Delta and Serrate expression in adjacent cells which can then signal back to the boundary cells to maintain Wingless expression in a positive feedback loop (de Celis and Bray, 1997). Wingless signalling also inhibits Notch signalling in the neighbouring cells through the interaction of Dishevelled with NICD (Axelrod et al., 1996; Munoz-Descalzo et al., 2010). This refines the domain of Wingless expression to a two cell wide stripe that straddles the dorso ventral boundary. However, in order to maintain the boundary, it is essential for the Wingless expressing cells not to respond to Wingless signalling. If these cells respond to Wingless by expressing Delta and Serrate, the high levels of these ligands will inhibit Notch signalling through cis-inhibition. Notch induced expression of the homeobox gene cut in Wingless expressing cells, in part, maintains the boundary cells by suppressing Delta and Serrate expression (de Celis and Bray, 1997; Micchelli et al., 1997). However, our inhibitory mechanism can also contribute by preventing β-catenin/TCF complexes inducing the expression of downstream target genes.

In conclusion, our results suggest that in vertebrates, NICD modulates Wnt signalling by blocking transcriptional activation at the level of β-catenin/TCF. This mechanism requires the NICD-induced transcription of Hes and Hey target genes. The induced Hey1 physically associates with β-catenin and TCF in the nucleus to inhibit their function.
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF

References


Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF


Supplementary Figure 4.1: Quantitative analysis of S45Fβ-catenin fluorescence intensity in the nucleus versus the cytosol. A. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells transfected with S45Fβ-catenin alone (as shown in Fig 4.1E). This graph is the same as on Chapter 2 Sup Fig 3.2, since it represents only one experiment where several pictures were taken. B. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells co-transfected with S45Fβ-catenin and NICD (as shown in Fig 4.1F). Note that in the presence of NICD the spectrum of intensities shifts towards the green and blue colours, suggesting that there is an accumulation of for β-catenin in the nucleus.
Supplementary Figure 4.1: Quantitative analysis of S45Fβ-catenin fluorescence intensity in the nucleus versus the cytosol. A. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells transfected with S45Fβ-catenin and NICD W1758A (as shown in Fig 4.2I). B. Graph showing nuclear to cytosol S45Fβ-catenin fluorescence intensity ratios of cells co-transfected with S45Fβ-catenin and NICD A2026V (as shown in Fig 4.1K).
Supplementary Figure 4.3: NICD activates expression of Hes/Hey family of proteins. HEK293T cells were transfected with 50 ng of NICD or with 50 ng of empty vector, per a 6 cm dish. mRNA was extracted from cells 48 hr post transfection. Expression of the Notch target genes *Hes1, Hes5, Hey1, Hey2* and *HeyL* was determined by quantitative PCR. *Hes5* and *Hey1* were the two most upregulated genes.
Supplementary Figure 4.4: Rbpj-VP16 R218H cannot activate a Rbpj-dependent reporter gene. HEK293T cells were transfected either with empty vector, 12.5 ng of Rbpj-VP16, or 12.5 ng of Rbpj-VP16 R218H, per well. Transcriptional activity was measured by transfecting 50 ng of 10xRbpj-luc Notch reporter plasmid. As a transfection control, 20 ng of pRL-CMV were used. Cells were lysed 48 hr post transfection. Transfections were performed in triplicate. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (**P<0.001; *P<0.05, one-way ANOVA and Tukey’s post-hoc test, N=1). Note that Rbpj-VP16 R218H activity was significantly decreased compared to Rbpj-VP16 and empty vector.
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF

![Graph showing fold change in RLU for Empty vector, Rbpj-VP16, and Rbpj-VP16 R218H. The graph indicates a significant difference (***).]
Supplementary Figure 4.5: Dominant negative forms of Hes and Hey proteins inhibit Hes and Hey repressor function. A. HEK293T cells were transfected with 12.5 ng of Rbpj-VP16 plasmid to activate Notch signalling. This activation was repressed by co-expression of 12.5 ng of Hey1 plasmid. Additionally, co-expression of increasing amounts of DNHey1 plasmid abolished Hey1 repressor function in a dose-dependent manner. To assess Hey1 and DNHey1 activity 50 ng of the Hey1-luc reporter were used, 20 ng of pRL-CMV were used as a transfection control. Transfections were performed in triplicate. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (**P<0.01; one-way ANOVA and Tukey’s post-hoc test, N=2). B. HEK293T cells were transfected with 12.5 ng of Rbpj-VP16 plasmid, per well, to activate Notch signalling. This activation was repressed by co-expression of 6.25 ng of Hes5 plasmid, per well. Additionally, co-expression of 18.75 ng of DNHes5 plasmid, per well, abolished Hes5 repressor function. To assess Hes5 and DNHes5 activity 50 ng of the Hes1-luc reporter were used, 20 ng of pRL-CMV were used as a transfection control. Transfections were performed in triplicate. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (***P<0.001; one-way ANOVA and Tukey’s post-hoc test, N=2). C. HEK293T cells were transfected with 12.5 ng of Rbpj-VP16 plasmid to activate Notch signalling. This activation was repressed by co-expression of 12.5 ng of Hey1 plasmid. Additionally, co-expression of 18.75 ng of DNHes5 plasmid abolished Hey1 repressor function. To measure the effect of DNHes5 on Hey1 repressor activity 50 ng of the Hey1-luc reporter were used, 20 ng of pRL-CMV were used as a transfection control. Transfections were performed in triplicate. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (***P<0.001; **P<0.01 one-way ANOVA and Tukey’s post-hoc test, N=1). D. HEK293T cells were transfected with 12.5 ng of Rbpj-VP16 plasmid to activate Notch signalling. This activation was repressed by co-expression of 6.25 ng of Hey5 plasmid. Additionally, co-expression of 6.25 or 12.5 ng of DNHey1 plasmid were not enough to abolish Hes5 repressor function. To measure the effect of DNHey1 on Hes5 repressor activity 50 ng of the Hes1-luc reporter were used and 20 ng of pRL-CMV were used as a transfection control. Transfections were performed in triplicate. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (NS P>0.05 one-way ANOVA and Tukey’s post-hoc test, N=2).
Chapter 4: Hey1 Inhibits Wnt Signalling by Associating with β-catenin and TCF

A. Hey1-luc

B. Hes1-luc

C. Hey1-luc

D. Hes1-luc

[Bar charts showing fold change in RU for different conditions involving Hey1 and Hes1 in association with β-catenin and TCF, with statistical significance indicated by asterisks or NS.]
Supplementary Figure 4.6: Notch target gene Hes5 regulates Wnt signalling. A. HEK293T cells were transfected with 12.5 ng of S45Fβ-catenin alone or in the presence of 12.5 ng of Hes5 or 12.5 ng of DNHes5. Wnt signalling was measured with TCFAdTATA reporter plasmid. Transfections were performed in triplicate. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (**P<0.01; one-way ANOVA and Tukey’s post-hoc test, N=3). B. HEK293T cells were transfected with 25 ng of S45Fβ-catenin alone or in the presence of 12.5 ng of Rbpj-VP16 or 12.5 ng of Rbpj-VP16 with 18.75 ng of DNHes5. DNHes5 abolishes the ability of Rbpj-VP16 to inhibit S45Fβ-catenin function. Transfections were performed in triplicate. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (***(P<0.001; one-way ANOVA and Tukey’s post-hoc test, N=1).
Supplementary Figure 4.7: Notch and the target gene Hey limit Wnt signalling. HEK293T cells were transfected with 12.5 ng of v2β-catenin alone or in the presence of 12.5 ng of TCFv1, along with either 25 ng of NICD or 25 ng of Hey1, per well. Wnt signalling was measured with TCFAdTATA reporter plasmid. Both NICD and Hey1 inhibit Wnt signalling even in the presence of ectopic TCF. Transfections were performed in triplicate. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (***P<0.001; one-way ANOVA and Tukey’s post-hoc test, N=1).
Supplementary Figure 4.8: The RFPHey1 and RFPDNHey1 can still cause a similar effect on S45FB-catenin transcriptional activity. Relative Wnt/β-catenin luciferase activity of HEK293T cells transfected with 25 ng of S45FB-catenin alone or in the presence of 12.5 ng of RFPHey1 or 12.5 ng of RFPDNHey1 was measured using 50 ng of TCFAdTATA reporter plasmid. Transfections were performed in triplicate. Data are presented as mean fold change (+/-SEM) in relative luciferase units (RLU) (***P<0.001; *P<0.05 one-way ANOVA and Tukey’s post-hoc test, N=1).
Chapter 5: General Discussion
5.1 Summary

The overall aim of this thesis was to identify the mechanisms that underpin the
crosstalk between the Notch and Wnt signalling pathways in vertebrates. Within Chapter 2,
reagents were identified that allowed the robust analysis of the crosstalk. This included the use
of a novel Wnt reporter plasmid, which cannot be affected by E-box dependent repression, as
well as, the use of two clearly distinct forms of Notch protein, one membrane-restricted and
other only localised in the nucleus. Using these reagents, we identified two independent
crosstalk mechanisms by which Notch negatively regulates Wnt signalling pathway at the level
of β-catenin. At the membrane, Notch reduces β-catenin transcriptional activation by 20%
(Chapter 3). This is likely to happen via Notch trafficking and degradation through the
endosomes with a component required for β-catenin activation, which could also include
activated β-catenin. In the nucleus, the active form of Notch, NICD, activates the transcription
of target genes, including members of the Hes and Hey family of transcriptional repressors.
Subsequently, Hey stops activation of Wnt target genes by physically associating with
β-catenin/TCF complex (Chapter 4). In summary, we have discovered two mechanisms for the
inhibitory crosstalk of Notch over β-catenin, one that is transcriptional independent and
membrane-restricted which is conserved from Drosophila (Hayward et al., 2005; Sanders et al.,
2009) and another novel mechanism that is dependent on the activation of NICD transcription.
The next section will briefly discuss the key findings of the work presented in this thesis from a
broader point of view and is followed by suggested future directions for the research and final
conclusions.

5.2 Notch and Wnt crosstalk mechanism

5.2.1 Notch and Wnt functional relationship

In this thesis a role for Notch protein as a modulator of Wnt signalling pathway has
been studied. We found that Notch inhibits Wnt signalling in vertebrates at the level of
β-catenin by two distinct mechanisms: one is transcription-independent and happens at the
membrane where Notch is present as a receptor; the other is dependent on NICD-induced CSL
transcription (Fig 5.1). The general view of this model is as follows: at the membrane in the
absence of Notch ligand, the Notch receptor is internalized, usually by Deltex, for trafficking
and recycling (Hori et al., 2004; Matsuno et al., 1995; Wilkin et al., 2008). Through this
trafficking route, Notch, can buffer Wnt signalling activation by degrading or sequestering a
component required for β-catenin activation or even activated forms of β-catenin present near
the membrane and have escaped the phosphorylation and subsequent degradation by the destruction complex (CK1, Axin, APC, GSK3β). This helps to stop possible spontaneous bursts of Wnt signalling at the wrong time. In the presence of the Notch ligand, NICD is released from the membrane, translocates to the nucleus where it forms a complex with the Rbpj transcription factor and the Mastermind-like transcriptional co-activator to induce the expression of target genes which include the Hairy and Enhancer of Split family of genes. Subsequently, Hes and Hey stop the expression of Wnt target genes by physically associating with the β-catenin/TCF complexes. The latter is a strong mechanism because it has to outcompete with Wnt signalling activation. In contrast, the membrane mechanism is weaker as it would happen in the absence of Wnt ligand, to avoid spontaneous Wnt signalling.

