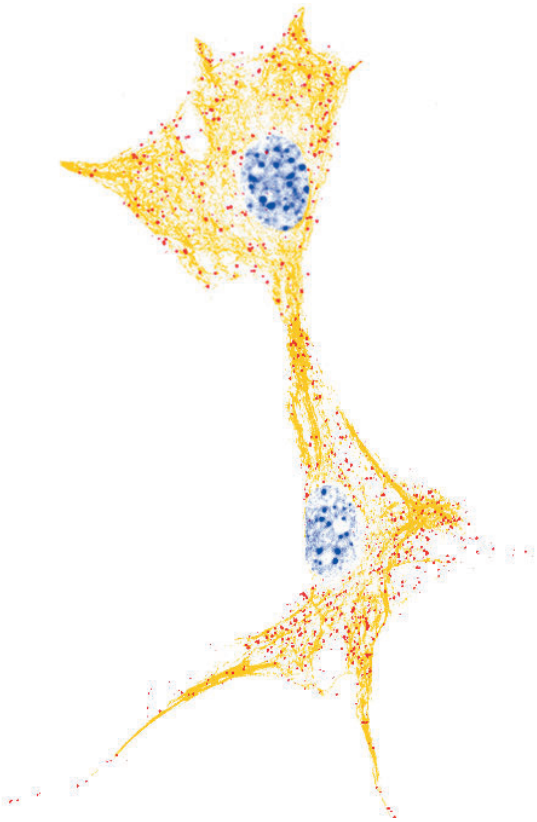
Åbo Akademi University

Daniel Antfolk

Regulation of Notch Signaling by Intracellular Trafficking



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Cover Image: "The vimentin man"

Vimentin shown in yellow in 3T3 mouse fibroblasts, with internalized Notch-peptides in red and nuclei in blue.

Profile picture by Solveig Eriksson

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To my family
and friends

TABLE OF CONTENTS

ABSTRACT iv
SAMMANFATTNING (ABSTRACT IN SWEDISH) v
LIST OF ORIGINAL PUBLICATIONS vi
AUTHOR CONTRIBUTION viii
ABBREVIATIONS ix
INTRODUCTION 1
REVIEW OF THE LITERATURE 2
1. The Notch signaling pathway 2
1.1 A brief history of Notch research 2
1.2 Notch receptors 4
1.3 Notch ligands 7
1.4 Activation of canonical Notch signaling 8
1.4.1 Non-canonical Notch 11
1.4.2 Cis-inhibition 12
1.5 Notch signaling during development 12
1.6 Notch signaling in disease 14
1.7 Notch signaling in the vasculature 15
1.7.1 Angiogenesis and Notch 16
2. Regulation of the Notch signaling pathway 19
2.1 The role of post-translational modifications in Notch signaling 19
2.1.1 The role of glycosylation in Notch signaling 21
2.1.2 The role of ubiquitination in Notch signaling 23
2.1.3 The role of phosphorylation in Notch signaling 25
2.2 Endocytosis 27
2.2.1 Endocytosis of Notch receptors 30
2.2.2 Endocytosis of Notch ligands 31
2.3 Intermediate filaments as regulators of cellular function 33
OUTLINE AND KEY AIMS OF THESIS 36
EXPERIMENTAL PROCEDURES 37

RESULTS AND DISCUSSION 39
1. Regulation of the Notch ligand Jagged1 by vimentin (I) 39
1.1 Vimentin regulates angiogenesis and correlates with Jagged1 39
1.2 Vimentin interacts with Jagged1 39
1.3 Vimentin affects Jagged1 endocytosis 42
1.4 Loss of vimentin disrupts angiogenesis but can be rescued by external Jagged1 44
2. Screening for regulators of Jagged1 endocytosis (II) 48
2.1 Dual labeled N1ECD peptides can be used to track and quantify endocytosed Jagged1 48
2.2 A cell spot microarray to identify new regulators of Jagged1 49
3. Phosphorylation by PKCζ regulates Notch trafficking (III) 52
3.1 PKCζ interacts with and phosphorylates Notch1 52
3.1.1 S1791 is identified as a PKCζ phosphorylation site on Notch1 52
3.2 PKCζ increases Notch activation 53
3.2.1 Site directed mutagenesis of S1791 54
3.3 PKCζ affects trafficking of Notch1 55
3.3.1 Internalization of activated Notch receptors 55
3.3.2 Recycling of inactive Notch receptors 56
3.4 S1791 regulates myogenic differentiation *in vitro* and PKCζ regulates neuronal differentiation *in vivo* 56
CONCLUDING REMARKS 59
ACKNOWLEDGEMENTS 62
REFERENCES 65
ORIGINAL PUBLICATIONS 93

ABSTRACT

The Notch signaling pathway is one of the essential mediators of cell-cell communication during development of multicellular organisms. Notch signaling is based on receptors and ligands that interact between neighboring cells. In recent years Notch has received a lot of interest as a promising therapeutic target in cancer and several approaches to intervene with the Notch pathway are under pre-clinical and clinical trials. The challenges are prominent side effects, lack of specificity and poor knowledge of the underlying mechanisms behind deregulated Notch activity. In this thesis, several aspects of Notch regulation have been investigated.

Intermediate filaments (IFs) are cytoskeletal proteins that regulate signaling activities in addition to providing structural support for the cell. In study I, we present a novel interaction between the intermediate filament vimentin and the Notch ligand Jagged1. The use of hybrid Jagged1-Dll4 ligands demonstrates a selective regulation of Notch ligands by vimentin. Mouse embryos lacking vimentin display delayed angiogenesis with reduced branching. *In vitro* and *ex vivo* angiogenesis assays show a reduced sprouting from vimentin deficient endothelial cells, a phenotype which can be rescued by addition of immobilized Jagged1 ligands. This work implies that IFs can selectively regulate Notch ligands to balance Notch activity. In the second study, we have initiated a screening approach to identify regulators of Jagged1. A dual label approach allows for visualization and measurement of endocytosed Notch extracellular domain peptides bound to Notch ligands. A pilot screen based on cell spot microarrays (CSMA) has generated a set of potential modulators of Jagged1 endocytosis for further validation and future research. In the final part of the thesis, I present the discovery of a novel PKCζ-mediated phosphorylation site on Notch1. Phosphorylation of the identified site, S1791, leads to enhanced trafficking of Notch to the nucleus and higher Notch signaling activity. Blocking PKCζ or using a phospho-deficient form of S1791 leads to less Notch activity and localization to intracellular endosomes. Our data also imply that PKCζ-mediated phosphorylation of Notch influences differentiation of myogenic cells *in vitro* and neuronal cells *in vivo*.

In summary, the work presented in this thesis characterizes various aspects of Notch signaling modifications within the context of endocytosis of Notch receptors and ligands. These findings contribute to a better understanding of the intricacies of Notch signaling regulation and may benefit future studies targeting Notch-related diseases. Additionally, the ligand tracking approach lays the groundwork for future work to identify new modulators of ligand endocytosis.

SAMMANFATTNING (ABSTRACT IN SWEDISH)

Notch-signaleringen är en av de huvudsakliga förmedlarna av kommunicering mellan närliggande celler under utvecklingen av flercelliga organismer. Notch-signaleringen baserar sig på Notch-receptorer och ligander vid cellmembranen. De senaste åren har Notch-signaleringen fått stor uppmärksamhet som ett potentiellt mål för nya behandlingsmetoder mot cancer och flera olika Notch inhiberare är i pre-kliniska och kliniska prövningar. De största utmaningarna är starka biverkningar, en brist på specificitet och en låg kunskap om de underliggande mekanismerna bakom en felreglerad Notch-aktivitet. I denna avhandling undersöks flera olika aspekter av Notch-regleringen.

Intermediärfilament är strukturella proteiner som även kan reglera olika signaleringsräckor. I den första studien presenterar vi för första gången en interaktion mellan intermediärfilamentet vimentin och Notch-liganden Jagged1. Genom att använda oss av hybrid-ligander bestående av liganderna Jagged1 och Dll4, visar vi att vimentin kan reglera dessa ligander på ett selektivt sätt. Vi visar också att möss som saknar vimentin har en fördröjd blodkärlsbildning under fosterutvecklingen. Endotelceller som saknar vimentin påvisade även färre förgreningar i våra blodkärlsbildningsanalyser. Detta kunde motverkas genom en tillsats av externa Jagged1-ligander. Detta arbete visar att intermediärfilament specifikt kan reglera olika Notch-ligander. I den andra studien har vi använt en högkapacitetsscreen för att identifiera nya Jagged1-reglerare. Med hjälp av en tvåfärgad analysmetod kunde vi visualisera och mäta Notch-peptider som binder till Notch-ligander som sedan endocyteras in i cellen. En primärscreen har genererat en uppsättning av potentiella reglerare av Jagged1-endocytos för framtida verifiering och forskning. I den sista delen av avhandlingen presenteras identifieringen av ett nytt PKCζ-medierat fosforyleringsställe på Notch1-receptorn. Genom att mutera det identifierade fosforyleringsstället kunde vi visa att receptorn tar olika rutter i cellen beroende på om den är aktiv eller inaktiv. Våra resultat antyder också att PKCζ-medierad fosforylering av Notch påverkar differentieringen av muskelceller *in vitro* och neuronala celler *in vivo*.

Sammanfattningsvis kan man säga att arbetet som presenteras i denna avhandling berör olika aspekter av Notch-regleringen med ett speciellt fokus på endocytos av Notch-receptorer och ligander. Dessa fynd bidrar till en bättre förståelse om de komplicerade modifieringar som berör Notch. Dessa kan komma att gynna framtida studier som berör Notch-relaterade sjukdomar. Analysmetoden som användes för att spåra Notch-ligander lägger grunden till att identifiera nya reglerare av ligand-endocytos i framtiden.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and a manuscript, which are referred to in the text by Roman numerals (I-III). In addition, unpublished results are included. The original publications have been reproduced with permission of the copyright holders.

I. **Antfolk D***., Sjöqvist M*., Cheng F., Isoniemi K., Duran CL., Rivero-Muller A., Antila C., Niemi R., Landor S., Bouten CVC., Bayless KJ., Eriksson JE., Sahlgren C. (2017) Selective regulation of Notch ligands during angiogenesis is mediated by vimentin. *Proceedings of the National Academy of Sciences U S A*, *114: 4574-4581*

II. **Antfolk D**., Arjonen A., Parikainen M., Ivaska J., Sahlgren C. Moves Like Jagged – High throughput cell arrays to identify novel regulators of Jagged trafficking. *Manuscript*

III. Sjöqvist, M*., **Antfolk D***., Ferraris S., Rraklli V., Haga C., Antila C., Mutvei A., Imanishi S., Holmberg J., Jin S., Eriksson JE., Lendahl U., Sahlgren C. (2014) PKCζ regulates Notch receptor routing and activity in a Notch signaling-dependent manner. *Cell Research, 24: 433-450*

*Equal contribution

PUBLICATIONS NOT INCLUDED IN THESIS

Antfolk D., Antila C., Kemppainen K., Landor S[#]., Sahlgren C[#]. (2019) Decoding the PTM-switchboard of Notch. *Biochimica et Biophysica Acta – Molecular Cell Research*

Van Engeland N*., Suarez-Rodriguez F*., Rivero-Muller A., Ristori T., Duran C., Stassen O., **Antfolk D**., Driessen R., Ruohonen S., Ruohonen S., Nuutinen S., Savontaus E., Loerakker S., Bayless K., Sjöqvist M., Bouten C., Eriksson J., Sahlgren C. (2019) Vimentin regulates Notch signaling strength and arterial remodeling in response to hemodynamic stress. *Scientific Reports*

Wilhelmsson U., Lebkuechner I., Leke R., Marasek P., Yang X., **Antfolk D**., Chen M., Mohseni M., Lasic E., Trkov S., Stenovec M., Zorec R., Nagy A., Sahlgren C., Pekna M., Pekny M. (2019) Nestin regulates neurogenesis through Notch signaling from astrocytes to neural stem cells. *Cerebral Cortex*

Rosenholm J., Gulin-Sarfraz T., Mamaeva V., Niemi R., Özliseli E., Desai D., **Antfolk D**., von Haartman E., Lindberg D., Prabhakar N., Näreoja T., Sahlgren C. (2016) Prolonged Dye Release from Mesoporous Silica-Based Imaging Probes Facilitates Long-Term Optical Tracking of Cell Populations In Vivo. *Small*

*Equal contribution
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AUTHOR CONTRIBUTION

I. The author contributed to designing research, performing research, analyzing data, and to writing the manuscript. Specifically, the author used the following experimental procedures: PLA with and without hybrid ligands, reporter activity assays, WB, FACS measurements of N1ECD endocytosis from agarose beads, ICC, *in vitro* angiogenesis assays, meta-analysis of GeneSapiens data, and IP. In addition, the author designed the addition of Jagged1-FC-beads into the *ex vivo* angiogenesis assay and designed the parameters for and visualized the CAM-experiment. From the results described in the thesis: F.C performed mouse work, embryo stainings and an initial *in vitro* angiogenesis assay. K.I made the original table from GeneSapiens, performed live-cell imaging of $N1ECD^{F}$, fingerprint assays and ICC. R.N performed the CAM-experiments. A.R.M designed the hybrid ligands. M.S performed surface biotinylation assays, WB and ICC. C.L.D performed *ex vivo* aortic ring assays and extracted endothelial cells from VimKO and VimWT mice. C.A performed qPCR of Lfng. M.S originally wrote the first draft of the manuscript. The author and C.S rewrote the manuscript for publication, with helpful comments from J.E.E. The author and C.S managed the revision, proofs and correspondence for publication.

II. The author contributed to designing research, performing research, analyzing data, and to writing the manuscript. The author, A.A, J.I and C.S conceived the project. The author designed the CSMA experiment together with A.A. The CSMA technology and arrays were previously made by J.R (acknowledgements). The author designed all other experiments, performed optimization and proof of concept experiments and employed the endocytosis assay in the screen. Array plates were measured by A.A. From the results described in the thesis: M.L assisted with particle analysis (acknowledgements). M.P performed the biotinylation assay, the metascape GO analysis and WB of ligand levels. The author analyzed the data, made the figures, and wrote the manuscript.

III. The author contributed to designing research, performing research, analyzing data, and to writing the manuscript. Specifically, the author used the following experimental procedures: WB, reporter activity assays, nuclear extractions, IP, co-localization analysis and ICC. S.J made the S1791 mutant constructs. The author together with M.S performed the experiments with S1791 Notch mutants, except the differentiation assay, which was performed by C.S. S.F performed most of the data involving dnPKC and pseudosubstrate, including ICC and the *in vitro* differentiation assay of primary myoblasts. S.I performed the mass spectrometry analysis and *in vitro* phosphorylation. The author and M.S performed all experiments during revision, except for the *in vivo* differentiation experiments. *In vivo* experiments were performed by V.R and J.H, including the CAG-Notch1ΔE-myc-IRES-EGFP constructs. The author and M.S contributed to writing the paper. C.S and U.L were the main contributors to writing the paper.

ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase
ANK	Ankyrin domain
aPKC	Atypical protein kinase C
caPKCζ	Constitutively active form of PKCζ
ARP2/3	Actin-related proteins 2/3
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CAM	Chorioallantoic membrane
CNS	Central nervous system
CSL	CBF1, Suppressor of Hairless, Lag-1
CSMA	Cell spot microarray
DAG	Diacylglycerol
DAPI	4', 6-diamidino-2-phenylindole
Dll	Delta-like ligand
dnPKCζ	Dominant negative form of PKCζ
DOS	Delta and OSM-11-like proteins
DSL	Delta/Serrate/LAG-2
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EMT	Epithelial-mesenchymal transition
EOGT	EGF Domain Specific O-Linked N-Acetylglucosamine Transferase
ER	Endoplasmic Reticulum
FACS	Fluorescence-activated cell sorting
FBXW7	F-box and WD repeat domain containing 7
FLN	Full length Notch
FNG	Fringe
GMC	Ganglion mother cell
GSI	Gamma secretase inhibitor
HD	Heterodimerization domain
HEK-293	Human embryonic kidney cell line
HES	Hairy and enhancer of split
HEY	Hairy/enhancer-of-split related YRPW motif protein
HIF	Hypoxia inducible factor
HUVEC	Human umbilical vein endothelial cells
ICC	Immunocytochemistry
IF	Intermediate filament

IP	Immunoprecipitation
KO	Knock out
LNR	Lin-12-Notch repeats
MAML	Mastermind-like
MIB	Mindbomb
MEF	Mouse embryonic fibroblast
MHC	Myosin heavy chain
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MVB	Multivesicular bodies
NECD	Notch extracellular domain
NEUR	Neuralized
NEXT	Notch extracellular truncation
NICD	Notch intracellular domain
NLS	Nuclear localisation signal
NMR	Nuclear magnetic resonance
NRR	Negative regulatory region
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDZ	PSD-95/Dlg/ZO-1
PEST	Proline-glutamic acid-serine-threonine rich domain
PLA	Proximity ligation assay
POGLUT	Protein O-Glucosyltransferase
POFUT	Protein O-Fucosyltransferase
PTM	Post translational modification
RAB	Small GTP-binding protein belonging to the Ras family
RAM	RBPJk-associated module
siRNA	Small interfering RNA
TAD	Transcriptional activation domain
T-ALL	T-cell acute lymphoblastic leukemia
ULF	Unit length filament
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Vim	Vimentin
VSMC	Vascular smooth muscle cell
WB	Western blotting
WT	Wild type

INTRODUCTION

Based on current understanding, a surge in the amount of multicellular organisms occurred around 600 million years ago during an increase in oxygen on earth (Grosberg and Strathmann, 2007). In order for multicellularity to work, individual cells had to be able to communicate with each other and coordinate their behavior. One of the signaling pathways that has played an essential role during metazoan development is Notch. The Notch pathway is based on receptors and ligands spanning across the cell membrane that can interact with each other during direct cell-to-cell contact between adjacent cells. The core pathway is deceptively simple, where ligand-mediated activation of the receptor leads to its proteolysis and subsequent release of a transcriptionally active Notch intracellular domain (NICD) that can translocate to the nucleus and activate downstream Notch target genes (Kopan and Ilagan, 2009). Despite the apparent simplicity, without secondary messengers and amplification steps, Notch is highly context dependent with multiple cellular phenotypes from seemingly similar Notch input. Accumulating evidence supports a view that Notch signaling is a highly regulated process with a myriad of interacting partners and modifications regulating activity and outcome. From the beginning of 2013, Notch related therapies have been in clinical trials for a number of different cancers in humans (Andersson and Lendahl, 2014). One of the challenges of therapeutic relevance of Notch today lies in how to specifically tune Notch signaling in the right context without adversly affecting normal tissue homeostasis, where Notch signaling is also crucial. Notch also represents a possible therapeutic target in cardiovascular diseases, although knowledge of the specific roles of dysregulated Notch signaling in the pathogenesis of vascular diseases is still lagging behind that of cancer research (Aquila et al., 2019).

Post-translational modifications and intracellular trafficking of Notch and its interacting partners critically regulate Notch signaling output. This thesis aims to advance the knowledge of Notch regulation, and proposes a new interaction between the cytoskeletal intermediate filament protein vimentin and the Notch ligand Jagged1. Post-translational modifications are also studied in the form of phosphorylation of the Notch receptor and its effect on receptor routing and Notch activity. Finally, a fluorescence based screening approach has been developed to identify more potential regulators of Notch ligand endocytosis that can serve as a basis for future research.

REVIEW OF THE LITERATURE

1. The Notch signaling pathway

Notch signaling is part of a select group of signaling pathways defining development of multicellular organisms. During development Notch orchestrates cell fate decisions by influencing differentiation, proliferation and apoptosis through physical interactions between Notch receptors and ligands on the membranes of neighboring cells. The Notch field emerged from genetic studies on development of the fruit fly *Drosophila melanogaster*. The importance of the core Notch pathway is implicated by conservation throughout evolution in all metazoans studied to date. More recently, human genetic studies have shown that Notch plays a role in numerous and diverse human diseases.

The Notch receptor is generated as a single precursor protein, but is cleaved by furin-like convertase in the Golgi during transport to the cell surface (S1 cleavage). At the cell surface, the receptor is presented as a noncovalently bound heterodimer that spans the cell membrane. In mammals, there are four Notch receptors (Notch1-4) and five Notch ligands (Jagged1-2, Dll1, 3-4). Notch signaling is initiated when a ligand from a juxtaposed cell binds the receptor and a sufficient force is generated, which pulls on the receptor and induces a conformational change, to allow for two proteolytic cleavages (S2 and S3 cleavage) that ultimately release the intracellular domain of the receptor. The intracellular part of Notch can then translocate to the nucleus where it forms a complex with CSL (CBF-1, Su(H), and Lag-1), Mastermind-like (MAML) and transcriptional co-activators to activate Notch target genes from the Hairy Enhancer of Split (HES) and Hes-related protein (HERP, also known as HEY) families (Kopan and Ilagan, 2009) (for further details see chapter 1.4). The complexity of Notch lies in its numerous levels of regulation that allow for varied and context-dependent outcomes of its activation. The implications of Notch in human disease, especially in cancer, has led to a promise of possible Notch therapies in the future, resulting in a wealth of studies relating to Notch during the last few decades.

1.1 A brief history of Notch research

The term Notch was first used over 100 years ago when John S. Dexter noticed that some fruit flies (*Drosophila melanogaster*) had an inheritable deformity, where the flies had

small notches in the tips of their wings (Dexter, 1914). This finding piqued the interest of geneticist and embryologist Thomas Hunt Morgan who subsequently identified the first Notch gene allele a few years later (Morgan, 1917). In the following years Morgan and members of his lab identified several more Notch alleles with various phenotypes, including the Notched wing phenotype (Figure 1), but also lethal phenotypes (Mohr, 1919). In the 1930s, Donald Poulson looked at broader phenotypic effects of removing entire chromosomes in fruit fly embryos. In his work, Poulson identified chromosomal defects, where parts of the chromosomes were mutated. One of these included the Notch gene locus. These mutations led to a disruption in development of the mesoderm and endoderm germ layers a few hours into embryogenesis. Poulson describing these Notch mutants were some of the first characterizations of how any specific gene, not only Notch, affects morphogenesis (Poulson, 1937). Poulson's discoveries were for many decades left without much follow up research. In the early 1980s, Spyros Artavanis-Tsakonas and Michael Young independently sequenced the Notch gene in *Drosophila* (Artavanis-Tsakonas et al., 1983; Kidd et al., 1983). By comparing the sequences from Notch cDNA to other proteins, Artavanis-Tsakonas speculated that Notch was a transmembrane protein and later work from his lab detailed that epidermal growth factor like repeats (EGF-repeats) extend outside of the cell (Wharton et al., 1985). This was at a time when the modern molecular biology revolution had just started with the discovery of the polymerase chain reaction (PCR) and in the years following, many of the key actors in the Notch signaling pathway were characterized. The idea of Notch being a cell-to-cell communication signal was sparked in 1987 when the Delta ligand was sequenced and discovered to be a transmembrane protein similar to the Notch receptor (Vässin et al., 1987). Serrate in *Drosophila* (corresponding to Jagged in mammals) was sequenced a few years later (Fleming et al., 1990). In the late 1980s, it was also shown that Notch regulates differentiation in both *C. elegans* and *Drosophila* (Kidd et al., 1989; Yochem et al., 1988). The first description of Notch in humans came in 1991 when Leif Ellisen sequenced a gene with a mutation of high occurrence in leukemia cells. This human sequence was remarkably similar to the *Notch* gene discovered in *Drosophila* and he further showed that truncated Notch1 proteins could contribute to cancer *in vitro* (Ellisen et al., 1991). Notch research gained great interest after this point and one of the most notable examples of Notch in human disease came when it was discovered that more than 50% of T-cell lymphoblastic leukemia (T-ALL) patient samples had Notch-activating mutations (Weng

et al., 2004). The implications of Notch in other cancer forms as well as various other diseases have been substantial since then (Aster et al., 2017; Louvi and Artavanis-Tsakonas, 2012). In the last few decades researchers have tried to understand how Notch can be so context dependent, how Notch is regulated, what the crosstalk is with other signaling pathways and if Notch can be targeted in disease. Combining research from *Drosophila*, *C. elegans* and mice together with clinical data from humans have established a detailed understanding of many aspects of Notch signaling. However, equally many questions remain in truly understanding the context dependent output of Notch and the finetuning of signaling activity within the pathway, as well as its interactions with other proteins.

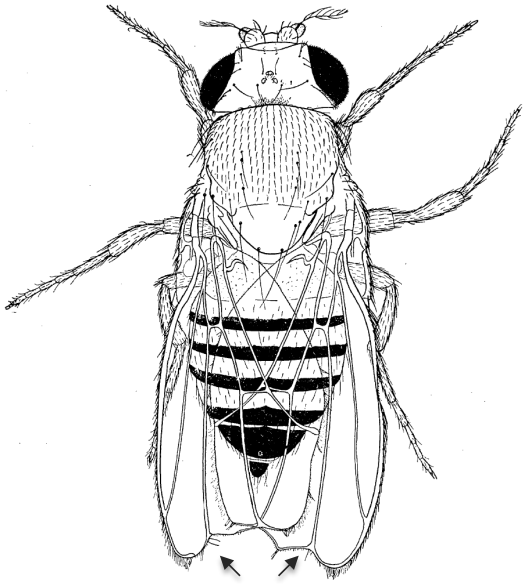

FIG. 1. Notch female.

Figure 1. The Notch mutant. Thomas Hunt Morgan describes a female fruit fly carrying a "notch" mutation in one of her X-chromosomes. The notched wing phenotype was a dominant trait and could only been seen in females, as half of her male offspring would get the mutant X-chromosome and die, while the other half would get the normal X-chromosome and develop normally, as described in "The theory of the gene", American Naturalist in 1917 (Adapted from Morgan, 1917).

1.2 Notch receptors

Notch receptors are large heterodimeric proteins spanning across the cell membrane. The general structure has been conserved throughout evolution, from the simplest invertebrates to humans. Much of the initial work on Notch was done in *Drosophila Melanogaster*, which has only one Notch receptor, in contrast to mammals that have four Notch receptors (Notch1-4) (Figure 2). Notch receptors are synthesized in the ER and processed further in the Golgi apparatus. During this processing step, the Notch precursor protein is

proteolytically cleaved by furin-like convertase (Site 1 or S1 cleavage), producing a heterodimer, which is further modified by glycosylation and other post-translational modifications before the receptor is presented at the cell membrane. The receptor at this point consists of an extracellular domain (ECD) and an intracellular domain (ICD) (Blaumueller et al., 1997; Gordon et al., 2008). The modification of the extracellular domain by *O*-linked glycans during synthesis is a key modification for the proper structure of the receptor and its interaction with its ligands. The EGF-repeats consist of approximately 40 aminoacids, including 6 conserved cysteines, which form three disulphid bonds between cysteines C1-C3, C2-C4 and C5-C6. The major glycosylation sites of the receptor are found on consensus sites between these cysteines (Harvey and Haltiwanger, 2018). Glycosylation, other post-translational modifications and their regulation of Notch are discussed further in chapter 2.1. The extracellular domain of Notch consists of 29-36 epidermal growth factor repeats (EGF), of which some interact directly with the Notch ligands, most notably EGF8-12 (Luca et al., 2017, 2015). Many of the individual EGF-domains bind calcium ions, which have been known to affect the structure of the receptor and affinity to its ligands. Recent structural studies surprisingly found a 90-degree angle occuring between EGF-repeat 5 and 6 in the Notch1 receptor structure; with EGF6 being a non calcium-binding repeat (Weisshuhn et al., 2015a). Nuclear magnetic resonance (NMR) spectroscopy analysis of other EGF-repeats confirmed that calcium binding repeats form rigid structures, while non-calcium binding may have different tilt angles, giving the overall NECD structure flexibility (Weisshuhn et al., 2015b). The model proposed for the structure based on X-ray crystallography of EGF4-13 and NMR data, consist of an L shaped 90 degree angle between EGF5-6 and a flexible region between EGF9-10, with more minor tilt angles possible between other EGF-repeats such as EGF11-12 and EGF12-13 (Weisshuhn et al., 2016). This gives updated insight into the shape of Notch receptors, which earlier have generally been depicted as a straight rods sticking out of the cell membrane. The EGF-repeats are followed by a negative regulatory region (NRR), which is composed of cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain (HD) (Gordon et al., 2007). The NRR plays a key role in preventing receptor activation when a ligand is not bound by hiding the S2 cleavage site deeply within the Notch HD domain, making it unavailable to ADAM/TACE-mediated cleavage (Gordon et al., 2007). Mutations in this region can

leave the receptors constitutively active without proper ligand activation, which can be seen in certain cancers (Malecki et al., 2006; Weng et al., 2004).

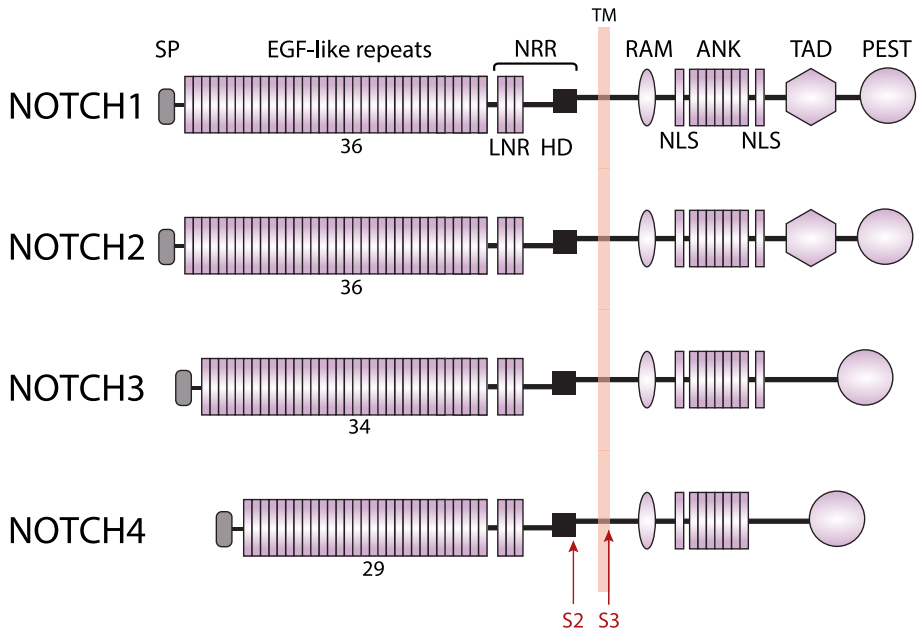

Figure 2. Domain organization of human Notch receptors. Mammalian Notch receptors include Notch1-4. The extracellular domain of the Notch receptor has a short signaling peptide, 29-36 epidermal growth factor like repeats (EGF), and a negative regulatory region (NRR). The NRR is composed of Lin-12-Notch repeats (LNR) and a heterodimerization domain (HD). The transmembrane domain (TM) leads to the Notch intracellular domain (NICD), which is composed of a RAM-domain (RBPjk associated module), two nuclear localisation signals (NLS) that flank seven ankyrin repeats (ANK). The C-terminal domain of Notch contains a transactivation domain (TAD) and a proline, glutamic acid, serine and threonine-rich domain (PEST). Notch3 and Notch4 lack the TAD domain and Notch4 lacks the second NLS. The S2 site in the HD domain and S3 site at the edge of the transmembrane domain (TM) indicate the proteolytical cleavage sites, mediated by ADAM-metalloproteases and γ-secretase, respectively.

The Notch intracellular domain (NICD) is composed of a RAM domain (RBPjk associated module) which can bind to the DNA binding protein CSL (CBF-1/RBPjk/Su(H)/Lag-1)(CSL, also commonly called RBPjk). Following the RAM domain is a nuclear localisation sequence (NLS) and seven ankyrin sequences, which are collectively called the ANK domain. CSL also interacts with the ANK domain. All mammalian Notch receptors, except Notch4, have another NLS following the ANK domain. A transactivation domain leads to the last part of the C-terminal end of the receptor, which is

the PEST domain. This domain is a conserved domain consisting of proline-glutamic acid-serine-threonine sequences which function as a degradation signal for NICD (Andersson et al., 2011). The PEST domain thereby affects the stability of NICD that has been released by receptor activation.

1.3 Notch ligands

The Notch ligands have structural similarities to Notch receptors. Notch ligands are also transmembrane proteins, which consist of EGF-like repeats in their ECD (D'Souza et al., 2008). The amount of EGF-repeats varies between 6-16 among the different ligands (D'Souza et al., 2008; Kopan and Ilagan, 2009) (Figure 3). There are two families of Notch ligands: Delta/Delta-like and Serrate/Jagged. Delta and Serrate are found in *Drosophila*, which has one of each ligand type. In mammals, the corresponding ligands are Delta-like (Dll) and Jagged, which include three Delta-like ligands (Dll1, Dll3 and Dll4) and two Jagged ligands (Jagged1-2) (D'Souza et al., 2008). Dll3 has been described as a decoy ligand, incapable of activating Notch receptors in *trans* (Ladi et al., 2005). In line with this, Dll3 knockout mice have higher Notch activity than WT mice (Chapman et al., 2011).

Canonical Notch ligands are recognized by their DSL domain (Delta, Serrate and Lag-2), which is a small domain similar to the EGF-repeats in structure. The interaction of the DSL domain with the Notch receptor EGF-repeats has been demonstrated to be required for the activation of Notch signaling (Becam et al., 2010; Luca et al., 2015; Takeuchi et al., 2018). Recently, the use of an engineered high affinity Dll4-Notch1 complex solved the longtime issue of producing a crystal structure of the normally low affinity ligand-receptor complex (Luca et al., 2015). Using immobilized EGF1-14 of the Notch receptor bound to magnetic beads with the EGF5 to the N-terminal region of Dll4, it was shown that the majority of the binding strength comes from the DSL domain of Dll4 binding to EGF11 of Notch1, and the C2 (MNNL) domain binding to EGF12 of Notch1 (Luca et al., 2015). Jagged1 was later shown to interact with EGF8-12 of Notch1 through its C2, DSL and EGF1-3 domains (Luca et al., 2017). Similarly to the Notch1 receptor, the crystal structure of Dll1 recently showed that the ligand makes a 90 degree bend around EGF4 and EGF5 (Kershaw et al., 2015).

Jagged ligands also have a cysteine-rich region in their ECD next to the transmembrane domain (Chillakuri et al., 2012). Furthermore, Jagged1, Dll1 and Dll4 have PDZ binding

motifs (PSD-95/Dlg/ZO-1) in their ICD, which can interact with the cytoskeleton, while Jagged1, Dll1 and Dll3 also have SH2 binding motifs that can interact with proteins containing SH2 domains in a phosphorylation dependent manner (Pintar et al., 2007).