Figure 5.1: Proposed model of the functional relationship between Notch and Wnt signalling pathways. **Mechanism 1:** The ligand independent traffic of Notch results in the sequestration, near the membrane, of a component (X) required for β-catenin activation (star). Thus, Notch trafficking can reduced Wnt-induced transcription. However, the amount of β-catenin available for activation at this point is very low due to the destruction complex (CK1, Axin, APC, GSK3β) which targets β-catenin for degradation by phosphorylation (P). **Mechanism 2:** Dishevelled (Dvl) is activated by the Wnt ligand inhibiting the destruction complex. This allows the accumulation of activated β-catenin in the cytosol and its translocation to the nucleus. Once in the nucleus β-catenin binds the TCF transcription factor to activate transcription. Meanwhile, Notch activation by Delta-like/Jagged (Dll/Jag) ligands releases NICD to the cytosol. Once in the nucleus NICD binds Rbpj and induces transcription of the Hes and Hey target genes. Subsequently, Hes and Hey physically associate with β-catenin/TCF complex to stop Wnt signalling.
At the outset of my PhD there were two big questions in the field that needed addressing. First, whether Notch inhibitory crosstalk on Wnt signalling required transcription of Notch or not. Second, whether this inhibitory crosstalk was conserved from invertebrates to vertebrates. I endeavoured to tackle these two questions directly with my experiments.

To differentiate between a transcriptional independent or dependent mechanism, a membrane-restricted form of Notch, ΔEGF_N1, was generated. Previously, membrane-tethered forms of Notch had been designed by fusing the Notch intracellular domain with the extracellular and transmembrane (TM) domain of other receptors, such as, the receptor tyrosine Kinase Torso (Hayward et al., 2005) or the CD8 protein (Sanders et al., 2009). In contrast, our ΔEGF_N1 molecule was generated from a murine full length Notch1 protein by removing the extracellular 36 EGF-like repeats required for ligand binding (Rebay et al., 1991). This prevents the consecutive S2 and S3 cleavages that release the NICD molecule for signalling (Brou et al., 2000; Mumm et al., 2000). However, our ΔEGF_N1 still conserves the original Notch TM domain and LNR repeats, meaning that will undergo S1 cleavage by Furin (Logeat et al., 1998) and will be present as a heterodimer on the cell surface, like the endogenous full length Notch1 protein (Blaumueller et al., 1997) (Chapter 2 Fig 2.4B, C, D).

Next, we chose NICD1 as a nucleus-restricted form of Notch. Usually Notch signalling is activated either with NΔE (Jarriault et al., 1995; Schroeter et al., 1998) or NICD1 (Coffman et al., 1993; Rebay et al., 1993). The NΔE construct is initially targeted to the cell membrane, where it is spontaneously cleaved by γ-secretase, releasing NICD into the cytosol. In contrast, the NICD construct is translated in the cytoplasm, and once synthesised it travels directly to the nucleus to activate transcription without localising to the membrane (Chapter 2 Fig 2.4B, C, E).

By using two functionally different forms of Notch: ΔEGF_N1 and NICD, we have specifically separated the investigation into signalling-inactive and signalling-active effects of Notch. This has allowed us to identify two different mechanisms by which Notch inhibits the ability of β-catenin to activate transcription. This is well illustrated with the W1758A and A2026V point mutations generated in both Notch constructs: ΔEGF_N1 and NICD, as they show opposite effects. While ΔEGF_N1 W1758A inhibited Wnt signalling significantly (Chapter 3 Fig 3.3A), NICD W1758A had no significant effect (Chapter 4 Fig 4.2F). In contrast, in the presence of ΔEGF_N1 A2026V, β-catenin activated Wnt signalling to similar levels as when it was transfected alone (Chapter 3 Fig 3.3A), but NICD A2026V inhibited Wnt activation to the same extent as NICD (Chapter 4 Fig 4.2G).
The Notch receptor at the membrane is constantly ubiquitinated by Deltex (Matsuno et al., 1995) and endocytosed into early endosomes for recycling or trafficking. In the early/sorting endosome the Notch intracellular domain stays within the cytosol, but as HOPS complex-dependent Rab5 to Rab7 conversion promotes endosome maturation into multivesicular bodies (or late endosomes), which eventually fuse with the lysosome, the Notch in internal vesicles is degraded. However, due to an acidic change in the pH of the vesicles the Notch present at the limiting membrane of the endosome can be cleaved releasing NICD (Wilkin et al., 2008). Our ΔEGF_N1 construct behaves as a normal Notch receptor. Therefore, through the evidence presented in this thesis we believe that in vertebrates Notch at the membrane inhibits Wnt/β-catenin transcriptional activity through Deltex-mediated trafficking. This mechanism is in agreement with observations in Drosophila (Hayward et al., 2005; Sanders et al., 2009) and mammalian cells (Kwon et al., 2011) since all studies agree that it is a DSL independent and CSL independent mechanism. However, the latter studies observe a degradation of β-catenin, whereas, we observed no obvious change in the cytoplasmic pool and an accumulation in the nucleus, suggesting that the protein is present but it is not functional. An explanation for this could be that Notch endocytoses a component for the activation of β-catenin; but we do not reject that it could be β-catenin itself. We have shown that the ΔEGF_N1 W1758A behaves as ΔEGF_N1. We believe that ΔEGF_N1 V1744G increases degradation of the factor required for the activation of β-catenin. This is possibly due to the presence of a Valine at position 1744 which disrupts the binding with γ-secretase, therefore this construct may be more easily internalised to the inner membrane of the multivesicular body and/or lysosome. The ΔEGF_N1 A2026V construct may bind Deltex so well that it holds Notch within the limiting membrane of the endosomes, preventing its entrance into the multivesicular bodies, by these means β-catenin can still be activated or released into the cytosol and signal (Fig 3.3).

Previously, genetic analysis had shown a cross-inhibitory effect between Notch and Wnt signalling pathways (Deregowski et al., 2006; Nicolas et al., 2003). For example, mice in which Notch function has been knocked out specifically in the skin demonstrate an increase in Wnt signalling within keratinocytes (Nicolas et al., 2003). Or in murine ST-2 cultures, NICD decreases the amount and transcriptional activity of an unphosphorylated form of β-catenin. However, in the latter study, activity levels were measured with a TOPflash Wnt reporter which can be inhibited by the repressor function of the Notch target genes Hes/Hey through binding to the E-boxes within the c-fos promoter of the TOPflash reporter. In contrast, we have chosen TCFAdTATA, a minimal reporter without E-boxes (Chapter 2). Derewgoski and
colleagues (2006), proposed a mechanism in which Hes1 interacts with Groucho and TCF in the nucleus to block Wnt transcription; however, evidence of a physical complex was not shown. A recent study has shown that injection of a Notch morpholino in developing Xenopus embryos shows an increase in nuclear $\beta$-catenin levels (Acosta et al., 2011). However, in our system NICD promotes the accumulation of S45F$\beta$-catenin in the nucleus (Sup Fig 4.1B and 4.2B). Acosta and colleagues (2011), also show that NICD inhibits the formation of a double axis generated by injection of ectopic $\beta$-catenin in developing embryos, through a degradation mechanism that is independent of GSK3$\beta$. In contrast, we show that Notch inhibition of $\beta$-catenin transcriptional activity requires Notch activation, and NICD Rbpj-dependent transcription of the target genes Hes and Hey (Fig 4.3). Additionally, we found that Hey1 stops Wnt signalling by physically interacting with the $\beta$-catenin/TCF complex in the nucleus (Fig 4.4). Interestingly, Hes/Hey do not use their transcriptional repressor function to cause this inhibition (Fig 4.4A). Furthermore, our experiments demonstrated that this crosstalk mechanism is conserved among vertebrate species (Chapter 3 Fig 3.6, Chapter 4 Fig 4.5). Therefore, our results are similar to the previous observations, however, we provide good evidence for a transcriptional-dependent mechanism.

**5.2.2 Notch inhibition of Wnt signalling in development**

One could ask, why Notch has two different mechanisms for the same crosstalk? A simple answer could be due to the two functions of Notch, at the membrane as a receptor, and in the nucleus as a transcription factor. Therefore, it is maybe not surprising that the Notch protein can have two distinct mechanisms to inhibit Wnt signalling.

The existence of two mechanisms also fits very well with some observations in Drosophila. For example, genetic experiments argue that there are two distinct times that Notch represses Wingless/Wnt during proneural cluster development. Proneural clusters are small groups of equivalent cells that all have the potential to develop into sense organ precursor (SOP) cells; the SOP will subsequently divide and differentiate to form all the cells of the final sense organ. However, typically only one or two cells will become SOPs. The cells adopting the SOP fate send a signal to the neighbouring cells causing them to differentiate into epithelial cells. This lateral inhibition signal is mediated by the canonical DSL ligand-induced Notch signal that induces the transcription of the Enhancer of split Complex (E(spl)C).

The identification and analysis of the $N^{Mcd}$ alleles suggests that the initial specification of the proneural clusters is suppressed by a membrane-restricted Notch function (Arias, 2002;
Ramain et al., 2001); similar results were also obtained with the Abruptex alleles of Notch (Brennan et al., 1999c). In these mutants, proneural cluster development is abolished. However, the mechanism is distinct from the ligand-induced transcription of members of the E(spl)C, since cluster development is not recovered by removing ligand or Su(H) function. In contrast, the clusters do develop when Deltex function is abolished or when there is a constitutive Wingless signal. This is very reminiscent of the molecular mechanism we have identified by which a membrane-restricted form of Notch can inhibit Wnt signalling in the absence of ligand binding and the interaction with Rbpj but requires the interaction with Deltex (Chapter 3). Functionally this membrane-restricted role of Notch appears to be required to prevent spontaneous Wingless/Armadillo signalling and is broken when Wingless is expressed ensuring the correct positioning of the proneural clusters (Ramain et al., 2001). In the absence of this initial repression, cluster development is precocious and poorly patterned (Brennan et al., 1999a; Brennan et al., 1999c; Martinez-Arias et al., 2002).

The resolution of the proneural cluster by lateral inhibition into one or two cells that adopt the SOP fate is mediated by a Delta-induced signal via Notch and Su(H) that initiates the transcription of members of the E(spl)C (Skeath and Carroll, 1991). Typically, the expressed members of the E(spl)C are thought to suppress SOP development by directly binding to the promoters of the proneural genes of the Achaete-Scute Complex repressing their expression (Skeath and Carroll, 1991). However, it is very likely that the interaction of the E(spl)C proteins with Armadillo and dTCF, as identified in Chapter 4, is also contributing. In many cases, the expression of the Achaete-Scute Complex is initiated by a Wingless/Armadillo signal (Cubas et al., 1991). Consequently, disrupting the Wingless signal will help resolve the proneural cluster.

The different environments in which the two crosstalk mechanisms occur could also explain their relative strengths. The membrane-restricted crosstalk mechanism is only required to prevent spontaneous Wingless/Armadillo signalling and must be overcome by a Wingless signal once Wingless is expressed (Brennan et al., 1999a; Brennan et al., 1999b; Lawrence et al., 2001). Therefore the crosstalk mechanism is only required to limit a weak Wingless/Armadillo signal. In contrast, the nuclear crosstalk mediated by the E(spl)C proteins must overcome an active Wingless signal and therefore must be much stronger (Heitzler and Simpson, 1991). In this case, it is interesting to note that the SOP development within the proneural cluster is biased with the SOP developing adjacent to the cells emitting the Wingless signal where Wingless/Armadillo signalling is presumably the strongest (Romani et al., 1989; Simpson, 1996). Similar Notch/Wnt interactions have also been observed in Drosophila during muscle
precursor specification (Brennan et al., 1999a) and during the expression of a visceral mesoderm-specific enhancer of the Ubx gene (Lawrence et al., 2001).

Recently two clear examples of Notch/Wnt crosstalk have also been described in vertebrates (Acosta et al., 2011; Kwon et al., 2011) that are proposed to be mediated by the membrane-restricted crosstalk mechanism to limit spontaneous Wnt/β-catenin signalling. In the first Acosta and colleagues (2011) show that maternal Notch degrades a form of β-catenin, that has escaped phosphorylation by GSK3β, through a non-transcriptional mechanism before mid blastula transition. This is important for the specification of the Xenopus signalling centres that will develop the brain and the embryonic dorsal midline structures. In the second, Kwon and colleagues (2011), show that Notch1 can titrate β-catenin protein levels, independently of GSK3β and the Notch ligands, to regulate the expansion of cardiac precursors.

However, Notch/Wnt antagonism has also been observed during vertebrate somitogenesis. Somitogenesis is the process by which the somites or segments, that will give rise to the anteroposterior body axis, are formed. The formation of somites is periodic and requires an oscillator (the segmentation clock) (Palmeirim et al., 1997). This oscillator is driven by cycles of Notch and Wnt induced gene expression, which are out of phase of each other (Pourquie, 2003). Notch signalling induces the expression of Hes/Hey family members in a wave across the presomitic mesoderm which resolves into a stripe in the posterior half of the forming somite to define the somite boundary (Jouve et al., 2000; Leimeister et al., 2000; Palmeirim et al., 1997). On the other hand, Wnt signalling regulates the expression of Axin2, which results in a negative feedback limiting the Wnt signal (Aulehla et al., 2003), and the Notch pathway components Lunatic Fringe (Aulehla et al., 2003) and Delta-like 1 (Galceran et al., 2004). The induction of Notch pathway components also provides an explanation as to why the Wnt and Notch signals are out of phase with each other, as the delay in transcribing and translating the Delta-like 1 gene will inevitably delay activation of the Notch pathway. In keeping with this, inhibiting the Wnt pathway pharmacologically disrupts the Notch signal and alters the periodicity of the somite clock (Gibb et al., 2009). However, the Notch inhibiting Wnt mechanisms described in chapters 3 and 4, along with the Wnt inhibiting Notch mechanism elucidated by Giovanna Collu in the laboratory, can also help explain the maintenance of the oscillations in Notch and Wnt signalling out of phase with each other during somite formation.
5.3 Future directions

5.3.1 Transcription-independent mechanism

At the membrane a Notch/β-catenin physical interaction would be in agreement with results observed in Drosophila (Hayward et al., 2005; Sanders et al., 2009), and stem and colon cancer cells (Kwon et al., 2011). Our results show a decrease in β-catenin transcriptional activity and point to either a sequestration of a component required for β-catenin activation, such as LRP5/6 or Dishevelled, or β-catenin itself. To investigate whether Notch and β-catenin (or any of the other candidate proteins) physically interact, a specific co-immunoprecipitation assay for membrane proteins, between ΔEGF-N1 or ΔEGF-N1 Δ425 and the candidate proteins, should be performed. The control for this experiment should be ΔEGF-N1 Δ671, because the latter construct does not affect the transcriptional function of β-catenin.

For the membrane mechanism we have observed a requirement for the Notch intracellular ANK domain. Furthermore, the results with ΔEGF-N1 A2026V suggest that this mechanism could be Deltex dependent (Chapter 3 Fig 3.3). This would be in agreement with previous experiments from Drosophila which have implicated Deltex in the Notch-mediated regulation of Wnt signalling ((Langdon et al., 2006; Ramain et al., 2001) reviewed in (Hayward et al., 2008)). However, other authors have suggested that the intracellular RAM domain is the one required, and that the mechanism at the membrane depends on the Numb adaptor protein (Kwon et al., 2011). Recently, it has been shown that Numb interacts with Notch 1 but not with Notch 3 (Beres et al., 2011). Therefore, it would be interesting to investigate the ability of β-catenin to induce transcription in the presence of ΔEGF-N3. This experiment will clarify whether, in our system, Notch cross-inhibitory mechanism on β-catenin is Numb dependent or not. Additionally, an immunoprecipitation of ΔEGF-N1 A2026V with Deltex in comparison with ΔEGF-N1 could be done.

It would be interesting to confirm whether Notch requires Dynamin-mediated endocytosis to limit Wnt signal. This could be easily studied by blocking endocytosis via expressing a dominant negative form of Dynamin and reporting Wnt activity by luciferase assays. Along these lines it should also be possible to determine whether ΔEGF-N1 and a Wnt pathway component, required for β-catenin activation, co-localise in the trafficking route. This could be done using immunofluorescence and markers for the different endocytic compartments, such as, Rab5 for endocytic vesicles, Rab 7 for late endosome or Rab4 and
Rab11 for recycling vesicles, or with dominant negative forms of the proteins and reporting Wnt activity by luciferase assays.

Given that quantitative analysis of the immunofluorescence images pointed that in the presence of membrane restricted Notch constructs S45Fβ-catenin accumulates in the nucleus (Chapter 3 Sup Fig 3.2, 3.3, 3.4), it would be interesting to perform a nuclear fractionation assay to verify what happens to S45Fβ-catenin in the nucleus.

Figure 5.2: Axis induction in Xenopus. A-D. *Xenopus laevis* embryos were injected with 500 pg of GFP (as a control, A & B) or 500 pg of GFPXβ-catenin (C & D). Embryos injected with GFP developed normally (A & B). Embryos injected with GFPXβ-catenin developed a double axis phenotype within 24 hr post injection (C & D). Images are presented in phase (A & C) or under UV light (B & D).

In order to provide a physiological relevance to this mechanism, it would be good to place our results into an *in vivo* context. Xenopus is a relatively simple system to use as phenotypic effects on the development of Xenopus embryos can be seen within 24 hr. A classic developmental biology experiment to assess Wnt signalling is the generation of a double axis in Xenopus embryos by injecting Wnt or β-catenin mRNA into one or both of the ventral blastomeres at the four cell stage (McMahon and Moon, 1989) (Fig 5.2). Inhibition of Wnt signalling can then be monitored by the suppression of the double axis by co-injecting mRNA for a second protein (Glinka et al., 1998; Niehrs, 2006). Similarly, a membrane restricted form of Xenopus Notch1 fused to RFP (for easy identification) (RFP-XΔEGF_N1) could be generated and injected along with Xβ-catenin to assess whether the membrane bound Notch could
inhibit the formation of the secondary axis, \textit{in vivo}, via inhibiting Wnt signalling. Additionally, we could inject RFP-XAEGF_N1 mRNA dorsally to study the inhibition of Wnt signalling during the inhibition on the formation of the primary body axis.

Finally, it would be interesting to address whether this mechanism can limit Wnt/\(\beta\)-catenin signalling in a cancerous cell line which has spontaneous high levels of \(\beta\)-catenin and Wnt signalling, for instance, a colorectal cancer cell line. This could point to a molecular mechanism that can be mimicked to limit Wnt/\(\beta\)-catenin signalling in the treatment of these cancers.

\textbf{5.3.2 Transcription-dependent mechanism.}

Previously, NICD has been shown to physically bind \(\beta\)-catenin in the cytosol of stem cells (after BIO treatment, to inhibit the destruction complex) and SW480 colorectal cancer cells (which contain high levels of endogenous active \(\beta\)-catenin) (Kwon et al., 2011). In our system, we have not seen an interaction between NICD and \(\beta\)-catenin in the cytosol (data not shown). Perhaps, a NICD/\(\beta\)-catenin interaction could simply reflect the interaction between the intracellular domain of Notch and \(\beta\)-catenin (Chapter 3). Once synthesized NICD is rapidly transported to the nucleus, therefore, NICD physiological levels in the cytosol are very low. We believe that an interaction in the cytosol is not very likely to happen. To address whether NICD and \(\beta\)-catenin physically interact or not, cells could be treated with 6-BIO (GSK3\(\beta\) inhibitor) to increase nuclear \(\beta\)-catenin levels and, therefore, the likelihood of \(\beta\)-catenin and NICD co-localisation, and then a Co-immunoprecipitation from nuclear samples should be performed.

Recently, it has been shown that during Sea urchin development TCF is exported from the nucleus of cells in the presence of Notch (Sethi et al., 2012). In our system, quantitative analysis of fluorescent images suggested that \(\beta\)-catenin accumulates in the nucleus in the presence of NICD (Chapter4 Sup Fig 4.1 and 4.2), though, we have not looked at TCF. In the case that \(\beta\)-catenin and TCF would not be exported from the nucleus, it would be interesting to perform a Chromatin Immunoprecipitation (ChIP) assay against TCF binding sites (Giese et al., 1991; Giese et al., 1992; van de Wetering et al., 1991), since \(\beta\)-catenin could be within the nucleus but not binding DNA. Additionally, a nuclear fractionation for NICD and Hes/Hey proteins when co-transfected with \(\beta\)-catenin would also help to confirm the immunofluorescence data and determine more insights about this mechanism.
We have also demonstrated that our Notch inhibitory mechanism in the nucleus requires binding to Rbpj and transcription of the Hes and Hey family of genes (Chapter 4 Fig 4.3); and that Hey1 physically associates with β-catenin/TCF complex to stop Wnt signalling activation (Chapter 4 Fig 4.4D). Although, we have not addressed whether the dominant negative form of Hey1 that we generated does not have any effect on Wnt signalling because it cannot interact with the β-catenin/TCF complex. This could easily be tested by repeating the Co-IP experiments shown in Chapter 4 Figure 4.4D using RFP-DNHey1.