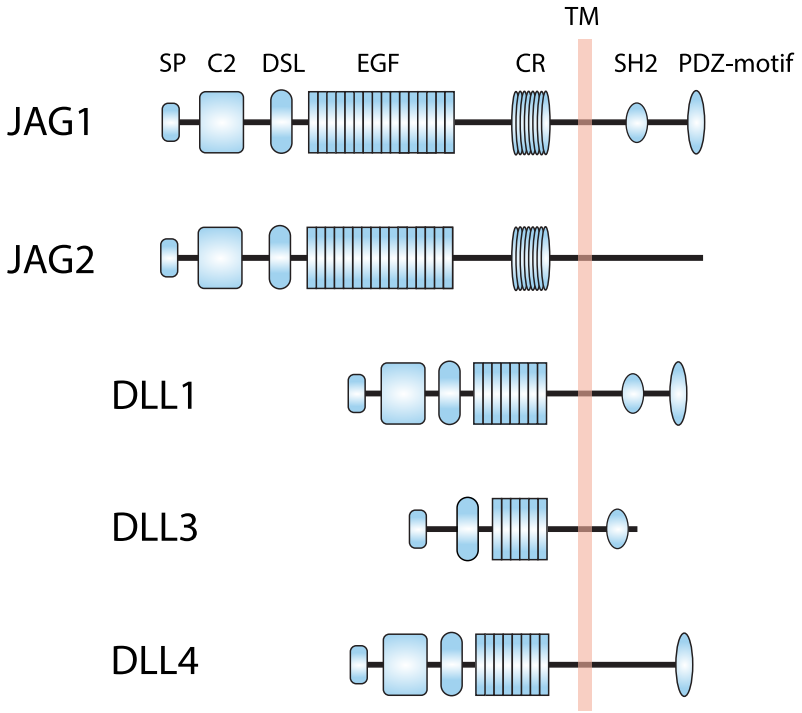

Figure 3. Domain organization of human DSL ligands (Delta, Serrate, Lag-2). Notch ligands in mammals include Dll1, 3 and 4, which are structural homologues of Delta in Drosophila, as well as Jagged 1, and 2, which are structural homologues of Serrate in Drosophila. Notch ligands are recognized by their Delta, Serrate, Lag-2 domain (DSL). N-terminally from the DSL domain is a short signal peptide and a C2 domain that until recently was known as the Module at the N-terminus of the ligand (MNNL). The extracellular domains also contain a variable number of EGF-like repeats (EGF). The two first EGF-repeats of Jagged1-2 and Dll1 have a slight difference in structure and are sometimes identified as the Delta/OSM-11 domain (DOS domain) (not shown in the figure). A cysteine-rich domain is also found in Jagged1-2. Jagged1, Dll1 and Dll4 have distinct PDZ-binding motifs in their intracellular domain. Dll3 lacks lysines in its intracellular domain making it unavailable for ubiquitination (Heuss et al., 2008).

1.4 Activation of canonical Notch signaling

Notch signaling is activated during cell-to-cell contact where Notch receptors interact with DSL ligands. Notch receptor activation normally occurs when Notch ligands on neighboring cells bind and activate the Notch receptor (Figure 4). The ligand-receptor

interaction leads to a change in the conformation of the Notch receptor, and the NRR is opened for S2 cleavage, which is followed by S3 cleavage in the transmembrane domain (Kovall et al., 2017). The mechanical force from endocytosis or cellular movement, which pulls on the Notch receptor, opens up the S2 cleavage site for ADAM-like metalloproteases such as ADAM17/TACE and ADAM10/Kuzbanian. Of these two, ADAM10 seems to be more relevant for canonical signaling physiologically, at least in Notch1 (van Tetering et al., 2009), but ADAM17 has been noted especially in aberrant ligand-independent signaling (Bozkulak and Weinmaster, 2009). The S2 site is located 12-13 amino acids before the transmembrane domain, which is well guarded by the negative regulatory domain (NRR) in non-activated cells (Gordon et al., 2009). The S2 cleavage also releases the NECD, which can be *trans*-endocytosed into the signal-sending cell together with the ligand (Gordon et al., 2008). γ-secretase is a protein enzyme complex consisiting of presinilin, nicastrin, PEN2 and APH1 and is required for S3 cleavage of the Notch receptor, which takes place just inside the cell membrane close to the cytoplasmic side (Bray, 2006). γ-secretase is not specific to Notch, as it is also responsible for cleavage of various other proteins such as APP, ErbB4 and CD44 (Selkoe and Wolfe, 2007). Inhibiting γ-secretase to curb Notch signaling in disease is therefore problematic, because of the wide range of functions for γ-secretase and clinical trials with γ-secretase inhibitors have shown unwanted side-effects (Andersson and Lendahl, 2014). The γ-secretase based S3 cleavage has also been found to occur in endosomes transporting NICD in the cell, as well as directly on the cell membrane (Sorensen and Conner, 2010; Tagami et al., 2008). Some studies imply that the γ-secretase activation can be even more efficient in endosomes than at the cell surface, due to the more acidic pH in late endosomes that is implicated to regulate γ-secretase activity (Vaccari et al., 2008; Windler and Bilder, 2010). The cleaved NICD can have a different half-life and activity depending on which amino acid is on the N-terminal end of NICD, which can be influenced by the location of the cleavage (Tagami et al., 2008). The most common cleavage site in humans is between amino acids Glu-1753 and Val-1754 (Val-1744 in mice) (Okochi et al., 2002). Released NICD can migrate to the cell nucleus where it can bind the DNA-binding protein CSL through its RAM and ANK domains. The ANK domain together with CSL recruits the co-activator Mastermind-like (MAML), which facilitates transcriptional activation of Notch target genes. Other co-activators such as p300 and PCAF can bind to this complex to activate Notch target genes.

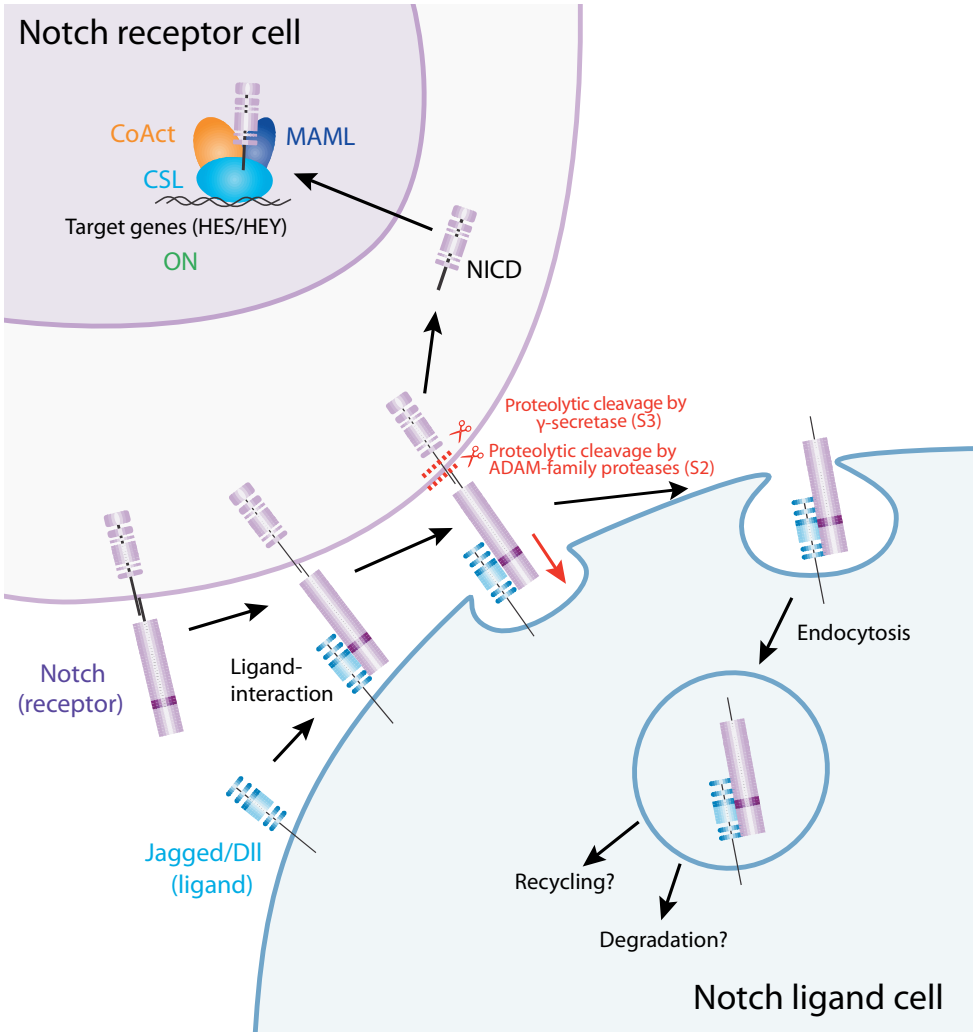

Figure 4. Notch activation overview. Canonical Notch signaling is initiated when a Notch ligand interacts with a Notch receptor from a neighboring cell. The tension generated (red arrow) during endocytosis of the ligand, changes the conformation of the bound Notch receptor to allow two subsequent proteolytic cleavage events, first by ADAM family metalloproteases and then by γ-secretase, which ultimately releases the transcriptionally active Notch ICD. NICD then translocates to the nucleus where it binds CSL, resulting in recruitment of co-activators, instead of co-repressors normally bound to CSL. This complex can then induce expression of gene families mainly relating to Hairy-Enhancer of Split (HES), and Hes-related proteins (HERP, also known as HEY), which themselves are transcription factors with broad effects on gene expression influencing various signaling pathways in the cell. During activation, the NECD is trans-endocytosed into the ligand cell. In addition to generating the conformational change of the receptor needed for activation, the effect of NECD endocytosed into the signal-sending cell is unclear. While presumably degraded, it is also possible that NECD and the ligand could dissociate from each other in the more acidic environment of endosomes in a way that could permit ligand recycling back to the cell membrane.

These genes include, notably, basic-helix-loop-helix (bHLH) transcription factors from the HES and HEY families (Bray, 2016). Other targets include MYC, cyclin D1 and Notch receptors and ligands themselves (Bray and Bernard, 2010). In the absence of active NICD, CSL is bound to DNA together with co-repressors and histonedeacetylases to function as a transcriptional repressor of Notch target genes (Borggrefe and Oswald, 2009; Xu et al., 2017). There is also evidence showing that CSL is not only present in the nucleus, but also exists in the cytoplasm where it can associate with NICD (Krejcí and Bray, 2007). New studies further show that Notch activation can affect chromatin remodeling and the amount of CSL binding to DNA (Castel et al., 2013; Gomez-Lamarca et al., 2018; Skalska et al., 2015). In contrast to models where CSL sits statically on DNA as a repressor and switches to an activator when associated to NICD, these studies advocate for a model of dynamic DNA binding by the NICD-CSL complex. *In vivo* responses of Notch-dependent transcriptional activation indicate that higher NICD levels increase burst duration of the transcriptional response (Falo-Sanjuan et al., 2019; Lee et al., 2019).

1.4.1 Non-canonical Notch

The mode of activation described in the previous chapter, based on DSL ligands that activate Notch receptors that then act through CSL, is known as canonical Notch signaling. There are also indications of Notch activation that do not fit within canonical Notch activation (Andersen et al., 2012). These non-canonical Notch modes include: responses without Jagged/Dll activation, CSL-independent effects of Notch, as well as Notch-independent CSL-effects (Ayaz and Osborne, 2014). CSL regulation without Notch has been studied in some detail in cases where CSL is switched from a repressor to an activator by viruses such as the Epstein-Barr virus EBNA2 in an NICD independent manner (Henkel et al., 1994; Zimber-Strobl and Strobl, 2001). CSL has also been found to form a complex together with the transcriptional regulators Ptf1a and p48 with effects on pancreatic development and differentiation in the nervous system (Beres et al., 2006; Hori et al., 2008; Masui et al., 2007; Obata et al., 2001). CSL-independent effects of NICD have been documented through the PI3K pathway, Wnt/B-Catenin and through IL-6 upregulation of NF-kB. Tumor cells have also been shown to escape apoptosis through Notch independently of CSL in several studies (Acosta et al., 2011; Jin et al., 2013; Kwon et al., 2011; Lee et al., 2013; Perumalsamy et al., 2009; Veeraraghavalu et al., 2005). Non-canonical ligands that are similar to DSL ligands have been shown to activate Notch and

include: Delta/Notch-like EGF related receptors (DNER), Delta-like 1 and 2 homologue (DLK1-2) as well as members of the contactin family (D'Souza et al., 2008; Eiraku et al., 2002; Greene et al., 2016; Traustadóttir et al., 2016). It is still unclear how effectively the non-canonical ligands can activate Notch compared to Jagged/Dll, or if they mainly inhibit canonical ligands through competitive inhibition. Nevertheless, examples such as these show that non-canonical signaling mechanisms may lead to outcomes that need to be taken into account for a detailed understanding of Notch.

1.4.2 Cis-inhibition

In addition to Notch *trans*-interactions i.e. when the activating ligand is present on another cell than the receptor, Notch receptors can also bind to ligands in *cis* or in the same cell (D'Souza et al., 2008). Ligands in *cis* can bind to the same receptors as *trans*-ligands and are considered inhibitory both in *Drosophila* development (Jacobsen et al., 1998), and in cell culture where *cis*-inhibition has been studied more meticulously by using controlled levels of receptor and ligand (Sprinzak et al., 2010). *Cis*-inhibition is most likely due to ligands and receptors sitting next to each other not being able to generate the force necessary to activate the receptor as in normal Notch *trans*-interactions whilst competitively inhibiting ligands from nearby cells (del Álamo et al., 2011). There is also a concept of *cis*-inhibition preventing unwanted ligand-independent activation and non-canonical ligands from activating Notch (Palmer et al., 2014). Further complicating interpretations of Notch signaling output and the theories of *cis*-inhibition, a new study reports *cis*-activation of Notch. Cells that expressed intermediate levels of Notch ligands Dll1 and Dll4 were able to activate Notch in a system with controlled ligand levels and cell-cell interactions, whereas only higher ligand levels led to *cis*-inhibition (Nandagopal et al., 2019).

1.5 Notch signaling during development

Notch is one of a small group of signaling pathways used extensively during development together with pathways such as Wnt, Sonic Hedgehog and TGF-β (Sanz-Ezquerro et al., 2017). Notch regulates developmental decisions through lateral inhibition, lateral induction and asymmetric cell division (Sjöqvist and Andersson, 2019). Lateral inhibition is when one cell prevents neighboring cells from adopting the same cell fate through a negative feedback loop. A well-known example of Notch in both lateral inhibition and

asymmetric cell division comes from neural development in *Drosophila,* where Notch influences cells to acquire different fates in a manner where high Notch cells remain either as undifferentiated progenitor cells or become cells of non-neural fate, while low Notch cells are able to progress through a more and more differentiated state ultimately giving rise to a neuron (Chitnis, 2009). The surrounding cells with higher levels of Notch become supportive cells such as glial cells. The first phase of the neural lineage decisions described above occurs due to lateral inhibition, when some cells within a proneural cluster (PNC) begin to express more Delta ligand, which then activates Notch in neighboring cells with subsequent activation of Notch target genes within the Enhancer of Split gene complex. This activation inhibits proneural gene expression and only the signal-sending cell adopts a path to become a neural cell. In the central nervous system (CNS) of *Drosophila* this initial neural cell fate is to become a neuroblast. The second phase of neural differentiation is an example of asymmetric cell division where the neuroblast divides with an asymmetric distribution of proteins, which includes the protein Numb – a negative regulator of Notch. Suppressing Notch in one of the daughter cells allow them to adopt distinct cell fates. The neuroblasts undergo repeated division to generate one new high Notch neuroblast and one low Notch ganglion mother cell (GMC). The GMC can then further divide to become neurons or glia. In settings where both are generated, the glial cell will have higher Notch and the neuron lower Notch levels (Chitnis, 2009). Without proper Notch signaling during these lineage decisions, too many cells become neural cells; an embryonically lethal phenotype termed the “neurogenic fate” (Artavanis-Tsakonas and Muskavitch, 2010; Lehmann et al., 1983).

During lateral induction, a feed forward response is created where the signal-receiving cell adopts the same cell fate as the sending cell, which goes on to induce the same fate in the next cell. One example of lateral induction is initiation of arterial wall formation through Jagged1 signaling, where endothelial cells of the vascular lumen expressing Jagged1 induce both vascular smooth muscle cell (VSMC) differentiation and upregulation of Jagged1 in the first VSMC layer, which then propagates the signal into the next layer of smooth muscle cells, generating an arterial wall of multiple layers of differentiated smooth muscle cells (Hoglund and Majesky, 2012; Manderfield et al., 2012). Similar Notch-dependent positive feedback responses have been observed during inner ear development and ocular lens fiber formation in mammals (Kiernan, 2013; Petrovic et al., 2014; Saravanamuthu et al., 2009).

1.6 Notch signaling in disease

Considering the importance of Notch signaling during development, it is not surprising that mutations in the Notch signaling pathway can contribute to a number of developmental phenotypes and disorders (Louvi and Artavanis-Tsakonas, 2012). These phenotypes affect the heart, vasculature, skeleton, kidney, liver, eye, nervous system and brain (Penton et al., 2012). Notch related congenital disorders include Alagille syndrome, spondylocostal dysostosis, Adams-Oliver syndrome, Dowling-Degos disease and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Aster et al., 2017). One of the first described congenital heart defects seen from *NOTCH1* mutations was bicuspid aortic valve, where the aortic valve has only two leaflets instead of three, leading to calcification of the aortic valve and aortic aneurysms (Garg et al., 2005; Preuss et al., 2016). Aortic aneurysms can also be seen in haploinsufficient *Notch1* mice (Koenig et al., 2017). Mutations of *NOTCH1* have recently been observed in patients with a wide variety of pathological cardiac phenotypes (Kerstjens-Frederikse et al., 2016).

Notch is important for homeostasis in adult tissues and both canonical and non-canonical Notch signaling modes have been implicated in several human cancers (Aster et al., 2017; Ayaz and Osborne, 2014). A landmark study of Notch in cancer demonstrated that more than 50% of T-ALL patients had activating *NOTCH1* mutations in their HD and PEST domains (Weng et al., 2004). We now know that Notch plays an essential role in many other hematological cancers as well and that active Notch can drive a survival bias in B and T cells, leading to uncontrolled cell division (Aster et al., 2017). Overexpression of NOTCH1 is also commonly found in solid tumors including breast, lung, prostate, colorectal and pancreatic cancers (Miele et al., 2006; Ranganathan et al., 2011b). However, in solid tumors Notch is not necessarily involved through activating Notch mutations as in many lymphatic diseases; instead Notch signaling can be deregulated in other ways, such as aberrant levels of receptors, ligands or other signaling modifiers (Andersson and Lendahl, 2014). Notch signaling has also been identified as a tumor suppressor in cancers such as head and neck carcinomas, squamous carcinomas of skin and lung, and pancreatic cancers (Avila and Kissil, 2013; Wang et al., 2011; Yap et al., 2015). Somatic mutations of Notch have recently been shown to accumulate during aging also in normal esophageal epithelial tissues, which surprisingly showed higher mutation rates than in esophageal

squamous cell carcinomas (Martincorena et al., 2018). In addition, Notch has been implicated to have different roles in different stages of tumor progression depending on context (Ranganathan et al., 2011b). Studies in pancreatic cancer, for example, have indicated that active Notch signaling can be tumor suppressive during initial stages of carcinogenesis (Hanlon et al., 2010) but needed for later stage tumor progression (Plentz et al., 2009). Notch signaling is also recognized as a promising target in tumor angiogenesis and metastasis as a consequence of the critical roles Notch plays in physiological angiogenesis and in vascular homeostasis (Boareto et al., 2015; Garcia and Kandel, 2012; Kofler et al., 2011; Li et al., 2007; Oon et al., 2017). Proposed clinical uses therefore have to be considering which part of the disease progression they are targeting. Notch inhibitors are currently in trials for combination therapies together with chemotherapeutic agents, as it has been shown that common chemotherapeutic treatments often increase Notch signaling in the tumor cells. Heightened Notch levels can lead to improved survival properties and concomitant resistance to chemotherapeutics, ultimately leading to relapse of the disease (Kamstrup et al., 2017). Combination therapies with Notch inhibitors have indeed been shown to have higher efficiency in killing cancer cells than either therapy alone as shown by Notch inhibition in combination with: doxyrubicin and trastuzumab in breast cancer, docetaxel in prostate cancer and temozolamide in glioma xenograft mice (Cui et al., 2015; Gilbert et al., 2010; Kim et al., 2015; Li et al., 2015; Osipo et al., 2008). Multiple excellent reviews on Notch in disease alluding to both present and future clinical therapies are available for the interested reader (Andersson and Lendahl, 2014; Aster et al., 2017; Louvi and Artavanis-Tsakonas, 2012; Ranganathan et al., 2011b; Tamagnone et al., 2018).

1.7 Notch signaling in the vasculature

The vascular system is crucial for development, homeostasis and disease. Cardiovascular diseases are currently the most common cause of mortality in humans (WHO, 2019). The vasculature provides all tissues with oxygen and nutrition, while allowing for removal of carbon dioxide and waste products. During early development, blood vessels are formed from endothelial precursor cells of mesodermal origin in a process termed vasculogenesis (Kolte, 2016). During vasculogenesis the vascular plexus is formed, which is a primitive endothelial cell vessel network. The vascular plexus is then divided into arteries, veins and capillaries during arteriovenous specification and further remodeled through branching of

the vasculature by angiogenesis to form a functional vasculature (Kolte, 2016). The emerging vasculature also attracts mural cells around the vessels, with vascular smooth muscle cells (VSMC) covering arteries/veins and pericytes covering the smaller venules/capillaries of the microcirculation.

There are many signaling pathways regulating vascular function including VEGF, Wnt, BMP, TGF-β, angiopoietin and Notch (Tetzlaff and Fischer, 2018). Notch signaling is distinct by being involved in all the key steps of vascular development; from initial vascular plexus formation, to arteriovenous patterning, to recruitment and maintenance of VSMCs and remodeling by angiogenesis (Gridley, 2010). Deletion of critical Notch components such as *Notch1* (Krebs et al., 2000; Limbourg et al., 2005), *Dll4* (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004), *Jag1* (Benedito et al., 2009; Xue et al., 1999), *CSL* (Krebs et al., 2004), Notch target genes *Hey1/Hey2* (Fischer et al., 2004), S2 cleavage mediator *Adam10* (Glomski et al., 2011), as well as γ-secretase components *nicastrin* (Li et al., 2003) and *presenilin* (Herreman et al., 1999) all lead to embryonic lethality in mice, with severe defects in the vasculature. The sensitive nature of Notch levels in the vasculature is further inferred from the fact that endothelial cell specific expression of constitutively active Notch1 (Krebs et al., 2010), Notch4 (Uyttendaele et al., 2001) and overexpression of endothelial Jag1 (Benedito et al., 2009) and Dll4 (Trindade et al., 2008) also lead to severe vascular defects and embryonic lethality in mice.

1.7.1 Angiogenesis and Notch

Angiogenesis is the development of new vessel branches from pre-existing blood vessels to maintain a functional circulatory system with adequate blood flow to all tissues. This process differs from *de novo* formation of blood vessels during vasculogenesis. Angiogenesis is activated during inflammation, mechanical stress, injury, hypoxia, low pH and during the pathophysiology of tumor growth. Especially a lack of oxygen (hypoxia) is easily sensed in tissues and leads to VEGF signaling through hypoxia inducible factor (HIF) (Kofler et al., 2011). Vascular endothelial cells express VEGF receptors, with VEGFR2 and VEGFR3 leading to endothelial sprouting. As VEGF-A (hereafter VEGF) is secreted in the hypoxic tissue, the nearby vessel sprouts begin to migrate towards the VEGF gradient (Figure 5). Angiogenesis is a multistep process, which includes an initial remodeling of the extracellular matrix, endothelial cell migration and tube formation. Endothelial cells in the pre-existing vessel initiate the process by migrating out a tip cell,

which is followed by stalk cells supporting the newly formed microvessel. The tip and stalk cell selection is regulated by Notch signaling in response to VEGF (Gerhardt et al., 2003). Endothelial cells have been reported to express Notch1, Notch4, Dll1, Dll4, Jag1 and Jag2 but in variable patterns depending on the vasculature analyzed and its state of development (Hofmann and Iruela-Arispe, 2007). Previous studies have identified important roles for especially Jag1, Dll4 and Notch1/4 (Kangsamaksin et al., 2015). In a similar manner as with knockout of key Notch components, deletion of VEGF or VEGFR is embryonically lethal due to vascular defects in mice (Carmeliet et al., 1996; Dumont et al., 1998; Ferrara et al., 1996). VEGFR1 is unique from VEFGR2/3 as it acts as a decoy receptor for VEGF (Meyer et al., 2006; Siekmann et al., 2013). VEGFR2/3 activate endothelial cells to extend filopodia and to invade the basement membrane by secreting matrix metalloproteinases (Arroyo and Iruela-Arispe, 2010). This breakdown is based on actin-based podosomes. The formation of functional podosomes is also regulated by VEGF and Notch (Spuul et al., 2016).

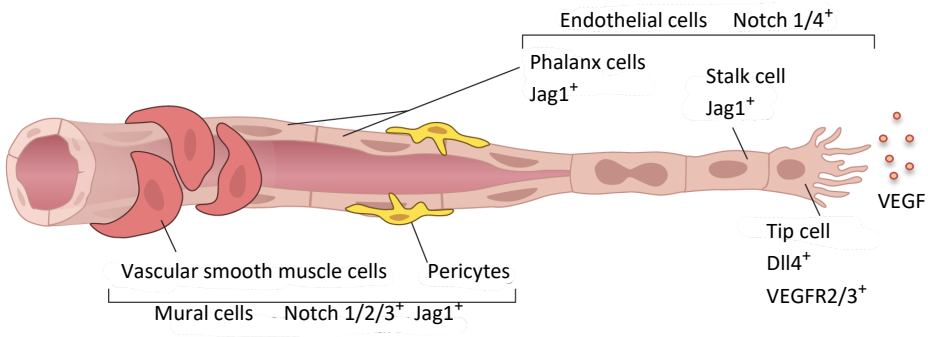

Figure 5. The Notch pathway in sprouting angiogenesis. During sprouting angiogenesis, migrating tip cells respond to vascular endothelial growth factor (VEGF) through VEGF receptor 2/3 (VEGFR2/3), which upregulates Dll4 expression in the tip cell. Dll4 then activates Notch1 in the trailing stalk cell, which downregulates VEGFR2/3, allowing for a proliferative phenotype that supports the formation of the growing vessel. In a mature vessel the stalk cells become quiescent phalanx cells. Endothelial cells recruit mural cells such as vascular smooth muscle cells and pericytes to support the new vessel sprout (Figure adapted from Masek and Andersson, 2017).

High levels of VEGF induce expression of the Notch ligand Dll4. Expression of Dll4 in tip cells activates Notch in neighboring cells which then downregulates expression of VEGFR2/3 (Lobov et al., 2007; Tammela et al., 2008; Taylor et al., 2002) and allows for these neighboring cells to adopt a stalk cell fate, forming the vascular lumen. The stalk

cells are more proliferative and migrate less than the tip cells. Stalk cells also recruit pericytes to support the newly formed vessel. ECs that have become quiescent have also been distinguished as phalanx cells. Activated Notch signaling in the adjacent stalk cells then decrease expression of VEGFR2/3 through Hey1 and Hey2 (Blanco and Gerhardt, 2013). In other words, the sprouting tip cells have been reported to have high Dll4 and VEGFR2/3 expression with low Notch signaling activity, while the proliferating stalk cells behind the tip cells have high Notch and low VEGFR2/3 (Figure 5).

EC specific *DLL4* knockouts and *DLL4* heterozygote mice show an excessive number of tip cells and sprouts with poorly perfused vessels (Hellström et al., 2007; Lobov et al., 2007; Suchting et al., 2007). Several studies have reported similar phenotypes during Notch inhibition and EC specific KO of Notch1 and CSL (Benedito et al., 2012, 2009; Hellström et al., 2007; Lim et al., 2019; Lobov et al., 2007; Ridgway et al., 2006). The Notch ligands function in distinct ways in the vasculature and Dll1 cannot compensate for Dll4 in the vasculature (Preuße et al., 2015). In addition to Dll4, Jagged1 is also expressed in the endothelium. Jagged1 is mostly expressed in the stalk cells and there are interpretations that Jagged1 can play an inhibitory role of Notch signaling in the tip cells by competing with more potent Dll4 ligands to lower Notch signaling in tip cells (Benedito et al., 2009). The theory of low Notch required in tip cells has been challenged, as loss of *Dll4* in endothelial sprouts did not affect tip cell identity in recent studies (Hasan et al., 2017; Pitulescu et al., 2017). Furthermore, live cell imaging of sprouting tip cells further demonstrated that Notch is first activated in the tip cell and then further increased over time (Hasan et al., 2017). A tumor angiogenesis study further suggests that Dll4 may be a dominant ligand over Jagged1, but that both are potent activators of Notch signaling in the endothelium and that competitive inhibition can not explain the roles of Jagged1 in that context (Oon et al., 2017).

2. Regulation of the Notch signaling pathway

Notch signaling combines ligand binding at the cell surface with transcriptional activation in the nucleus, in a regulatory pathway based on proteolytic cleavage of the receptor. This mode of signaling has a couple of interesting attributes. Because the proteolytical cleavage at S2 physically separates the ligand bound extracellular domain from the intracellular domain, which upon release, has a limited half-life, means that each receptor can signal only once. This means that the level of signaling activity and output is highly dependent on the amount of receptors and ligands on the cell surface, as well as their activation potential and the subsequent fate of the NICD in the cell. Previous studies have shown that both the receptors and the ligands undergo endocytosis, and the trafficking of these proteins may regulate the amount of Notch components at the cell surface. Endocytosis is also part of the activation mechanism of Notch signaling as endocytosis of Notch ligands have been shown to generate the force needed to physically pull on the receptor to change its conformation and thus enable proteolytic cleavage by ADAM/TACE (Meloty-Kapella et al., 2012; Musse et al., 2012). Furthermore endocytosis of the Notch receptor can control the fate of NICD upon activation, and aberrant endosomal trafficking of the receptor has also been shown to lead to cleavage of the receptor without ligand activation – a form of non-canonical activation of Notch (Vaccari et al., 2008).

Post-translational modifications (PTMs) of Notch and its interacting partners also have an important role in the signaling output. Notch receptor glycosylation is required for efficient binding by its ligands, and modifies which ligand interaction is preferred, whereas other PTMs regulate the intracellular domain to affect the half-life of NICD (Borggrefe et al., 2016). The proteins interacting with Notch do not only block or terminate active signaling but also provide the tools needed to fine-tune and control Notch-mediated cellular processes.

2.1 The role of post-translational modifications in Notch signaling

The core Notch signaling pathway exhibits a deceiving simplicity, with its one-to-one, ligand-to-receptor, linear activation mechanism. Despite this, the Notch signaling pathway has been shown to be particularly context-dependent with multiple possible outcomes from its receptor activation. PTMs regulate the functional response of proteins by addition and removal of functional groups to proteins. It is now clear that Notch is tightly regulated

and numerous reports over the last few decades point to PTMs being key players in increasing the diversity of Notch sigaling output. In addition to their role in influencing endosomal trafficking of Notch, some PTMs directly affect the binding of NICD to the transcriptional complex, while others modify and alter ligand-receptor preferences (Kakuda and Haltiwanger, 2017; Le Bras et al., 2011; Ranganathan et al., 2011a). Different PTMs can further act together to fine-tune the response of active NICD in the cell (Lee et al., 2015). Notch activity is modified by PTMs such as sumoylation, methylation, acetylation, hydroxylation, glycosylation, phosphorylation and ubiquitination (Antfolk et al., 2019; Pfeffer et al., 2019) (Figure 6-7). As a consequence of the linearity of the pathway, the output is therefore also highly dependent on the half-life of NICD. The longevity or stability of NICD affects not only how long NICD stays bound to CSL on the DNA allowing for continued transcriptional activation, but also determines if Notch is stable enough to enter the nucleus before it is degraded. The ubiquitin-based modifications are key regulators of the degradation of NICD (Borggrefe et al., 2016; Fryer et al., 2004). In the next chapters, glycosylation, phosphorylation and ubiquitination will be briefly described, as they relate most closely to the results presented in this thesis.

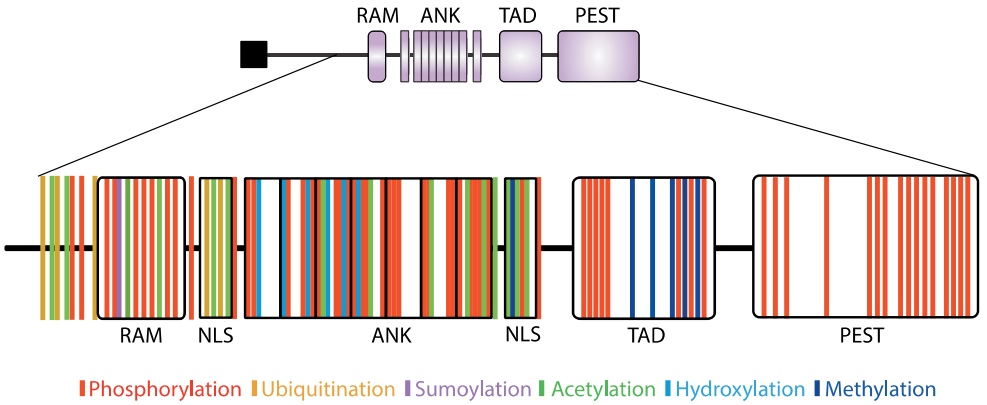

Figure 6. Schematic of post-translational modifications of the Notch intracellular domain (NICD). The intracellular domain of the Notch receptor can be modified by phosphorylation, ubiquitination, sumoylation, acetylation, hydroxylation and methylation, which affect the signaling output of receptor activation. Many of the identified sites still have unknown functional consequences. A highlight of functionally important PTM-sites that are also linked to disease are shown in table 1. (Figure adapted from Antfolk et al., 2019).

Table 1. Highlight of important post-translational modifications of Notch that are also linked to disease. (For a complete list of PTMs in Notch signaling and their functions, see Antfolk et al., 2019).