The majority of the data presented in the thesis has been generated with an overexpression system. Therefore, it would be desirable to have an assay where we can demonstrate that this mechanism occurs endogenously. For example, an easy experiment to perform in HEK293T cells would be to knock down Notch endogenous promoters and then measure the levels of Wnt target genes, to observe whether they are up or down regulated. This could be done by performing a quantitative PCR against Wnt target genes, such as Axin2 (Jho et al., 2002). Based on our mechanism, one would expect Wnt target genes to be upregulated. Given that in human cells there are four Notch genes, it would seem more appropriate to knockdown the transcription factor Rbpj (from which there is only one gene) or ideally, the primary Notch target genes Hey1 or Hes. However, there would still be several members of the Hes/Hey family which could also be causing a mild inhibitory effect. We could then investigate whether if we transfect RFPHey1 in the Notch knockdown background Wnt target genes go down. As a control for this experiment RFPDNHey1 could be used.

Having established that Hey1 could inhibit Xenopus Wnt signalling in vitro (Chapter 4 Fig 4.5) we could establish whether Hey1 inhibits Wnt signalling during embryonic development. This experiment could be easily performed by injecting Xβ-catenin mRNA along with XHey1 mRNA into one of the ventral blastomeres of the 4 cell Xenopus embryo, to assess double axis formation and inhibition. An obvious control to use would be XDNHey1. For this it would be necessary to verify expression of XHey and XDNHey1 proteins in the injected embryos by western blot. Additionally, dorsal injections of XHey or XDNHey1 before axis formation could be performed. This will help to determine whether Hey has an effect on the formation of the primary axis which is established in response to Wnt signalling in the early embryo. We should assume that the double axis effects will not be completely reverted by co-injection of Xβ-catenin with XHey1, therefore, an index of double axis phenotypes should be used. A similar experiment to the knockdown assay in cell culture would be to inject Rbpj morpholino in the developing Xenopus embryos. Therefore, if there would be an increase in
Wnt signalling activation we would probably see the formation of a double axis, meaning that Notch target genes are required for the normal development.

5.4 Final conclusions

We have unraveled two crosstalk mechanisms by which Notch regulates Wnt signalling. At the membrane Notch inhibits β-catenin possibly by degrading it or another Wnt pathway component through the endosomal route. In the nucleus, NICD-induced transcription of Hes and Hey target genes stops Wnt signalling by associating into a physical complex between Hey, β-catenin and TCF. These mechanisms seem to be conserved across vertebrates. Taking together previous reports and our findings it seems that this new role of Notch as a modulator of Wnt signalling pathway functions to “fine-tune” the activity of β-catenin during cell fate decisions in development. It is possible that this mechanism occurs in any type of cell, however, it will be dependent on the cell specific environment. In the same way that we fine-tune an instrument, signalling pathways have developed their own tuning mechanisms, such as inhibitory crosstalks. The generation of a tuning system is not an easy task and finding a successful combination of tuning requires its own characteristics depending on the context.
References


Matsuno, K., Diederich, R. J., Go, M. J., Blaumueller, C. M. and Artavanis-Tsakonas, S. (1995). Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* 121, 2633-44.


Chapter 6:
Supplementary Materials & Methods
6.1 Cell culture

6.1.1 Cell lines

Human embryonic kidney cells that stably express the large T antigen of simian virus 40 (HEK293T) were obtained from Dr Anthony Brown (Weill Medical College, Cornell University, New York, USA) and from Dr Valerie Kouskoff (Paterson Institute for Cancer Research, Manchester, UK). NIH Swiss mouse embryo fibroblast (NIH-3T3) cell line was a kind gift from Dr Anthony Brown (Weill Medical College of Cornell University, New York, USA). The Chinese hamster ovary K1 variant (CHO-K1) cell line was obtained from Dr John Gallager (Paterson Institute for Cancer Research, Manchester, UK). Mouse embryonic fibroblast (MEF) cells were obtained from Dr Andrew Gilmore (Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester, UK). HEK293 cells were a gift from Dr Charles Streuli (Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester, UK).

6.1.2 Cell culture reagents

Dulbecco’s modified Eagle’s medium (DMEM) and Eagle’s minimal essential medium (EMEM) were obtained from Lonza Group Ltd (Switzerland). Ham’s F12 nutrient mixture was purchased from Gibco (Invitrogen). Phosphate buffer saline (PBS) without calcium and magnesium (1 x), dimethyl sulphoxide (DMSO), trypsin ethyldiamine tetra-acetic acid (EDTA) solution (1 x), penicillin/streptomycin antibiotic solution, insulin and hydrocortisone were purchased from Sigma Chemicals Co. Ltd (Dorset, UK). Foetal bovine serum (FBS) was obtained from BioSera (East Sussex, UK). Tissue culture dishes, vials, multi-well plates and plastic pipettes were purchased from Corning Incorporated (NY, USA).

6.1.3 Cell growth conditions and culture media

Cells were maintained at 37°C and 5% CO₂ in a humidified incubator. HEK293T, NIH-3T3 and MEF cells were grown in DMEM 4.5 g/L glucose, with L-glutamine supplemented with 10% FBS, 50 μg/ml penicillin and 50 μg/ml streptomycin. CHO-K1 cells were cultured in Ham’s F12 medium supplemented with 10% FBS, 1% non-essential amino acids, 50 μg/ml penicillin and 50 μg/ml streptomycin. HEK293 cells were cultured in EMEM medium supplemented with 10% FBS, 1% non-essential amino acids, 1% Na Pyruvate, 50 μg/ml
penicillin and 50 μg/ml streptomycin. All supplemented media are hereafter referred to as growth medium.

### 6.1.4 Sub-culture of cells

Cells were sub-cultured when 95% confluent. Prior to trypsinisation, cells were washed with 1 x PBS. Then 1 ml trypsin, for a 10 ml dish, was added to cells and incubated for 1 min at room temperature in the laminar flow hood for detachment. After trypsin aspiration, cells were re-suspended in fresh growth medium and sub-cultured into a new dish at the required dilution, ranging from 1:2 to 1:20.

### 6.1.5 Freezing of cells

Confluent cells were detached as described in section 6.1.4 with PBS and trypsin and re-suspended in fresh growth medium. Cells were then centrifuged at 220 x g for 3 min, the supernatant was aspirated and the pelleted cells re-suspended in the required volume of growth medium, for example 4 ml from 7 ml of a confluent 10 cm dish, with 10% DMSO. Subsequently, 1 ml of the cell suspension was transferred into cryovials and slowly frozen at -80°C overnight. Vials were transferred afterwards to liquid nitrogen for long-term storage.

### 6.1.6 Defrosting of cells

Cryovials containing frozen cells were quickly thawed in a water bath at 37°C and directly re-suspended in fresh growth medium. The cell suspension was then centrifuged at 220 x g for 3 min. The supernatant was aspirated and the cell pellet re-suspended in 4 ml of fresh growth medium and plated into a 60 mm tissue culture dish. Once the cells were attached to the surface of the dish (24 to 48 h later) the medium was replaced with fresh medium, to remove dead cells.

### 6.1.7 Counting of cells

Following detachment and re-suspension in fresh culture medium, cells were transferred to a 15 ml centrifuge tube. The density of the cells in the suspension was determined using an Improved Neubauer haemocytometer, 0.1 mm cell depth, 1/400 mm², (Hawksley) under a light microscope (Olympus CK X31 microscope). To calculate cell concentration per ml, the mean number of cells per quadrant was multiplied by 1 x 10⁴.
6.1.8 Transfection of cells

DNA was transfected into cells using Lipofectamine and PLUS reagent (Invitrogen) or X-tremeGENE 9 DNA transfection reagent (Roche) according to manufacturer’s instructions.

HEK293T cells were seeded at a density of $2 \times 10^5$ per well in 24-well plates or $4 \times 10^5$ per well in 6-well plates with growth medium and incubated overnight. Transfections were performed in triplicate with a total of 250 ng of DNA per well of a 24 well dish. Therefore, a cocktail for 4 wells was prepared in one tube with a total of 1 μg of DNA, 100 μl of serum and antibiotic free (SF) medium with 6 μl of the plus reagent. The cocktail was incubated for 15 min at room temperature. In a separate tube, 100 μl of SF medium were incubated with 4 μl of lipofectamine for 15 min at room temperature. Both solutions were then mixed and incubated for an additional 15 min at room temperature. In the mean time, cells were washed in 1 ml (per well of a 24 well plate) or 2 ml (per well of a six well plate) of SF medium and replaced with a minimal volume of SF medium, 0.5 ml or 1 ml of SF medium, respectively. Finally, the transfection mixture was added drop-wise to the cells. To maintain transfections with a constant amount of 1 μg of total DNA per cocktail, pcDNA3.1(+) was used. For larger dishes, the total amount of DNA was scaled up accordingly. Cells were incubated for 3 h with the transfection mixture in a humidified incubator, at 37°C and 5% CO₂, until medium was replaced with fresh growth medium. Medium was replaced once more 24 h later to minimize the toxic effects of the transfection medium on the cells. Cells were harvested 24 to 48 hr after transfection.

When using X-tremeGENE 9 DNA transfection reagent, transfections were also performed in triplicate, therefore, for a 24 well dish a cocktail for 4 wells was prepared with a total of 1 μg of DNA. This cocktail was added to 100 μl of serum and antibiotic free (SF) medium with 3 μl of the X-tremeGENE 9 DNA transfection reagent and incubated for 25 min at room temperature. Shortly before transfection, the old medium was removed and fresh growth medium was added to cells. Cells were transfected with 25 μl of the cocktail (250 μg of DNA) added drop-wise per well.

6.1.9 Fluorescence-activated cell sorting

To perform fluorescence-activated cell sorting (FACS), HEK 293T cells were washed once with 1 x PBS, trypsinised and gently resuspended with SF medium containing HEPES at a final concentration of 25 mM. The single cell suspension was then filtered through a 50 μm cup type Filcon (BD Biosciences, #340630) prior to FACS performance by the University of
Manchester flow cytometry lab. The sorted cells were collected in 1 x PBS and incubated on ice until used.

6.2 Protein techniques

6.2.1 Reagents

All chemicals were AnalaR grade or better and were purchased from Merck Chemicals Ltd (Nottingham, UK) unless otherwise stated.

6.2.2 Luciferase assay

Luciferase assays were performed 24 or 48 hr post transfection. Cells were washed twice in 0.5 ml of 1 x PBS, per well, lysed in 100 μl of passive lysis buffer (Promega) and vigorously shaken for 30 min at room temperature. Lysates were directly used for luciferase assays or stored at -20°C to analyse at a later date. Luciferase assays were carried out using the dual Luciferase reporter assay system (Promega), a MicroLumatPlus top plate reader (Berthold Technologies), and black 96 well plates. In all assays, 5 μl of cell lysate were added per well and the plate was inserted into the illuminometer. The automated program settings were as follow: Initially, 50 μl of Luciferase Assay Reagent II (LAR II) were added per well and firefly luciferase activity measured over 10 seconds. Subsequently, 50 μl of Stop and Glo solution were added to quench firefly luciferase activity and activate renilla luciferase, whose activity was also measured over 10 seconds. All experiments were performed in triplicate and the ratio of firefly activity to renilla was calculated using Excel (Microsoft). Data were presented as mean fold change (+/-SEM) in relative luciferase units (RLU), in relation to the corresponding positive control. Statistical analysis was performed with GraphPad Prism program (GraphPad Software, Inc.) using one-way ANOVA and Tukey’s post-hoc tests or with a Student T-test for data sets of two samples.

6.2.3 Whole cell lysis

Cells were washed in 1 x PBS and lysed in 250 μl of boiling (100°C) sodium dodecylsholphate (SDS) lysis buffer (50 mM Tris-HCl pH 7.4, 2% SDS) by scraping. Collected lysates were denatured at 100°C for three min and reaction stopped on ice. Genomic DNA was sheared by continuous sonication at an amplification of 003 watts (RMS) for 5 sec in a Microson XL-2000 ultrasonic cell disruptor (Misonix). Lysates were stored at -20°C.
6.2.4 S100/P100 cytosolic fractionation

Cells were washed twice in 3 ml of 1 x TBS (10 mM Tris-HCl pH 7.4, 140 mM NaCl) + 2 mM CaCl$_2$, placed on ice and lysed in 1 ml of lysis buffer (1 x TBS + protease inhibitor cocktail set (Calbiochem Cat# 539131)) + 10 µl of 100 mM PMSF. Subsequently, cells were dislodged from the plate using a pre-chilled cell scraper and the cell suspension was poured into a cold 2 ml dounce homogenizer. Cells were sheared with 30 strokes of the homogenizer and the cell lysate was poured into a cold 1.5 ml eppendorf. Cell lysates were centrifuged at 1500 x g for 5 min at 4°C. The supernatant was carefully poured into a cold Beckman ultracentrifuge tube (Polyallomer 11 x 60 mm) which was balanced and spun for 90 min at 100000 x g in a TLA 110 rotor in a Beckman Coulter Optima TLX-120 Ultracentrifuge at 4°C. After the spin, the S100/P100 fraction was split, 100 µl of the supernatant representing the cytosolic fraction was mixed with 100 µl of 2 x Laemmli buffer (100 mM Tris -HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue and 20% glycerol), denatured for 3 min and stored at -20°C; the rest of the supernatant (around 500 µl) was stored at -80°C. The pellet containing the membrane fraction was washed twice in 1 x TBS, resuspended in 1 ml of 2 x Laemmli buffer, denatured for 3 min and kept at -80°C.

6.2.5 Protein assay

Protein concentration of samples was determined by a bicinchoninic acid (BCA) protein assay following manufacturer’s instructions (Pierce). A standard curve of known concentrations: 0.1, 0.2, 0.4, 0.6, 0.8, 1 mg/ml was produced using serial dilutions of a 2 mg/ml stock of bovine serum albumin (BSA) (Pierce). Subsequently, 10 µl of each standard and 2 µl of sample were pipetted per well of a clear 96 well plate, in triplicate. Wells were completed up to 200 µl with the BSA reagent (a 50:1 mix of solution A: solution B) (Pierce). The plate was covered with parafilm and incubated at 37°C for 30 min. Protein concentration was determined by measuring absorbance at 562 nm using a 96 well spectrophotometric plate reader (Biohit BP 808) with Elisa-XL software.

6.2.6 Western blot

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were loaded with a consistent total amount of protein, for example 50 µg, or in relation to renilla activity (as required), diluted in 5 x Laemmli buffer (250 mM Tris-HCl pH 6.8, 500 mM DTT, 10% SDS, 0.5% bromophenol blue, 50% glycerol).
Gels ranged from 6 to 12% acrylamide depending on the size of the protein to be separated, although, the majority were 10%. SDS-PAGE gels are made of a resolving and a stacking gel. The resolving gel comprised the appropriate percentage of acrylamide/bis solution (37:5:1) (BioRad), 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% ammonium persulphate (APS) (BDH), and 0.0001% TEMED (BioRad). The stacking gel comprised 5% acylamide/bis solution (37:5:1), 125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulphate (APS) (BDH), and 0.001% TEMED. Prior to loading, samples were denatured by boiling at 100°C in a heat block for 3 min. Protein separation was performed in a BioRad mini PROTEAN III electrophoresis system (BioRad) with constant current of 35 mA per gel, in running buffer (25 mM Tris base, 250 mM glycine, 0.1% SDS) for 1 h 30 min or until bromophenol blue run out of the bottom of the gel. Samples were run alongside 5 μl of spectra multicolor broad range protein ladder (Fermentas) to estimate the molecular weight of the proteins of interest.

Separated proteins were electrophoretically transferred to a nitrocellulose membrane (BioRad) using a mini Trans-Blot system (BioRad) with a constant voltage of 100 V for 90 min, in transfer buffer (48 mM Tris base, 39 mM glycine, 20% methanol). When required membranes were stained with Ponceau solution (Sigma) to see that the proteins had transferred and cut to allow probing with a number of different antibodies. Next the nitrocellulose membrane was incubated for 30 min at room temperature in blocking buffer (5% skimmed dry milk (Premier brands) in 1 x TBS-T (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% v/v Tween 20 (Sigma)) to block the non-specific protein binding sites. Membranes were incubated overnight at 4°C with the appropriate primary antibody (table 6.1) diluted in TBS-T. The following day, the membrane was washed four times for 5 min in TBS-T, to remove unbound primary antibody and incubated with Horseradish peroxidise (HRP)-conjugated secondary antibody (table 6.1) diluted in TBS-T for 45 min at room temperature. Subsequently, the membrane was washed four times for 5 min in TBS-T, to remove unbound secondary antibody. Visualization of HRP-conjugated immune complexes on fuji medical X-ray film super RX (Fujifilm) was carried out using SuperSignal west pico or femto chemiluminescence substrates (Pierce) according to the manufacturer’s instructions.

6.2.7 Stripping and re-probing of membranes

Membranes were rinsed in distilled water and incubated in 0.2 M NaOH for 5 min at room temperature with vigorous shake, followed by a rinse and a wash in distilled water for 5 min. Membranes were finally incubated in TBS for 15 min and stored at 4°C until further use.
6.2.8 Nuclear Immunoprecipitation (IP)

For the immunoprecipitation of RFP-fusion proteins, RFP-Trap_A beads (Chromo Tek GmbH, Martinsried, Germany) were used according to the manufacturer’s instructions. Cells were washed in 1 x PBS and lysed in 500 μl IP lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 1 x protease inhibitor cocktail set (Calbiochem Cat# 539131) freshly added). The lysate was clarified by centrifugation at 21,000 x g for 10 min at 4°C. For subsequent western blot analysis, 50 μl of the supernatant were retained as a total cell lysate sample, the rest was discarded. Pellet was re-suspended in 500 μl of nuclear lysis buffer (10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 0.5% NP-40, 1 x protease inhibitor cocktail set (Calbiochem Cat# 539131) freshly added) and incubated with gentle end-over-end mixing for 10 min at 4°C to swell the nucleus. Lyophilised DNase I was added to a final concentration of 1μg/μl along with NaCl to a final concentration of 150 mM and the tube was incubated with gentle end-over-end mixing for 30 min at 4°C. The lysate was clarified by centrifugation at 20,000 x g for 10 min at 4°C and 50 μl of supernatant were retained as a nuclear input sample for subsequent western blot analysis, the remaining 450 μl were mixed with 20 μl of beads slurry previously equilibrated in 500 μl of lysis buffer. The mixture was incubated with gentle end-over-end mixing for 2 hr at 4°C. Beads were separated by centrifugation at 2700 x g for 4 min at 4°C. For western blot analysis 50 μl of supernatant were retained as a non-bound sample, the rest was discarded. The pellet was washed once in lysis buffer and twice in wash buffer (10 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.5 mM EDTA), re-suspended in 2 x Laemmli buffer and heated at 60°C for 10 min, this sample was the bound fraction.

6.2.9 Immunofluorescence

Nitric acid treated coverslips stored in 100% ethanol were placed in to wells of 6 well plates 30 min prior to seeding of cells. Ethanol was allowed to evaporate for 30 min and wells were washed once with 1 x PBS before seeding of cells. 24 to 48 hr post transfection, cells were washed in 1 x PBS and then fixed in 4% formaldehyde for 10 min at room temperature. Following 3 x washing with 1 x PBS, coverslips were incubated with primary antibody (see Table 6.1) diluted 1:100 in blocking solution (3% goat serum (Biosera, Sussex, UK), 0.1% Triton-X100, 0.05% NaN₃ in TBS) in a humidified chamber for 1 hr, washed and incubated with fluorescence-conjugated secondary antibody (see Table 6.1) in blocking solution. Coverslips were mounted with VECTASHIELD mounting medium for fluorescence with DAPI H-1200 (Vector Laboratories). Images were captured with a Zeiss LSM 700, AxioObserver flexible confocal microscope (Carl Zeiss MicroImaging GmbH, Germany) and a (Plan-Apochromat
63x/1.40 Oil DIC M27) objective using the Zeiss ZEN 2011 software (Carl Zeiss MicroImaging GmbH, Germany). The confocal settings were as follows: pinhole 89.66 µm (for Notch constructs) and 83.39 µm (for S45Fβ-catenin), format 8 Bit, image size 512 x 512 pixels. Images were collected using the 405 nm (45%), 488 nm (11%), and 555 nm (26%) laser lines, respectively. When acquiring 3D optical stacks, the confocal software was used to determine the optimal number of Z sections. Either maximum intensity projections or single z-sections are shown in the results.

6.2.10 Quantitative Image analysis

The fluorescence intensity of nuclear S45Fβ-catenin was determined with image J software (open source software, National Institute of Health, USA) by measuring the integrated fluorescence intensity of the nucleus and cytoplasm of cells transfected with different plasmids. Using the free drawing tool a region was drawn around each nucleus and cell to be measured. Three similar size circles were drawn in an area without cells and the average fluorescent intensity of these three was used as a background reading. The integrated density of each cell was measured at the middle plane of a z series (this was usually the plane cutting through the middle of most cells). Cells that were dividing were not included in the measurements. The maximum nuclear to cytosol ratio was set to 0.75 (as this was the maximum intensity observed when S45Fβ-catenin was transfected alone). Negative nuclear to cytosol ratios (ie: when nuclear corrected total fluorescence was negative) were set to zero. At least 25 cells were measured per condition. Nuclear to cytosol ratio of S45Fβ-catenin fluorescence intensities were plotted at 0.1 intervals. For nuclear to cytosol ratio measurements, the following formulas were used:

Whole cell signal = Integrated density (sum of the intensity of the pixels for one cell)

Nuclear signal = Integrated density of the nucleus of a cell

Cytosol fluorescence = whole cell signal – nuclear signal

Corrected total fluorescence (CTF) = Integrated density – (Area of selected cell or nucleus x Mean fluorescence of background readings).