Enzyme	PTM	Domain	Functional importance / Disease	Reference
POFUT1	Glycosylation	EGF-repeats	Essential for Notch function and ligand-receptor interactions / Dowling-Degos disease	(Li et al., 2013)
POGLUT1	Glycosylation	EGF-repeats	Essential for Notch function and ligand-receptor interactions / Dowling-Degos disease	(Basmanav et al., 2014)
EOGT	Glycosylation	EGF-repeats	Adams-Oliver syndrome	(Cohen et al., 2014)
Lunatic Fringe	Glycosylation	EGF-repeats	Regulates ligand binding / Spondylocostal Dysostosis	(Sparrow et al., 2006)
Cyclin C - CDK3, 8, 19	Phosphorylation	PEST-domain	Enhances FBXW7-dependent ubiquitination and degradation / T-ALL	(Li et al., 2014)
PIM	Phosphorylation	Second NLS	Enhances nuclear localization and transactivation of NICD / Breast cancer, prostate cancer	(Santio et al., 2016)
FBXW7	Ubiquitination	PEST-domain	Induces degradation of Notch / T-ALL	(O'Neil et al., 2007)

2.1.1 The role of glycosylation in Notch signaling

The extracellular part of Notch can be modified by several different types of sugar modifications, which alter the structure and function of Notch (Figure 7). Serine or threonine linked *O*-glycosylations in particular have been shown to be crucial for Notch signaling and several human diseases are also linked to mutations in the glycosyltransferases carrying out *O*-glycan modifications, with multiple Notch linked phenotypes (Harvey and Haltiwanger, 2018). These complex sugar modifications can be attached to the extracellular domain of Notch during receptor processing in the endoplasmic reticulum (ER). There are three main types of *O*-glycosylation of EGF-repeats of Notch, which include: *O*-fucosylation (*O*-fucose), *O*-glucosylation (*O*-glucose) and *O*-GlcNAcylation (*O*-linked β-*N*-acetylglucoseamine) (Takeuchi and Haltiwanger, 2014). Each individual EGF repeat consists of approximately 40 amino acids, which include 6 conserved cysteine residues (Kovall et al., 2017). *O*-glucosylation by Protein *O*-Glucosyltransferase 1 (POGLUT1) can occur between the first and the second cysteine residues at C^1-X-S-X-(P/A)-C^2 consensus sequences (where X is any amino acid, and the modified serine underlined), *O*-fucosylation by Protein *O*-Fucosyltransferase 1 (POFUT1) can occur between the second and the third cysteine at the consensus sequence C^2-X-X-X-

X-(S/T)-C[3], and *O*-GlcNAcylation by EGF-domain specific *O*-GlcNAc Transferase (EOGT) between the fifth and sixth conserved cysteine residues at the consensus sequence C[5]-X-X-X-(F/W/Y)-(T/S)-G-X-X-C[6] (Takeuchi and Haltiwanger, 2014). Recently, *O*-glucosylation between cysteines 3 and 4 by POGLUT2/3 was also identified (Takeuchi et al., 2018) after the discovery of a non-POGLUT1 consensus sequence being glucosylated in the crystal structure of the DLL4-NOTCH1 complex (Luca et al., 2015). Loss of *Poglut1* or *Pofut1* in mice leads to embryonic lethality with Notch-linked phenotypes (Fernandez-Valdivia et al., 2011; Shi and Stanley, 2003). Loss of *Pofut1* is similar to loss of CSL, which has more severe phenotypes than the loss of any single Notch receptor (Shi and Stanley, 2003).

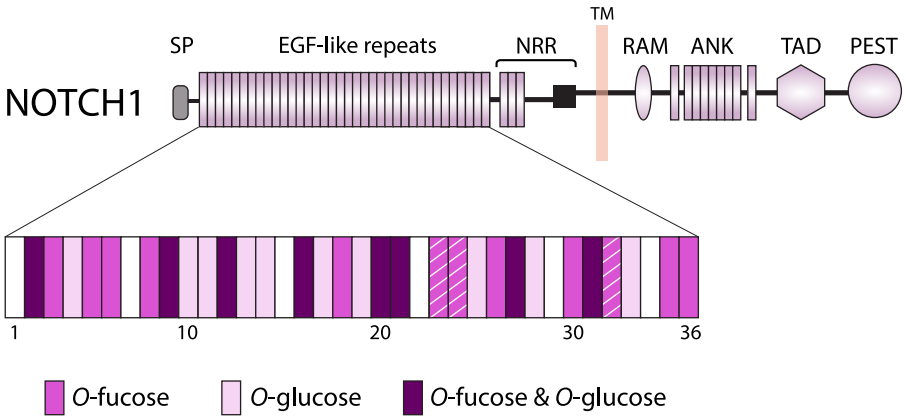

Figure 7. Glycosylation sites on mammalian NOTCH1 EGF-repeats. The extracellular domain of the Notch receptor is modified by glycosylation. O-fucosylation by POFUT1 and O-glucosylation by POGLUT1 crucially regulate Notch structure and the ability to be activated by ligands. EGF-repeats predicted to be modified by POFUT1 and POGLUT1-3 on NOTCH1 are indicated above. All predicted sites have been confirmed by glycoproteomic methods (Kakuda and Haltiwanger, 2017; Rana et al., 2011) except for repeats with diagonal white lines (EGF23, 24 & 32). The only NOTCH1 EGF-repeat modified by POGLUT2/3 is EGF11 (Takeuchi et al., 2018) (Figure adapted from Antfolk et al., 2019).

The *O*-linked glycans can be further elongated to di-, tri- or tetra-saccharides, most notably: elongation of *O*-glucose by xyloses (xylosyltransferases), and *O*-fucose by GlcNAc (Fringe enzymes) (Brückner et al., 2000; Moloney et al., 2000; Rampal et al., 2005; Sethi et al., 2010; Taylor et al., 2014). In Drosophila, deletion of *Fringe* leads to a notched phenotype of its wings and increases activation by Delta while decreasing activation by Serrate (Jagged in mammals) (Irvine and Wieschaus, 1994; Panin et al.,

1997). In mammals, three Fringe orthologs add to the complexity of glycan modifications. All three (Lfng, Mfng and Rfng) potentiate Dll1 to Notch1 signaling. Rfng also potentiates Jagged1 to Notch1 signaling, but Mfng and Lfng decrease activation of Notch1 through Jagged1 (Yang et al., 2005). Recent glycoproteomic analysis indicate that the differential effects of the three Fringes may stem from which EGF-repeats they are able to modify (Kakuda and Haltiwanger, 2017). Even though all three mammalian Fringes are expressed in many tissues during development, only the lack of Lfng has substantial effects on viability and fertility of mice (Evrard et al., 1998; Moran et al., 2009, 1999). By contrast all three Fringes have been shown to contribute to proper development of B and T cells and surprisingly, *Lfng/Mfng/Rfng* triple knockouts could be rescued by any single Fringe allele in this context (Song et al., 2016).

2.1.2 The role of ubiquitination in Notch signaling

Ubiquitination (also known as ubiquitylation) is a process of attaching ubiquitin residues to target proteins by E1 ubiquitin-activating enzymes, E2 ubiquitin conjugating-enzymes and E3 ubiquitin ligases. Ubiquitin E3 ligases recognize the target site and recruit E2 enzymes to facilitate transfer of the ubiquitin onto a target lysine. Adaptors such as epsins can then recognize these ubiquitin tags (Sen et al., 2012). Ubiquitin can be added as monomers or extended to poly-ubiquitin chains, where specific lysines of ubiquitin are further modified (Komander and Rape, 2012). Although poly-ubiquitination of certain lysines is a common tag for proteasomal degradation in the cell, different types of ubiquitin modification have various other effects on the targeted protein, such as regulation of its location through endocytosis (Swatek and Komander, 2016).

To generate pulling force by endocytosis, there is a requirement for mono-ubiquitination of Notch ligands by the E3 ligases Neuralized (Neur) and Mindbomb 1 (Mib1) in *Drosophila* (Itoh et al., 2003; Lai et al., 2001). Due to differential expression during development, the loss of either one gives rise to the neurogenic phenotype, which is similar to a loss of Notch phenotype (Le Borgne et al., 2005; Wang and Struhl, 2005). Neur and Mib1 are localized to the cell membrane and loss of either, lead to an accumulation of signaling incompetent ligands on the cell surface (Itoh et al., 2003; Lai et al., 2001; Le Borgne and Schweisguth, 2003). In settings where both are expressed in the same cells, deletion of both are required for loss of Notch phenotypes (Lai et al., 2005; Wang and Struhl, 2005). However, Mib1 cannot rescue the neurogenic phenotype of Neur

in flies (Le Borgne et al., 2005). Neur and Mib1 ubiquitinate both Delta and Serrate, thereby affecting ligand endocytosis and *trans*-activation of both *Drosophila* ligands (Lai et al., 2005; Le Borgne et al., 2005). A second Mib homologue (Mib2) exists in *Drosophila* and mammals (Koo et al., 2005b) and although there is no requirement for Mib2 for viability in mice, Mib2 is important for muscle integrity, which cannot be rescued by Mib1 or Neur (Nguyen et al., 2007). Mammals have two Neur orthologs (NEURL1 and NEURL1B) and one Mib1 ortholog (MIB1). Cell culture assays show that all three can regulate Notch *in vitro* (Koutelou et al., 2008; Teider et al., 2010) but in contrast to *Drosophila* development, only loss of *Mib1* has adverse effects on Notch signaling in mice (Barsi et al., 2005; Koo et al., 2007). Mice defective for all three *Neur1, Neur1b* and *Mib2* have no obvious phenotypes while knock out of *Mib1* alone produces the characteristic loss of Notch like phenotypes (Koo et al., 2005a; Koo et al., 2007).

Ubiquitination of Notch also affects trafficking of the receptor, and several E3 ligases such as Deltex, AIP4/Itch (human/mouse), Nedd4, Fbxw7 and c-Cbl ubiquitinate Notch (Le Bras et al., 2011). The regulatory effects of ubiquitination of the Notch receptor vary from stimulatory to inhibitive. Deltex, for instance, can activate Notch in certain settings in flies that are linked to ligand-independent γ-secretase cleavage, but promote its degradation when complexed with the β-arrestin homolog Kurtz (Hori et al., 2011; Matsuno et al., 1995; Mukherjee et al., 2005). Downregulation of Deltex1 in mammalian cells has been reported to increase Notch activity and enhance Notch receptor levels at the cell surface (Zheng et al., 2013). As an example of degradation of NICD, the E3 ligase Fbxw7 can ubiquitinate a specific phosphodegron region of the PEST domain as a degradation signal, if it has first been phosphorylated by a kinase such as Cdk8 (Fryer et al., 2004; Wu et al., 2001). In line with this, mutations in Fbxw7 that decrease its activity, can lead to increased amounts of NICD (Aydin et al., 2014; Malyukova et al., 2007; Mansour et al., 2009). Mutations in the *FBXW7* gene have been found in different cancers such as T-ALL, small cell lung cancer and melanoma (Aydin et al., 2014; George et al., 2015; Larson Gedman et al., 2009). PEST mutations that truncate the domain so that the phosphodegron site is lost have a similar effect as inactivating *FBWX7* mutations. Although an RNAi screen identified several deubiquitinases that can regulate Notch (Zhang et al., 2012), their effects on Notch signaling have only recently started to be uncovered. One example comes from the deubiquitinase Usp28, which negates Fbxw7 ubiquitination and thereby increases Notch levels (Diefenbacher et al., 2015). In addition,

USP10 has been shown to affect angiogenesis through regulation of NICD turnover in endothelial cells (Lim et al., 2019).

2.1.3 The role of phosphorylation in Notch signaling

Phosphorylation is an essential PTM, commonly used for regulation of protein activity. During phosphorylation, phosphate groups can be added to specific amino acid residues that include serine, threonine and tyrosine residues. The addition of phosphate groups, in a reaction catalyzed by enzymes called kinases, can change the conformation and functionality of a protein in an ATP dependent process that can later be reversed by the action of phosphatases. Several kinases that phosphorylate NICD have been identified in various systems (Borggrefe et al., 2016). Notably, many of them affect the stability of active NICD by phosphorylating the PEST domain for Fbxw7-mediated degradation. Kinases that have been shown to prime PEST for ubiquitin based degradation include; CDK1, CDK2, CDK3, CDK8, CDK19 and ILK (Carrieri et al., 2019; Fryer et al., 2004; Li et al., 2014; Mo et al., 2007). GSK3-β has been found to phosphorylate N1ICD in several studies. One of these studies reported increased stability of N1ICD (Foltz et al., 2002), while another showed reduced Notch activity in two different cell lines in vitro (Jin et al., 2009). A reduction in Notch activity has also been shown in Notch2 (Espinosa et al., 2003). Many other kinases, such as Nemo kinase can affect the interaction with the transcriptional complex by NICD phosphorylation (Ishitani et al., 2010). In a similar manner CK2 has been shown to inhibit N1ICD from forming a complex with CSL and MAML, reducing transcriptional activity (Ranganathan et al., 2011a). Src kinase has been shown to act in a similar fashion by inhibiting MAML recruitment (LaFoya et al., 2018). Phosphorylation of N1ICD by Akt (also known as protein kinase B, PKB) has been reported to inhibit nuclear localization (Song et al., 2008) with similar findings in Notch4 where Akt inhibited translocation to the nucleus through 14-3-3 (Ramakrishnan et al., 2015). PIM kinases on the other hand have been shown to increase nuclear localization of N1- and N3ICD (Santio et al., 2016). Global proteomic studies have identified many other NICD phosphorylation sites, but the majority of sites discovered are without insight into which specific kinases are involved or the outcome of the modifications (Antfolk et al., 2019) (Figure 6).

Phosphorylation of other Notch signaling components has also been shown to regulate signaling. In mammals, atypical Protein Kinase C (aPKC) regulates endocytosis of Notch

through directly influencing Notch regulatory protein Numb (Sato et al., 2011; Smith et al., 2007), as well as Mindbomb indirectly through PAR-1 (Ossipova et al., 2009). PKC is a family of serine/threonine protein kinases, which are divided into three subfamilies that include classical, novel and atypical PKCs that all have different requirements for their activation (Newton, 2018). The PKC family share high similarities in their catalytic domains with Akt (Facchinetti et al., 2008; Franke et al., 1994). Novel PKC isoforms require diacylglycerol (DAG) for their activation, classical PKC isoforms require DAG and calcium while atypical PKC isoforms require neither calcium nor DAG but do require a phosphatidyl serine for their activation (Figure 8). The different subfamilies have slight differences in their domains, with aPKCs completely lacking the calcium activated C2 domain while also having a DAG insensitive C1 domain (Pu et al., 2006) (Figure 8). The C1 domain is still important for aPKCs because it affects the localization of aPKC in the cell (Pu et al., 2006). Atypical PKCs also have a PB1 domain, which activates aPKCs when bound to protein scaffolds such as PAR6 or p62 (Tobias and Newton, 2016).

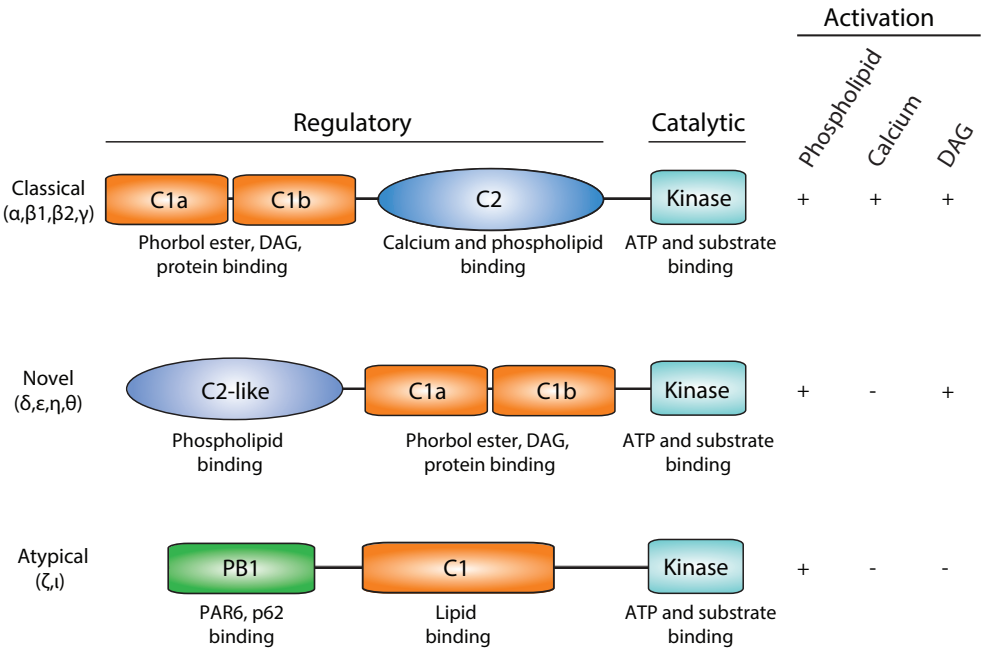

Figure 8. Schematic of PKC isozyme domains and their activation. Classical PKC isoforms require diacylglycerol (DAG) and calcium for their activation, novel PKC isoforms require DAG, while atypical PKC isoforms require neither calcium nor DAG. Phorbol esters activate classical and novel isoforms. Atypical PKCs are not activated by phorbol esters but they do require a phosphatidyl serine for their activation. (Figure based on Garg et al., 2014)

The DAG sensitive PKC isoforms have gathered special attention since it became clear that they could also be activated by tumor promoting phorbol esters, which at the time characterized PKCs as potential oncogenes (Newton, 2018). It has now become evident that PKCs can function as both tumor promoters and tumor suppressors, with the majority of data supporting that the correct function of PKC is tumor suppressive (Ali et al., 2016; Antal et al., 2015; Dowling et al., 2016; Justilien et al., 2014; Parker et al., 2014; Uhlen et al., 2017). Even though phorbol esters activate classical and novel PKC isoforms, they also leave their conformation irreversibly open to dephosphorylation and ubiquitin based degradation (Hansra et al., 1999; Jaken et al., 1981). This could be an explanation for the minimal success achieved with PKC inhibitors in clinical trials over the years (Dowling et al., 2016; Zhang et al., 2015).

Atypical PKC isoforms include PKCζ (*zeta*) and PKCλ/ι (*lamda/iota*) (human/mouse) and they are especially known for their role as part of a PAR-3/PAR-6/aPKC complex, which has key functions during cellular polarity with additional roles in organization of cellular junctions and ARP2/3 linked endocytosis (Chen and Zhang, 2013; Georgiou et al., 2008; Hapak et al., 2018; Leibfried et al., 2008; Nishizuka, 1992). Atypical PKCs are involved in migration and wound healing where they can be found at the leading edge of the cell (Etienne-Manneville and Hall, 2001; Xiao and Liu, 2013). Both atypical PKC isoforms are linked to cancer. Their roles include invasion promoting properties exemplified by both upregulation and activation of matrix metalloproteinase 9 (MMP-9) by PKCζ (Estève et al., 2002; Xiao et al., 2010). Similarly, PKCι has also been found to drive growth and invasion of lung cancer cells through MMP-10 by Rac1 activation (Frederick et al., 2008). However, PKCζ has been indicated to have both tumor suppressive and promoting roles. While PKCζ overexpression has been reported in cancers such as prostate and bladder cancer (Dhanasekaran et al., 2005; Sanchez-Carbayo et al., 2006), downregulation of PKCζ has been indicated in lung, pancreatic and kidney cancers (Galvez et al., 2009; Lenburg et al., 2003; Selbie et al., 1993).

2.2 Endocytosis

Endocytosis is the process where vesicles bud off from the plasma membrane through invagination. Endocytosis controls important processes such as recycling of membrane components, protein and nutrient uptake, retargeting and degradation of proteins, signal

transduction, cell polarity and migration (Di Fiore and von Zastrow, 2014; Doherty and McMahon, 2009). Membrane proteins and lipids can be endocytosed through clathrin-dependent and clathrin-independent mechanisms (Doherty and McMahon, 2009; Ferreira and Boucrot, 2018). Receptor-mediated endocytosis is used for uptake of extracellular molecules and trans-membrane proteins based on receptor-ligand binding and the use of clathrin attached to the plasma membrane to form clathrin coated pits (McMahon and Boucrot, 2011) (Figure 9). Membrane receptors can accumulate in these coated pits. This form of transport is very common and more than 20 different receptors have been shown to internalize through this endocytic pathway (Xu et al., 2017b).

Dynamin is a protein that has a key role in pinching off the internalized vesicles from the cell membrane. After invagination the clathrin coat is removed by Hsc70 together with auxilin (Eisenberg and Greene, 2007). After the vesicles have internalized, the receptors can enter a network of endocytic trafficking pathways (Spang, 2009). Endocytosed vesicles normally fuse with early endosomes, which can be described as sorting stations for which endocytic route the cargo will enter (Jovic et al., 2010). At this stage, proteins may be recycled to the plasma membrane, sorted to the lysosomes for degradation or delivered to the trans-Golgi network (Jovic et al., 2010). Endosomal trafficking can be tracked by analyzing Rab GTPases (RABs), that specify distinct endocytic compartments and are heavily involved in trafficking and fusion of vesicles (Zerial and McBride, 2001). Early and late endosomes can be identified based on their specific types, where RAB5 specify early endosomes and RAB7 late endosomes (Rink et al., 2005). Consequently, when vesicles transition from early to late endosomes the amount of RAB7 increases, while RAB5 is removed at the vesicle membrane (Wandinger-Ness and Zerial, 2014). Early endosomes are acidic with a pH in the range of 6.0-6.2, which can lead to the dissociation of many ligands from their receptors. Late endosomes are more acidic still and further fuse with lysosomal vesicles, which carry acidic hydrolases that degrade endocytosed materials (pH 5). The early to late endosomal transition also leads to a repackaging of the transmembrane proteins into multivesicular bodies (MVBs) (Doherty and McMahon, 2009). Some membrane receptors also show specific signaling from endosomes compared to the plasma membrane (Alanko et al., 2015). Proteins that recycle back to the plasma membrane normally enter recycling endosomes before returning to the cell membrane through compartments commonly including RAB11 and a multiprotein complex called the exosyst (Emery et al., 2005; Jafar-Nejad et al., 2005). Some

transmembrane proteins can also recycle back to the cell membrane faster by returning directly from the early endosomes in a RAB4-dependent manner (Grant and Donaldson, 2009).

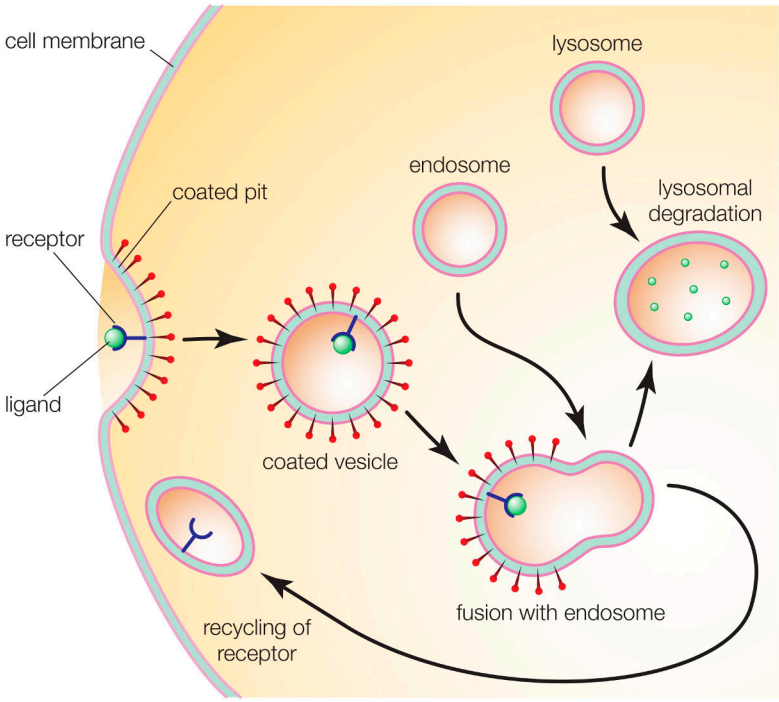

Figure 9. Receptor-mediated endocytosis. In receptor-mediated endocytosis, ligands bind to receptors on the cell membrane, which then recruit adaptor proteins and clathrin to form clathrin-coated pits. Invagination of the plasma membrane produces a clathrin-coated vesicle. Internalization is followed by uncoating of the vesicle and fusion with sorting/early endosomes. The endocytosed proteins can then be sorted for recycling or fuse with lysosomes for degradation. (Figure adapted from Britannica, 2019)

Endocytosis and endosomal trafficking of Notch receptors and Notch ligands control Notch signaling pathway activation. The importance of endocytosis of Notch was first shown in Drosophila, where cells from shibire mutant flies (shibire is a homolog of mammalian dynamin) were defective in both sending and receiving lateral communication through Notch signaling (Seugnet et al., 1997). Further work in *Drosophila* demonstrated how endocytosis of Delta in the signal sending cell is mandatory for activation of Notch in adjacent cells, and that the extracellular domain of Notch co-localizes with Delta in the signal sending cell upon activation (Parks et al., 2000). Studies have also shown that the

force generated by ligand endocytosis is in the same order of magnitude as the force required for Notch activation (Gordon et al., 2015; Meloty-Kapella et al., 2012). This form of activation is in contrast to general receptor-mediated endocytosis depicted in Figure 9, where ligands are internalized with the receptors. In addition to the pulling force model of Notch activation, endocytosis and recycling of both Notch ligands and receptors might allow for re-distribution of Notch components within the cell membrane to facilitate increased receptor activation, as seen with other receptors (Simons and Toomre, 2000). Finally, endocytosis can also negatively regulate Notch, by removing ligands and receptors from the surface of the cell and target them for degradation in lysosomes and proteasomes (Conner, 2016).

2.2.1 Endocytosis of Notch receptors

Notch receptors that are not interacting with ligands, have been shown to be constantly endocytosed, and then either recycled or degraded (Jehn et al., 2002; McGill et al., 2009). Cleavage of the receptor without ligand activation can occur in cases where endocytic trafficking from early or late endosomes is restricted by interferences in the fusion to late endosomes or lysosomes (Fortini and Bilder, 2009). Such unwanted activation can have severe consequences, with similar results as constitutively active mutations of Notch. Consequently, ligand-independent activation following endosomal defects has been considered as a cause of cancer (Tanaka et al., 2008). It is still unclear whether the S3 cleavage occurs directly on the cell membrane after the S2 cleavage or predominantly later in endosomes, and which alternative is more efficient in generating productive NICD. The activity of γ-secretase may be higher in endocytic vesicles where pH is lower (Pasternak et al., 2003), which is supported by the fact that mutations in proteins which limit the acidification of endosomes, lead to accumulation of Notch in enlarged endosomes in *Drosophila* (Yan et al., 2009). Blocking Rab5 in *Drosophila* leads to accumulation of Notch at the cell surface with an accompanying reduction in NICD produced, which could be due to less effective γ-secretase activity at the cell surface (Vaccari et al., 2008), although other possibilities also exist. However, studies with Notch extracellular truncation (NEXT) peptides (S2 cleaved, but not S3 cleaved Notch) have shown that WT NEXT cleaved at the plasma membrane generates mostly the more stable Val-1744 form of NICD while cleavage in endosomes generates mostly the unstable Ser-1747 cleavage product (Tagami et al., 2008). NEXT fragments harboring a point mutation at K1749R

that inhibits monoubiquitination has been considered evidence that K1749 ubiquitination and endocytosis is required for activation of Notch (Gupta-Rossi et al., 2004). Tagami and colleagues later showed that K1749R does not inhibit Notch receptor activation and that NEXT can still be cleaved at the plasmamembrane by y-secretase, but it generates mostly the unstable Ser-1747 NICD and even more unstable forms like Arg-1749 that are rapidly degraded (Tagami et al., 2008). NICD cleavage has also been observed in late endosomes without ligand activation in cases where the acidity has led to a separation of the Notch heterodimer (Wilkin et al., 2008).

In addition to Fbxw7-mediated ubiquitination and proteosomal degradation of NICD, AIP4/Itch can also ubiquitinate Notch1 without ligand activation to facilitate lysosomal degradation in mammals (Chastagner et al., 2008). Binding of Numb to AIP4/Itch seems to further promote its degradation in lysosomes (McGill et al., 2009). Nedd4 and c-Cbl are predicted to function in a similar manner as AIP4/Itch by leading to trafficking of Notch and subsequent degradation of non-activated Notch in lysosomes (Le Bras et al., 2011). ESCRT proteins regulate the routing of Notch through multivesicular endosomes to lysosomes, and loss-of-function ESCRT mutations increase Notch signaling activity (Conner, 2016).

2.2.2 Endocytosis of Notch ligands

Already in early studies in *Drosophila*, Delta ligands were found in intracellular compartments (Kooh et al., 1993) and blocking endocytosis of DSL ligands led to impaired Notch signaling activity in *Drosophila* (Parks et al., 2000). During receptor activation and S2 cleavage, the Notch extracellular domain (NECD) is released and can be endocytosed into the signal-sending cell together with the ligand (Parks et al., 2000). NECD is then presumably degraded together with the ligand in the lysosomes (Hansson et al., 2010). It has also been shown that the NECD can be endocytosed into the ligand cell upon pulling force even if ADAM metalloproteases have been inhibited (Nichols et al., 2007). This indicates that the Notch extracellular domain is not released by ADAM cleavage but by the mechanical pulling force, which in turn allows the receptor to be cleaved at S2. Structural studies show that the S2 cleavage site is buried within the NRR, and mutations affecting NRR structure in a way that exposes the S2 site, are consistently active without the need for ligand activation (Gordon et al., 2007; Henrique and Schweisguth, 2019). Early studies also showed that recombinant secreted ligands interact

with Notch, but are not able to activate Notch signaling and instead block signaling by acting as decoys (Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1997). Immobilized ligands, however, do activate Notch signaling in cell culture, which also supports tension or force being required for Notch activation (Varnum-Finney et al., 2000). More detailed work with different molecular force measurement systems in mammalian cells have elucidated the actual forces required for the conformational change of the receptor leading to its activation. The pulling force model has been further strengthened by the use of controlled mechanical force to generate the conformational change needed to allow for S2 cleavage. The forces documented have been in the 5 to 10 piconewton (pN) range, as measured with different molecular force measurement systems (Chowdhury et al., 2016; Gordon et al., 2015; Seo et al., 2016). Although forces generated by ligand endocytosis have not been determined with comparable detail, optical tweezer studies have shown that Dll1 endocytosis can generate forces up to 10 pN (Meloty-Kapella et al., 2012). Ubiquitinated ligands can be bound by endocytic adaptor proteins of the epsin family, which are known to facilitate both clathrin-dependent and clathrin-independent endocytosis (Chen et al., 1998; Sigismund et al., 2005). Epsin has been identified as a critical adaptor for endocytosis of Notch ligands in *Drosophila* and mice (Chen et al., 2009; Langridge and Struhl, 2017; Meloty-Kapella et al., 2012; Wang and Struhl, 2004). Epsin is also linked to regulation of the actin cytoskeleton (Horvath et al., 2007).

In addition to ligand endocytosis inducing activation of Notch signaling through force, ligands have also been found in endosomes of cells that are unable to signal (Wang and Struhl, 2005, 2004). It has therefore been considered that ligands are endocytosed constitutively without ligand-receptor interactions. Yamamoto and colleagues have shown that although Mib1 is required for ligands to activate Notch, a large amount of Jag1 inside endosomes do not require Mib (Yamamoto et al., 2010). It has further been shown that mutated Dll1 ligands lacking intracellular lysines are still internalized but they are unable to recycle back to the cell surface (Heuss et al., 2008). It has been debated whether endocytosis only functions to control ligand levels at the cell membrane, if recycling is necessary for the ligands to become signaling competent or if it functions to move ligands into specific membrane compartments such as specialized lipid domains (Heuss et al., 2008; Suckling et al., 2017; Yamamoto et al., 2010). There have been no descriptions of what an “activated” signaling competent ligand would look like compared to a “non-activated” ligand. Also, studies in *Drosophila* cell lines show that loss of Rab5 or Rab11

has no effect on Delta-mediated activation of the receptor. As transitioning from early endosomes to recycling endosomes requires Rab5 and recycling requires Rab11, this indicates that ligand activation through recycling is not required to produce active ligands, at least in *Drosophila* (Windler and Bilder, 2010). There are however studies showing that ligands can be relocated to specific parts of the cell through endocytic recycling (Benhra et al., 2010; Rajan et al., 2009).

2.3 Intermediate filaments as regulators of cellular function

The cytoskeleton is a complex network of protein fibers, which allow cells to maintain shape and mechanical integrity, keep organelles in their positions, aid in cellular movement and contribute to vesicle and protein trafficking within the cell. In addition, various components of the cytoskeleton transmit signaling from outside the cell and can help the cell respond to outside stress (Toivola et al., 2010). The cytoskeleton is comprised of microtubules, intermediate filaments and microfilaments (also known as actin filaments). Microtubules form the thickest filaments with hollow tubes that can guide organelle movement and pull the chromosomes apart during cell division. Microfilaments have the smallest diameter size of their fibers and they form the cellular cortex and function in transport of cellular components, cellular movement and cell division. They also maintain the microvilli structures and are key components to functioning muscle cells and their contraction (Bezanilla et al., 2015).

Intermediate filaments (IFs) have fiber sizes (10 nm) in between those of microfilaments (7 nm) and microtubules (25 nm) (Buehler, 2013). IFs associate with the plasma membrane and provide the cell with structural support and also organize the microtubule and actin filament networks (Gruenbaum and Aebi, 2014). IF subunits self assemble into nonpolar ropelike structures without a need for ATP/GTP, which is in contrast to microtubule and actin filament formation (Herrmann and Aebi, 2016). IF monomers consist of a conserved rod domain, flanked by an N-terminal head domain and a C-terminal tail domain, that vary in structure depending on IF (Kim and Coulombe, 2007). Two rod-domains connect to form a dimer, two dimers form a tetramer. The rope like fiber is made up from eight tetramers, which is called a unit length filament (ULF), which can self assemble into long intermediate filaments (Herrmann and Aebi, 2016) (Figure 10). The IF structure observed in the cell is highly dynamic, branched and connects to other

parts of the cytoskeleton through binding partners such as plectins (Wiche et al., 2015). Phosphorylation of the IF subunits influence assembly and properties of the IF network (Eriksson et al., 2004; Snider and Omary, 2014).

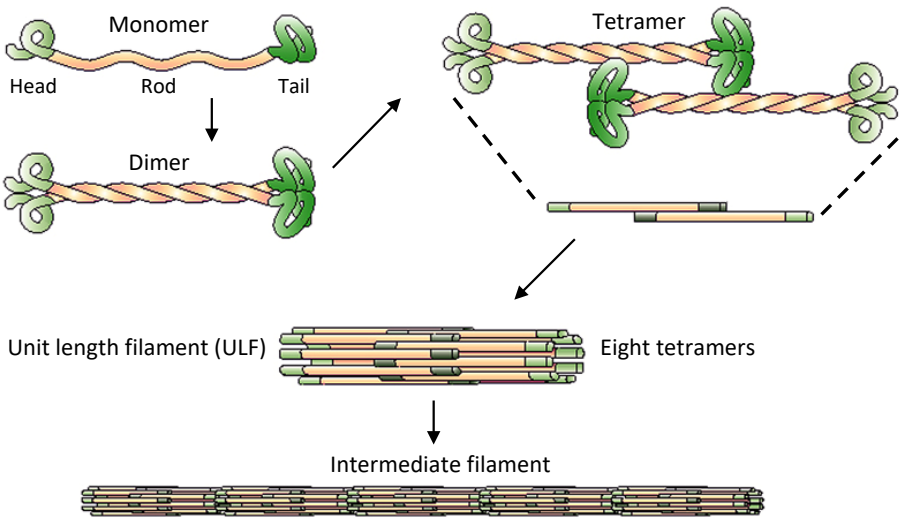

Figure 10. Schematic of IF assembly. IF monomers, consisting of a rod domain flanked by a head and a tail domain, form coiled-coil dimers. Two dimers form anti-parallel tetramers and eight tetramers form a unit-length filament (ULF). ULFs assemble to form an intermediate filament by annealing (Figure modified from Dunleavy et al., 2019).