Nuclear to cytosol ratio = Nuclear CTF / Cytosol CTF
6.3 Molecular biology techniques

6.3.1 Restriction enzyme digestion

Both single and double restriction enzyme digests were performed as required. Diagnostic digests, to identify successful clones containing fragments in the correct orientation, were carried out with 300 ng of miniprep DNA, 1 x appropriate restriction endonuclease buffer, 5 units of the required restriction endonuclease and distilled water as required to make the final volume 20 μl. Reactions to generate inserts for cloning contained 5 μg of DNA, 1 x appropriate restriction endonuclease buffer, 15 units of the required restriction enzyme and distilled water as required to make the final volume 50 μl. All digests were incubated at 37°C (or the required temperature) for 2 hr and restriction products separated on a 1% agarose gel to check digest completion and size of fragments. Vector fragments were generated in 50 μl reactions as described above but with the addition of 2 units of alkaline phosphatase to remove 5' phosphate groups. All enzymes and buffers were supplied by Roche or New England Biolabs.

6.3.2 Agarose gel electrophoresis

DNA fragments were separated on 1% (w/v) agarose gels prepared in 1 x Tris-acetate EDTA (TAE) buffer (40 mM Tris-acetate, 1 mM EDTA) and 0.5 μg/ml ethidium bromide (Sigma) or 1:25000 SYBR Safe DNA gel stain (#s33102, Invitrogen). DNA samples were diluted with 6 x loading buffer (10 mM Tris-HCl pH 7.6, and 0.15% Orange G (Sigma), 60% glycerol, 60 mM EDTA) and water prior to loading. GeneRuler 1 kb plus DNA ladder (Fermentas) was loaded onto the gel to estimate the size of fragments based upon their migration distance. Electrophoresis was carried out at 100 V on a 6 cm tray in 1 x TAE buffer. DNA was visualized using an ultraviolet transilluminator at a wavelength of 312 nm or if the DNA was to be purified, at 365 nm, or a blue light transilluminator when using SYBR Safe and documented on a Kodak Gel logic 100 imaging documentation system.

RNA fragments were separated on 0.6% (w/v) agarose gels prepared in 1 x MOPS buffer (50 mM MOPS, 150 mM NaAc, 25 mM EDTA, adjust to pH 7 with NaOH, DEPC-treated water was used in all steps.), 0.03% formaldehyde in DEPC-T water and Safe View RNA gel stain (NBS Biologicals Ltd). RNA samples were denatured at 65°C for 5 min and diluted with RNA loading buffer (62.5% deionised formamide, 1.14 M formaldehyde, 1.25 x MOPS buffer, 200 μg/ml bromophenol blue, 200 μg/ml Orange G (Sigma), and 50 μg/ml ethidium bromide or
1:25000 Safe View RNA gel stain (#NBS-SV1, NBS Biologicals Ltd) prior to loading. Electrophoresis was carried out at 100 V on a 6 cm tray in 1 x MOPS buffer. RNA was visualized using a blue light transilluminator, and documented on a Kodak Gel logic 100 imaging documentation system.

### 6.3.3 DNA purification

Gel purification of DNA for cloning was carried out to isolate the correctly-sized restriction fragments using a QIAquick gel extraction kit (Qiagen) following manufacturer’s instructions. DNA fragments were excised from the gel under a UV lamp at an absorbance of 365 nm with a razor blade or using a blue light transilluminator. Gel fragments were dissolved in 3 volumes of buffer QG at 50°C for 10 min and 1 volume of isopropanol was added. This solution was passed through a QIAquick spin column at 13000 x g for 1 min and then washed with 750 μl of PE buffer. DNA was finally eluted from the spin column membrane with 50 μl of EB buffer in a final centrifugation step at 13000 x g for 1 min. DNA samples were stored at -20°C.

Plasmid DNA was purified using either QIAprep spin miniprep or HiSpeed plasmid maxi kits (Qiagen) as per manufacturer’s instructions, briefly outlined below:

**Miniprep:** 5 ml of Luria Bertani (LB) medium (10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, and 10 g/l NaCl) containing 100 μg/ml ampicillin were inoculated with one colony from a culture plate and incubated at 37°C overnight in a shaking incubator. Cells were harvested by centrifugation at 13000 x g for 3 min, and resuspended in 250 μl of buffer P1 containing RNase A. The same volume of alkaline lysis buffer P2 was added and incubated at room temperature for 5 min to lyse cells. This suspension was neutralised by addition of 350 μl of buffer N3. Precipitated bacterial cellular debris and genomic DNA was removed by centrifugation at 13000 x g for 10 min. The supernatant was loaded onto a QIAprep mini column and centrifuged at maximum speed for 1 min. DNA bound to the membrane of the column was washed, with 500 μl of buffer PB and 750 μl of buffer PE, at maximum speed for 1 min and eluted with 50 μl of buffer TE.

**Maxiprep:** 2.5 ml of LB medium containing 100 μg/ml ampicillin inoculated with a single colony and grown at 37°C for 8 hr in a shaking incubator (200 rpm), was used to inoculate a 200 ml LB culture containing 100 μg/ml ampicillin and incubated overnight at 37°C. Bacteria were harvested by centrifugation at 4200 x g for 15 min at 4°C and pellet re-suspended in 10 ml of buffer P1 containing RNase A. The same volume of alkaline lysis buffer P2 was added and
incubated at room temperature for 5 min to lyse cells. This suspension was neutralised by addition of 10 ml of buffer P3, transferred to a QIAfilter cartridge-syringe filter and incubated at room temperature for 10 min. The lysis solution was filtered to remove cell debris and genomic DNA into an equilibrated HiSpeed QIAGen Maxi Tip. DNA bound to this maxi column was then washed with QC buffer and eluted with QF buffer. DNA precipitation was carried out by addition of 0.7 volumes of isopropanol. Precipitated DNA was bound to a QIACartrige, washed in 70% ethanol and eluted in 1ml of EB buffer.

6.3.4 DNA quantification

Purified DNA from restriction endonuclease digests was quantified following agarose gel electrophoresis by comparing the intensity of the purified DNA band to a DNA band of known concentration within the GeneRuler 1kb plus DNA ladder, or with a nanodrop spectrophotometer (Thermo Scientific). Plasmid DNA was quantified with a nanodrop spectrophotometer.

6.3.5 Ligation

Ligation reactions were performed in a total volume of 20 μl containing 0.01 pmol of vector, 0.02 pmol of insert, 1 x T4 DNA ligase buffer, 1 unit of T4 DNA ligase and distilled water to make the final volume. Reactions were incubated at room temperature for 2 hr or overnight.

6.3.6 DNA blunting

DNA blunting was performed when required to convert DNA fragments with 5'- and 3'-overhanging ends to DNA fragments with blunt ends using T4 DNA polymerase (Roche). This reactions were performed with 5 μg of DNA, 10 μl of 10 x T4 DNA polymerase buffer (Roche), 5 μl of 2 mM dNTP, 5 μl 1 mg/ml BSA and 10 units of T4 DNA polymerase (Roche) in a total volume of 100 μl of water. The blunting reaction was incubated at 12°C for 20 min and heat-inactivated at 95°C for 10 min.

6.3.7 Bacterial transformation

_E. coli_ DH5α competent bacteria (Invitrogen) or JM109 (Stratagene) were thaw on ice for 15 min and then 50 μl were gently mixed with 300 ng of plasmid DNA or 10 μl of ligation reaction. This mixture was incubated on ice for 15 min and then heat-shocked at 42°C for 1 min followed by incubation on ice for 2 min. After addition of 150 μl of SOC medium (Invitrogen), the mixture was incubated at 37°C for 45 to 50 min. Transformed cells were
selected by plating 100 μl onto a sterile agar plate (LB medium with 15 g/l of Bacto-agar and 100 μg/ml of Ampicillin (Sigma) or 100 μg/ml of Kanamycin) which was incubated at 37°C overnight.

6.3.8 Mutagenesis

Single point mutations were generated with the QuickChange Lightning Multi Site-Directed Mutagenesis Kit or the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer’s instructions. In brief, PCR-based amplification of the entire plasmid was performed using specific mutagenesis oligos, followed by a fast Dpn I digestion of the methylated parental DNA and transformation of the mutated plasmids into competent cells for nick repair. Primers used are detailed in Table 6.2. Per reaction, 100 ng of ds-DNA template and 0.50 μl of QuickSolution were used.

6.3.9 DNA Sequencing

Sequencing reactions were performed with 300 ng of the DNA template, 3.2 pmol of the desired primers, 2 μl of the Big Dye Terminator mix version 3.1 (Applied Biosystems), 4 μl of the 5 x sequencing buffer (Applied Biosystems), and completed to a total volume of 20 μl with distilled water. Primers used included the standard primers SP6 and T7, and the gene specific primers detailed in Table 6.3. The reactions were run on a PCR machine using the following program:

Step 1: 96°C 1min
Step 2: 96°C 30 sec
Step 3: decrease to 48°C at 1°C sec⁻¹
Step 4: 50°C 10 sec
Step 5: increase to 60°C at 1°C sec⁻¹
Step 6: 60°C 4 min
Step 7: go to Step 2 x 34
Step 8: 4°C forever

After the sequencing reaction finished, samples were ethanol precipitated. Samples were incubated for 30 min at room temperature with 2.5 volumes of 96% ethanol and 0.1 volumes of 3 M sodium acetate pH 5.2. Samples were subsequently centrifuged at 21000 x g for 20 min and the supernatant was discarded. The precipitate was washed in 12.5 volumes of 75% ethanol and centrifuged at 21000 x g for 15 min. The supernatant was discarded and the
precipitate was air-dried for 10 min. Samples were then dispatched for sequencing using an Applied Biosystems 3730 DNA Analyzer at the University of Manchester Sequencing Facility. The sequencing results were analysed using the ContigExpress assembly module within Vector NTI (Invitrogen), DNA Dynamo software (Blue Tractor Software), or Four peaks software (Mekentosj).