IFs can be classified into six different types based on sequence similarity, where type I and type II keratins that are expressed in epithelial cells represent the largest group of IF genes. Keratins form heteropolymers consisting of type I and type II filaments (Jacob et al., 2018). Type III IFs can form both homo- and heteropolymer filaments and include vimentin, desmin, GFAP and peripherin. Of type III IFs, vimentin is by far the most widely expressed filament and found in mesenchymal cells, leukocytes, endothelial cells of blood vessels and only occasionally in epithelial cells (Battaglia et al., 2018). The abundant expression of vimentin in cells of mesenchymal origin such as fibroblasts, is often used as a marker for epithelial to mesenchymal transition (EMT) (Ivaska, 2011; Mendez et al., 2010), a process occuring during development and wound healing that is highly correlated to the invasive migrating phenotype of cancer cells at the leading edge of carcinomas. There are hopes of targeting vimentin in mesenchymal cancers and a small molecule (FiVe1) targeting vimentin organization and phosphorylation was recently

identified as a potent inhibitor of breast cancer cell lines that had transitioned through EMT (Bollong et al., 2017). In the past decade, evidence has emerged alluding to important regulatory functions of intermediate filaments (Battaglia et al., 2018; Gregor et al., 2014; Ivaska et al., 2007). Although the vimentin knock out mouse is viable without obvious phenotypes at a first look (Colucci-Guyon et al., 1994), vimentin has been found to be involved in numerous processes, including differentiation, proliferation, migration and invasion (Boraas and Ahsan, 2016; Cheng et al., 2016; Nieminen et al., 2006; Richardson et al., 2018). Vimentin knock out mice have defects in wound healing in various different tissues (Bargagna-Mohan et al., 2012; Cheng et al., 2016; dos Santos et al., 2015). Other defects related to VimKO mice are varied and include: mammary gland development (Peuhu et al., 2017), glia development (Colucci-Guyon et al., 1999), inflammation (dos Santos et al., 2015), steroidogenesis (Shen et al., 2012) and myelination of peripheral nerves (Triolo et al., 2012). Astrocytes from GFAP and vimentin double knock out mice have been previously reported to have an effect on Notch signaling through Jagged1 (Wilhelmsson et al., 2012). Vimentin is also linked to vascular processes, including endothelial sprouting, vascular tuning and arterial remodeling (Dave and Bayless, 2014; van Engeland et al., 2019). The wide expression profile, extensive interaction network and involvement in EMT suggest that the role of vimentin is important in highly dynamic processes and may be especially important during various forms of cell stress.

OUTLINE AND KEY AIMS OF THESIS

The communication of neighboring cells via the Notch signaling pathway is crucial during development and homeostasis in all multicellular organisms that have been studied to this day. The key aim of this thesis is to study the regulation and activation of the Notch signaling pathway with a special focus on endocytosis and trafficking of Notch receptors and ligands. Endocytosis is a key regulator of Notch signaling activation. Endocytosis of Notch ligands can generate the force to activate the cleavage of the receptor on the cell membrane. During receptor activation the intracellular domain (ICD) of the receptor also undergoes endocytosis, which ultimately leads to the relocation of Notch ICD to the nucleus and activation of target genes. The studies in the thesis include regulation of Notch ligands during angiogenesis and Notch receptors during differentiation. A dual label screening approach is also developed and optimized to identify further regulators of Notch ligand endocytosis.

Key aims of this thesis:

- Determine how the intermediate filament vimentin regulates Notch ligand-receptor interactions in the context of angiogenesis

- Set up a method for visualization and measurement of Notch ligand endocytosis to enable identification of new regulators of Jagged1

- Study the effects of Notch receptor phosphorylation by PKCζ on the trafficking and activity of Notch receptors.

EXPERIMENTAL PROCEDURES

More detailed information on materials and methods can be found in the original articles and the manuscript.

Table 2. Methods used in the studies of this thesis.

Method	Study
Aortic ring assay	I
Biotinylation assay	I, II, III
CAM-model	I
Cell culture	I, II, III
Cell spot microarray	II
Cyclohexamide chase experiment	I, III
Fingerprint assay	I
Fluorescence activated cell sorting (FACS)	I, III
High throughput fluorescence plate reading	II
Image analysis	I, II, III
Immunocytochemistry & immunohistochemistry	I, II, III
Immunoprecipitation	I, II, III
In vitro phosphorylation	III
In vivo transfection	III
Ligand trans-endocytosis	I, II
Live cell imaging	I
Luciferase reporter assay	I, III
Mass spectrometry	III
Microscopy	I, II, III
Proximity ligation assay	I
Quantitative reverse transcription PCR	I, III
Recycling assay	I
SDS-PAGE and western blotting	I, II, III
siRNA interference	I, II
Spheroid angiogenesis assay	I
Statistical analysis	I, II, III
Transfection	I, II, III
Ubiquitination assay	III

Table 3. Cell lines used in the studies of this thesis.

Cell line	Type	Study
SW-13	Human adrenal carcinoma	I
HEK293	Human embryonic kidney	II, III
HEK293 FLN	Human embryonic kidney stable FLN1 overexpression	I, III
HEK293 JAG	Human embryonic kidney stable JAG1 overexpression	II
HeLa	Human cervical cancer cells	III
HUVEC	Human umbilical vein endothelial cells	I
C2C12	Mouse myoblasts	III
3T3 JAG	Mouse fibroblasts stable JAG1 overexpression	I
MEF VimWT	Mouse embryonic fibroblast	I
MEF VimKO	Mouse embryonic fibroblast from Vim knock out mice	I

Table 4. Primary antibodies used in the studies of this thesis and their applications.

Antibody	Company	Application	Study
β-actin	Cell Signaling	WB	I, II, III
β-tubulin	Cell Signaling	ICC	II
CD31 PECAM-1	BD Pharmingen	IHC	I
Cleaved Notch1 Val1744	Cell Signaling	IP, WB	I, III
Delta C20	Santa Cruz Biotechnology	WB	I, II
Anti-Dll4	Sigma-Aldrich	ICC, IP, PLA, WB	I
GFP	Clontech	WB	III
α-GFP	Invitrogen	WB	III
HA1.1	Covance	IP	III
Hsc70	StressGen	WB	III
Jagged1 28H8	Cell Signaling	ICC, IP, PLA, WB	I, II
Jagged1 H-66	Santa Cruz Biotechnology	PLA	I
Lamp1	Abcam	WB	III
Myosin hc	Santa Cruz Biotechnology	IF, WB	III
Manic Fringe	Abcam	WB	I
Notch1	Sigma-Aldrich	WB	III
Notch C20	Santa Cruz Biotechnology	ICC, IP, WB	I, III
PECAM-1 C20	Santa Cruz Biotechnology	ICC	I
PKCζ	Santa Cruz Biotechnology	ICC, IP, WB	III
VE-Cadherin	Enzo Life Sciences	ICC	I
Vimentin D21H3	Cell Signaling	ICC, IP, WB	I
Vimentin V9	Sigma-Aldrich	ICC, PLA	I, II

RESULTS AND DISCUSSION

1. Regulation of the Notch ligand Jagged1 by vimentin (I)

1.1 Vimentin regulates angiogenesis and correlates with Jagged1

During the last few decades intermediate filaments (IF) have been established as signaling modulators, in addition to their structural functions (Battaglia et al., 2018; Pallari and Eriksson, 2006). Vimentin is an IF found primarily in mesenchymal cells and is abundant in endothelial cells lining the blood vessels (Dave and Bayless, 2014). During angiogenesis, endothelial cells in the pre-existing blood vessel initiate the process by migrating out a tip cell, which is then followed by stalk cells to form a new vessel sprout (Figure 4). Tip and stalk cell identity has been shown to be regulated by Notch signaling downstream of VEGF (Benedito and Hellström, 2013; Gerhardt et al., 2003).

In study I, we used the vimentin knock out mouse (VimKO) to study if and how vimentin regulates Notch signaling. VimKO embryos show delayed angiogenesis during embryonal day 12.5 (E12.5) of mouse development (I, Fig 4D) and placental tissue from VimKO mice at E11.5 also showed a disturbed or delayed surface vascularisation pattern compared to VimWT (I, Fig 4C). As previously described, the main Notch ligands regulating angiogenesis are Jagged1 and Dll4 (Benedito and Hellström, 2013). Moreover, mice lacking Jagged1 display disrupted remodeling of the vasculature in the embryo and the yolk sack, which leads to lethality around E10 (Xue et al., 1999). In our study, meta-analysis of human gene transcripts from the GeneSapiens database showed that Jagged1 and vimentin correlate strongly in several tissues, which include the heart and blood vessels (I, Fig S1A). This analysis also showed a correlation between Jagged1 and vimentin in several cancers (I, Fig S1B). Co-expression in several tissues pointed to a possibility of a functional link between Jagged1 and vimentin.

1.2 Vimentin interacts with Jagged1

Proximity ligation assay (PLA) uses two primary antibodies of distinct species, to target two unique proteins, combined with oligonucleotide-labeled secondary antibodies (PLA probes). Hybridizing connector oligos can then join the probes only if the two proteins are in close proximity. Joining the PLA probes to form a loop, is required for rolling circle

amplification and finally a detectable PLA signal (Alam, 2018). Using mouse embryonal fibroblast (MEF) cells from VimWT mice in PLAs with primary antibodies against vimentin, Jag1 and Dll4, we demonstrated that the PLA signal between Jagged1 and vimentin was higher than between Dll4 and vimentin (I, Fig 1A-B). Complementary to this, immunoprecipitation (IP) of vimentin consistently showed interactions with Jagged1 in MEF WT cells (I, Fig 1C). No interaction with Dll4 could be detected (not shown). VimKO MEF cells were used as negative controls (I, Fig 1C). It is important to emphasize that the targeting of Jagged1 in these PLAs and IPs was done with an antibody that binds to the intracellular domain of Jagged1 (Jagged1 28H8, Cell Signaling Technology).

The observed lack of interaction with Dll4 could in theory have been due to differences in affinity between the Jagged1 and Dll4 antibodies. The PLA assay in particular, could give misleading results if the Dll4 antibody is significantly weaker in its ability to bind its target or if the amount of Dll4 is significantly lower than Jagged1 in these cells. However, Dll4 has been readily detected with the same antibody previously in our lab and in human umbilical vein endothelial cells (HUVECs) in this study (I, Fig 6B). There is also a possibility that the PLA signal could be lower from Dll4 if vimentin covers the epitope for the Dll4 antibody, if the distance between the antibodies is different, or if the Dll4 antibody would be in the wrong orientation.

In order to provide more convincing evidence for the specific interaction of vimentin with Jagged1, and to elucidate if the interaction with vimentin is mediated through the intracellular domain of Jagged1, we decided to generate hybrid ligands of Jagged1 and Dll4, where we swapped the intracellular domain of the two ligands. By comparing Jagged1 ligands, with chimeric ligands consisting of the extracellular domain of Jagged1 and the intracellular domain of Dll4 (Jagged1ECD-Dll4ICD), we were able to evaluate the impact of the ICDs of these two ligands. Importantly, by using an antibody that binds to the extracellular domain of Jagged1 (Jagged1 H-66, Santa Cruz Biotechnology) and thereby using the same antibody for both types of ligands, we removed the uncertainty of potential differences in antibody affinity (I, Fig 1D). The expression levels of the Jagged1 and the hybrid ligand constructs were controlled by detection of an incorporated turboGFP tag and live-cell imaging showed similar localization patterns in transfected cells (not shown). Swapping the ICD of Jagged1 to the ICD of Dll4, led to a near complete loss of PLA signal (I, Fig 1D). Furthermore, the use of the Jagged1-ECD antibody in this

experiment, gave significantly increased amounts of PLA signal compared to the intracellular antibody used in I, Fig 1A. Although it is possible that the Jagged1 H-66 ECD-antibody is simply better at detecting Jagged1 than the Jagged1 28H8 ICD-antibody, our previous experiences do not support this, and in this case, it is more likely that the extracellular Jagged1 H-66 antibody and the vimentin V9 antibody are more optimally spaced to generate consistently robust PLA signals. PLA interactions occurring between 0-10 nm can be detected only if the two primary antibodies are not sterically hindered from binding to their respective sites (Sigma-Aldrich, 2019). If the interaction occurs directly between the intracellular domain of Jagged1 and vimentin, as our results indicate, it is possible that the antibody binding to the extracellular domain of Jagged1 provides less hindrance with the vimentin antibody, compared to the intracellular Jagged1 antibody that may be binding close to the interaction site. Antibody epitopes near the protein-protein interaction site may also lead to similar results. Although we did not pursue any experiments to determine the actual binding region of these proteins, it is attractive to speculate that the binding of vimentin to Jagged1 could occur near Glu1140, which is the epitope that the Jagged 28H8 antibody recognizes in the intracellular domain of Jagged1. As a reference, the PDZ-motif of Jagged1, which has previously been shown to make indirect interactions with the cytoskeleton, is at the C-terminus between residues 1213-1218 (Popovic et al., 2011). The protein MPDZ has later been shown to interact with the intracellular domains of Dll1 and Dll4 through their PDZ-binding motif and influence Dll4 localization and activity to regulate sprouting angiogenesis (Tetzlaff and Fischer, 2018). PDZ-domain proteins may therefore function as a link also between intermediate filaments and Notch-ligands, although this remains to be seen in the future.

For potential interaction studies on the vimentin side, the hyaluronan receptor CD44 has previously been shown to interact with the head domain of vimentin at the cell membrane of endothelial cells (Päll et al., 2011). More interestingly, integrins have been shown to bind directly to the vimentin head region, specifically between amino acids 21-45 (Kim et al., 2016). Vimentin is composed of 466 amino acids and the head domain includes residues 1-77 (Tomiyama et al., 2017). This region of vimentin could also serve as a starting point for any future studies detailing the interaction between Jagged1 and vimentin. Taken together, our results as demonstrated by PLA and IP provide evidence of a novel interaction between the intracellular domain of Jagged1 and the intermediate filament vimentin. This interaction is interesting, as both vimentin and Jagged1 have

strong links to tumor progression and metastasis (Bednarz-Knoll et al., 2016; Kidd et al., 2014; Satelli and Li, 2011; Sethi et al., 2011; Wang et al., 2018; Zhang et al., 2019; Zhu et al., 2013).

1.3 Vimentin affects Jagged1 endocytosis

As endocytosis of Notch ligands is important for Notch activation through *trans*-endocytosis, and since inactive ligands are constantly recycled, we wanted to explore how Notch ligands behave in VimWT and VimKO cells. Alexa Fluor 488-conjugated recombinant extracellular Notch1 peptides ($N1ECD^F$) that bind to Notch ligands on the cell surface and internalize through endocytosis, were differently distributed in VimWT and VimKO cells (I, Fig 2C). Moreover, the amount of endocytosed $N1ECD^F$ into VimKO MEFs was increased (I, Fig 2B). In these MEFs, the total amount of Jagged1 was similar in WT and KO cells (I, Fig 2A). $N1ECD^F$-uptake in MEFs transfected with Jagged1, followed by live cell imaging for 1 minute further revealed that the loss of vimentin increases directional mobility of endocytosed $N1ECD^F$-vesicles (I, Fig 2D-F). Surface biotinylation assays and immunocytochemistry also showed increased amounts of Jagged1 on the surface of MEF KO cells compared to WT (I, Fig 3B-C). Subsequently we used a fingerprint assay to determine if the increased amounts of Jagged1 on the surface of these cells could still bind Notch effectively. WT and KO MEFs were cultured on coverslips coated with $N1ECD^F$, or FC-control (not shown). A crosslinking agent was used to preserve any $N1ECD^F$-Jagged1 interactions, followed by an extraction of the cells. Crosslinked Jagged1 was then immunolabelled and the results showed more Jagged1 bound to the VimKO coverslips (I, Fig 3D). The increased binding is in line with the higher accumulation on the surface, which indicates that Jagged1 on the surface of VimKO cells is able to bind Notch receptors.

To measure Notch activity, we used a luciferase reporter assay based on multimerized CSL binding sites in a co-culture system using WT or KO MEFs cultured with HEK-293 cells expressing the 12xCSL-luciferase reporter constructs. Surprisingly, the Notch activity was not enhanced in the Notch reporter cells when activated by VimKO cells, and when related to the amount of Jagged1 on the surface of these cells, the activation potential was significantly lower (I, Fig 3E). Reintroducing vimentin in VimKO cells, increased the Notch signaling activity in the reporter cells to correspond closely with

Jagged1 ligand levels on the surface of VimKO cells (I, Fig 3E). Previous work from the Weinmaster group indicates that constant endocytosis of Notch ligands is distinct from the endocytosis required for *trans*-activation of Notch by pulling force (Meloty-Kapella et al., 2012; Musse et al., 2012). Pulling NECD-peptides from non-covalently bound agarose beads, required different endocytic proteins, such as EPS1/2 and actin, in comparison to uptake of free NECD-peptides (Meloty-Kapella et al., 2012). Using a similar setup, with $N1ECD^{F}$ bound to protein A agarose beads, we show that the uptake of $N1ECD^{F}$ from beads was significantly lowered in VimKO cells (I, Fig 3F), which also supports the lower activity in the Notch reporter assay in I, Fig 3E.

Vimentin may influence endocytosis of Jagged1 directly, but it could also be through interactions between vimentin and actin. Actin has been well established as a regulator of endocytosis and identified as necessary for Dll1-mediated Notch *trans*-activation (Ferguson et al., 2017; Meloty-Kapella et al., 2012). Looking at the endocytic mechanisms of other cell membrane receptors may also provide a clue to endocytosis of Notch receptors and ligands. Ligand-induced activation of GPCRs lead to clustering in clathrin-coated pits. In these pits, the cytoplasmic tail of the receptor can bind to the actin network through their PDZ-domains, which slows down endocytosis (Puthenveedu and von Zastrow, 2006). Actin may therefore influnce Notch endocytosis independently of vimentin as well. We decided not to pursue potential actin dynamics in our system, although we did look for an interaction with the actin-related protein-2/3 (ARP2/3) complex in our IPs of Jagged1, which could not be detected (not shown, Figure S2A). Nevertheless, looking at actin polymerization and its role in potential force generation would be highly interesting in the future as actomyosin contractility has recently been shown to contribute to the mechanichal tension during Notch activation (Hunter et al., 2019).

In conclusion, our data indicate that VimKO MEF cells express similar amounts of total Jagged1 protein compared to WT, but display higher Jagged1 levels at the cell membrane, resulting in higher uptake of free NECD-peptides. Despite this, VimKO MEFs are not stronger activators of Notch signaling in co-culture or more effective at endocytosing NECD bound to beads, which mimics the physical strain during Notch activation by *trans*-endocytosis. This suggests that vimentin promotes Jagged1-mediated *trans*-activation potential and pulling force strength and without vimentin, signaling incompetent Jagged1

ligands are accumulated at the cell membrane. The recent elucidation of the crystal structure of the binding interface between Jagged1 and Notch1, indicated that Notch ligands and receptors form so called catch bonds, where the binding strength increases with increased pulling force, and that Jagged1 requires a higher tension treshold compared to Dll4 (Luca et al., 2017). Vimentin may therefore, through such a mechanism, influence and tune the Notch activation process by improving the force generation of Jagged1. This influence may be further increased during shear stress, such as blood flow.

1.4 Loss of vimentin disrupts angiogenesis but can be rescued by external Jagged1

In addition to data from mouse embryos showing delayed angiogenesis (I, Fig 4C-D), we decided to implement *in vitro* and *ex-vivo* angiogenesis assays to see if vimentin also affects angiogenesis in more controlled settings. We used human umbilical vein endothelial cells (HUVECs) in an *in vitro* spheroid assay, where round bottom wells (96 well plates) were coated with agarose to prevent cells from attaching to the well and instead promote spheroid formation of the endothelial cells. Spheroids were then mixed with fibrinogen, which was allowed to polymerize. Endothelial sprouts were imaged during the next 4 days (I, Fig 6H). Knock down of vimentin, through short hairpin-mediated RNA interference (shVim) (I, Fig 6I), significantly inhibited sprout formation in this assay (I, Fig 6H). Jagged1 and Dll4 have been previously shown to regulate angiogenic sprouting, where Dll4 has been described as anti-angiogenic and Jagged1 as pro-angiogenic (Benedito et al., 2009; Pedrosa et al., 2015; Suchting et al., 2007; Xue et al., 1999). Interestingly, mixing in immobilized Jagged1-FC-beads with the spheroids in the fibrinogen gel was able to rescue the sprouting defect of shVim treated HUVECs (I, Fig 6H). Immobilized FC and Dll4-FC had no observable effect on endothelial sprouting from shVim spheroids (not shown).

To analyze endothelial sprouting directly from VimKO and WT mice, we used a three-dimensional *ex vivo* angiogenesis assay (Baker et al., 2012), where a slice of the mouse aorta was excised and embedded in collagen. Addition of VEGF allow for the endothelial cells lining the inside of the aortic ring to form sprouts (I, Fig 5A). This method has several advantages, which include the generation of many aortic rings from one mouse aorta to help facilitate quantification, and the simplicity of the assay. More importantly,

the assay is more physiologically relevant than *in vitro* assays, as the vessels form a proper lumen and also recruit supporting cells such as vascular smooth muscle cells and pericytes to associate with the developing endothelial tube, following a timeline that is similar to angiogenesis *in vivo*. These experiments revealed that aortic rings from VimKO mice have fewer sprouts per ring and a reduction in sprout length compared to WT (I, Fig 5A-B). Aortic rings from Vim heterozygote mice displayed an intermediate sprouting phenotype, indicating that the amount of vimentin can tune this process (I, Fig 5A-B). The sprouting endothelial cell tubes were confirmed by immunofluorescence of VE-Cad and PECAM-1 (I, Fig 5C). In line with the results from the *in vitro* angiogenesis assay, the addition of immobilized Jagged1, but not Dll4 peptides, in the collagen gel, rescued the amount of endothelial sprouts initiated from VimKO aortic rings, although in this case the sprout length was not significantly affected (I, Fig 6E-G).

These results challenge the argument that the proangiogenic role of Jagged1 in angiogenesis and in stalk cells is merely a result of inhibiting more potent Dll4 signaling in tip cells through competitive inhibition (Benedito et al., 2009). Unless Jagged1 inhibited endothelial Dll4 *cis*-activation in our assays, it is hard to envision how the addition of external Jagged1 peptides would be competing with other ligands, when they are interacting with endothelial cells in the aortic rings and spheroids from the outside. I hypothesize that a more probable explanation would be that Jagged1 can regulate endothelial sprouting differently than Dll4, perhaps in a similar manner as the ligand discrimination recently described between Dll1 generating pulsatile and Dll4 sustained Notch activation, leading to distinct gene responses (Nandagopal et al., 2018). Distinct responses are substantiated by the fact that Dll4 has been unable to replace the function of Dll1 in other systems and Dll4 cannot compensate for Dll1 function when knocked into the Dll1 locus in mice (Preuße et al., 2015). Nandagopal and colleagues argue that the discrete pulsatile effect from Dll1 stem from assembly of ligand-receptor clusters that release a burst of NICD when the cluster reaches a critical size, while Dll4 does not require clustering and thereby generates a more sustained signaling response (Nandagopal et al., 2018). It was further shown that pulsatile bursts favor *Hes1* activation while sustained Notch activated predominantly *Hey1/L*. If the closely related Dll1 and Dll4 can generate such distinct responses, it is easy to visualize that Jagged1 could also generate distinct responses from Dll4 in a similar way. In view of this, the Jagged1 peptides used in

our angiogenesis assays would have already been pre-clustered by virtue of being immobilized to agarose beads.

The effects of vimentin on Notch signaling may be further modified and balanced by Fringe glycosyltransferases, as immunoblotting of VimKO primary endothelial cells and shVim HUVECs both showed increased levels of MFNG compared to wild type (I, Fig 6B-C). qPCR of VimKO cells also showed an increase in Lfng (I, Fig 6D). Lfng and Mfng were previously implicated in balancing Dll4 and Jagged1 during sprouting of retinal vasculature (Benedito et al., 2009). Both Lfng and Mfng promote Dll-mediated activation of Notch *in vitro* at the expense of Jagged signaling (Kakuda and Haltiwanger, 2017). We did not analyze Fringe-mediated effects any further, nor look at Rfng. As Rfng has been shown to promote Jagged1-Notch1 activation in addition to Dll-mediated activation, it is still unclear if downregulation of vimentin upregulates all Fringes, or only Dll-Notch promoting Mfng and Lfng. If only Lfng and Mfng are upregulated, the data would suggest that Jagged1 signaling from VimKO cells is impaired due to loss of *trans*-endocytosis of Jagged1, while Dll signaling to VimKO is further increased due to sugar modifications by Fringes that promote Dll activation at the expense of Jagged1 signaling.

Endothelial specific knock down of Jagged1 imply that Jagged1-mediated vascular defects stem from both disrupted angiogenesis and vascular smooth muscle cell differentiation (High et al., 2008). We have recently continued our work on vimentin and Jagged1 to determine their effects on vascular smooth muscle cell differentiation. We showed that vimentin regulates Notch signaling and VSMC differentiation in response to hemodynamic force (van Engeland et al., 2019). During conditions of shear stress, vimentin phosphorylation of serine 38 was augmented. Notch signaling activity was also increased under these conditions, and the use of a phospho-mimicking mutant of VimS38 increased Notch activation in signaling assays (van Engeland et al., 2019). These results imply that site-specific modifications of vimentin under hemodynamic forces may influence the effects of vimentin on Notch ligands and Notch signaling activity. The increase in Jagged1 activity during shear stress is likely a factor in the regulation of angiogenesis as well.

Taken together, we show that vimentin affects the signal sending potential of Jagged1-mediated Notch activation by interacting with the intracellular domain of Jagged1. The lack of angiogenesis from vimentin null endothelial cells can be rescued by Jagged1-

mediated Notch signaling. Jagged1 and Dll4 have distinct roles during angiogenesis and the use of hybrid Jagged1-Dll4 ligands demonstrate a selective regulation of Notch ligands by vimentin. The effects on Notch signaling by a lack of vimentin may be increased by downstream effectors that upregulate Fringe glycosyltransferases to further potentiate Dll-mediated activation of Notch. These results have implications not only in angiogenesis and vascular homeostasis, but also in tumorigenesis as both vimentin and Jagged1 are linked to tumor progression and metastasis.

2. Screening for regulators of Jagged1 endocytosis (II)

2.1 Dual labeled N1ECD peptides can be used to track and quantify endocytosed Jagged1

We expect that there are other proteins in addition to vimentin that can selectively regulate Notch ligands. Previous studies on Notch ligand endocytosis have been focused primarily on endocytosis of Delta and Delta-like ligands (Couturier and Schweisguth, 2014; Meloty-Kapella et al., 2012; Overstreet et al., 2004; Windler and Bilder, 2010). To facilitate identification of new regulators of Jagged endocytosis we wanted to design a method where we can track Notch ligand endocytosis and then use this setup to track Jagged1 specifically. We adapted a dual label setup of N1ECD peptides by first introducing primary amine NHS labels (555) and combining the pre-labeled N1ECD-555 peptides with Alexa Fluor 488 secondary antibody labeling as used previously (Study I). By quenching one label (488) at the surface after endocytosis, we now had a screening setup that could be used to quantify endocytosis by high-throughput plate readers and by confocal microscopy (II, Fig 2A) (Arjonen et al., 2012). Dual label N1ECD peptides ($N1ECD^{D}$) were incubated on ice with HEK-293 cells stably overexpressing Jagged1 (hereafter, 293-JAG). As expected, Jagged1 expression is drastically higher in these cells than other Notch ligands (II, Fig S1). A one hour incubation of $N1ECD^{D}$ peptides on ice followed by fixation shows $N1ECD^{D}$ bound to the surface of the cells and a strong overlap of 488 and 555 fluorophores (II, Fig 2B). Incubating the cells in 37 °C after attaching $N1ECD^{D}$ to cells on ice, allowed for endocytosis to resume in the cells. A 30 min incubation in 37 °C, followed by quenching of the Alexa Fluor 488 antibody at the cell membrane by an anti-Alexa 488 blocking antibody, effectively quenched green fluorescence while leaving red fluorescence unaffected at the cell membrane. This facilitated the distinction between endocytosed $N1ECD^{D}$, and $N1ECD^{555}$ on the cell membrane (II, Fig 2C, 3C). Omitting the endocytosis step with subsequent quenching of 488 at the cell membrane showed the effectiveness of the surface quenching (II, Fig 2D). As another control, the use of dynasore to block dynamin, completely abolished the endocytosis of $N1ECD^{D}$ peptides (II, Fig 2E). Dynamin is used in both clathrin dependent and clathrin independent endocytosis to pinch of vesicles at the cell membrane. In our imaging based proof of concept, the amount of endocytosis was quantified by automated image analysis using a ComDet 4.2 plugin in ImageJ (II, Fig 3B-C). Analysis of different

time points (0, 15, 30, 60 min) of endocytosis indicated that the amount of endocytosed N1ECD^{D} peptides peak at 15 or 30 minutes before decreasing or becoming harder to detect (II, Fig 3A-C). Comparison of this assay with an endocytosis assay based on biotinylated cell surface proteins that were immunoprecipitated by streptavidin and blotted for Jagged1 displayed similar kinetics as the image based assay, but with a slightly lower end point rate at 60 min (II, Fig 3B, D).

2.2 A cell spot microarray to identify new regulators of Jagged1

We then employed our N1ECD^{D}-based endocytosis assay in a cell spot microarray (CSMA) based on a Qiagen druggable genome library v1.0 (Arjonen et al., 2012; Pellinen et al., 2012; Rantala et al., 2011). This library includes siRNAs against genes that are considered targets for druggable treatment and include GPCRs, kinases, phosphatases and calcium channel receptors. The CSMA technique is based on reverse transfection, where the lipid-based transfection reagent and the siRNA are mixed with Matrigel and subsequently printed on array plates. CSMAs have several advantages for high-throughput screening (HTS) purposes compared to traditional plates. These include no well-to-well variation and less reagents needed (50 pg/spot siRNA vs 10 ng/well for 384 well plate). The arrays allow for multiparametric analysis and readouts through high-content imaging and microarray scanners (II, Fig 4A-B). Using a restricted cell adhesion time of 15 min allowed for adherence of 293-JAG cells to the array spots (II, Fig S2). After 48 hours of reverse transfection the cell arrays were incubated with N1ECD^{D} on ice, allowed to endocytose for 30 min, followed by quenching of Alexa Fluor 488 at the cell surface as described previously. Cells were then fixed and analyzed by laser microarray scanning (Tecan LS400). The CSMA technology was available through a collaboration with Professor Johanna Ivaskas group, where the same library and CSMA design had been used previously to identify regulators of β1 integrins (Pellinen et al., 2012; Rantala et al., 2011). This was our first application of the N1ECD^{D}-based assay in a high-throughput setting. As a consequence of the array being predesigned, it lacked some of the relevant positive controls for Notch endocytosis, including dynamin that we used earlier as our proof of concept. Nevertheless, we reasoned that this setup can give an interesting set of potential effector targets that can serve as a starting point for hits to be included and verified in smaller secondary screens in the future. Validation with individual siRNAs is crucial also when using all the appropriate controls. In a previous screen for active β1 integrin, with an

identical CSMA design, the secondary validation confirmed 23 out of 50 top hits, and 5 genes turned out to have the opposite effect on integrin activity compared to what was shown in the primary screen (Pellinen et al., 2012).

From our preliminary data we present 20 targets (individual siRNAs) that had the highest increase in $N1ECD^D$ endocytosis and 20 targets that had the highest decrease in $N1ECD^D$ endocytosis when knocked down (II, Fig 4C). Gene ontology (GO) analysis of clusters including a minimum of three genes from the same biological process (BP), molecular function (MF) or cellular component (CC) identified kinase activity (MF) as the most significant cluster of hits (II, Fig 4D). Other overrepresented clusters of targets that led to a reduction of $N1ECD^D$ internalization when knocked down included targets previously linked to endocytosis and intracellular signaling pathways (II, Fig 4D). These three clusters of genes with roles as positive regulators of endocytosis, receptor-mediated endocytosis and intracellular receptor signaling pathways indicate the validity of many of our top hits. This is promising, as the risk of false positives is high within a single screen. Targets that increased endocytosis in this assay formed less significant clusters with the most notable one being related to cell junction assembly (BP). This is not an unreasonable finding as Notch is often found in various cell junctions and has also been shown to be involved in their remodeling (Batchuluun et al., 2017; Benhra et al., 2011; Grammont, 2007; Hatakeyama et al., 2014; Sasaki et al., 2007). This cluster contains CDC-42, which is also important for clathrin-independent endocytosis (Ferreira and Boucrot, 2018). The highest individual hit upregulating endocytosis of N1ECD in our screen was PDZD2 (PDZ domain-containing intracellular PDZ protein 2). On the other hand, MAGI2, another PDZ domain-containing protein was a hit with a negative effect on endocytosis. MAGI2 has also been shown previously to interact with other Notch ligands (Pfister et al., 2003). In Study I, we speculate that the differential regulation of Jagged1 from Dll4 could be due to PDZ binding motif interactions with vimentin, as vimentin has previously found to interact to SCRBBL through its PDZ domain (Phua et al., 2009). This group of proteins require further validation as it may point to a player in Notch ligand regulation in both study I and within these preliminary hits in study II. With over 300 proteins containing a PDZ-domain, there are plenty of potential interaction studies in the future (Pintar et al., 2007). Within our research group we have recently generated Jagged1 constructs lacking the intracellular PDZ-binding motif to further study potential differences in interaction between Jagged1 and other proteins. Targets from the screen in study II that have been

shown to be related to Notch signaling activity or ligand endocytosis include at least the following: LRRK (Imai et al., 2015), HIPK4 (Lee et al., 2009), PAK4 (Santiago-Gómez et al., 2019), MAGI2 (Pfister et al., 2003), SNAI1 (Morel et al., 2003; Saad et al., 2010), MAML2 (Lin et al., 2002), PLG (Shimizu et al., 2011) and CDC-42 (Balklava et al., 2007). Further analysis is best performed on hits that are validated with more stringent requirements in upcoming secondary screens. During these screens and during validation with individual siRNAs we could also incorporate the agarose beads from study I to mimic the strain required for *trans*-endocytosis of Notch. This would then allow for identification of potential differences between constant endocytosis of ligands and Notch-activating *trans*-endocytosis.

3. Phosphorylation by PKCζ regulates Notch trafficking (III)

3.1 PKCζ interacts with and phosphorylates Notch1

Post-translational modifications such as phosphorylation increase the functional diversity of proteins. Phosphorylation is a reversible modification and can thereby function to deliver rapid control of protein and cellular function. Although technological advances have allowed for global profiling of the proteome and many Notch phosphorylation sites have been identified, only a number of sites have been linked to specific functional outcomes and kinases (Antfolk et al., 2019).

In study III, we investigated the role of atypical protein kinase C zeta (PKCζ). PKCζ has previously been shown to regulate differentiation in mammals (McCaffrey and Macara, 2009). We first used C2C12 mouse myoblasts to determine PKCζ-mediated effects on Notch. C2C12 cells are able to proliferate at a high rate under conditions of high serum but undergo differentiation to myoblasts under low serum conditions. We found that PKCζ interacts with endogenous Notch1 in both differentiated and undifferentiated C2C12 cells (III, Fig 2A). PKCζ also interacted with transfected Full Length Notch1 (FLN1) in C2C12 cells and a membrane-tethered active form of Notch1 (Notch1ΔE) in HeLa cells (III, Fig 2B-C). Moreover, blocking Notch S3 cleavage by a γ-secretase inhibitor (GSI) led to a slight increase in interaction (III, Fig 2B-C). We did not detect an interaction by IP when using an antibody that recognizes only cleaved NICD (III, Fig 2D) nor when transfecting NICD and using an antibody that recognizes both full length and cleaved forms of Notch (III, Fig 2D right and bottom). These data suggest that Notch interacts with PKCζ when Notch is tethered to the cell membrane, as GSI treatment results in more Notch1ΔE stuck at the membrane, hence more interaction, while transfected NICD is never localized to the cell membrane, resulting in no visible interaction. These findings are supported by specific features of atypical PKCs. Atypical PKCs (PKCζ and PKCι/λ) have a mutation in their C1 domain rendering them insensitive to DAG. The same mutation also changes the electric potential of the C1 domain leading to an increased interaction with the negatively charged plasma membrane (Pu et al., 2006).