6.3.10 Quantitative RT-PCR

Total RNA was extracted from cells using peqGOLD TriFast solution (peqlab Biotechnologie GmbH) according to manufacturer’s instructions. RNA was separated on 0.6% RNA gel (see section 6.3.2) to confirm its integrity. A total of 2 µg of RNA were reverse transcribed to cDNA in 20 µl reactions using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), following manufacturer’s instructions. The resultant cDNA was diluted 10-fold and 2 µl were used as template for quantitative PCR (qPCR).

qPCR was performed in triplicate, in a total volume of 20 µl, using the primers listed in Table 6.3, and Fast SYBR Green PCR Master Mix (Applied Biosystems) according to manufacturer’s instructions. PCR products were separated on 2% agarose gels (as described on section 6.3.2) to verify size, and absence of non-specific products and primer dimers. The relative amounts of the PCR products were analysed using the comparative RQ method and using PPIA gene as an internal normalization control.

6.4 Description of the construct used in this thesis

6.4.1 Existing constructs in the lab

The following plasmids were kind gifts: The pLNCX + mWnt1 was obtained from Dr Anthony Brown (Weill Medical College, Cornell University, New York, USA). mDvl2 and mβ-catenin cDNAs were obtained from Geneservice (Cambridge, UK) (I.M.A.G.E. clones 6402000 and 5709247, respectively). The pcDNA3 + mN1 was obtained from Dr Jeff Nye (Northwestern University Medical School, Chicago, USA). The pEGFP-N1 + mNICD was a gift from Dr Vincent Zecchini (The University of Cambridge, UK). The pcDNA3.1 Zeo + TCF4v1 and pcDNA3.1 Zeo + v2β-catenin were obtained from Dr Claudia Wellbrook (Faculty of Life
Sciences, University of Manchester, UK). The $\beta$-catenin plasmid was a gift from Dr Louise Howe (Weill Medical College of Cornell University, New York, USA). The pCS107XtHey1 plasmid was obtained from Dr Nancy Papalopulu (Faculty of Life Sciences, University of Manchester, UK). The plasmid pcDNA3.1(+) used as an empty vector in transfections, was obtained from Invitrogen. The p10xCBF1-luc Notch reporter plasmid was obtained from Dr Grahame MacKenzie (Lorantis, Cambridge, UK). The pTOPflash Wnt reporter was obtained from Dr Louise Howe (Weill Medical College, Cornell University, New York, USA). The pRLCMV reporter plasmid was obtained from Promega. The pmRFP-C1 plasmid was obtained from Dr Andrew Gilmore (Faculty of Life Sciences, University of Manchester, UK).

6.4.2 Constructs generated by cloning

pSecTagNC + ΔEGF_mN1: the cDNA encoding mouse Notch 1 molecule that lacks all 36 EGF-like repeats was generated by cloning a PCR fragment (using mN1 4409F and mN1 4901R primers) digested HindIII/SacI along with a SacI/HindIII fragment of the mN1 cDNA into pSecTagNC+ΔEGF+LNR_mN1 (HindIII) (KB, unpublished data).

pSecTagNC + ΔEGF + mLNR_N1: was generated by cloning a PCR fragment (using primers mN1 5042F, mN1 5589R) digested HindIII/BclI with a 3570 bp fragment of mN1 digested with BclI/EcoRI, into pSecTagNC (HindIII/EcoRI). This plasmid encodes a Notch molecule lacking the extracellular EGF-like and Lin-12/Notch repeats. (KB, unpublished data)

pSecTagNC + ΔN-mN1: encoding the extracellular juxtamembrane, transmembrane and intracellular domains of mN1, was cloned as HindIII/BclI-digested PCR product (amplified using the primers mN1 5210F and mN1 5589R from the pcDNA3+mN1 template) and a BclI/EcoRI restriction fragment of pcDNA3+mN1. These two fragments were ligated into pSecTagNC digested with HindIII/EcoRI (KB, unpublished data).

pSecTagNC ΔN_mN1 Δ425: was generated by cloning the HindIII(blunted)/EcoRI fragment from pSecTagNC ΔN_mN1 Δ425 into pSecTagNC (AHS, unpublished data).

pSecTagNC + ΔEGF_N1 Δ425: was generated by cloning the BspEI/EcoRI fragment containing the C-terminal deletion from pSecTagNC + ΔN_mN1 Δ425 into pSecTagNC + ΔEGF_mN1 (AHS, unpublished data).

pSecTagNC + ΔEGF_mN1 Δ671: was generated by cloning a 153 bp BsmBI/Bsu36I (blunted) mN1 fragment and a 1261 bp SpeI (blunted)/EcoRI mN1 fragment into
pSecTagNC + ΔEGF_mN1 digested with BsmBI/EcoRI. In this ligation, the Bsu36I and the SpeI sites fused to recreate the SpeI site (AHS, unpublished data).

pSecTagNC + mΔEGF_N1 Δ764: was generated by cloning a 955 bp HindIII/Bsu36I (blunted) mN1 fragment from pSecTagNC + ΔEGF_mN1 and a 1261 bp SpeI (blunted)/EcoRI mN1 fragment into pSecTagNC digested with HindIII/EcoRI. In this ligation, the Bsu36I and the SpeI sites fused to recreate the SpeI site (AHS, unpublished data).

pcDNA3 + mNICD: the cDNA encoding myc tagged mNICD was generated by cloning the KpnI/BspEI fragment from pEGFP-N1 + mNICD into pcDNA3 + mN1 (KpnI/BspEI) (KB, unpublished data).

cmpDNA3.1(+) + VP16/Rbpj: was generated by digesting pcDNA3.1(+) KpnI/EcoRI (blunted) and ligated to remove the restriction sites from HindIII to EcoRI in the pcDNA3.1(+) multiple cloning site. The VP16-Tag was cloned from pCMX-N + VP16/Rbpj (HindIII). The Rbpj cDNA was cloned from pCMX-N + VP16/Rbpj (EcoRI). (Spyros Stylianou and KB, unpublished data).

cmpDNA3.1(+) + VP16/Rbpj R218H: was generated by cloning the R218H mutation from pCMX + CBF1 R218H digested with SacII/KpnI into pcDNA3.1(+) + VP16/Rbpj (SacII/KpnI) (AHS, unpublished data).

pcDNA3.1(+) + myc-hHey1: was generated by digesting pcDNA3.1(+) with PmeI and inserting a fragment containing the following sequences: Kozak, myc-tag epitope, EcoRI, HindIII, and BamHI (see below), and then cloning hHey1 cDNA (I.M.A.G.E. BC001873) as an EcoRI/BamHI-digested PCR fragment generated using hHey1 98F and hHey1 11077R primers (KB, unpublished data).

G GTT TAA ACT GCC ACC ATG GAG CAG AAG CTG ATC TCC GAG GAG GAC CTG AAT TCA AGC TTG GAT CCT GAG GTT TAA ACG CTAG

pcDNA3.1(+) + myc-mHes5: was generated by digesting pcDNA3.1(+) with PmeI and inserting a fragment containing the following sequences: Kozak, myc-tag epitope, EcoRI, HindIII, and BamHI (as above), and then cloning mHes5 cDNA as an EcoRI/BamHI-digested PCR fragment generated using mHes5 73F and mHes5 663R primers (KB, unpublished data).

pmRFP + hHey1 & pmRFP + DNhHey1: these cDNAs were generated by digesting pcDNA3.1 + myc-hHey1 and pcDNA3.1 + myc-DNhHey1 with EcoRI/BamHI and inserting the fragment into pmRFP-C1 (EcoRI/BamHI) (AHS, unpublished data).
**pcDNA6 V5/HisA + mDvl2:** was generated by cloning mDvl2 cDNA from pYx-Asc (Geneservice) into pcDNA6 V5-his as an EcoRI/Sall restriction fragment and a Sall/Xbal-digested PCR fragment generated using mDvl2 1834F and mDvl2 2331R primers. (Giovanna Collu, unpublished data).

**pcDNA3.1 myc/HisA + mβ-catenin:** was generated by cloning mβ-catenin from pYx-Asc (Geneservice) into pcDNA3.1myc-hisA (digested KpnI/XhoI) as a KpnI/SacI restriction fragment and a SacI/XhoI digested PCR fragment using mβ-catenin 1914F and mβ-catenin 2555R primers (KB, unpublished data).

**pcDNA6 V5/HisA + S45Fmβ-catenin:** was generated by excising S45Fmβ-catenin from pcDNA3.1(+)/myc-HisA + S45Fmβ-catenin (generated by KB using site directed mutagenesis, primers mβ-catenin S45F F, mβ-catenin S45F R) as a KpnI/Xhol fragment and inserting it into pcDNA6/V5-HisA vector (KpnI/Xhol) (AHS, unpublished data).

**pcDNA3.1 myc/HisA + TCF4:** was generated by cloning the TCF4 cDNA from pcDNA3.1 Zeo + TCF4v1 as an EcoRI/Clal (blunted) fragment into pcDNA3.1 myc/HisA digested with EcoRI/Xhol (blunted) (AHS, unpublished data).

**pcDNA6 V5/HisA + TCF4:** was generated by cloning the TCF4 cDNA from pcDNA3.1 Zeo + TCF4v1 as an EcoRI/Clal (blunted) fragment into pcDNA6 V5/HisA digested with EcoRI/Xhol (blunted) (AHS, unpublished data).

**pcDNA3.1 + myc-XtHey1:** was generated by cloning a PCR fragment encoding XtHey1 (amplified using XtHey1 F and XtHey1 R primers and pCS107XtHey1 as template) digested EcoRI/BamHI into pcDNA3.1 + myc-hHey1 (digested EcoRI/BamHI) to replace the hHey1 cDNA (AHS, unpublished data).

**pGL3basic + TCFAdTATA:** this Wnt reporter plasmid was generated in two steps. Initially, p10xCBF1-luc was digested with Xhol (blunted)/BglII (blunted) and religated to form pGL3basic + AdTATA. pGL3basic + AdTATA was then digested with Xhol and the Sall fragment containing the 4 TCF sites from pTOPFlash was introduced (Elena Garusi and KB unpublished data).