3.1.1 S1791 is identified as a PKCζ phosphorylation site on Notch1

We found that immunoprecipitated Notch1 is phosphorylated *in vitro* by recombinant PKCζ, as shown by autoradiography of radioactive phosphorus-32-ATP (III, Fig 2E). To

identify PKCζ-mediated phosphorylation sites on Notch1, we performed mass spectrometry analysis of immunoprecipitated Notch1 that was phosphorylated *in vitro* by PKCζ (III, Fig S3A). Serine 1791 was identified as the predominant phosphorylation site on mouse Notch1 (III, Fig S3A) (Figure 11). Further analysis with atypical PKCι showed that both atypical PKC isoforms are capable of phosphorylating mNotch1 on S1791 (III, Fig S3B). The identified serine is conserved in many species including *Homo sapiens, Rattus norvegicus, Mus musculus*, *Xenopus laevis* and *Danio rerio*, but not in *Drosophila melanogaster* or *Caenorhabditis elegans* (III, Fig S4).

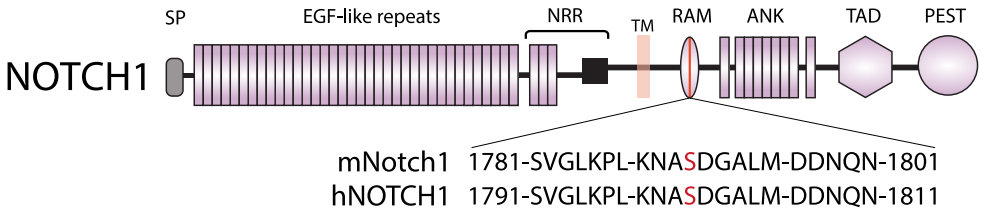

Figure 11. S1791 is phosphorylated on Notch1. Mass spectrometry identified S1791 in the intracellular domain of mNotch1 as the predominant phosphorylation site of atypical PKCζ (zeta) and ι (iota). S1791 on mouse Notch1 corresponds to S1801 on human NOTCH1.

3.2 PKCζ increases Notch activation

Transfection of a constitutively active form of PKCζ (caPKCζ) led to an increase in Notch activation in Notch reporter assays based on 12xCSL-Luc constructs, where Full Length Notch (FLN) overexpressing cells were activated by immobilized ligands (III, Fig 3A). Similarly, caPKCζ increased the reporter signal from transfected Notch1ΔE (III, Fig 3B). In contrast, caPKCζ had no effect on transfected NICD in these cells (III, Fig 3C). These results are in agreement with the lack of interaction between PKCζ and NICD (III, Fig 2D). Moreover, caPKCζ did not enhance the low reporter activity measured from mutant Notch1ΔE^{K1749R} (III, Fig 3D). The point mutation of K1749R has previously been shown to generate highly unstable forms of NICD, which are rapidly degraded before any activation of transcription can occur (Tagami et al., 2008). Our results are consistent with this model. Transfecting caPKCζ or caPKCι in different concentrations together with Notch1ΔE also led to a dose dependent increase in NICD levels (III, Fig 3E).

3.2.1 Site directed mutagenesis of S1791

To determine the specific role of the identified aPKC phosporylation site S1791, we employed site directed mutagenesis to generate phosphorylation-deficient and phosporylation-mimicking forms of Notch1ΔE. In the phospho-deficient form, the serine at 1791 was substituted for alanine (Notch1ΔE^{S1791A}), and in the mimicking form for glutamic acid (Notch1ΔE^{S1791E}) (III, Fig 4A) (Figure 12). We also generated a phospho-mimicking form of full-length Notch, Notch1FLN^{S1791E} (III, Fig 4A).

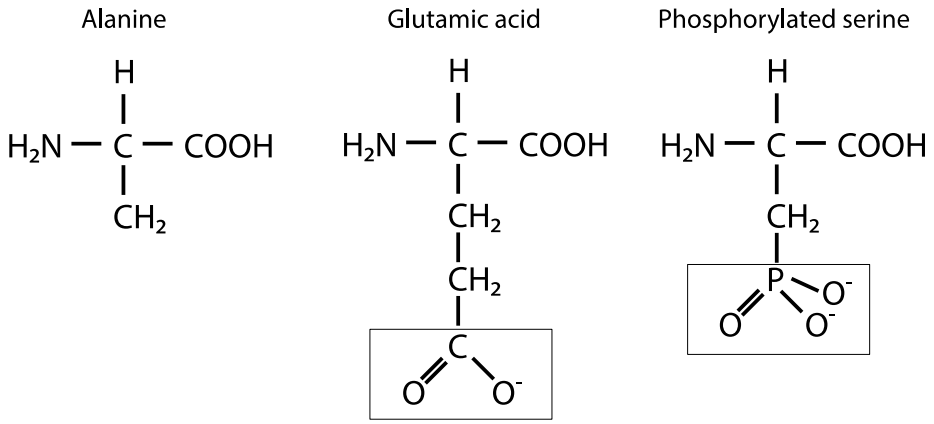

Figure 12. Mutations of S1791. Substituting the serine 1791 for an alanine residue, renders the S1791 site unavailable for phosphorylation resulting in a phospho-deficient or phospho-dead mutant. Glutamic acid (E) has a negatively charged side group, which can mimic some of the functions of a phosphorylated serine in the cell.

Transfecting WT, 1791A and 1791E mutant versions of Notch1ΔE and blocking S3 cleavage by GSI show equal amounts of Notch1ΔE from the different constructs, and low levels of NICD as expected when inhibiting S3 cleavage (III, Fig 4B upper). Without GSI, expression of Notch1ΔE^{S1791E} resulted in increased levels of NICD compared to WT, while expression of Notch1ΔE^{S1791A} resulted in lower NICD levels than WT (III, Fig 4B lower). 12xCSL-Luc reporter assays support these findings and show enhanced signaling from Notch1ΔE^{S1791E} and decreased signaling from Notch1ΔE^{S1791A} (III, Fig 4C). Lower reporter activation from Notch1ΔE^{S1791A} could not be compensated or increased by caPKCζ (III, Fig 4D). On the contrary, we found that expression of caPKCζ resulted in even lower activity from Notch1ΔE^{S1791A} (III, Fig 4D). This could be due to downregulation of endogenous Notch upon transfection with Notch1ΔE forms, or direct or

indirect repressive effects of PKCζ on Notch when S1791 is not available for phosphorylation. Expression of the Notch1FLN^{S1791E} mutant produced higher reporter activity when activated by immobilized ligands, which is in agreement with the Notch1ΔE^{S1791E} results (III, Fig 4E-F). The increase in NICD levels were not due to different degradation rates of NICD as determined by cyclohexamide chase experiments, where we compared NICD levels at 0, 3 and 7 hours of cyclohexamide treatment (III, Fig S8A).

3.3 PKCζ affects trafficking of Notch1

3.3.1 Internalization of activated Notch receptors

Immunostaining of Notch1ΔE, Notch1ΔE^{S1791A} and Notch1ΔE^{S1791E} indicated that Notch1ΔE^{S1791E} and Notch1ΔE localize more to the nucleus compared to the phospho-deficient Notch1ΔE^{S1791A} (III, Fig 5A). Western blotting of nuclear extracts also pointed to increased levels of NICD from Notch1ΔE^{S1791E}, and lower levels from Notch1ΔE^{S1791A}, compared to Notch1ΔE (III, Fig 5B). Our experiments further showed increased nuclear staining of Notch in different cell lines when caPKCζ was expressed together with Notch1ΔE (III, Fig 3H-I). The use of a dominant negative PKCζ mutant (dnPKCζ), with a defective catalytic domain showed an opposite effect on Notch, with less nuclear Notch reactivity and no increase in NICD production (III, Fig 3F, 3H-I). This result indicates that the kinase activity of PKCζ is important for the observed effects on Notch signaling.

All three forms of Notch1ΔE interacted with Rab5 and Rab7, representing early and late endosomal localizations, respectively. The interaction between the phospho-mimetic Notch1ΔE^{S1791E} and Rab7 was slightly decreased, indicating that this form of Notch1ΔE may be released from the late endosomes or from early endosomes during its transition to late endosomes. This implies that less Notch/NICD is being transferred to lysosomes (III, Fig 5D-E). Moreover, downregulation of PKCζ by siRNA increased the interaction between Notch and lysosomal associated protein LAMP-1 (III, Fig 5G). A similar result was obtained when PKCζ was inhibited by a pseudosubstrate, which instead led to strong co-localization between Notch1 and LAMP-1 (III, Fig 5H).

In conclusion, these results suggest that when PKCζ is active or a phospho-mimicking mutation of S1791 is used, the amount of NICD generated is increased (III, Fig 3E, 4B

lower) and more active Notch is translocated to the nucleus (III, Fig 3H-I, 5A-B) leading to higher Notch activity (III, Fig 4C, E-F). Conversely, when PKCζ is inhibited more Notch is shifted through late endosomes to lysosomes, leading to a degradation of Notch, resulting in lower observed levels of NICD (III, Fig 3E-F, I, 4B lower, 5B) and decreased Notch signaling activity (III, Fig 4C-D).

3.3.2 Recycling of inactive Notch receptors

The results from previous sections describe the impact of PKCζ on activated Notch receptors. Although our study focused on activated receptors, we made some interesting observations on the effects of PKCζ on non-active receptors. When Notch receptors are inactive by the lack of cell-cell interactions in sparsely cultured cells without immobilized ligand activation, PKCζ does not promote higher levels of NICD or signaling activity. In these cells, caPKCζ expression results in an initial shift of Notch from the cell membrane to intracellular vesicles (III, Fig 7A-C). In contrast to activated receptors, this transfer did not lead to a higher NICD production (III, Fig 7D). Instead we observed an increase in the ubiquitination of Notch (III, Fig 7E) and an accompanied increase in interaction between Notch1 and the ubiquitin binding protein Hrs, which functions as an endosomal sorting protein (III, Fig 7F). The internalization, ubiquitination, and endosomal sorting was not linked to an increased degradation of Notch (III, Fig 7G). Our best explanation is that non-activated receptors are recycled back to the cell membrane supported by other reports describing constant recycling to and from the cell membrane (Johnson et al., 2016; McGill et al., 2009; Yamamoto et al., 2010).

3.4 S1791 regulates myogenic differentiation *in vitro* and PKCζ regulates neuronal differentiation *in vivo*

We then decided to examine if PKCζ inhibition can affect the differentiation in our C2C12 mouse myoblast cell line and in primary myoblasts, as myogenic differentiation has been previously shown to be regulated by Notch signaling (Buas and Kadesch, 2010; Nofziger et al., 1999). We used the myosin heavy chain (MHC) as a marker for myogenic differentiation. Primary mouse myoblasts treated with pseudosubstrate show significantly higher differentiation rates compared to cells treated with scrambled control (III, Fig 6 A-B). Similarly, down regulation of PKCζ by siRNA also resulted in earlier differentiation of C2C12 cells and conversely, expression of caPKCζ resulted in a delayed differentiation

(III, Fig 6C-D). Expression of Notch1ΔE^{S1791A} further showed that 71% of cells expressing the phospho-deficient S1791A were positive for MHC compared to only 14% for Notch1ΔE after 72 h of differentiation (III, Fig 6E).

Finally we employed a previously used model to study differentiation of the developing chick central nervous system (CNS) (Holmberg et al., 2008). We used an engineered Notch1ΔE construct with an incorporated myc-tag, and an IRES-EGFP to track expression of nuclear Notch. We first tested the CAG-Notch1ΔE-Myc-IRES-EGFP construct by expressing it together with a 12xCSL-dsRED reporter construct and confirmed strong activation of Notch signaling (III, Fig 1A-C). We then co-expressed CAG-Notch1ΔE-Myc-IRES-EGFP with a myristylated pseudosubstrate to inhibit PKCζ in stage 10 chick embryos (Hamburger Hamilton stage 10). We looked at the amount of Tuj-1 (neuronal lineage marker) positive neuronal cells. Expressing the Notch construct on one side of the neural tube led to a significant reduction in Tuj-1 positive cells as compared to the other side of the neural tube without the active Notch construct (III, Fig 1D, F). This is in accordance with the established function of Notch as an inhibitor of differentiation in the CNS (Borghese et al., 2010; Holmberg et al., 2008; Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005). When we then used the pseudosubstrate inhibitor of PKCζ together with the Notch construct, we identified more Notch1 positive cells (tracked by EGFP) in the marginal zone of the neural tube, with cells being positive for both Notch1 and Tuj-1 (III, Fig 1E). This suggests that the cells were able to undergo differentiation despite the high Notch1 expression from the active construct. Our analysis also revealed differences in intracellular localization of Notch1 when we examined Notch immunoreactivity through the incorporated myc-tag. Notch expression without the PKCζ inhibitor produced both nuclear and cytoplasmic Notch-myc reactivity, but addition of the PKCζ inhibitor reduced the nuclear staining (III, Fig S1A-B). We also quantified the ratio of nuclear to cytoplasmic myc reactivity, which indicated that approximately half as many cells show nuclear reactivity during PKCζ inhibition (III, Fig 1G-I).

Our results on PKCζ-mediated effects on differentiation are in agreement with a previous study, which also identified PKCζ as a suppressor of neuronal differentiation (Ossipova et al., 2009). Ossipova and colleagues proposed that PKCζ excerts its effects on neurogenesis through Notch by linking PKCζ-mediated regulation of PAR-1 to critical regulation of the E3 ligase Mib. This is another possible function of PKCζ-mediated

regulation. However, studies with PAR-1 knock out mice show no Notch-related phenotypes (Hurov et al., 2001). We suggest a more direct regulation of Notch by PKCζ, which is most likely determining the outcome of Notch in combination with other effects of PKCζ phosphorylation, such as general regulation of endocytosis or the aforementioned regulation of Mib. It is likely that PKCζ can regulate endocytosis in other ways to internalize Notch receptors independently of S1791 when caPKCζ is expressed, but perhaps phosphorylation of S1791 further specifies the Notch signaling outcome. This would explain why Notch1ΔE^{S1791A} in some settings could be downregulated by caPKCζ (III, Fig 2D) if some parts of the general PKCζ-mediated internalized endosomes are targeted for degradation. Again, actin or vimentin may be involved in the mechanisms of influencing the general endocytosis through PKCs. At least one novel PKC isoform has been shown to influence the generation of a large actin-based lamellum allowing for efficient Notch cleavage by ADAM10 (Britton et al., 2017). Regulation of vimentin by PKCs has been previously documented to affect integrin recycling (Ivaska et al., 2005) and more recently, both atypical isoforms PKCζ and PKCι have been shown to influence vimentin assembly (Ratnayake et al., 2018).

CONCLUDING REMARKS

During the last few decades many key aspects of Notch signaling have been described in both development and disease. Still researchers struggle to understand the context-dependent output of Notch. The aim of this thesis has been to provide new insights into the regulation of the Notch signaling pathway. These aims involve phosphorylation of Notch and endocytosis of Jagged ligands. In our first study, Jagged1 ligands accumulated at the surface of cells lacking vimentin, but with a compromised signal sending ability. Vimentin interacts with the intracellular domain of Jagged1, but not with Dll4 as demonstrated by swapping the intracellular domain of Jagged1 to Dll4-ICD, where the interaction was lost. We then studied the vimentin-Jagged1 axis in the context of angiogenesis. A lack of vimentin negatively affected the ability of endothelial cells to form angiogenic sprouts. Addition of external Jagged1 ligands rescued the angiogenic defects of vimentin ablation, which also challenges the prevailing argument that the main role of Jagged1 in angiogenesis is to inhibit more potent Dll4-Notch signaling. Recent studies on tip cell dynamics combined with our study highlight a more complex role for Jagged1 during angiogenesis, where it might not be low Notch that drives initial tip cell sprouting but that Jagged1 and Dll4 may activate Notch in distinct ways. Our findings further add to the important roles of intermediate filaments as signaling modulators and the interplay between vimentin and Jagged1 may also be amenable to therapeutic intervention in areas such as tumor angiogenesis and metastasis. Future studies will likely determine how and which Notch ligands can be regulated by IFs. With keratin IFs recently shown to interact with Notch receptors (Lähdeniemi et al., 2017), these studies can easily be extended to Notch receptors as well.

Accumulating evidence point to deregulated Jagged1 in several different cancers, with an overexpression of JAGGED1 associated with increased metastasis and poor survival in women with breast cancer (Bednarz-Knoll et al., 2016; Sethi et al., 2011). Similarly, higher expression of JAGGED1 has been identified in prostate cancer patients with especially aggressive tumors (Zhu et al., 2013). Our dual label approach to track endocytosis of Notch ligands in study II should allow us to identify and validate other new Jagged1 modulators in the future. In addition, the hits from the cell spot microarray pilot screen have given us a short list of targets to include in our upcoming efforts to detail Notch ligand endocytosis.

Finally, we identify atypical protein kinase C zeta (PKCζ) as a regulator of Notch trafficking and activation. PKCζ interacts with membrane-tethered Notch1 receptors and phosphorylates S1791 in the intracellular domain of the receptor. The signaling from active Notch receptors is further enhanced by PKCζ, leading to relocation of more NICD to the nucleus and higher activation in reporter assays. When PKCζ was blocked, we found that the opposite was true, and observed less Notch signaling activity, less NICD in the nucleus, and a relocation of Notch to late endosomes. We then generated phospho-deficient (S1791A) and phospho-mimicking (S1791E) mutants of active Notch1ΔE. In agreement with our previous findings, expression of the phospho-mimicking Notch1ΔE^{1791E} led to increased signaling and NICD with more Notch localized to the nucleus, while the phospho-deficient Notch1ΔE^{1791A} led to lower signaling, less NICD and more Notch localized in intracellular vesicles. We also report that blockage of PKCζ leads to more neuronal differentiation *in vivo* in the developing chicken spinal cord. Corroborating these results we found that C2C12 cells expressing phospho-deficient Notch1ΔE^{1791A} consistently differentiated to myotubes. These results suggest that phosphorylation by PKCζ can influence differentiation through Notch1.

Taken together, this thesis shows for the first time an interaction between the intermediate filament vimentin and Jagged1 in the signal sending cell as well as PKCζ and Notch1 in the signal receiving cell (summarized in figure 13). These results also provide insight into the regulation of Notch during angiogenesis and differentiation. Combining the data from these studies paint a picture of a potential network between PKCζ, vimentin, Jagged and Notch. Determining the interplay between these components should provide for plenty of research opportunities in the future.

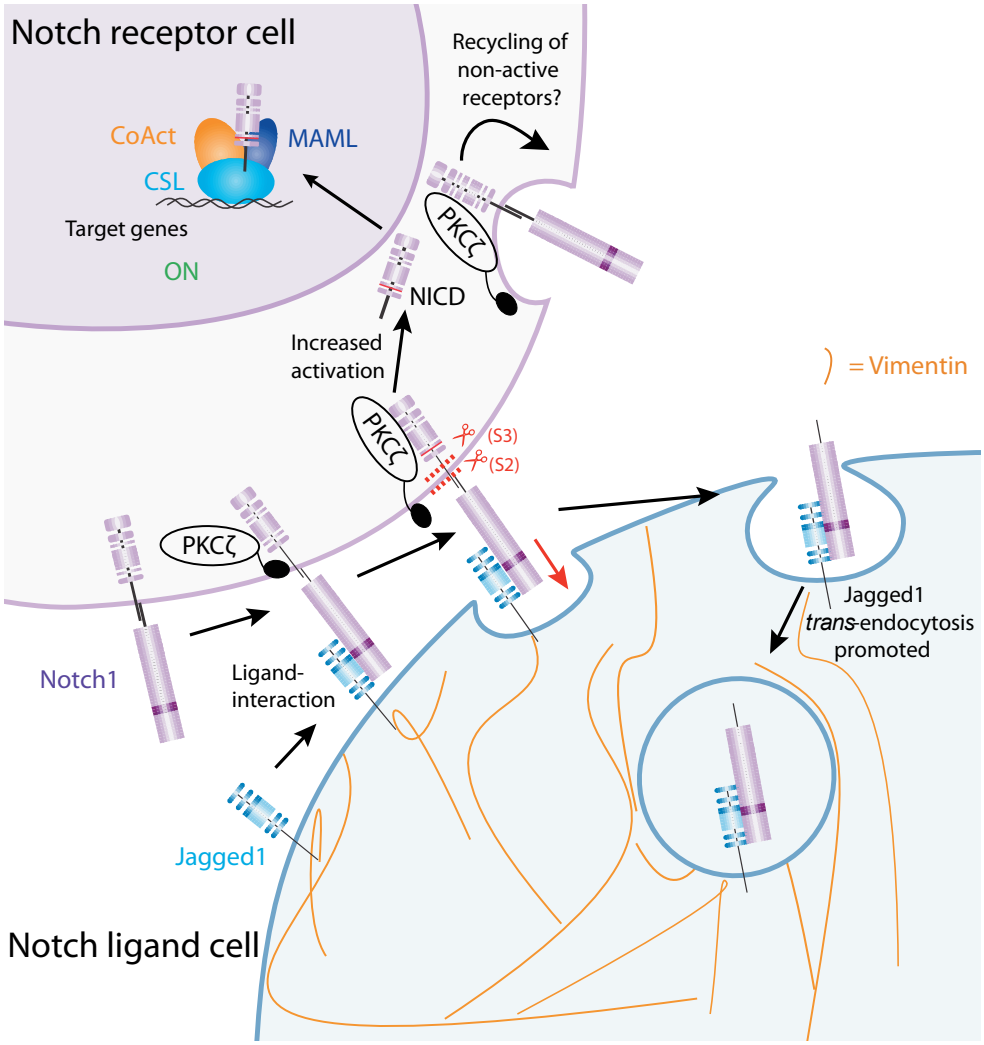

Figure 13. Schematic of the Notch signaling pathway and the main findings in this thesis. Vimentin promotes force generation and *trans*-activation potential of Jagged1 in the signal-sending cell (Notch ligand cell). In the signal-receiving cell, PKCζ interacts with membrane-bound Notch1 and phosphorylates S1791 in the intracellular domain of Notch1, which further enhances active Notch signaling. When Notch is inactive, PKCζ leads to an initial internalization of Notch receptors but without an increase in either signaling or degradation. The majority of these receptors may be recycled back to the cell membrane.

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REFERENCES

Acosta, H., López, S.L., Revinski, D.R., Carrasco, A.E., 2011. Notch destabilises maternal beta-catenin and restricts dorsal-anterior development in Xenopus. Development 138, 2567–79. https://doi.org/10.1242/dev.061143

Alam, M.S., 2018. Proximity Ligation Assay (PLA). Curr. Protoc. Immunol. 123, e58. https://doi.org/10.1002/cpim.58

Alanko, J., Mai, A., Jacquemet, G., Schauer, K., Kaukonen, R., Saari, M., Goud, B., Ivaska, J., 2015. Integrin endosomal signalling suppresses anoikis. Nat. Cell Biol. 17, 1412–1421. https://doi.org/10.1038/ncb3250

Ali, S.A., Justilien, V., Jamieson, L., Murray, N.R., Fields, A.P., 2016. Protein Kinase Cι Drives a NOTCH3-dependent Stem-like Phenotype in Mutant KRAS Lung Adenocarcinoma. Cancer Cell 29, 367–378. https://doi.org/10.1016/j.ccell.2016.02.012

Andersen, P., Uosaki, H., Shenje, L.T., Kwon, C., 2012. Non-canonical Notch signaling: emerging role and mechanism. Trends Cell Biol. 22, 257–265. https://doi.org/10.1016/j.tcb.2012.02.003

Andersson, E.R., Lendahl, U., 2014. Therapeutic modulation of Notch signalling--are we there yet? Nat. Rev. Drug Discov. 13, 357–78. https://doi.org/10.1038/nrd4252

Andersson, E.R., Sandberg, R., Lendahl, U., 2011. Notch signaling: simplicity in design, versatility in function. Development 138, 3593–612. https://doi.org/10.1242/dev.063610

Antal, C.E., Hudson, A.M., Kang, E., Zanca, C., Wirth, C., Stephenson, N.L., Trotter, E.W., Gallegos, L.L., Miller, C.J., Furnari, F.B., Hunter, T., Brognard, J., Newton, A.C., 2015. Cancer-Associated Protein Kinase C Mutations Reveal Kinase's Role as Tumor Suppressor. Cell 160, 489–502. https://doi.org/10.1016/j.cell.2015.01.001

Antfolk, D., Antila, C., Kemppainen, K., Landor, S.K.-J., Sahlgren, C., 2019. Decoding the PTM-switchboard of Notch. Biochim. Biophys. Acta - Mol. Cell Res. 118507. https://doi.org/10.1016/j.bbamcr.2019.07.002

Aquila, G., Kostina, A., Vieceli Dalla Sega, F., Shlyakhto, E., Kostareva, A., Marracino, L., Ferrari, R., Rizzo, P., Malaschicheva, A., 2019. The Notch pathway: a novel therapeutic target for cardiovascular diseases? Expert Opin. Ther. Targets 23, 695–710. https://doi.org/10.1080/14728222.2019.1641198

Arjonen, A., Alanko, J., Veltel, S., Ivaska, J., 2012. Distinct recycling of active and inactive β1 integrins. Traffic 13, 610–25. https://doi.org/10.1111/j.1600-0854.2012.01327.x

Arroyo, A.G., Iruela-Arispe, M.L., 2010. Extracellular matrix, inflammation, and the angiogenic response. Cardiovasc. Res. 86, 226–235. https://doi.org/10.1093/cvr/cvq049

Artavanis-Tsakonas, S., Muskavitch, M.A., Yedvobnick, B., 1983. Molecular cloning of Notch, a locus affecting neurogenesis in Drosophila melanogaster. Proc. Natl. Acad. Sci. 80, 1977–1981. https://doi.org/10.1073/pnas.80.7.1977

Artavanis-Tsakonas, S., Muskavitch, M.A.T., 2010. Notch: The Past, the Present, and the Future. pp. 1–29. https://doi.org/10.1016/S0070-2153(10)92001-2

Aster, J.C., Pear, W.S., Blacklow, S.C., 2017. The Varied Roles of Notch in Cancer. Annu. Rev. Pathol. Mech. Dis. 12, 245–275. https://doi.org/10.1146/annurev-pathol-052016-100127

Avila, J.L., Kissil, J.L., 2013. Notch signaling in pancreatic cancer: oncogene or tumor suppressor? Trends Mol. Med. 19, 320–327. https://doi.org/10.1016/j.molmed.2013.03.003

Ayaz, F., Osborne, B.A., 2014. Non-Canonical Notch Signaling in Cancer and Immunity. Front. Oncol. 4. https://doi.org/10.3389/fonc.2014.00345

Aydin, I.T., Melamed, R.D., Adams, S.J., Castillo-Martin, M., Demir, A., Bryk, D., Brunner, G., Cordon-Cardo, C., Osman, I., Rabadan, R., Celebi, J.T., 2014. FBXW7 Mutations in Melanoma and a New Therapeutic Paradigm. JNCI J. Natl. Cancer Inst. 106, dju107. https://doi.org/10.1093/jnci/dju107

Baker, M., Robinson, S.D., Lechertier, T., Barber, P.R., Tavora, B., D'Amico, G., Jones, D.T., Vojnovic, B., Hodivala-Dilke, K., 2012. Use of the mouse aortic ring assay to study angiogenesis. Nat. Protoc. 7, 89–104. https://doi.org/10.1038/nprot.2011.435

Balklava, Z., Pant, S., Fares, H., Grant, B.D., 2007. Genome-wide analysis identifies a general requirement for polarity proteins in endocytic traffic. Nat. Cell Biol. 9, 1066–1073. https://doi.org/10.1038/ncb1627

Bargagna-Mohan, P., Paranthan, R.R., Hamza, A., Zhan, C.-G., Lee, D.-M., Kim, K.B., Lau, D.L., Srinivasan, C., Nakayama, K., Nakayama, K.I., Herrmann, H., Mohan, R., 2012. Corneal Antifibrotic Switch Identified in Genetic and Pharmacological Deficiency of Vimentin. J. Biol. Chem. 287, 989–1006. https://doi.org/10.1074/jbc.M111.297150

Barsi, J.C., Rajendra, R., Wu, J.I., Artzt, K., 2005. Mind bomb1 is a ubiquitin ligase essential for mouse embryonic development and Notch signaling. Mech. Dev. 122, 1106–1117. https://doi.org/10.1016/j.mod.2005.06.005

Basmanav, F.B., Oprisoreanu, A.M., Pasternack, S.M., Thiele, H., Fritz, G., Wenzel, J., Größer, L., Wehner, M., Wolf, S., Fagerberg, C., Bygum, A., Altmüller, J., Rütten, A., Parmentier, L., El Shabrawi-Caelen, L., Hafner, C., Nürnberg, P., Kruse, R., Schoch, S., Hanneken, S., Betz, R.C., 2014. Mutations in POGLUT1, encoding protein o-glucosyltransferase 1, cause autosomal-dominant dowling-degos disease. Am. J. Hum. Genet. 94, 135–143. https://doi.org/10.1016/j.ajhg.2013.12.003

Batchuluun, K., Azuma, M., Yashiro, T., Kikuchi, M., 2017. Notch signaling-mediated cell-to-cell interaction is dependent on E-cadherin adhesion in adult rat anterior pituitary. Cell Tissue Res. https://doi.org/10.1007/s00441-016-2540-5

Battaglia, R.A., Delic, S., Herrmann, H., Snider, N.T., 2018. Vimentin on the move: new developments in cell migration. F1000Research 7, 1796. https://doi.org/10.12688/f1000research.15967.1

Becam, I., Fiuza, U.-M., Arias, A.M., Milán, M., 2010. A role of receptor Notch in ligand cis-inhibition in Drosophila. Curr. Biol. 20, 554–60. https://doi.org/10.1016/j.cub.2010.01.058

Bednarz-Knoll, N., Efstathiou, A., Gotzhein, F., Wikman, H., Mueller, V., Kang, Y., Pantel, K., 2016. Potential Involvement of Jagged1 in Metastatic Progression of Human Breast Carcinomas. Clin. Chem. 62, 378–386. https://doi.org/10.1373/clinchem.2015.246686

Benedito, R., Hellström, M., 2013. Notch as a hub for signaling in angiogenesis. Exp. Cell Res. 319, 1281–1288. https://doi.org/10.1016/j.yexcr.2013.01.010

Benedito, R., Roca, C., Sörensen, I., Adams, S., Gossler, A., Fruttiger, M., Adams, R.H., 2009. The Notch Ligands Dll4 and Jagged1 Have Opposing Effects on Angiogenesis. Cell 137, 1124–1135. https://doi.org/10.1016/j.cell.2009.03.025

Benedito, R., Rocha, S.F., Woeste, M., Zamykal, M., Radtke, F., Casanovas, O., Duarte, A., Pytowski, B., Adams, R.H., 2012. Notch-dependent VEGFR3 upregulation allows angiogenesis without VEGF–VEGFR2 signalling. Nature 484, 110–114. https://doi.org/10.1038/nature10908

Benhra, N., Lallet, S., Cotton, M., Le Bras, S., Dussert, A., Le Borgne, R., 2011. AP-1 controls the trafficking of notch and sanpodo toward E-cadherin junctions in sensory organ precursors. Curr. Biol. https://doi.org/10.1016/j.cub.2010.12.010

Benhra, N., Vignaux, F., Dussert, A., Schweisguth, F., Le Borgne, R., 2010. Neuralized Promotes Basal to Apical Transcytosis of Delta in Epithelial Cells. Mol. Biol. Cell 21, 2078–2086. https://doi.org/10.1091/mbc.e09-11-0926

Beres, T.M., Masui, T., Swift, G.H., Shi, L., Henke, R.M., MacDonald, R.J., 2006. PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L. Mol. Cell. Biol. 26, 117–30. https://doi.org/10.1128/MCB.26.1.117-130.2006

Bezanilla, M., Gladfelter, A.S., Kovar, D.R., Lee, W.-L., 2015. Cytoskeletal dynamics: A view from the membrane. J. Cell Biol. 209, 329–337. https://doi.org/10.1083/JCB.201502062

Blanco, R., Gerhardt, H., 2013. VEGF and Notch in Tip and Stalk Cell Selection. Cold Spring Harb. Perspect. Med. 3, a006569–a006569. https://doi.org/10.1101/cshperspect.a006569

Blaumueller, C.M., Qi, H., Zagouras, P., Artavanis-Tsakonas, S., 1997. Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell 90, 281–91.

Boareto, M., Jolly, M.K., Ben-Jacob, E., Onuchic, J.N., 2015. Jagged mediates differences in normal and tumor angiogenesis by affecting tip-stalk fate decision. Proc. Natl. Acad. Sci. U. S. A. 112, E3836-44. https://doi.org/10.1073/pnas.1511814112

Bollong, M.J., Pietilä, M., Pearson, A.D., Sarkar, T.R., Ahmad, I., Soundararajan, R., Lyssiotis, C.A., Mani, S.A., Schultz, P.G., Lairson, L.L., 2017. A vimentin binding small molecule leads to mitotic disruption in mesenchymal cancers. Proc. Natl. Acad. Sci. U. S. A. 114, E9903–E9912. https://doi.org/10.1073/pnas.1716009114

Boraas, L.C., Ahsan, T., 2016. Lack of vimentin impairs endothelial differentiation of embryonic stem cells. Sci. Rep. 6, 30814. https://doi.org/10.1038/srep30814

Borggrefe, T., Lauth, M., Zwijsen, A., Huylebroeck, D., Oswald, F., Giaimo, B.D., 2016. The Notch intracellular domain integrates signals from Wnt, Hedgehog, TGFβ/BMP and hypoxia pathways. Biochim. Biophys. Acta - Mol. Cell Res. 1863, 303–313. https://doi.org/10.1016/j.bbamcr.2015.11.020

Borggrefe, T., Oswald, F., 2009. The Notch signaling pathway: transcriptional regulation at Notch target genes. Cell. Mol. Life Sci. 66, 1631–46. https://doi.org/10.1007/s00018-009-8668-7

Borghese, L., Dolezalova, D., Opitz, T., Haupt, S., Leinhaas, A., Steinfarz, B., Koch, P., Edenhofer, F., Hampl, A., Brüstle, O., 2010. Inhibition of Notch Signaling in Human Embryonic Stem Cell-Derived Neural Stem Cells Delays G1/S Phase Transition and Accelerates Neuronal Differentiation In Vitro and In Vivo. Stem Cells 28, 955–964. https://doi.org/10.1002/stem.408

Bozkulak, E.C., Weinmaster, G., 2009. Selective use of ADAM10 and ADAM17 in activation of Notch1 signaling. Mol. Cell. Biol. 29, 5679–95. https://doi.org/10.1128/MCB.00406-09

Bray, S., Bernard, F., 2010. Notch Targets and Their Regulation, in: Current Topics in Developmental Biology. pp. 253–275. https://doi.org/10.1016/S0070-2153(10)92008-5

Bray, S.J., 2016. Notch signalling in context. Nat. Rev. Mol. Cell Biol. 17, 722–735. https://doi.org/10.1038/nrm.2016.94

Bray, S.J., 2006. Notch signalling: a simple pathway becomes complex. Nat. Rev. Mol. Cell Biol. 7, 678–689. https://doi.org/10.1038/nrm2009

Britannica, E., 2019. Giant cell | Britannica.com [WWW Document]. URL https://www.britannica.com/science/giant-cell (accessed 12.31.19).

Britton, G.J., Ambler, R., Clark, D.J., Hill, E. V, Tunbridge, H.M., McNally, K.E., Burton, B.R., Butterweck, P., Sabatos-Peyton, C., Hampton-O'Neil, L.A., Verkade, P., Wülfing, C., Wraith, D.C., 2017. PKCθ links proximal T cell and Notch signaling through localized regulation of the actin cytoskeleton. Elife 6. https://doi.org/10.7554/eLife.20003

Brückner, K., Perez, L., Clausen, H., Cohen, S., 2000. Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. Nature 406, 411–5. https://doi.org/10.1038/35019075

Buas, M.F., Kadesch, T., 2010. Regulation of skeletal myogenesis by Notch. Exp. Cell Res. 316, 3028–33. https://doi.org/10.1016/j.yexcr.2010.05.002

Buehler, M.J., 2013. Mechanical players-The role of intermediate filaments in cell mechanics and organization. Biophys. J. 105, 1733–4. https://doi.org/10.1016/j.bpj.2013.08.050

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., Nagy, A., 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380, 435–439. https://doi.org/10.1038/380435a0

Carrieri, F.A., Murray, P.J., Ditsova, D., Ferris, M.A., Davies, P., Dale, J.K., 2019. CDK1 and CDK2 regulate NICD1 turnover and the periodicity of the segmentation clock. EMBO Rep. 20, e46436. https://doi.org/10.15252/embr.201846436

Castel, D., Mourikis, P., Bartels, S.J.J., Brinkman, A.B., Tajbakhsh, S., Stunnenberg, H.G., 2013. Dynamic binding of RBPJ is determined by Notch signaling status. Genes Dev. 27, 1059–71. https://doi.org/10.1101/gad.211912.112

Chapman, G., Sparrow, D.B., Kremmer, E., Dunwoodie, S.L., 2011. Notch inhibition by the ligand Delta-Like 3 defines the mechanism of abnormal vertebral segmentation in spondylocostal dysostosis. Hum. Mol. Genet. 20, 905–916. https://doi.org/10.1093/hmg/ddq529

Chastagner, P., Israël, A., Brou, C., 2008. AIP4/Itch Regulates Notch Receptor Degradation in the Absence of Ligand. PLoS One 3, e2735. https://doi.org/10.1371/journal.pone.0002735

Chen, H., Fre, S., Slepnev, V.I., Capua, M.R., Takei, K., Butler, M.H., Di Fiore, P.P., De Camilli, P., 1998. Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. Nature 394, 793–797. https://doi.org/10.1038/29555

Chen, H., Ko, G., Zatti, A., Di Giacomo, G., Liu, L., Raiteri, E., Perucco, E., Collesi, C., Min, W., Zeiss, C., De Camilli, P., Cremona, O., 2009. Embryonic arrest at midgestation and disruption of Notch signaling produced by the absence of both epsin 1 and epsin 2 in mice. Proc. Natl. Acad. Sci. U. S. A. 106, 13838–43. https://doi.org/10.1073/pnas.0907008106

Chen, J., Zhang, M., 2013. The Par3/Par6/aPKC complex and epithelial cell polarity. Exp. Cell Res. 319, 1357–1364. https://doi.org/10.1016/j.yexcr.2013.03.021

Cheng, F., Shen, Y., Mohanasundaram, P., Lindström, M., Ivaska, J., Ny, T., Eriksson, J.E., 2016. Vimentin coordinates fibroblast proliferation and keratinocyte differentiation in wound healing via TGF-β–Slug signaling. Proc. Natl. Acad. Sci. 113, E4320–E4327. https://doi.org/10.1073/pnas.1519197113

Chillakuri, C.R., Sheppard, D., Lea, S.M., Handford, P.A., 2012. Notch receptor–ligand binding and activation: Insights from molecular studies. Semin. Cell Dev. Biol. 23, 421–428. https://doi.org/10.1016/j.semcdb.2012.01.009

Chitnis, A., 2009. Notch Pathway: Lateral Inhibition. Encycl. Neurosci. 1249–1257. https://doi.org/10.1016/B978-008045046-9.01027-5

Chowdhury, F., Li, I.T.S., Ngo, T.T.M., Leslie, B.J., Kim, B.C., Sokoloski, J.E., Weiland, E., Wang, X., Chemla, Y.R., Lohman, T.M., Ha, T., 2016. Defining Single Molecular Forces Required for Notch Activation Using Nano Yoyo. Nano Lett. 16, 3892–7. https://doi.org/10.1021/acs.nanolett.6b01403

Cohen, I., Silberstein, E., Perez, Y., Landau, D., Elbedour, K., Langer, Y., Kadir, R., Volodarsky, M., Sivan, S., Narkis, G., Birk, O.S., 2014. Autosomal recessive Adams-Oliver syndrome caused by homozygous mutation in EOGT, encoding an EGF domain-specific O-GlcNAc transferase. Eur. J. Hum. Genet. 22, 374–378. https://doi.org/10.1038/ejhg.2013.159

Colucci-Guyon, E., Giménez Y Ribotta, M., Maurice, T., Babinet, C., Privat, A., 1999. Cerebellar defect and impaired motor coordination in mice lacking vimentin. Glia 25, 33–43.

Colucci-Guyon, E., Portier, M.-M., Dunia, I., Paulin, D., Pournin, S., Babinet, C., 1994. Mice lacking vimentin develop and reproduce without an obvious phenotype. Cell 79, 679–694. https://doi.org/10.1016/0092-8674(94)90553-3

Conner, S.D., 2016. Regulation of Notch Signaling Through Intracellular Transport. Int. Rev. Cell Mol. Biol. 323, 107–127. https://doi.org/10.1016/BS.IRCMB.2015.12.002

Couturier, L., Schweisguth, F., 2014. Antibody Uptake Assay and In Vivo Imaging to Study Intracellular Trafficking of Notch and Delta in Drosophila, in: Methods in Molecular Biology (Clifton, N.J.). pp. 79–86. https://doi.org/10.1007/978-1-4939-1139-4_6

Cui, D., Dai, J., Keller, J.M., Mizokami, A., Xia, S., Keller, E.T., 2015. Notch Pathway Inhibition Using PF-03084014, a -Secretase Inhibitor (GSI), Enhances the Antitumor Effect of Docetaxel in Prostate Cancer. Clin. Cancer Res. 21, 4619–4629. https://doi.org/10.1158/1078-0432.CCR-15-0242

D'Souza, B., Miyamoto, A., Weinmaster, G., 2008. The many facets of Notch ligands. Oncogene 27, 5148–5167. https://doi.org/10.1038/onc.2008.229

Dave, J.M., Bayless, K.J., 2014. Vimentin as an Integral Regulator of Cell Adhesion and Endothelial Sprouting. Microcirculation 21, 333–344. https://doi.org/10.1111/micc.12111

del Álamo, D., Rouault, H., Schweisguth, F., 2011. Mechanism and Significance of cis-Inhibition in Notch Signalling. Curr. Biol. 21, R40–R47. https://doi.org/10.1016/j.cub.2010.10.034

Dexter, J.S., 1914. The Analysis of a Case of Continuous Variation in Drosophila by a Study of Its Linkage Relations. Am. Nat. https://doi.org/10.2307/2455888

Dhanasekaran, S.M., Dash, A., Yu, J., Maine, I.P., Laxman, B., Tomlins, S.A., Creighton, C.J., Menon, A., Rubin, M.A., Chinnaiyan, A.M., 2005. Molecular profiling of human prostate tissues: Insights into gene expression patterns of prostate development during puberty. FASEB J. 19, 243–245. https://doi.org/10.1096/fj.04-2415fje

Di Fiore, P.P., von Zastrow, M., 2014. Endocytosis, signaling, and beyond. Cold Spring Harb. Perspect. Biol. 6. https://doi.org/10.1101/cshperspect.a016865

Diefenbacher, M.E., Chakraborty, A., Blake, S.M., Mitter, R., Popov, N., Eilers, M., Behrens, A., 2015. Usp28 counteracts Fbw7 in intestinal homeostasis and cancer. Cancer Res. 75, 1181–6. https://doi.org/10.1158/0008-5472.CAN-14-1726

Doherty, G.J., McMahon, H.T., 2009. Mechanisms of Endocytosis. Annu. Rev. Biochem. 78, 857–902. https://doi.org/10.1146/annurev.biochem.78.081307.110540

dos Santos, G., Rogel, M.R., Baker, M.A., Troken, J.R., Urich, D., Morales-Nebreda, L., Sennello, J.A., Kutuzov, M.A., Sitikov, A., Davis, J.M., Lam, A.P., Cheresh, P., Kamp, D., Shumaker, D.K., Budinger, G.R.S., Ridge, K.M., 2015. Vimentin regulates activation of the NLRP3 inflammasome. Nat. Commun. 6, 6574. https://doi.org/10.1038/ncomms7574

Dowling, C.M., Phelan, J., Callender, J.A., Cathcart, M.C., Mehigan, B., McCormick, P., Dalton, T., Coffey, J.C., Newton, A.C., O'Sullivan, J., Kiely, P.A., 2016. Protein kinase C beta II suppresses colorectal cancer by regulating IGF-1 mediated cell survival. Oncotarget 7, 20919–33. https://doi.org/10.18632/oncotarget.8062

Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., Costa, L., Henrique, D., Rossant, J., 2004. Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev. 18, 2474–2478. https://doi.org/10.1101/gad.1239004

Dumont, D.J., Jussila, L., Taipale, J., Lymboussaki, A., Mustonen, T., Pajusola, K., Breitman, M., Alitalo, K., 1998. Cardiovascular Failure in Mouse Embryos Deficient in VEGF Receptor-3. Science (80-.). 282, 946–949. https://doi.org/10.1126/science.282.5390.946

Dunleavy, J.E.M., O'Bryan, M.K., Stanton, P.G., O'Donnell, L., 2019. The cytoskeleton in spermatogenesis. Reproduction. https://doi.org/10.1530/REP-18-0457

Eiraku, M., Hirata, Y., Takeshima, H., Hirano, T., Kengaku, M., 2002. Delta/notch-like epidermal growth factor (EGF)-related receptor, a novel EGF-like repeat-containing protein targeted to dendrites of developing and adult central nervous system neurons. J. Biol. Chem. 277, 25400–7. https://doi.org/10.1074/jbc.M110793200

Eisenberg, E., Greene, L.E., 2007. Multiple Roles of Auxilin and Hsc70 in Clathrin-Mediated Endocytosis. Traffic 8, 640–646. https://doi.org/10.1111/j.1600-0854.2007.00568.x

Ellisen, L.W., Bird, J., West, D.C., Soreng, A.L., Reynolds, T.C., Smith, S.D., Sklar, J., 1991. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 66, 649–61.

Emery, G., Hutterer, A., Berdnik, D., Mayer, B., Wirtz-Peitz, F., Gaitan, M.G., Knoblich, J.A., 2005. Asymmetric Rab 11 endosomes regulate delta recycling and specify cell fate in the Drosophila nervous system. Cell 122, 763–73. https://doi.org/10.1016/j.cell.2005.08.017

Eriksson, J.E., He, T., Trejo-Skalli, A. V, Härmälä-Braskén, A.-S., Hellman, J., Chou, Y.-H., Goldman, R.D., 2004. Specific in vivo phosphorylation sites determine the assembly dynamics of vimentin intermediate filaments. J. Cell Sci. 117, 919–932. https://doi.org/10.1242/jcs.00906

Espinosa, L., Inglés-Esteve, J., Aguilera, C., Bigas, A., 2003. Phosphorylation by Glycogen Synthase Kinase-3β Down-regulates Notch Activity, a Link for Notch and Wnt Pathways. J. Biol. Chem. 278, 32227–32235. https://doi.org/10.1074/jbc.M304001200

Estève, P.O., Chicoine, E., Robledo, O., Aoudjit, F., Descoteaux, A., Potworowski, E.F., St-Pierre, Y., 2002. Protein kinase C-zeta regulates transcription of the matrix metalloproteinase-9 gene induced by IL-1 and TNF-alpha in glioma cells via NF-kappa B. J. Biol. Chem. 277, 35150–5. https://doi.org/10.1074/jbc.M108600200

Etienne-Manneville, S., Hall, A., 2001. Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCζ. Cell 106, 489–498. https://doi.org/10.1016/S0092-8674(01)00471-8

Evrard, Y.A., Lun, Y., Aulehla, A., Gan, L., Johnson, R.L., 1998. lunatic fringe is an essential mediator of somite segmentation and patterning. Nature 394, 377–381. https://doi.org/10.1038/28632

Facchinetti, V., Ouyang, W., Wei, H., Soto, N., Lazorchak, A., Gould, C., Lowry, C., Newton, A.C., Mao, Y., Miao, R.Q., Sessa, W.C., Qin, J., Zhang, P., Su, B., Jacinto, E., 2008. The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. EMBO J. 27, 1932–43. https://doi.org/10.1038/emboj.2008.120

Falo-Sanjuan, J., Lammers, N.C., Garcia, H.G., Bray, S.J., 2019. Enhancer Priming Enables Fast and Sustained Transcriptional Responses to Notch Signaling. Dev. Cell 50, 411-425.e8. https://doi.org/10.1016/j.devcel.2019.07.002

Ferguson, J.P., Huber, S.D., Willy, N.M., Aygün, E., Goker, S., Atabey, T., Kural, C., 2017. Mechanoregulation of clathrin-mediated endocytosis. J. Cell Sci. 130, 3631–3636. https://doi.org/10.1242/jcs.205930

Fernandez-Valdivia, R., Takeuchi, H., Samarghandi, A., Lopez, M., Leonardi, J., Haltiwanger, R.S., Jafar-Nejad, H., 2011. Regulation of mammalian Notch signaling and embryonic development by the protein O-glucosyltransferase Rumi. Development 138, 1925–1934. https://doi.org/10.1242/dev.060020

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., Moore, M.W., 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380, 439–442. https://doi.org/10.1038/380439a0

Ferreira, A.P.A., Boucrot, E., 2018. Mechanisms of Carrier Formation during Clathrin-Independent Endocytosis. Trends Cell Biol. https://doi.org/10.1016/j.tcb.2017.11.004

Fischer, A., Schumacher, N., Maier, M., Sendtner, M., Gessler, M., 2004. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev. 18, 901–911. https://doi.org/10.1101/gad.291004

Fleming, R.J., Scottgale, T.N., Diederich, R.J., Artavanis-Tsakonas, S., 1990. The gene Serrate encodes a putative EGF-like transmembrane protein essential for proper ectodermal development in Drosophila melanogaster. Genes Dev. 4, 2188–2201. https://doi.org/10.1101/gad.4.12a.2188

Foltz, D.R., Santiago, M.C., Berechid, B.E., Nye, J.S., 2002. Glycogen synthase kinase-3beta modulates notch signaling and stability. Curr. Biol. 12, 1006–11.

Fortini, M.E., Bilder, D., 2009. Endocytic regulation of Notch signaling. Curr. Opin. Genet. Dev. 19, 323–8. https://doi.org/10.1016/j.gde.2009.04.005

Franke, T.F., Tartof, K.D., Tsichlis, P.N., 1994. The SH2-like Akt homology (AH) domain of c-akt is present in multiple copies in the genome of vertebrate and invertebrate eucaryotes. Cloning and characterization of the Drosophila melanogaster c-akt homolog Dakt1. Oncogene 9, 141–8.

Frederick, L.A., Matthews, J.A., Jamieson, L., Justilien, V., Thompson, E.A., Radisky, D.C., Fields, A.P., 2008. Matrix metalloproteinase-10 is a critical effector of protein kinase Ciota-Par6alpha-mediated lung cancer. Oncogene 27, 4841–53. https://doi.org/10.1038/onc.2008.119

Fryer, C.J., White, J.B., Jones, K.A., 2004. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol. Cell 16, 509–20. https://doi.org/10.1016/j.molcel.2004.10.014

Gale, N.W., Dominguez, M.G., Noguera, I., Pan, L., Hughes, V., Valenzuela, D.M., Murphy, A.J., Adams, N.C., Lin, H.C., Holash, J., Thurston, G., Yancopoulos, G.D., 2004. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. Proc. Natl. Acad. Sci. U. S. A. 101, 15949–54. https://doi.org/10.1073/pnas.0407290101

Galvez, A.S., Duran, A., Linares, J.F., Pathrose, P., Castilla, E.A., Abu-Baker, S., Leitges, M., Diaz-Meco, M.T., Moscat, J., 2009. Protein kinase Czeta represses the interleukin-6 promoter and impairs tumorigenesis in vivo. Mol. Cell. Biol. 29, 104–15. https://doi.org/10.1128/MCB.01294-08

Garcia, A., Kandel, J.J., 2012. Notch: a key regulator of tumor angiogenesis and metastasis. Histol. Histopathol. 27, 151–6. https://doi.org/10.14670/HH-27.151

Garg, R., Benedetti, L.G., Abera, M.B., Wang, H., Abba, M., Kazanietz, M.G., 2014. Protein kinase C and cancer: What we know and what we do not. Oncogene. https://doi.org/10.1038/onc.2013.524

Garg, V., Muth, A.N., Ransom, J.F., Schluterman, M.K., Barnes, R., King, I.N., Grossfeld, P.D., Srivastava, D., 2005. Mutations in NOTCH1 cause aortic valve disease. Nature 437, 270–274. https://doi.org/10.1038/nature03940

George, J., Lim, J.S., Jang, S.J., Cun, Y., Ozretić, L., Kong, G., Leenders, F., Lu, X., Fernández-Cuesta, L., Bosco, G., Müller, C., Dahmen, I., Jahchan, N.S., Park, K.-S., Yang, D., Karnezis, A.N., Vaka, D., Torres, A., Wang, M.S., Korbel, J.O., Menon, R., Chun, S.-M., Kim, D., Wilkerson, M., Hayes, N., Engelmann, D., Pützer, B., Bos, M., Michels, S., Vlasic, I., Seidel, D., Pinther, B., Schaub, P., Becker, C., Altmüller, J., Yokota, J., Kohno, T., Iwakawa, R., Tsuta, K., Noguchi, M., Muley, T., Hoffmann, H., Schnabel, P.A., Petersen, I., Chen, Y., Soltermann, A., Tischler, V., Choi, C., Kim, Y.-H., Massion, P.P., Zou, Y., Jovanovic, D., Kontic, M., Wright, G.M., Russell, P.A., Solomon, B., Koch, I., Lindner, M., Muscarella,

L.A., la Torre, A., Field, J.K., Jakopovic, M., Knezevic, J., Castaños-Vélez, E., Roz, L., Pastorino, U., Brustugun, O.-T., Lund-Iversen, M., Thunnissen, E., Köhler, J., Schuler, M., Botling, J., Sandelin, M., Sanchez-Cespedes, M., Salvesen, H.B., Achter, V., Lang, U., Bogus, M., Schneider, P.M., Zander, T., Ansén, S., Hallek, M., Wolf, J., Vingron, M., Yatabe, Y., Travis, W.D., Nürnberg, P., Reinhardt, C., Perner, S., Heukamp, L., Büttner, R., Haas, S.A., Brambilla, E., Peifer, M., Sage, J., Thomas, R.K., 2015. Comprehensive genomic profiles of small cell lung cancer. Nature 524, 47–53. https://doi.org/10.1038/nature14664

Georgiou, M., Marinari, E., Burden, J., Baum, B., 2008. Cdc42, Par6, and aPKC Regulate Arp2/3-Mediated Endocytosis to Control Local Adherens Junction Stability. Curr. Biol. 18, 1631–1638. https://doi.org/10.1016/j.cub.2008.09.029

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., Jeltsch, M., Mitchell, C., Alitalo, K., Shima, D., Betsholtz, C., 2003. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J. Cell Biol. 161, 1163–1177. https://doi.org/10.1083/jcb.200302047

Gilbert, C.A., Daou, M.-C., Moser, R.P., Ross, A.H., 2010. Gamma-secretase inhibitors enhance temozolomide treatment of human gliomas by inhibiting neurosphere repopulation and xenograft recurrence. Cancer Res. 70, 6870–9. https://doi.org/10.1158/0008-5472.CAN-10-1378

Glomski, K., Monette, S., Manova, K., De Strooper, B., Saftig, P., Blobel, C.P., 2011. Deletion of Adam10 in endothelial cells leads to defects in organ-specific vascular structures. Blood 118, 1163–74. https://doi.org/10.1182/blood-2011-04-348557

Gomez-Lamarca, M.J., Falo-Sanjuan, J., Stojnic, R., Abdul Rehman, S., Muresan, L., Jones, M.L., Pillidge, Z., Cerda-Moya, G., Yuan, Z., Baloul, S., Valenti, P., Bystricky, K., Payre, F., O'Holleran, K., Kovall, R., Bray, S.J., 2018. Activation of the Notch Signaling Pathway In Vivo Elicits Changes in CSL Nuclear Dynamics. Dev. Cell 44, 611-623.e7. https://doi.org/10.1016/j.devcel.2018.01.020

Gordon, W.R., Arnett, K.L., Blacklow, S.C., 2008. The molecular logic of Notch signaling - a structural and biochemical perspective. J. Cell Sci. 121, 3109–3119. https://doi.org/10.1242/jcs.035683

Gordon, W.R., Vardar-Ulu, D., Histen, G., Sanchez-Irizarry, C., Aster, J.C., Blacklow, S.C., 2007. Structural basis for autoinhibition of Notch. Nat. Struct. Mol. Biol. 14, 295–300. https://doi.org/10.1038/nsmb1227

Gordon, W.R., Vardar-Ulu, D., L'Heureux, S., Ashworth, T., Malecki, M.J., Sanchez-Irizarry, C., McArthur, D.G., Histen, G., Mitchell, J.L., Aster, J.C., Blacklow, S.C., 2009. Effects of S1 Cleavage on the Structure, Surface Export, and Signaling Activity of Human Notch1 and Notch2. PLoS One 4, e6613. https://doi.org/10.1371/journal.pone.0006613

Gordon, W.R., Zimmerman, B., He, L., Miles, L.J., Huang, J., Tiyanont, K., McArthur, D.G., Aster, J.C., Perrimon, N., Loparo, J.J., Blacklow, S.C., 2015. Mechanical Allostery: Evidence for a Force Requirement in the Proteolytic Activation of Notch. Dev. Cell 33, 729–36. https://doi.org/10.1016/j.devcel.2015.05.004

Grammont, M., 2007. Adherens junction remodeling by the Notch pathway in Drosophila melanogaster oogenesis. J. Cell Biol. 177, 139–150. https://doi.org/10.1083/jcb.200609079

Grant, B.D., Donaldson, J.G., 2009. Pathways and mechanisms of endocytic recycling. Nat. Rev. Mol. Cell Biol. 10, 597–608. https://doi.org/10.1038/nrm2755

Greene, M., Lai, Y., Pajcini, K., Bailis, W., Pear, W.S., Lancaster, E., 2016. Delta/Notch-Like EGF-Related Receptor (DNER) Is Not a Notch Ligand. PLoS One 11, e0161157. https://doi.org/10.1371/journal.pone.0161157

Gregor, M., Osmanagic-Myers, S., Burgstaller, G., Wolfram, M., Fischer, I., Walko, G., Resch, G.P., Jörgl, A., Herrmann, H., Wiche, G., 2014. Mechanosensing through focal adhesion-

anchored intermediate filaments. FASEB J. 28, 715–729. https://doi.org/10.1096/fj.13-231829

Gridley, T., 2010. Notch signaling in the vasculature. Curr. Top. Dev. Biol. 92, 277–309. https://doi.org/10.1016/S0070-2153(10)92009-7

Grosberg, R.K., Strathmann, R.R., 2007. The Evolution of Multicellularity: A Minor Major Transition? Annu. Rev. Ecol. Evol. Syst. 38, 621–654. https://doi.org/10.1146/annurev.ecolsys.36.102403.114735

Gruenbaum, Y., Aebi, U., 2014. Intermediate filaments: a dynamic network that controls cell mechanics. F1000Prime Rep. 6, 54. https://doi.org/10.12703/P6-54

Gupta-Rossi, N., Six, E., LeBail, O., Logeat, F., Chastagner, P., Olry, A., Israël, A., Brou, C., 2004. Monoubiquitination and endocytosis direct gamma-secretase cleavage of activated Notch receptor. J. Cell Biol. 166, 73–83. https://doi.org/10.1083/jcb.200310098

Hanlon, L., Avila, J.L., Demarest, R.M., Troutman, S., Allen, M., Ratti, F., Rustgi, A.K., Stanger, B.Z., Radtke, F., Adsay, V., Long, F., Capobianco, A.J., Kissil, J.L., 2010. Notch1 functions as a tumor suppressor in a model of K-ras-induced pancreatic ductal adenocarcinoma. Cancer Res. 70, 4280–6. https://doi.org/10.1158/0008-5472.CAN-09-4645

Hansra, G., Garcia-Paramio, P., Prevostel, C., Whelan, R.D., Bornancin, F., Parker, P.J., 1999. Multisite dephosphorylation and desensitization of conventional protein kinase C isotypes. Biochem. J. 342 (Pt 2), 337–44.

Hansson, E.M., Lanner, F., Das, D., Mutvei, A., Marklund, U., Ericson, J., Farnebo, F., Stumm, G., Stenmark, H., Andersson, E.R., Lendahl, U., 2010. Control of Notch-ligand endocytosis by ligand-receptor interaction. J. Cell Sci. 123, 2931–2942. https://doi.org/10.1242/jcs.073239

Hapak, S.M., Rothlin, C. V, Ghosh, S., 2018. PAR3-PAR6-atypical PKC polarity complex proteins in neuronal polarization. Cell. Mol. Life Sci. 75, 2735–2761. https://doi.org/10.1007/s00018-018-2828-6

Harvey, B.M., Haltiwanger, R.S., 2018. Regulation of Notch Function by O-Glycosylation, in: Advances in Experimental Medicine and Biology. pp. 59–78. https://doi.org/10.1007/978-3-319-89512-3_4

Hasan, S.S., Tsaryk, R., Lange, M., Wisniewski, L., Moore, J.C., Lawson, N.D., Wojciechowska, K., Schnittler, H., Siekmann, A.F., 2017. Endothelial Notch signalling limits angiogenesis via control of artery formation. Nat. Cell Biol. 19, 928–940. https://doi.org/10.1038/ncb3574

Hatakeyama, J., Wakamatsu, Y., Nagafuchi, A., Kageyama, R., Shigemoto, R., Shimamura, K., 2014. Cadherin-based adhesions in the apical endfoot are required for active Notch signaling to control neurogenesis in vertebrates. Development 141, 1671–1682. https://doi.org/10.1242/dev.102988

Hellström, M., Phng, L.-K., Hofmann, J.J., Wallgard, E., Coultas, L., Lindblom, P., Alva, J., Nilsson, A.-K., Karlsson, L., Gaiano, N., Yoon, K., Rossant, J., Iruela-Arispe, M.L., Kalén, M., Gerhardt, H., Betsholtz, C., 2007. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 445, 776–780. https://doi.org/10.1038/nature05571

Henkel, T., Ling, P.D., Hayward, S.D., Peterson, M.G., 1994. Mediation of Epstein-Barr virus EBNA2 transactivation by recombination signal-binding protein J kappa. Science 265, 92–5.

Henrique, D., Schweisguth, F., 2019. Mechanisms of Notch signaling: a simple logic deployed in time and space. Development 146, dev172148. https://doi.org/10.1242/dev.172148

Herreman, A., Hartmann, D., Annaert, W., Saftig, P., Craessaerts, K., Serneels, L., Umans, L., Schrijvers, V., Checler, F., Vanderstichele, H., Baekelandt, V., Dressel, R., Cupers, P., Huylebroeck, D., Zwijsen, A., Leuven, F. Van, Strooper, B. De, 1999. Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein

processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. Proc. Natl. Acad. Sci. U. S. A. 96, 11872. https://doi.org/10.1073/PNAS.96.21.11872

Herrmann, H., Aebi, U., 2016. Intermediate Filaments: Structure and Assembly. Cold Spring Harb. Perspect. Biol. 8, a018242. https://doi.org/10.1101/cshperspect.a018242

Heuss, S.F., Ndiaye-Lobry, D., Six, E.M., Israël, A., Logeat, F., 2008. The intracellular region of Notch ligands Dll1 and Dll3 regulates their trafficking and signaling activity. Proc. Natl. Acad. Sci. U. S. A. 105, 11212–7. https://doi.org/10.1073/pnas.0800695105

High, F.A., Lu, M.M., Pear, W.S., Loomes, K.M., Kaestner, K.H., Epstein, J.A., 2008. Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. Proc. Natl. Acad. Sci. U. S. A. 105, 1955–9. https://doi.org/10.1073/pnas.0709663105

Hofmann, J.J., Iruela-Arispe, M.L., 2007. Notch Signaling in Blood Vessels. Circ. Res. 100, 1556–1568. https://doi.org/10.1161/01.RES.0000266408.42939.e4

Hoglund, V.J., Majesky, M.W., 2012. Patterning the Artery Wall by Lateral Induction of Notch Signaling. Circulation 125, 212–215. https://doi.org/10.1161/CIRCULATION-AHA.111.075937

Holmberg, J., Hansson, E., Malewicz, M., Sandberg, M., Perlmann, T., Lendahl, U., Muhr, J., 2008. SoxB1 transcription factors and Notch signaling use distinct mechanisms to regulate proneural gene function and neural progenitor differentiation. Development 135, 1843–1851. https://doi.org/10.1242/dev.020180

Hori, K., Cholewa-Waclaw, J., Nakada, Y., Glasgow, S.M., Masui, T., Henke, R.M., Wildner, H., Martarelli, B., Beres, T.M., Epstein, J.A., Magnuson, M.A., Macdonald, R.J., Birchmeier, C., Johnson, J.E., 2008. A nonclassical bHLH Rbpj transcription factor complex is required for specification of GABAergic neurons independent of Notch signaling. Genes Dev. 22, 166–78. https://doi.org/10.1101/gad.1628008

Hori, K., Sen, A., Kirchhausen, T., Artavanis-Tsakonas, S., 2011. Synergy between the ESCRT-III complex and Deltex defines a ligand-independent Notch signal. J. Cell Biol. 195, 1005–1015. https://doi.org/10.1083/jcb.201104146

Horvath, C.A.J., Vanden Broeck, D., Boulet, G.A.V., Bogers, J., De Wolf, M.J.S., 2007. Epsin: Inducing membrane curvature. Int. J. Biochem. Cell Biol. 39, 1765–1770. https://doi.org/10.1016/j.biocel.2006.12.004

Hukriede, N.A., Gu, Y., Fleming, R.J., 1997. A dominant-negative form of Serrate acts as a general antagonist of Notch activation. Development 124, 3427–37.

Hunter, G.L., He, L., Perrimon, N., Charras, G., Giniger, E., Baum, B., 2019. A role for actomyosin contractility in Notch signaling. BMC Biol. 17, 12. https://doi.org/10.1186/s12915-019-0625-9

Hurov, J.B., Stappenbeck, T.S., Zmasek, C.M., White, L.S., Ranganath, S.H., Russell, J.H., Chan, A.C., Murphy, K.M., Piwnica-Worms, H., 2001. Immune system dysfunction and autoimmune disease in mice lacking Emk (Par-1) protein kinase. Mol. Cell. Biol. 21, 3206–19. https://doi.org/10.1128/MCB.21.9.3206-3219.2001

Imai, Y., Kobayashi, Y., Inoshita, T., Meng, H., Arano, T., Uemura, K., Asano, T., Yoshimi, K., Zhang, C.L., Matsumoto, G., Ohtsuka, T., Kageyama, R., Kiyonari, H., Shioi, G., Nukina, N., Hattori, N., Takahashi, R., 2015. The Parkinson's Disease-Associated Protein Kinase LRRK2 Modulates Notch Signaling through the Endosomal Pathway. PLoS Genet. https://doi.org/10.1371/journal.pgen.1005503

Irvine, K.D., Wieschaus, E., 1994. Cell intercalation during Drosophila germband extension and its regulation by pair-rule segmentation genes. Development 120, 827–41.

Ishitani, T., Hirao, T., Suzuki, M., Isoda, M., Ishitani, S., Harigaya, K., Kitagawa, M., Matsumoto, K., Itoh, M., 2010. Nemo-like kinase suppresses Notch signalling by interfering with

formation of the Notch active transcriptional complex. Nat. Cell Biol. 12, 278–285. https://doi.org/10.1038/ncb2028

Itoh, M., Kim, C.-H., Palardy, G., Oda, T., Jiang, Y.-J., Maust, D., Yeo, S.-Y., Lorick, K., Wright, G.J., Ariza-McNaughton, L., Weissman, A.M., Lewis, J., Chandrasekharappa, S.C., Chitnis, A.B., 2003. Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. Dev. Cell 4, 67–82.

Ivaska, J., 2011. Vimentin: Central hub in EMT induction? Small GTPases 2, 51–53. https://doi.org/10.4161/sgtp.2.1.15114

Ivaska, J., Pallari, H.-M., Nevo, J., Eriksson, J.E., 2007. Novel functions of vimentin in cell adhesion, migration, and signaling. Exp. Cell Res. 313, 2050–2062. https://doi.org/10.1016/j.yexcr.2007.03.040

Ivaska, J., Vuoriluoto, K., Huovinen, T., Izawa, I., Inagaki, M., Parker, P.J., 2005. PKCε-mediated phosphorylation of vimentin controls integrin recycling and motility. EMBO J. 24, 3834–3845. https://doi.org/10.1038/sj.emboj.7600847

Jacob, J.T., Coulombe, P.A., Kwan, R., Omary, M.B., 2018. Types I and II Keratin Intermediate Filaments. Cold Spring Harb. Perspect. Biol. 10, a018275. https://doi.org/10.1101/cshperspect.a018275

Jacobsen, T.L., Brennan, K., Arias, A.M., Muskavitch, M.A., 1998. Cis-interactions between Delta and Notch modulate neurogenic signalling in Drosophila. Development 125, 4531–40.

Jafar-Nejad, H., Andrews, H.K., Acar, M., Bayat, V., Wirtz-Peitz, F., Mehta, S.Q., Knoblich, J.A., Bellen, H.J., 2005. Sec15, a component of the exocyst, promotes notch signaling during the asymmetric division of Drosophila sensory organ precursors. Dev. Cell 9, 351–63. https://doi.org/10.1016/j.devcel.2005.06.010

Jaken, S., Tashjian, A.H., Blumberg, P.M., 1981. Characterization of phorbol ester receptors and their down-modulation in GH4C1 rat pituitary cells. Cancer Res. 41, 2175–81.