**6.4.3 Constructs generated by mutagenesis**

**pMOPFlash:** this Wnt reporter plasmid was generated by QuikChange Site-Directed mutagenesis kit (Stratagene) using pTOPFlash as a template to mutate the two E-boxes within
the c-fos promoter with the primers MCFOS 556F MUT, MCFOS 590R MUT, MCFOS 549R MUT, and MCFOS 516F MUT (Giovanna Collu, unpublished data).

pSecTagNC + ΔEGF_mN1 W1758A, pSecTagNC + ΔEGF_mN1 V1744G and pSecTagNC + ΔEGF_mN1 A2026V: these plasmids were generated by PCR-based mutagenesis using the QuikChange Lightning Multi Site-Directed mutagenesis kit (Stratagene) using pSecTagNC + ΔEGF_mN1 as a template and primers: W1758A F, V1744G F, and A2026VmutF, respectively (AHS, unpublished data).

cDNA3 + mNICD W1758A: this plasmid was generated by PCR-based mutagenesis using the QuikChange Site-Directed mutagenesis kit (Stratagene) using pcDNA3 + mNICD as a template and the primers W1758A F and W1758A R (Laura Bayston and KB, unpublished data).

cDNA3 + mNICD A2026V: this plasmid was generated by PCR-based mutagenesis using the QuikChange Lightning Multi Site-Directed mutagenesis kit (Stratagene) using pcDNA3 + mNICD as a template and the primer A2026VmutF (AHS, unpublished data).

cDNA3.1(+)+ DNHHey1, pcDNA3.1(+)+ DNmHes5, and pcDNA3.1(+)+ DNXtHey1: these plasmids were generated by mutating the conserved DNA binding domain Glu-Lys-X-X-Arg (EK**R) to Alanines. This generates a non-functional protein that when transfected can dimerise with endogenous proteins disrupting their function. These DN proteins were generated by PCR-based mutagenesis using the QuikChange Lightning Multi Site-Directed mutagenesis kit (Stratagene) using pcDNA3.1(+)+ myc-Hey1, pcDNA3.1(+)+ myc-mHes5, and pcDNA3.1(+)+ myc-XtHey1 as templates and primers: hHey1 E58AK59AR62A, mHes5 E25AK26AR29A, and XtHey1 E57AK58AR61A, respectively (AHS, unpublished data).

6.5 In vivo work

6.5.1 Injection of *Xenopus laevis* embryos

*Xenopus laevis* embryos were obtained from Dr Karel Dorey’s laboratory, dejellied and raised as previously described (Chalmers et al., 2002). The Ambion mMessage mMachine SP6 kit (Invitrogen) was used to synthesize the capped mRNAs for injection. Embryos were injected at the 4-cell stage dorsally with 500 pg of GFP mRNA as a control and 500 pg GFPXβ-catenin mRNA. Embryos were analysed 24 hr post injection at around stage 25 (Nieuwkoop and Faber, 1967).
# PRIMARY ANTIBODIES WB

<table>
<thead>
<tr>
<th>Host species</th>
<th>Immunogen</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Cat. Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>α-Tubulin</td>
<td>1:1000</td>
<td>Gift K.Gull Uni of Mcr</td>
<td>N/A</td>
</tr>
<tr>
<td>Mouse</td>
<td>β-catenin</td>
<td>1:2000</td>
<td>BD Transduction lab</td>
<td>610154</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Calnexin</td>
<td>1:2000</td>
<td>Bioquote</td>
<td>SPC-108A/B</td>
</tr>
<tr>
<td>Mouse</td>
<td>GSK3β</td>
<td>1:2500</td>
<td>Sigma</td>
<td>H1009</td>
</tr>
<tr>
<td>Mouse</td>
<td>Myc (clone 4A6)</td>
<td>1:1000</td>
<td>Upstate</td>
<td>05-724</td>
</tr>
<tr>
<td>Mouse</td>
<td>Renilla luciferase</td>
<td>1:500</td>
<td>Cell Signaling</td>
<td>2272</td>
</tr>
<tr>
<td>Rabbit</td>
<td>RFP</td>
<td>1:1000</td>
<td>MBI</td>
<td>PM005</td>
</tr>
<tr>
<td>Mouse</td>
<td>V5</td>
<td>1:1000</td>
<td>Invitrogen</td>
<td>R960-25</td>
</tr>
<tr>
<td>Mouse</td>
<td>VP16</td>
<td>1:200</td>
<td>Santa Cruz</td>
<td>sc-7545</td>
</tr>
</tbody>
</table>

# PRIMARY ANTIBODIES IF

<table>
<thead>
<tr>
<th>Host species</th>
<th>Immunogen</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Cat. Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>Myc-Tag</td>
<td>1:400</td>
<td>Cell signaling</td>
<td>#2272</td>
</tr>
<tr>
<td>Mouse</td>
<td>V5-Tag: Alexa 488</td>
<td>1:100</td>
<td>AbD SeroTec</td>
<td>MCA1360A488</td>
</tr>
</tbody>
</table>

# SECONDARY ANTIBODIES WB

<table>
<thead>
<tr>
<th>Host species</th>
<th>Immunogen</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Cat. Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donkey</td>
<td>Mouse IgG</td>
<td>1:10000</td>
<td>Jackson</td>
<td>715-035-150</td>
</tr>
<tr>
<td>Donkey</td>
<td>Rabbit IgG</td>
<td>1:10000</td>
<td>Jackson</td>
<td>715-035-152</td>
</tr>
</tbody>
</table>

# SECONDARY ANTIBODY IF

<table>
<thead>
<tr>
<th>Host species</th>
<th>Immunogen</th>
<th>Dilution</th>
<th>Supplier</th>
<th>Cat. Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat</td>
<td>Rabbit Alexa 594</td>
<td>1:400</td>
<td>Molecular Probes</td>
<td>A11037</td>
</tr>
</tbody>
</table>

Table 6.1: Antibodies used for western blot and immunofluorescence.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mβ-cat S45FF</td>
<td>ACCACCAACAGCTCCTTCTGTGACGTCGGCAAGGCG</td>
</tr>
<tr>
<td>mβ-cat S45FR</td>
<td>GCCGTTGCAACTCAAGGAGCTGTGAGTT</td>
</tr>
<tr>
<td>W1758A F</td>
<td>CAGCATGGCCAGCTCGCTGGTTGGCTAGGAGGGTTGG</td>
</tr>
<tr>
<td>W1758A R</td>
<td>GAAACCCTCAGGGAACGCAGCTGGCCATGCTG</td>
</tr>
<tr>
<td>V1744G F</td>
<td>CTTTGTGGGCTGTGGGGCTGTGGTCCCAGGAAG</td>
</tr>
<tr>
<td>A2026VmutF</td>
<td>TTTGCATTGGGCGGACGGTTTTGGGAAGAATGAGGATG</td>
</tr>
<tr>
<td>hHey1E58AK59AR62A</td>
<td>GGAGAGGAATATTGCGCGCGCGCGAGCAAGCGGATCAATAAC</td>
</tr>
<tr>
<td>mHes5E25AK26AR29A</td>
<td>GGAAGCCGTTGTTGCGCGCGCGAGATCGTGGCGCACCGATCAACAGC</td>
</tr>
<tr>
<td>XtHey1 E57AK58AR61A</td>
<td>CGCAGAGGGATTATTTGGCGCGCGCGCGACAGAGGATTAATAC</td>
</tr>
</tbody>
</table>

Table 6.2: Mutagenesis primers.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mN1 4409F</td>
<td>GGTAAAGCTTCAGATTGAGGAGGCATGTGAG</td>
</tr>
<tr>
<td>mN1 4901R</td>
<td>GCTTGAAAGACCACCTTGTGGT</td>
</tr>
<tr>
<td>mN1 5042F</td>
<td>TAGTAAGCTTGAGCTTGACCCTATGGACAT</td>
</tr>
<tr>
<td>mN1 5589R</td>
<td>TGCTGCTGAGTCCACTGTCT</td>
</tr>
<tr>
<td>mN1 5210F</td>
<td>TAGTAAGCTTGAGAAGGATGAGCCGGTGG</td>
</tr>
<tr>
<td>mHes5 73F</td>
<td>TAGCGAATTCTGGCATGGCACCTAGTACCCTGG</td>
</tr>
<tr>
<td>mHes5 663R</td>
<td>TCGTGGATCCTGAACCTGCTGGGGGAATGGTC</td>
</tr>
<tr>
<td>hHey1 98 F</td>
<td>TAGCGAATTCTATGAAGCGTCTATGAAACCTAGG</td>
</tr>
<tr>
<td>hHey1 1077 R</td>
<td>TAGCGGATCTTATTACAGTACCAGCAGCCCA</td>
</tr>
<tr>
<td>mDvl2 1834F</td>
<td>CTTCTTTCGTAACCTATGGTGGAG</td>
</tr>
<tr>
<td>mDvl2 2331 R</td>
<td>GGTATCTAGACATAACATCTACATACAAAAACTCACTG</td>
</tr>
<tr>
<td>mβ-cat 1914F</td>
<td>GAGATAGTAGAGGAGGGGTACTG</td>
</tr>
<tr>
<td>mβ-cat 2555R</td>
<td>CCTACTCGAGGTCATGATCAAACCAGG</td>
</tr>
<tr>
<td>XtHey1 F</td>
<td>TGACGAATTCTATGAAGCGGACGACATTAC</td>
</tr>
<tr>
<td>XtHey1 R</td>
<td>TGATGGATCCCTAAAGGCTCCGATTTCGTC</td>
</tr>
</tbody>
</table>

**Table 6.3: PCR and sequencing primers**
## Table 6.4: Quantitative PCR primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hPygopus1-a F</td>
<td>GTTTCCTCGCATGGTGGTGA</td>
</tr>
<tr>
<td>hPygopus1-a R</td>
<td>TGGATTCGGTGGTGAGCAT</td>
</tr>
<tr>
<td>hPygopus2-a F</td>
<td>GCAAGGCCCCTCTGCAAATG</td>
</tr>
<tr>
<td>hPygopus2-a R</td>
<td>GGTTGGTGCAAACCTCGTCA</td>
</tr>
<tr>
<td>hBcl9-a F</td>
<td>TGTGGCCAGCTCAGATGACG</td>
</tr>
<tr>
<td>hBcl9-a R</td>
<td>AACCACGGGTTTGGACCTG</td>
</tr>
<tr>
<td>hHey1 1-449 F</td>
<td>AAAGCGTGAGCGGATCAG</td>
</tr>
<tr>
<td>hHey1 1-449 R</td>
<td>CTCGTCGCGCTTTCAAT</td>
</tr>
<tr>
<td>hHey1-real5'</td>
<td>GGCAGAGGGAAAGTTACT</td>
</tr>
<tr>
<td>hHey1-real3'</td>
<td>GCTGGGAAGGGTGTGGTT</td>
</tr>
<tr>
<td>hHey2-real5'</td>
<td>AAGATGCTTCAGGCAACAG</td>
</tr>
<tr>
<td>hHes1-real5'</td>
<td>TCTGAGCCAGCTGAAAACAC</td>
</tr>
<tr>
<td>hHes1-real3'</td>
<td>CTCGGTACTTCCCAGCAC</td>
</tr>
<tr>
<td>hHes5-real5'-new</td>
<td>CCCAAAGAGAAAAACCGACTG</td>
</tr>
<tr>
<td>hHes5-real3'-new</td>
<td>GCTTGGAGGGGTGGTGT</td>
</tr>
<tr>
<td>hHeyL-real5'</td>
<td>AGACCGCATCAACAGTACC</td>
</tr>
<tr>
<td>hHeyL-real3'</td>
<td>AAAGAATCTGTCCCACA</td>
</tr>
<tr>
<td>hPPIA-real5'</td>
<td>ATGCTGGACCAACACAA</td>
</tr>
<tr>
<td>hPPIA-real3'</td>
<td>TTTCACCTTGGCCAACACCA</td>
</tr>
</tbody>
</table>
References