Jehn, B.M., Dittert, I., Beyer, S., von der Mark, K., Bielke, W., 2002. c-Cbl binding and ubiquitin-dependent lysosomal degradation of membrane-associated Notch1. J. Biol. Chem. 277, 8033–40. https://doi.org/10.1074/jbc.M108552200

Jin, S., Mutvei, A.P., Chivukula, I. V, Andersson, E.R., Ramsköld, D., Sandberg, R., Lee, K.L., Kronqvist, P., Mamaeva, V., Ostling, P., Mpindi, J.-P., Kallioniemi, O., Screpanti, I., Poellinger, L., Sahlgren, C., Lendahl, U., 2013. Non-canonical Notch signaling activates IL-6/JAK/STAT signaling in breast tumor cells and is controlled by p53 and IKKα/IKKβ. Oncogene 32, 4892–902. https://doi.org/10.1038/onc.2012.517

Jin, Y.H., Kim, H., Oh, M., Ki, H., Kim, K., 2009. Regulation of Notch1/NICD and Hes1 expressions by GSK-3α/β. Mol. Cells 27, 15–19. https://doi.org/10.1007/s10059-009-0001-7

Johnson, S.A., Zitserman, D., Roegiers, F., 2016. Numb regulates the balance between Notch recycling and late-endosome targeting in Drosophila neural progenitor cells. Mol. Biol. Cell 27, 2857. https://doi.org/10.1091/MBC.E15-11-0751

Jovic, M., Sharma, M., Rahajeng, J., Caplan, S., 2010. The early endosome: a busy sorting station for proteins at the crossroads. Histol. Histopathol. 25, 99–112. https://doi.org/10.14670/HH-25.99

Justilien, V., Walsh, M.P., Ali, S.A., Thompson, E.A., Murray, N.R., Fields, A.P., 2014. The PRKCI and SOX2 Oncogenes Are Coamplified and Cooperate to Activate Hedgehog Signaling in Lung Squamous Cell Carcinoma. Cancer Cell 25, 139–151. https://doi.org/10.1016/j.ccr.2014.01.008

Kakuda, S., Haltiwanger, R.S., 2017. Deciphering the Fringe-Mediated Notch Code: Identification of Activating and Inhibiting Sites Allowing Discrimination between Ligands. Dev. Cell 40, 193–201. https://doi.org/10.1016/j.devcel.2016.12.013

Kamstrup, M.R., Biskup, E., Manfè, V., Savorani, C., Liszewski, W., Wirèn, J., Specht, L., Gniadecki, R., 2017. Chemotherapeutic treatment is associated with Notch1 induction in cutaneous T-cell lymphoma. Leuk. Lymphoma 58, 171–178. https://doi.org/10.1080/10428194.2016.1180681

Kangsamaksin, T., Murtomaki, A., Kofler, N.M., Cuervo, H., Chaudhri, R.A., Tattersall, I.W., Rosenstiel, P.E., Shawber, C.J., Kitajewski, J., 2015. NOTCH Decoys That Selectively Block DLL/NOTCH or JAG/NOTCH Disrupt Angiogenesis by Unique Mechanisms to Inhibit Tumor Growth. Cancer Discov. 5, 182–197. https://doi.org/10.1158/2159-8290.CD-14-0650

Kershaw, N.J., Church, N.L., Griffin, M.D.W., Luo, C.S., Adams, T.E., Burgess, A.W., 2015. Notch ligand delta-like1: X-ray crystal structure and binding affinity. Biochem. J. 468, 159–166. https://doi.org/10.1042/BJ20150010

Kerstjens-Frederikse, W.S., van de Laar, I.M.B.H., Vos, Y.J., Verhagen, J.M.A., Berger, R.M.F., Lichtenbelt, K.D., Klein Wassink-Ruiter, J.S., van der Zwaag, P.A., du Marchie Sarvaas, G.J., Bergman, K.A., Bilardo, C.M., Roos-Hesselink, J.W., Janssen, J.H.P., Frohn-Mulder, I.M., van Spaendonck-Zwarts, K.Y., van Melle, J.P., Hofstra, R.M.W., Wessels, M.W., 2016. Cardiovascular malformations caused by NOTCH1 mutations do not keep left: data on 428 probands with left-sided CHD and their families. Genet. Med. 18, 914–923. https://doi.org/10.1038/gim.2015.193

Kidd, M.E., Shumaker, D.K., Ridge, K.M., 2014. The role of Vimentin intermediate filaments in the progression of lung cancer. Am. J. Respir. Cell Mol. Biol. https://doi.org/10.1165/rcmb.2013-0314TR

Kidd, S., Baylies, M.K., Gasic, G.P., Young, M.W., 1989. Structure and distribution of the Notch protein in developing Drosophila. Genes Dev. 3, 1113–29. https://doi.org/10.1101/GAD.3.8.1113

Kidd, S., Lockett, T.J., Young, M.W., 1983. The Notch locus of Drosophila melanogaster. Cell 34, 421–433. https://doi.org/10.1016/0092-8674(83)90376-8

Kiernan, A.E., 2013. Notch signaling during cell fate determination in the inner ear. Semin. Cell Dev. Biol. 24, 470–479. https://doi.org/10.1016/J.SEMCDB.2013.04.002

Kim, B., Stephen, S.L., Hanby, A.M., Horgan, K., Perry, S.L., Richardson, J., Roundhill, E.A., Valleley, E.M.A., Verghese, E.T., Williams, B.J., Thorne, J.L., Hughes, T.A., 2015. Chemotherapy induces Notch1-dependent MRP1 up-regulation, inhibition of which sensitizes breast cancer cells to chemotherapy. BMC Cancer 15, 634. https://doi.org/10.1186/s12885-015-1625-y

Kim, J., Yang, C., Kim, E.J., Jang, J., Kim, S.-J., Kang, S.M., Kim, M.G., Jung, H., Park, D., Kim, C., 2016. Vimentin filaments regulate integrin–ligand interactions by binding to the cytoplasmic tail of integrin β3. J. Cell Sci. 129, 2030–2042. https://doi.org/10.1242/jcs.180315

Kim, S., Coulombe, P.A., 2007. Intermediate filament scaffolds fulfill mechanical, organizational, and signaling functions in the cytoplasm. Genes Dev. 21, 1581–97. https://doi.org/10.1101/gad.1552107

Koenig, S.N., LaHaye, S., Feller, J.D., Rowland, P., Hor, K.N., Trask, A.J., Janssen, P.M.L., Radtke, F., Lilly, B., Garg, V., 2017. Notch1 haploinsufficiency causes ascending aortic aneurysms in mice. JCI Insight 2. https://doi.org/10.1172/jci.insight.91353

Kofler, N.M., Shawber, C.J., Kangsamaksin, T., Reed, H.O., Galatioto, J., Kitajewski, J., 2011. Notch signaling in developmental and tumor angiogenesis. Genes Cancer 2, 1106–16. https://doi.org/10.1177/1947601911423030

Kolte, D., 2016. Vasculogenesis and Angiogenesis. Transl. Res. Coron. Artery Dis. 49–65. https://doi.org/10.1016/B978-0-12-802385-3.00006-1

Komander, D., Rape, M., 2012. The Ubiquitin Code. Annu. Rev. Biochem. 81, 203–229. https://doi.org/10.1146/annurev-biochem-060310-170328

Koo, B.-K., Lim, H.-S., Song, R., Yoon, M.-J., Yoon, K.-J., Moon, J.-S., Kim, Y.-W., Kwon, M.-C., Yoo, K.-W., Kong, M.-P., Lee, J., Chitnis, A.B., Kim, C.-H., Kong, Y.-Y., 2005. Mind bomb 1 is essential for generating functional Notch ligands to activate Notch. Development 132, 3459–3470. https://doi.org/10.1242/dev.01922

Koo, Bon-Kyoung, Yoon, K.-J., Yoo, K.-W., Lim, H.-S., Song, R., So, J.-H., Kim, C.-H., Kong, Y.-Y., 2005. Mind Bomb-2 Is an E3 Ligase for Notch Ligand. J. Biol. Chem. 280, 22335–22342. https://doi.org/10.1074/jbc.M501631200

Koo, B.-K., Yoon, M.-J., Yoon, K.-J., Im, S.-K., Kim, Y.-Y., Kim, C.-H., Suh, P.-G., Jan, Y.N., Kong, Y.-Y., 2007. An obligatory role of mind bomb-1 in notch signaling of mammalian development. PLoS One 2, e1221. https://doi.org/10.1371/journal.pone.0001221

Kooh, P.J., Fehon, R.G., Muskavitch, M.A., 1993. Implications of dynamic patterns of Delta and Notch expression for cellular interactions during Drosophila development. Development 117, 493–507.

Kopan, R., Ilagan, M.X.G., 2009. The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. Cell 137, 216–233. https://doi.org/10.1016/j.cell.2009.03.045

Koutelou, E., Sato, S., Tomomori-Sato, C., Florens, L., Swanson, S.K., Washburn, M.P., Kokkinaki, M., Conaway, R.C., Conaway, J.W., Moschonas, N.K., 2008. Neuralized-like 1 (Neurl1) Targeted to the Plasma Membrane by *N* -Myristoylation Regulates the Notch Ligand Jagged1. J. Biol. Chem. 283, 3846–3853. https://doi.org/10.1074/jbc.M706974200

Kovall, R.A., Gebelein, B., Sprinzak, D., Kopan, R., 2017. The Canonical Notch Signaling Pathway: Structural and Biochemical Insights into Shape, Sugar, and Force. Dev. Cell 41, 228–241. https://doi.org/10.1016/j.devcel.2017.04.001

Krebs, L.T., Shutter, J.R., Tanigaki, K., Honjo, T., Stark, K.L., Gridley, T., 2004. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. Genes Dev. 18, 2469–2473. https://doi.org/10.1101/gad.1239204

Krebs, L.T., Starling, C., Chervonsky, A. V., Gridley, T., 2010. Notch1 activation in mice causes arteriovenous malformations phenocopied by ephrinB2 and EphB4 mutants. genesis 48, NA-NA. https://doi.org/10.1002/dvg.20599

Krebs, L.T., Xue, Y., Norton, C.R., Shutter, J.R., Maguire, M., Sundberg, J.P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., Smith, G.H., Stark, K.L., Gridley, T., 2000. Notch signaling is essential for vascular morphogenesis in mice. Genes Dev. 14, 1343–52.

Krejcí, A., Bray, S., 2007. Notch activation stimulates transient and selective binding of Su(H)/CSL to target enhancers. Genes Dev. 21, 1322–7. https://doi.org/10.1101/gad.424607

Kwon, C., Cheng, P., King, I.N., Andersen, P., Shenje, L., Nigam, V., Srivastava, D., 2011. Notch post-translationally regulates β-catenin protein in stem and progenitor cells. Nat. Cell Biol. 13, 1244–51. https://doi.org/10.1038/ncb2313

Ladi, E., Nichols, J.T., Ge, W., Miyamoto, A., Yao, C., Yang, L.-T., Boulter, J., Sun, Y.E., Kintner, C., Weinmaster, G., 2005. The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. J. Cell Biol. 170, 983–92. https://doi.org/10.1083/jcb.200503113

LaFoya, B., Munroe, J.A., Pu, X., Albig, A.R., 2018. Src kinase phosphorylates Notch1 to inhibit MAML binding. Sci. Rep. 8, 15515. https://doi.org/10.1038/s41598-018-33920-y

Lähdeniemi, I.A.K., Misiorek, J.O., Antila, C.J.M., Landor, S.K.-J., Stenvall, C.-G.A., Fortelius, L.E., Bergström, L.K., Sahlgren, C., Toivola, D.M., 2017. Keratins regulate colonic epithelial cell differentiation through the Notch1 signalling pathway. Cell Death Differ. 24, 984–996. https://doi.org/10.1038/cdd.2017.28

Lai, E.C., Deblandre, G.A., Kintner, C., Rubin, G.M., 2001. Drosophila neuralized is a ubiquitin ligase that promotes the internalization and degradation of delta. Dev. Cell 1, 783–94.

Lai, E.C., Roegiers, F., Qin, X., Jan, Y.N., Rubin, G.M., 2005. The ubiquitin ligase Drosophila Mind bomb promotes Notch signaling by regulating the localization and activity of Serrate and Delta. Development 132, 2319–2332. https://doi.org/10.1242/dev.01825

Langridge, P.D., Struhl, G., 2017. Epsin-Dependent Ligand Endocytosis Activates Notch by Force. Cell 171, 1383-1396.e12. https://doi.org/10.1016/j.cell.2017.10.048

Larson Gedman, A., Chen, Q., Kugel Desmoulin, S., Ge, Y., LaFiura, K., Haska, C.L., Cherian, C., Devidas, M., Linda, S.B., Taub, J.W., Matherly, L.H., 2009. The impact of NOTCH1, FBW7 and PTEN mutations on prognosis and downstream signaling in pediatric T-cell acute lymphoblastic leukemia: a report from the Children's Oncology Group. Leukemia 23, 1417–1425. https://doi.org/10.1038/leu.2009.64

Le Borgne, R., Remaud, S., Hamel, S., Schweisguth, F., 2005. Two distinct E3 ubiquitin ligases have complementary functions in the regulation of delta and serrate signaling in Drosophila. PLoS Biol. 3, e96. https://doi.org/10.1371/journal.pbio.0030096

Le Borgne, R., Schweisguth, F., 2003. Unequal segregation of Neuralized biases Notch activation during asymmetric cell division. Dev. Cell 5, 139–48. https://doi.org/10.1016/s1534-5807(03)00187-4

Le Bras, S., Loyer, N., Le Borgne, R., 2011. The Multiple Facets of Ubiquitination in the Regulation of Notch Signaling Pathway. Traffic 12, 149–161. https://doi.org/10.1111/j.1600-0854.2010.01126.x

Lee, C., Shin, H., Kimble, J., 2019. Dynamics of Notch-Dependent Transcriptional Bursting in Its Native Context. Dev. Cell 50, 426-435.e4. https://doi.org/10.1016/j.devcel.2019.07.001

Lee, H.J., Kim, M.Y., Park, H.S., 2015. Phosphorylation-dependent regulation of Notch1 signaling: The fulcrum of Notch1 signaling. BMB Rep. 48, 431–437. https://doi.org/10.5483/BMBRep.2015.48.8.107

Lee, K.-S., Wu, Z., Song, Y., Mitra, S.S., Feroze, A.H., Cheshier, S.H., Lu, B., 2013. Roles of PINK1, mTORC2, and mitochondria in preserving brain tumor-forming stem cells in a noncanonical Notch signaling pathway. Genes Dev. 27, 2642–7. https://doi.org/10.1101/gad.225169.113

Lee, W., Andrews, B.C., Faust, M., Walldorf, U., Verheyen, E.M., 2009. Hipk is an essential protein that promotes Notch signal transduction in the Drosophila eye by inhibition of the global co-repressor Groucho. Dev. Biol. 325, 263–272. https://doi.org/10.1016/j.ydbio.2008.10.029

Lehmann, R., Jiménez, F., Dietrich, U., Campos-Ortega, J.A., 1983. On the phenotype and development of mutants of early neurogenesis inDrosophila melanogaster. Wilhelm Roux's Arch. Dev. Biol. 192, 62–74. https://doi.org/10.1007/BF00848482

Leibfried, A., Fricke, R., Morgan, M.J., Bogdan, S., Bellaiche, Y., 2008. Drosophila Cip4 and WASp Define a Branch of the Cdc42-Par6-aPKC Pathway Regulating E-Cadherin Endocytosis. Curr. Biol. 18, 1639–1648. https://doi.org/10.1016/j.cub.2008.09.063

Lenburg, M.E., Liou, L.S., Gerry, N.P., Frampton, G.M., Cohen, H.T., Christman, M.F., 2003. Previously unidentified changes in renal cell carcinoma gene expression identified by parametric analysis of microarray data. BMC Cancer 3. https://doi.org/10.1186/1471-2407-3-31

Li, J.-L., Sainson, R.C.A., Shi, W., Leek, R., Harrington, L.S., Preusser, M., Biswas, S., Turley, H., Heikamp, E., Hainfellner, J.A., Harris, A.L., 2007. Delta-like 4 Notch Ligand Regulates Tumor Angiogenesis, Improves Tumor Vascular Function, and Promotes Tumor Growth *In vivo*. Cancer Res. 67, 11244–11253. https://doi.org/10.1158/0008-5472.CAN-07-0969

Li, J., Fici, G.J., Mao, C.-A., Myers, R.L., Shuang, R., Donoho, G.P., Pauley, A.M., Himes, C.S., Qin, W., Kola, I., Merchant, K.M., Nye, J.S., 2003. Positive and Negative Regulation of the γ-Secretase Activity by Nicastrin in a Murine Model. J. Biol. Chem. 278, 33445–33449. https://doi.org/10.1074/jbc.M301288200

Li, M., Cheng, R., Liang, J., Yan, H., Zhang, H., Yang, L., Li, Chengrang, Jiao, Q., Lu, Z., He, J., Ji, J., Shen, Z., Li, Chunqi, Hao, F., Yu, H., Yao, Z., 2013. Mutations in POFUT1, encoding protein O-fucosyltransferase 1, cause generalized Dowling-Degos disease. Am. J. Hum. Genet. 92, 895–903. https://doi.org/10.1016/j.ajhg.2013.04.022

Li, N., Fassl, A., Chick, J., Inuzuka, H., Li, X., Mansour, M.R., Liu, L., Wang, H., King, B., Shaik, S., Gutierrez, A., Ordureau, A., Otto, T., Kreslavsky, T., Baitsch, L., Bury, L., Meyer, C.A., Ke, N., Mulry, K.A., Kluk, M.J., Roy, M., Kim, S., Zhang, X., Geng, Y., Zagozdzon, A., Jenkinson, S., Gale, R.E., Linch, D.C., Zhao, J.J., Mullighan, C.G., Harper, J.W., Aster, J.C., Aifantis, I., von Boehmer, H., Gygi, S.P., Wei, W., Look, A.T., Sicinski, P., 2014. Cyclin C is a haploinsufficient tumour suppressor. Nat. Cell Biol. 16, 1080–1091. https://doi.org/10.1038/ncb3046

Li, Z.-L., Chen, C., Yang, Y., Wang, C., Yang, T., Yang, X., Liu, S.-C., 2015. Gamma secretase inhibitor enhances sensitivity to doxorubicin in MDA-MB-231 cells. Int. J. Clin. Exp. Pathol. 8, 4378–87.

Lim, R., Sugino, T., Nolte, H., Andrade, J., Zimmermann, B., Shi, C., Doddaballapur, A., Ong, Y.T., Wilhelm, K., Fasse, J.W.D., Ernst, A., Kaulich, M., Husnjak, K., Boettger, T., Guenther, S., Braun, T., Krüger, M., Benedito, R., Dikic, I., Potente, M., 2019. Deubiquitinase USP10 regulates Notch signaling in the endothelium. Science 364, 188–193. https://doi.org/10.1126/science.aat0778

Limbourg, F.P., Takeshita, K., Radtke, F., Bronson, R.T., Chin, M.T., Liao, J.K., 2005. Essential role of endothelial Notch1 in angiogenesis. Circulation 111, 1826–32. https://doi.org/10.1161/01.CIR.0000160870.93058.DD

Lin, S.E., Oyama, T., Nagase, T., Harigaya, K., Kitagawa, M., 2002. Identification of new human mastermind proteins defines a family that consists of positive regulators for notch signaling. J. Biol. Chem. https://doi.org/10.1074/jbc.M209529200

Lobov, I.B., Renard, R.A., Papadopoulos, N., Gale, N.W., Thurston, G., Yancopoulos, G.D., Wiegand, S.J., 2007. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc. Natl. Acad. Sci. 104, 3219–3224. https://doi.org/10.1073/pnas.0611206104

Louvi, A., Artavanis-Tsakonas, S., 2012. Notch and disease: A growing field. Semin. Cell Dev. Biol. 23, 473–480. https://doi.org/10.1016/j.semcdb.2012.02.005

Louvi, A., Artavanis-Tsakonas, S., 2006. Notch signalling in vertebrate neural development. Nat. Rev. Neurosci. 7, 93–102. https://doi.org/10.1038/nrn1847

Luca, V.C., Jude, K.M., Pierce, N.W., Nachury, M. V., Fischer, S., Garcia, K.C., 2015. Structural basis for Notch1 engagement of Delta-like 4. Science (80-.). 347, 847–853. https://doi.org/10.1126/science.1261093

Luca, V.C., Kim, B.C., Ge, C., Kakuda, S., Wu, D., Roein-Peikar, M., Haltiwanger, R.S., Zhu, C., Ha, T., Garcia, K.C., 2017. Notch-Jagged complex structure implicates a catch bond in tuning ligand sensitivity. Science (80-.). 355, 1320–1324. https://doi.org/10.1126/science.aaf9739

Malecki, M.J., Sanchez-Irizarry, C., Mitchell, J.L., Histen, G., Xu, M.L., Aster, J.C., Blacklow, S.C., 2006. Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. Mol. Cell. Biol. 26, 4642–51. https://doi.org/10.1128/MCB.01655-05

Malyukova, A., Dohda, T., von der Lehr, N., Akhoondi, S., Akhondi, S., Corcoran, M., Heyman, M., Spruck, C., Grandér, D., Lendahl, U., Sangfelt, O., 2007. The tumor suppressor gene hCDC4 is frequently mutated in human T-cell acute lymphoblastic leukemia with functional consequences for Notch signaling. Cancer Res. 67, 5611–6. https://doi.org/10.1158/0008-5472.CAN-06-4381

Manderfield, L.J., High, F.A., Engleka, K.A., Liu, F., Li, L., Rentschler, S., Epstein, J.A., 2012. Notch activation of Jagged1 contributes to the assembly of the arterial wall. Circulation 125, 314–23. https://doi.org/10.1161/CIRCULATIONAHA.111.047159

Mansour, M.R., Sulis, M.L., Duke, V., Foroni, L., Jenkinson, S., Koo, K., Allen, C.G., Gale, R.E., Buck, G., Richards, S., Paietta, E., Rowe, J.M., Tallman, M.S., Goldstone, A.H., Ferrando, A.A., Linch, D.C., 2009. Prognostic implications of NOTCH1 and FBXW7 mutations in adults with T-cell acute lymphoblastic leukemia treated on the MRC UKALLXII/ECOG E2993 protocol. J. Clin. Oncol. 27, 4352–6. https://doi.org/10.1200/JCO.2009.22.0996

Martincorena, I., Fowler, J.C., Wabik, A., Lawson, A.R.J., Abascal, F., Hall, M.W.J., Cagan, A., Murai, K., Mahbubani, K., Stratton, M.R., Fitzgerald, R.C., Handford, P.A., Campbell, P.J., Saeb-Parsy, K., Jones, P.H., 2018. Somatic mutant clones colonize the human esophagus with age. Science (80-.). 362, 911–917. https://doi.org/10.1126/science.aau3879

Masui, T., Long, Q., Beres, T.M., Magnuson, M.A., MacDonald, R.J., 2007. Early pancreatic development requires the vertebrate Suppressor of Hairless (RBPJ) in the PTF1 bHLH complex. Genes Dev. 21, 2629–43. https://doi.org/10.1101/gad.1575207

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

McCaffrey, L.M., Macara, I.G., 2009. The Par3/aPKC interaction is essential for end bud remodeling and progenitor differentiation during mammary gland morphogenesis. Genes Dev. 23, 1450–1460. https://doi.org/10.1101/gad.1795909

McGill, M.A., Dho, S.E., Weinmaster, G., McGlade, C.J., 2009. Numb Regulates Post-endocytic Trafficking and Degradation of Notch1. J. Biol. Chem. 284, 26427–26438. https://doi.org/10.1074/jbc.M109.014845

McMahon, H.T., Boucrot, E., 2011. Molecular mechanism and physiological functions of clathrin-mediated endocytosis. Nat. Rev. Mol. Cell Biol. 12, 517–533. https://doi.org/10.1038/nrm3151

Meloty-Kapella, L., Shergill, B., Kuon, J., Botvinick, E., Weinmaster, G., 2012. Notch Ligand Endocytosis Generates Mechanical Pulling Force Dependent on Dynamin, Epsins, and Actin. Dev. Cell 22, 1299–1312. https://doi.org/10.1016/j.devcel.2012.04.005

Mendez, M.G., Kojima, S.-I., Goldman, R.D., 2010. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. FASEB J. 24, 1838–1851. https://doi.org/10.1096/fj.09-151639

Meyer, R.D., Mohammadi, M., Rahimi, N., 2006. A Single Amino Acid Substitution in the Activation Loop Defines the Decoy Characteristic of VEGFR-1/FLT-1. J. Biol. Chem. 281, 867–875. https://doi.org/10.1074/jbc.M506454200

Miele, L., Golde, T., Osborne, B., 2006. Notch Signaling in Cancer. Curr. Mol. Med. 6, 905–918. https://doi.org/10.2174/156652406779010830

Mo, J.-S., Kim, M.-Y., Han, S.-O., Kim, I.-S., Ann, E.-J., Lee, K.S., Seo, M.-S., Kim, J.-Y., Lee, S.-C., Park, J.-W., Choi, E.-J., Seong, J.Y., Joe, C.O., Faessler, R., Park, H.-S., 2007. Integrin-Linked Kinase Controls Notch1 Signaling by Down-Regulation of Protein Stability through Fbw7 Ubiquitin Ligase. Mol. Cell. Biol. 27, 5565–5574. https://doi.org/10.1128/MCB.02372-06

Mohr, O.L., 1919. Character Changes Caused by Mutation of an Entire Region of a Chromosome in Drosophila. Genetics 4, 275–82.

Moloney, D.J., Panin, V.M., Johnston, S.H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K.D., Haltiwanger, R.S., Vogt, T.F., 2000. Fringe is a glycosyltransferase that modifies Notch. Nature 406, 369–75. https://doi.org/10.1038/35019000

Moran, J.L., Levorse, J.M., Vogt, T.F., 1999. Limbs move beyond the Radical fringe. Nature 399, 742–743. https://doi.org/10.1038/21560

Moran, J.L., Shifley, E.T., Levorse, J.M., Mani, S., Ostmann, K., Perez-Balaguer, A., Walker, D.M., Vogt, T.F., Cole, S.E., 2009. Manic fringe is not required for embryonic development, and fringe family members do not exhibit redundant functions in the axial skeleton, limb, or hindbrain. Dev. Dyn. 238, 1803–1812. https://doi.org/10.1002/dvdy.21982

Morel, V., Le Borgne, R., Schweisguth, F., 2003. Snail is required for Delta endocytosis and Notch-dependent activation of single-minded expression. Dev. Genes Evol. 213, 65–72. https://doi.org/10.1007/s00427-003-0296-x

Morgan, T.H., 1917. The theory of the gene. Am. Nat. https://doi.org/10.2307/2455888

Mukherjee, A., Veraksa, A., Bauer, A., Rosse, C., Camonis, J., Artavanis-Tsakonas, S., 2005. Regulation of Notch signalling by non-visual β-arrestin. Nat. Cell Biol. 7, 1191–1201. https://doi.org/10.1038/ncb1327

Musse, A.A., Meloty-Kapella, L., Weinmaster, G., 2012. Notch ligand endocytosis: Mechanistic basis of signaling activity. Semin. Cell Dev. Biol. 23, 429–436. https://doi.org/10.1016/j.semcdb.2012.01.011

Nandagopal, N., Santat, L.A., Elowitz, M.B., 2019. Cis-activation in the Notch signaling pathway. Elife 8. https://doi.org/10.7554/eLife.37880

Nandagopal, N., Santat, L.A., LeBon, L., Sprinzak, D., Bronner, M.E., Elowitz, M.B., 2018. Dynamic Ligand Discrimination in the Notch Signaling Pathway. Cell 172, 869-880.e19. https://doi.org/10.1016/j.cell.2018.01.002

Newton, A.C., 2018. Protein kinase C: perfectly balanced. Crit. Rev. Biochem. Mol. Biol. 53, 208–230. https://doi.org/10.1080/10409238.2018.1442408

Nguyen, H.T., Voza, F., Ezzeddine, N., Frasch, M., 2007. *Drosophila mind bomb2* is required for maintaining muscle integrity and survival. J. Cell Biol. 179, 219–227. https://doi.org/10.1083/jcb.200708135

Nichols, J.T., Miyamoto, A., Olsen, S.L., D'Souza, B., Yao, C., Weinmaster, G., 2007. DSL ligand endocytosis physically dissociates Notch1 heterodimers before activating proteolysis can occur. J. Cell Biol. 176, 445–458. https://doi.org/10.1083/jcb.200609014

Nieminen, M., Henttinen, T., Merinen, M., Marttila–Ichihara, F., Eriksson, J.E., Jalkanen, S., 2006. Vimentin function in lymphocyte adhesion and transcellular migration. Nat. Cell Biol. 8, 156–162. https://doi.org/10.1038/ncb1355

Nishizuka, Y., 1992. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258, 607–14.

Nofziger, D., Miyamoto, A., Lyons, K.M., Weinmaster, G., 1999. Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. Development 126, 1689–702.

O'Neil, J., Grim, J., Strack, P., Rao, S., Tibbitts, D., Winter, C., Hardwick, J., Welcker, M., Meijerink, J.P., Pieters, R., Draetta, G., Sears, R., Clurman, B.E., Look, A.T., 2007. FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to γ-secretase inhibitors. J. Exp. Med. 204, 1813–1824. https://doi.org/10.1084/jem.20070876

Obata, J., Yano, M., Mimura, H., Goto, T., Nakayama, R., Mibu, Y., Oka, C., Kawaichi, M., 2001. p48 subunit of mouse PTF1 binds to RBP-Jkappa/CBF-1, the intracellular mediator of Notch signalling, and is expressed in the neural tube of early stage embryos. Genes Cells 6, 345–60.

Okochi, M., Steiner, H., Fukumori, A., Tanii, H., Tomita, T., Tanaka, T., Iwatsubo, T., Kudo, T., Takeda, M., Haass, C., 2002. Presenilins mediate a dual intramembranous gamma-secretase cleavage of Notch-1. EMBO J. 21, 5408–16.

Oon, C.E., Bridges, E., Sheldon, H., Sainson, R.C.A., Jubb, A., Turley, H., Leek, R., Buffa, F., Harris, A.L., Li, J.-L., Oon, C.E., Bridges, E., Sheldon, H., Sainson, R.C.A., Jubb, A., Turley, H., Leek, R., Buffa, F., Harris, A.L., Li, J.-L., 2017. Role of Delta-like 4 in Jagged1-induced tumour angiogenesis and tumour growth. Oncotarget 8, 40115–40131. https://doi.org/10.18632/oncotarget.16969

Osipo, C., Patel, P., Rizzo, P., Clementz, A.G., Hao, L., Golde, T.E., Miele, L., 2008. ErbB-2 inhibition activates Notch-1 and sensitizes breast cancer cells to a gamma-secretase inhibitor. Oncogene 27, 5019–32. https://doi.org/10.1038/onc.2008.149

Ossipova, O., Ezan, J., Sokol, S.Y., 2009. PAR-1 phosphorylates Mind bomb to promote vertebrate neurogenesis. Dev. Cell 17, 222–33. https://doi.org/10.1016/j.devcel.2009.06.010

Overstreet, E., Fitch, E., Fischer, J.A., 2004. Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. Development 131, 5355–66. https://doi.org/10.1242/dev.01434

Päll, T., Pink, A., Kasak, L., Turkina, M., Anderson, W., Valkna, A., Kogerman, P., 2011. Soluble CD44 Interacts with Intermediate Filament Protein Vimentin on Endothelial Cell Surface. PLoS One 6, e29305. https://doi.org/10.1371/journal.pone.0029305

Pallari, H.-M., Eriksson, J.E., 2006. Intermediate Filaments as Signaling Platforms. Sci. STKE 2006, pe53–pe53. https://doi.org/10.1126/stke.3662006pe53

Palmer, W.H., Jia, D., Deng, W.-M., 2014. Cis-interactions between Notch and its ligands block ligand-independent Notch activity. Elife 3. https://doi.org/10.7554/eLife.04415

Panin, V.M., Papayannopoulos, V., Wilson, R., Irvine, K.D., 1997. Fringe modulates Notch–ligand interactions. Nature 387, 908–912. https://doi.org/10.1038/43191

Parker, P.J., Justilien, V., Riou, P., Linch, M., Fields, A.P., 2014. Atypical Protein Kinase Cι as a human oncogene and therapeutic target. Biochem. Pharmacol. 88, 1–11. https://doi.org/10.1016/j.bcp.2013.10.023

Parks, A.L., Klueg, K.M., Stout, J.R., Muskavitch, M.A., 2000. Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. Development 127, 1373–85.

Pasternak, S.H., Bagshaw, R.D., Guiral, M., Zhang, S., Ackerley, C.A., Pak, B.J., Callahan, J.W., Mahuran, D.J., 2003. Presenilin-1, nicastrin, amyloid precursor protein, and gamma-secretase activity are co-localized in the lysosomal membrane. J. Biol. Chem. 278, 26687–94. https://doi.org/10.1074/jbc.m304009200

Pedrosa, A.-R., Trindade, A., Carvalho, C., Graça, J., Carvalho, S., Peleteiro, M.C., Adams, R.H., Duarte, A., 2015. Endothelial Jagged1 promotes solid tumor growth through both pro-angiogenic and angiocrine functions. Oncotarget 6, 24404–23. https://doi.org/10.18632/oncotarget.4380

Pellinen, T., Rantala, J.K., Arjonen, A., Mpindi, J.-P., Kallioniemi, O., Ivaska, J., 2012. A functional genetic screen reveals new regulators of β1-integrin activity. J. Cell Sci. 125, 649–61. https://doi.org/10.1242/jcs.090704

Penton, A.L., Leonard, L.D., Spinner, N.B., 2012. Notch signaling in human development and disease. Semin. Cell Dev. Biol. 23, 450–457. https://doi.org/10.1016/j.semcdb.2012.01.010

Perumalsamy, L.R., Nagala, M., Banerjee, P., Sarin, A., 2009. A hierarchical cascade activated by non-canonical Notch signaling and the mTOR-Rictor complex regulates neglect-induced

death in mammalian cells. Cell Death Differ. 16, 879–89. https://doi.org/10.1038/cdd.2009.20

Petrovic, J., Formosa-Jordan, P., Luna-Escalante, J.C., Abello, G., Ibanes, M., Neves, J., Giraldez, F., 2014. Ligand-dependent Notch signaling strength orchestrates lateral induction and lateral inhibition in the developing inner ear. Development 141, 2313–2324. https://doi.org/10.1242/dev.108100

Peuhu, E., Virtakoivu, R., Mai, A., Wärri, A., Ivaska, J., 2017. Epithelial vimentin plays a functional role in mammary gland development. Development 144, 4103–4113. https://doi.org/10.1242/dev.154229

Pfeffer, I., Brewitz, L., Krojer, T., Jensen, S.A., Kochan, G.T., Kershaw, N.J., Hewitson, K.S., McNeill, L.A., Kramer, H., Münzel, M., Hopkinson, R.J., Oppermann, U., Handford, P.A., McDonough, M.A., Schofield, C.J., 2019. Aspartate/asparagine-β-hydroxylase crystal structures reveal an unexpected epidermal growth factor-like domain substrate disulfide pattern. Nat. Commun. 10. https://doi.org/10.1038/s41467-019-12711-7

Pfister, S., Przemeck, G.K.H., Gerber, J.-K., Beckers, J., Adamski, J., de Angelis, M.H., 2003. Interaction of the MAGUK Family Member Acvrinp1 and the Cytoplasmic Domain of the Notch Ligand Delta1. J. Mol. Biol. 333, 229–235. https://doi.org/10.1016/J.JMB.2003.08.043

Phua, D.C.Y., Humbert, P.O., Hunziker, W., 2009. Vimentin regulates scribble activity by protecting it from proteasomal degradation. Mol. Biol. Cell 20, 2841–55. https://doi.org/10.1091/mbc.e08-02-0199

Pintar, A., De Biasio, A., Popovic, M., Ivanova, N., Pongor, S., 2007. The intracellular region of Notch ligands: does the tail make the difference? Biol. Direct 2, 19. https://doi.org/10.1186/1745-6150-2-19

Pitulescu, M.E., Schmidt, I., Giaimo, B.D., Antoine, T., Berkenfeld, F., Ferrante, F., Park, H., Ehling, M., Biljes, D., Rocha, S.F., Langen, U.H., Stehling, M., Nagasawa, T., Ferrara, N., Borggrefe, T., Adams, R.H., 2017. Dll4 and Notch signalling couples sprouting angiogenesis and artery formation. Nat. Cell Biol. 19, 915–927. https://doi.org/10.1038/ncb3555

Plentz, R., Park, J.-S., Rhim, A.D., Abravanel, D., Hezel, A.F., Sharma, S. V, Gurumurthy, S., Deshpande, V., Kenific, C., Settleman, J., Majumder, P.K., Stanger, B.Z., Bardeesy, N., 2009. Inhibition of gamma-secretase activity inhibits tumor progression in a mouse model of pancreatic ductal adenocarcinoma. Gastroenterology 136, 1741–9.e6. https://doi.org/10.1053/j.gastro.2009.01.008

Popovic, M., Bella, J., Zlatev, V., Hodnik, V., Anderluh, G., Barlow, P.N., Pintar, A., Pongor, S., 2011. The interaction of Jagged-1 cytoplasmic tail with afadin PDZ domain is local, folding-independent, and tuned by phosphorylation. J. Mol. Recognit. 24, 245–253. https://doi.org/10.1002/jmr.1042

Poulson, D.F., 1937. Chromosomal Deficiencies and the Embryonic Development of Drosophila Melanogaster. Proc. Natl. Acad. Sci. U. S. A. 23, 133–7.

Preuss, C., Capredon, M., Wünnemann, F., Chetaille, P., Prince, A., Godard, B., Leclerc, S., Sobreira, N., Ling, H., Awadalla, P., Thibeault, M., Khairy, P., Samuels, M.E., Andelfinger, G., Andelfinger, G., 2016. Family Based Whole Exome Sequencing Reveals the Multifaceted Role of Notch Signaling in Congenital Heart Disease. PLOS Genet. 12, e1006335. https://doi.org/10.1371/journal.pgen.1006335

Preuße, K., Tveriakhina, L., Schuster-Gossler, K., Gaspar, C., Rosa, A.I., Henrique, D., Gossler, A., Stauber, M., 2015. Context-Dependent Functional Divergence of the Notch Ligands DLL1 and DLL4 In Vivo. PLOS Genet. 11, e1005328. https://doi.org/10.1371/journal.pgen.1005328

Pu, Y., Peach, M.L., Garfield, S.H., Wincovitch, S., Marquez, V.E., Blumberg, P.M., 2006. Effects on ligand interaction and membrane translocation of the positively charged arginine residues situated along the C1 domain binding cleft in the atypical protein kinase C isoforms. J. Biol. Chem. 281, 33773–88. https://doi.org/10.1074/jbc.M606560200

Puthenveedu, M.A., von Zastrow, M., 2006. Cargo Regulates Clathrin-Coated Pit Dynamics. Cell 127, 113–124. https://doi.org/10.1016/j.cell.2006.08.035

Rajan, A., Tien, A.-C., Haueter, C.M., Schulze, K.L., Bellen, H.J., 2009. The Arp2/3 complex and WASp are required for apical trafficking of Delta into microvilli during cell fate specification of sensory organ precursors. Nat. Cell Biol. 11, 815–824. https://doi.org/10.1038/ncb1888

Ramakrishnan, G., Davaakhuu, G., Chung, W.C., Zhu, H., Rana, A., Filipovic, A., Green, A.R., Atfi, A., Pannuti, A., Miele, L., Tzivion, G., 2015. AKT and 14-3-3 regulate Notch4 nuclear localization. Sci. Rep. 5, 8782. https://doi.org/10.1038/srep08782

Rampal, R., Li, A.S.Y., Moloney, D.J., Georgiou, S.A., Luther, K.B., Nita-Lazar, A., Haltiwanger, R.S., 2005. Lunatic fringe, manic fringe, and radical fringe recognize similar specificity determinants in O-fucosylated epidermal growth factor-like repeats. J. Biol. Chem. 280, 42454–63. https://doi.org/10.1074/jbc.M509552200

Rana, N.A., Nita-Lazar, A., Takeuchi, H., Kakuda, S., Luther, K.B., Haltiwanger, R.S., 2011. O-glucose trisaccharide is present at high but variable stoichiometry at multiple sites on mouse Notch1. J. Biol. Chem. 286, 31623–31637. https://doi.org/10.1074/jbc.M111.268243

Ranganathan, P., Vasquez-Del Carpio, R., Kaplan, F.M., Wang, H., Gupta, A., VanWye, J.D., Capobianco, A.J., 2011a. Hierarchical Phosphorylation within the Ankyrin Repeat Domain Defines a Phosphoregulatory Loop That Regulates Notch Transcriptional Activity. J. Biol. Chem. 286, 28844–28857. https://doi.org/10.1074/jbc.M111.243600

Ranganathan, P., Weaver, K.L., Capobianco, A.J., 2011b. Notch signalling in solid tumours: a little bit of everything but not all the time. Nat. Rev. Cancer 11, 338–351. https://doi.org/10.1038/nrc3035

Rantala, J.K., Mäkelä, R., Aaltola, A.-R., Laasola, P., Mpindi, J.-P., Nees, M., Saviranta, P., Kallioniemi, O., 2011. A cell spot microarray method for production of high density siRNA transfection microarrays. BMC Genomics 12, 162. https://doi.org/10.1186/1471-2164-12-162

Ratnayake, W.S., Apostolatos, C.A., Apostolatos, A.H., Schutte, R.J., Huynh, M.A., Ostrov, D.A., Acevedo-Duncan, M., 2018. Oncogenic PKC-ι activates Vimentin during epithelial-mesenchymal transition in melanoma; a study based on PKC-ι and PKC-ζ specific inhibitors. Cell Adh. Migr. 12, 447–463. https://doi.org/10.1080/19336918.2018.1471323

Richardson, A.M., Havel, L.S., Koyen, A.E., Konen, J.M., Shupe, J., Wiles, W.G., Martin, W.D., Grossniklaus, H.E., Sica, G., Gilbert-Ross, M., Marcus, A.I., 2018. Vimentin Is Required for Lung Adenocarcinoma Metastasis via Heterotypic Tumor Cell–Cancer-Associated Fibroblast Interactions during Collective Invasion. Clin. Cancer Res. 24, 420–432. https://doi.org/10.1158/1078-0432.CCR-17-1776

Ridgway, J., Zhang, G., Wu, Y., Stawicki, S., Liang, W.-C., Chanthery, Y., Kowalski, J., Watts, R.J., Callahan, C., Kasman, I., Singh, M., Chien, M., Tan, C., Hongo, J.-A.S., de Sauvage, F., Plowman, G., Yan, M., 2006. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. Nature 444, 1083–1087. https://doi.org/10.1038/nature05313

Rink, J., Ghigo, E., Kalaidzidis, Y., Zerial, M., 2005. Rab Conversion as a Mechanism of Progression from Early to Late Endosomes. Cell 122, 735–749. https://doi.org/10.1016/j.cell.2005.06.043

Saad, S., Stanners, S.R., Yong, R., Tang, O., Pollock, C.A., 2010. Notch mediated epithelial to mesenchymal transformation is associated with increased expression of the Snail transcription factor. Int. J. Biochem. Cell Biol. https://doi.org/10.1016/j.biocel.2010.03.016

Sanchez-Carbayo, M., Socci, N.D., Lozano, J., Saint, F., Cordon-Cardo, C., 2006. Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. J. Clin. Oncol. 24, 778–789. https://doi.org/10.1200/JCO.2005.03.2375

Santiago-Gómez, A., Kedward, T., Simões, B.M., Dragoni, I., NicAmhlaoibh, R., Trivier, E., Sabin, V., Gee, J.M., Sims, A.H., Howell, S.J., Clarke, R.B., 2019. PAK4 regulates stemness and progression in endocrine resistant ER-positive metastatic breast cancer. Cancer Lett. https://doi.org/10.1016/j.canlet.2019.05.014

Santio, N.M., Landor, S.K.J., Vahtera, L., Ylä-Pelto, J., Paloniemi, E., Imanishi, S.Y., Corthals, G., Varjosalo, M., Manoharan, G.B., Uri, A., Lendahl, U., Sahlgren, C., Koskinen, P.J., 2016. Phosphorylation of Notch1 by Pim kinases promotes oncogenic signaling in breast and prostate cancer cells. Oncotarget 7, 43220–43238. https://doi.org/10.18632/oncotarget.9215

Sanz-Ezquerro, J.J., Münsterberg, A.E., Stricker, S., 2017. Editorial: Signaling Pathways in Embryonic Development. Front. cell Dev. Biol. 5, 76. https://doi.org/10.3389/fcell.2017.00076

Saravanamuthu, S.S., Gao, C.Y., Zelenka, P.S., 2009. Notch signaling is required for lateral induction of Jagged1 during FGF-induced lens fiber differentiation. Dev. Biol. 332, 166–176. https://doi.org/10.1016/j.ydbio.2009.05.566

Sasaki, N., Sasamura, T., Ishikawa, H.O., Kanai, M., Ueda, R., Saigo, K., Matsuno, K., 2007. Polarized exocytosis and transcytosis of Notch during its apical localization in Drosophila epithelial cells. Genes to Cells. https://doi.org/10.1111/j.1365-2443.2007.01037.x

Satelli, A., Li, S., 2011. Vimentin in cancer and its potential as a molecular target for cancer therapy. Cell. Mol. Life Sci. https://doi.org/10.1007/s00018-011-0735-1

Sato, K., Watanabe, T., Wang, S., Kakeno, M., Matsuzawa, K., Matsui, T., Yokoi, K., Murase, K., Sugiyama, I., Ozawa, M., Kaibuchi, K., 2011. Numb controls E-cadherin endocytosis through p120 catenin with aPKC. Mol. Biol. Cell 22, 3103–19. https://doi.org/10.1091/mbc.E11-03-0274

Selbie, L.A., Schmitz-Peiffer, C., Sheng, Y., Biden, T.J., 1993. Molecular cloning and characterization of PKC iota, an atypical isoform of protein kinase C derived from insulin-secreting cells. J. Biol. Chem. 268, 24296–302.

Selkoe, D.J., Wolfe, M.S., 2007. Presenilin: running with scissors in the membrane. Cell 131, 215–21. https://doi.org/10.1016/j.cell.2007.10.012

Sen, A., Madhivanan, K., Mukherjee, D., Aguilar, R.C., 2012. The epsin protein family: coordinators of endocytosis and signaling. Biomol. Concepts 3, 117–126. https://doi.org/10.1515/bmc-2011-0060

Seo, D., Southard, K.M., Kim, J.-W., Lee, H.J., Farlow, J., Lee, J.-U., Litt, D.B., Haas, T., Alivisatos, A.P., Cheon, J., Gartner, Z.J., Jun, Y.-W., 2016. A Mechanogenetic Toolkit for Interrogating Cell Signaling in Space and Time. Cell 165, 1507–1518. https://doi.org/10.1016/j.cell.2016.04.045

Sethi, M.K., Buettner, F.F.R., Krylov, V.B., Takeuchi, H., Nifantiev, N.E., Haltiwanger, R.S., Gerardy-Schahn, R., Bakker, H., 2010. Identification of Glycosyltransferase 8 Family Members as Xylosyltransferases Acting on *O* -Glucosylated Notch Epidermal Growth Factor Repeats. J. Biol. Chem. 285, 1582–1586. https://doi.org/10.1074/jbc.C109.065409

Sethi, N., Dai, X., Winter, C.G., Kang, Y., 2011. Tumor-Derived Jagged1 Promotes Osteolytic Bone Metastasis of Breast Cancer by Engaging Notch Signaling in Bone Cells. Cancer Cell 19, 192–205. https://doi.org/10.1016/j.ccr.2010.12.022

Seugnet, L., Simpson, P., Haenlin, M., 1997. Requirement for dynamin during Notch signaling in Drosophila neurogenesis. Dev. Biol. 192, 585–98. https://doi.org/10.1006/dbio.1997.8723

Shen, W.-J., Zaidi, S.K., Patel, S., Cortez, Y., Ueno, M., Azhar, R., Azhar, S., Kraemer, F.B., 2012. Ablation of Vimentin Results in Defective Steroidogenesis. Endocrinology 153, 3249–3257. https://doi.org/10.1210/en.2012-1048

Shi, S., Stanley, P., 2003. Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. Proc. Natl. Acad. Sci. 100, 5234–5239. https://doi.org/10.1073/pnas.0831126100

Shimizu, M., Cohen, B., Goldvasser, P., Berman, H., Virtanen, C., Reedijk, M., 2011. Plasminogen activator uPA is a direct transcriptional target of the JAG1-notch receptor signaling pathway in breast cancer. Cancer Res. https://doi.org/10.1158/0008-5472.CAN-10-2523

Siekmann, A.F., Affolter, M., Belting, H.-G., 2013. The tip cell concept 10 years after: new players tune in for a common theme. Exp. Cell Res. 319, 1255–63. https://doi.org/10.1016/j.yexcr.2013.01.019

Sigismund, S., Woelk, T., Puri, C., Maspero, E., Tacchetti, C., Transidico, P., Di Fiore, P.P., Polo, S., 2005. Clathrin-independent endocytosis of ubiquitinated cargos. Proc. Natl. Acad. Sci. 102, 2760–2765. https://doi.org/10.1073/pnas.0409817102

Sigma-Aldrich, 2019. Duolink® PLA Troubleshooting Guide | Sigma-Aldrich [WWW Document]. URL https://www.sigmaaldrich.com/technical-documents/protocols/biology/duolink-trouble-shooting-guide.html (accessed 11.28.19).

Simons, K., Toomre, D., 2000. Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. 1, 31–39. https://doi.org/10.1038/35036052

Sjöqvist, M., Andersson, E.R., 2019. Do as I say, Not(ch) as I do: Lateral control of cell fate. Dev. Biol. 447, 58–70. https://doi.org/10.1016/j.ydbio.2017.09.032

Skalska, L., Stojnic, R., Li, J., Fischer, B., Cerda-Moya, G., Sakai, H., Tajbakhsh, S., Russell, S., Adryan, B., Bray, S.J., 2015. Chromatin signatures at Notch-regulated enhancers reveal large-scale changes in H3K56ac upon activation. EMBO J. 34, 1889–1904. https://doi.org/10.15252/embj.201489923

Smith, C.A., Lau, K.M., Rahmani, Z., Dho, S.E., Brothers, G., She, Y.M., Berry, D.M., Bonneil, E., Thibault, P., Schweisguth, F., Le Borgne, R., McGlade, C.J., 2007. aPKC-mediated phosphorylation regulates asymmetric membrane localization of the cell fate determinant Numb. EMBO J. 26, 468–80. https://doi.org/10.1038/sj.emboj.7601495

Snider, N.T., Omary, M.B., 2014. Post-translational modifications of intermediate filament proteins: mechanisms and functions. Nat. Rev. Mol. Cell Biol. 15, 163–177. https://doi.org/10.1038/nrm3753

Song, J., Park, S., Kim, M., Shin, I., 2008. Down-regulation of Notch-dependent transcription by Akt in vitro. FEBS Lett. 582, 1693–1699. https://doi.org/10.1016/j.febslet.2008.04.024

Song, Y., Kumar, V., Wei, H.-X., Qiu, J., Stanley, P., 2016. Lunatic, Manic, and Radical Fringe Each Promote T and B Cell Development. J. Immunol. 196, 232–243. https://doi.org/10.4049/jimmunol.1402421

Sorensen, E.B., Conner, S.D., 2010. γ-secretase-dependent cleavage initiates notch signaling from the plasma membrane. Traffic 11, 1234–45. https://doi.org/10.1111/j.1600-0854.2010.01090.x

Spang, A., 2009. On the fate of early endosomes. Biol. Chem. 390, 753–9. https://doi.org/10.1515/BC.2009.056

Sparrow, D.B., Chapman, G., Wouters, M.A., Whittock, N. V., Ellard, S., Fatkin, D., Turnpenny, P.D., Kusumi, K., Sillence, D., Dunwoodie, S.L., 2006. Mutation of the LUNATIC FRINGE gene in humans causes spondylocostal dysostosis with a severe vertebral phenotype. Am. J. Hum. Genet. 78, 28–37. https://doi.org/10.1086/498879

Sprinzak, D., Lakhanpal, A., LeBon, L., Santat, L.A., Fontes, M.E., Anderson, G.A., Garcia-Ojalvo, J., Elowitz, M.B., 2010. Cis-interactions between Notch and Delta generate mutually exclusive signalling states. Nature 465, 86–90. https://doi.org/10.1038/nature08959

Spuul, P., Daubon, T., Pitter, B., Alonso, F., Fremaux, I., Kramer, Ij., Montanez, E., Génot, E., 2016. VEGF-A/Notch-Induced Podosomes Proteolyse Basement Membrane Collagen-IV during Retinal Sprouting Angiogenesis. Cell Rep. 17, 484–500. https://doi.org/10.1016/j.celrep.2016.09.016

Suchting, S., Freitas, C., le Noble, F., Benedito, R., Breant, C., Duarte, A., Eichmann, A., 2007. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc. Natl. Acad. Sci. 104, 3225–3230. https://doi.org/10.1073/pnas.0611177104

Suckling, R.J., Korona, B., Whiteman, P., Chillakuri, C., Holt, L., Handford, P.A., Lea, S.M., 2017. Structural and functional dissection of the interplay between lipid and Notch binding by human Notch ligands. EMBO J. 36, 2204–2215. https://doi.org/10.15252/embj.201796632

Sun, X., Artavanis-Tsakonas, S., 1997. Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in Drosophila. Development 124, 3439–48.

Swatek, K.N., Komander, D., 2016. Ubiquitin modifications. Cell Res. 26, 399–422. https://doi.org/10.1038/cr.2016.39

Tagami, S., Okochi, M., Yanagida, K., Ikuta, A., Fukumori, A., Matsumoto, N., Ishizuka-Katsura, Y., Nakayama, T., Itoh, N., Jiang, J., Nishitomi, K., Kamino, K., Morihara, T., Hashimoto, R., Tanaka, T., Kudo, T., Chiba, S., Takeda, M., 2008. Regulation of Notch Signaling by Dynamic Changes in the Precision of S3 Cleavage of Notch-1. Mol. Cell. Biol. 28, 165–176. https://doi.org/10.1128/MCB.00863-07

Takeuchi, H., Haltiwanger, R.S., 2014. Significance of glycosylation in Notch signaling. Biochem. Biophys. Res. Commun. 453, 235–42. https://doi.org/10.1016/j.bbrc.2014.05.115

Takeuchi, H., Schneider, M., Williamson, D.B., Ito, A., Takeuchi, M., Handford, P.A., Haltiwanger, R.S., 2018. Two novel protein O-glucosyltransferases that modify sites distinct from POGLUT1 and affect Notch trafficking and signaling. Proc. Natl. Acad. Sci. U. S. A. 115, E8395–E8402. https://doi.org/10.1073/pnas.1804005115

Tamagnone, L., Zacchigna, S., Rehman, M., 2018. Taming the Notch Transcriptional Regulator for Cancer Therapy. Molecules 23, 431. https://doi.org/10.3390/molecules23020431

Tammela, T., Zarkada, G., Wallgard, E., Murtomäki, A., Suchting, S., Wirzenius, M., Waltari, M., Hellström, M., Schomber, T., Peltonen, R., Freitas, C., Duarte, A., Isoniemi, H., Laakkonen, P., Christofori, G., Ylä-Herttuala, S., Shibuya, M., Pytowski, B., Eichmann, A., Betsholtz, C., Alitalo, K., 2008. Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. Nature 454, 656–660. https://doi.org/10.1038/nature07083

Tanaka, N., Kyuuma, M., Sugamura, K., 2008. Endosomal sorting complex required for transport proteins in cancer pathogenesis, vesicular transport, and non-endosomal functions. Cancer Sci. 99, 1293–303. https://doi.org/10.1111/j.1349-7006.2008.00825.x

Taylor, K.L., Henderson, A.M., Hughes, C.C.W., 2002. Notch Activation during Endothelial Cell Network Formation in Vitro Targets the Basic HLH Transcription Factor HESR-1 and Downregulates VEGFR-2/KDR Expression. Microvasc. Res. 64, 372–383. https://doi.org/10.1006/mvre.2002.2443

Taylor, P., Takeuchi, H., Sheppard, D., Chillakuri, C., Lea, S.M., Haltiwanger, R.S., Handford, P.A., 2014. Fringe-mediated extension of O-linked fucose in the ligand-binding region of Notch1 increases binding to mammalian Notch ligands. Proc. Natl. Acad. Sci. U. S. A. 111, 7290–7295. https://doi.org/10.1073/pnas.1319683111

Teider, N., Scott, D.K., Neiss, A., Weeraratne, S.D., Amani, V.M., Wang, Y., Marquez, V.E., Cho, Y.-J., Pomeroy, S.L., 2010. Neuralized1 causes apoptosis and downregulates Notch target genes in medulloblastoma. Neuro. Oncol. 12, 1244–1256. https://doi.org/10.1093/neuonc/noq091

Tetzlaff, F., Fischer, A., 2018. Control of Blood Vessel Formation by Notch Signaling, in: Advances in Experimental Medicine and Biology. pp. 319–338. https://doi.org/10.1007/978-3-319-89512-3_16

Tobias, I.S., Newton, A.C., 2016. Protein Scaffolds Control Localized Protein Kinase Cζ Activity. J. Biol. Chem. 291, 13809–13822. https://doi.org/10.1074/jbc.M116.729483

Toivola, D.M., Strnad, P., Habtezion, A., Omary, M.B., 2010. Intermediate filaments take the heat as stress proteins. Trends Cell Biol. 20, 79. https://doi.org/10.1016/J.TCB.2009.11.004

Tomiyama, L., Kamino, H., Fukamachi, H., Urano, T., 2017. Precise epitope determination of the anti-vimentin monoclonal antibody V9. Mol. Med. Rep. 16, 3917–3921. https://doi.org/10.3892/mmr.2017.7102

Traustadóttir, G.Á., Jensen, C.H., Thomassen, M., Beck, H.C., Mortensen, S.B., Laborda, J., Baladrón, V., Sheikh, S.P., Andersen, D.C., 2016. Evidence of non-canonical NOTCH signaling: Delta-like 1 homolog (DLK1) directly interacts with the NOTCH1 receptor in mammals. Cell. Signal. 28, 246–54. https://doi.org/10.1016/j.cellsig.2016.01.003

Trindade, A., Kumar, S.R., Scehnet, J.S., Lopes-da-Costa, L., Becker, J., Jiang, W., Liu, R., Gill, P.S., Duarte, A., 2008. Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos. Blood 112, 1720–9. https://doi.org/10.1182/blood-2007-09-112748

Triolo, D., Dina, G., Taveggia, C., Vaccari, I., Porrello, E., Rivellini, C., Domi, T., La Marca, R., Cerri, F., Bolino, A., Quattrini, A., Previtali, S.C., 2012. Vimentin regulates peripheral nerve myelination. Development 139, 1359–67. https://doi.org/10.1242/dev.072371

Uhlen, M., Zhang, C., Lee, S., Sjöstedt, E., Fagerberg, L., Bidkhori, G., Benfeitas, R., Arif, M., Liu, Z., Edfors, F., Sanli, K., von Feilitzen, K., Oksvold, P., Lundberg, E., Hober, S., Nilsson, P., Mattsson, J., Schwenk, J.M., Brunnström, H., Glimelius, B., Sjöblom, T., Edqvist, P.-H., Djureinovic, D., Micke, P., Lindskog, C., Mardinoglu, A., Ponten, F., 2017. A pathology atlas of the human cancer transcriptome. Science (80-.). 357, eaan2507. https://doi.org/10.1126/science.aan2507

Uyttendaele, H., Ho, J., Rossant, J., Kitajewski, J., 2001. Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. Proc. Natl. Acad. Sci. U. S. A. 98, 5643–8. https://doi.org/10.1073/pnas.091584598

Vaccari, T., Lu, H., Kanwar, R., Fortini, M.E., Bilder, D., 2008. Endosomal entry regulates Notch receptor activation in Drosophila melanogaster. J. Cell Biol. 180, 755–62. https://doi.org/10.1083/jcb.200708127

van Engeland, N.C.A., Suarez Rodriguez, F., Rivero-Müller, A., Ristori, T., Duran, C.L., Stassen, O.M.J.A., Antfolk, D., Driessen, R.C.H., Ruohonen, S., Ruohonen, S.T., Nuutinen, S., Savontaus, E., Loerakker, S., Bayless, K.J., Sjöqvist, M., Bouten, C.V.C., Eriksson, J.E., Sahlgren, C.M., 2019. Vimentin regulates Notch signaling strength and arterial remodeling in response to hemodynamic stress. Sci. Rep. 9, 12415. https://doi.org/10.1038/s41598-019-48218-w

van Tetering, G., van Diest, P., Verlaan, I., van der Wall, E., Kopan, R., Vooijs, M., 2009. Metalloprotease ADAM10 Is Required for Notch1 Site 2 Cleavage. J. Biol. Chem. 284, 31018–31027. https://doi.org/10.1074/jbc.M109.006775

Varnum-Finney, B., Wu, L., Yu, M., Brashem-Stein, C., Staats, S., Flowers, D., Griffin, J.D., Bernstein, I.D., 2000. Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. J. Cell Sci. 113 Pt 23, 4313–8.

Vässin, H., Bremer, K.A., Knust, E., Campos-Ortega, J.A., 1987. The neurogenic gene Delta of Drosophila melanogaster is expressed in neurogenic territories and encodes a putative transmembrane protein with EGF-like repeats. EMBO J. 6, 3431–40.

Veeraraghavalu, K., Subbaiah, V.K., Srivastava, S., Chakrabarti, O., Syal, R., Krishna, S., 2005. Complementation of human papillomavirus type 16 E6 and E7 by Jagged1-specific Notch1-phosphatidylinositol 3-kinase signaling involves pleiotropic oncogenic functions independent of CBF1;Su(H);Lag-1 activation. J. Virol. 79, 7889–98. https://doi.org/10.1128/JVI.79.12.7889-7898.2005

Wandinger-Ness, A., Zerial, M., 2014. Rab proteins and the compartmentalization of the endosomal system. Cold Spring Harb. Perspect. Biol. 6, a022616. https://doi.org/10.1101/cshperspect.a022616

Wang, N.J., Sanborn, Z., Arnett, K.L., Bayston, L.J., Liao, W., Proby, C.M., Leigh, I.M., Collisson, E.A., Gordon, P.B., Jakkula, L., Pennypacker, S., Zou, Y., Sharma, M., North, J.P., Vemula, S.S., Mauro, T.M., Neuhaus, I.M., LeBoit, P.E., Hur, J.S., Park, K., Huh, N., Kwok, P.-Y., Arron, S.T., Massion, P.P., Bale, A.E., Haussler, D., Cleaver, J.E., Gray, J.W., Spellman, P.T., South, A.P., Aster, J.C., Blacklow, S.C., Cho, R.J., 2011. Loss-of-function mutations in Notch receptors in cutaneous and lung squamous cell carcinoma. Proc. Natl. Acad. Sci. 108, 17761–17766. https://doi.org/10.1073/pnas.1114669108

Wang, W., Struhl, G., 2005. Distinct roles for Mind bomb, Neuralized and Epsin in mediating DSL endocytosis and signaling in Drosophila. Development 132, 2883–94. https://doi.org/10.1242/dev.01860

Wang, W., Struhl, G., 2004. Drosophila Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. Development 131, 5367–80. https://doi.org/10.1242/dev.01413

Wang, W., Yi, M., Zhang, R., Li, J., Chen, S., Cai, J., Zeng, Z., Li, X., Xiong, W., Wang, L., Li, G., Xiang, B., 2018. Vimentin is a crucial target for anti-metastasis therapy of nasopharyngeal carcinoma. Mol. Cell. Biochem. 438, 47–57. https://doi.org/10.1007/s11010-017-3112-z

Weisshuhn, P.C., Handford, P.A., Redfield, C., 2015a. 1H, 13C and 15N assignments of EGF domains 4 to 7 of human Notch-1. Biomol. NMR Assign. 9, 275–279. https://doi.org/10.1007/s12104-014-9591-x

Weisshuhn, P.C., Handford, P.A., Redfield, C., 2015b. 1H, 13C and 15N assignments of EGF domains 8–11 of human Notch-1. Biomol. NMR Assign. 9, 375–379. https://doi.org/10.1007/s12104-015-9613-3

Weisshuhn, P.C., Sheppard, D., Taylor, P., Whiteman, P., Lea, S.M., Handford, P.A., Redfield, C., 2016. Non-Linear and Flexible Regions of the Human Notch1 Extracellular Domain Revealed by High-Resolution Structural Studies. Structure 24, 555–566. https://doi.org/10.1016/j.str.2016.02.010

Weng, A.P., Ferrando, A.A., Lee, W., Morris, J.P., Silverman, L.B., Sanchez-Irizarry, C., Blacklow, S.C., Look, A.T., Aster, J.C., 2004. Activating Mutations of NOTCH1 in Human T Cell Acute Lymphoblastic Leukemia. Science (80-.). 306, 269–271. https://doi.org/10.1126/science.1102160

Wharton, K.A., Johansen, K.M., Xu, T., Artavanis-Tsakonas, S., 1985. Nucleotide sequence from the neurogenic locus Notch implies a gene product that shares homology with proteins containing EGF-like repeats. Cell 43, 567–581. https://doi.org/10.1016/0092-8674(85)90229-6

WHO, 2019. Cardiovascular diseases (CVDs) [WWW Document]. URL https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) (accessed 11.29.19).

Wiche, G., Osmanagic-Myers, S., Castañón, M.J., 2015. Networking and anchoring through plectin: a key to IF functionality and mechanotransduction. Curr. Opin. Cell Biol. 32, 21–29. https://doi.org/10.1016/j.ceb.2014.10.002

Wilhelmsson, U., Faiz, M., de Pablo, Y., Sjöqvist, M., Andersson, D., Widestrand, Å., Potokar, M., Stenovec, M., Smith, P.L.P., Shinjyo, N., Pekny, T., Zorec, R., Ståhlberg, A., Pekna, M., Sahlgren, C., Pekny, M., 2012. Astrocytes Negatively Regulate Neurogenesis Through the Jagged1-Mediated Notch Pathway. Stem Cells 30, 2320–2329. https://doi.org/10.1002/stem.1196

Wilkin, M., Tongngok, P., Gensch, N., Clemence, S., Motoki, M., Yamada, K., Hori, K., Taniguchi-Kanai, M., Franklin, E., Matsuno, K., Baron, M., 2008. Drosophila HOPS and AP-3 Complex Genes Are Required for a Deltex-Regulated Activation of Notch in the Endosomal Trafficking Pathway. Dev. Cell 15, 762–772. https://doi.org/10.1016/j.devcel.2008.09.002

Windler, S.L., Bilder, D., 2010. Endocytic internalization routes required for delta/notch signaling. Curr. Biol. 20, 538–43. https://doi.org/10.1016/j.cub.2010.01.049

Wu, G., Lyapina, S., Das, I., Li, J., Gurney, M., Pauley, A., Chui, I., Deshaies, R.J., Kitajewski, J., 2001. SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. Mol. Cell. Biol. 21, 7403–15. https://doi.org/10.1128/MCB.21.21.7403-7415.2001

Xiao, H., Bai, X.-H., Kapus, A., Lu, W.-Y., Mak, A.S., Liu, M., 2010. The Protein Kinase C Cascade Regulates Recruitment of Matrix Metalloprotease 9 to Podosomes and Its Release and Activation. Mol. Cell. Biol. 30, 5545–5561. https://doi.org/10.1128/mcb.00382-10

Xiao, H., Liu, M., 2013. Atypical protein kinase C in cell motility. Cell. Mol. Life Sci. https://doi.org/10.1007/s00018-012-1192-1

Xu, T., Park, S., Giaimo, B.D., Hall, D., Ferrante, F., Ho, D.M., Hori, K., Anhezini, L., Ertl, I., Bartkuhn, M., Zhang, H., Milon, E., Ha, K., Conlon, K.P., Kuick, R., Govindarajoo, B., Zhang, Y., Sun, Y., Dou, Y., Basrur, V., Elenitoba-Johnson, K.S., Nesvizhskii, A.I., Ceron, J., Lee, C., Borggrefe, T., Kovall, R.A., Rual, J., 2017. RBPJ/CBF1 interacts with L3MBTL3/MBT1 to promote repression of Notch signaling via histone demethylase KDM1A/LSD1. EMBO J. 36, 3232–3249. https://doi.org/10.15252/embj.201796525

Xu, Y., Xia, J., Liu, S., Stein, S., Ramon, C., Xi, H., Wang, L., Xiong, X., Zhang, L., He, D., Yang, W., Zhao, X., Cheng, X., Yang, X., Wang, H., 2017. Endocytosis and membrane receptor internalization: implication of F-BAR protein Carom. Front. Biosci. (Landmark Ed. 22, 1439–1457. https://doi.org/10.2741/4552

Xue, Y., Gao, X., Lindsell, C.E., Norton, C.R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E.B., Weinmaster, G., Gridley, T., 1999. Embryonic Lethality and Vascular Defects in Mice Lacking the Notch Ligand Jagged1. Hum. Mol. Genet. 8, 723–730. https://doi.org/10.1093/hmg/8.5.723

Yamamoto, S., Charng, W.-L., Bellen, H.J., 2010. Endocytosis and Intracellular Trafficking of Notch and Its Ligands, in: Current Topics in Developmental Biology. pp. 165–200. https://doi.org/10.1016/S0070-2153(10)92005-X

Yan, Y., Denef, N., Schüpbach, T., 2009. The Vacuolar Proton Pump, V-ATPase, Is Required for Notch Signaling and Endosomal Trafficking in Drosophila. Dev. Cell 17, 387–402. https://doi.org/10.1016/j.devcel.2009.07.001

Yang, L.-T., Nichols, J.T., Yao, C., Manilay, J.O., Robey, E.A., Weinmaster, G., 2005. Fringe Glycosyltransferases Differentially Modulate Notch1 Proteolysis Induced by Delta1 and Jagged1. Mol. Biol. Cell 16, 927–942. https://doi.org/10.1091/mbc.e04-07-0614

Yap, L., Lee, D., Khairuddin, A., Pairan, M., Puspita, B., Siar, C., Paterson, I., 2015. The opposing roles of NOTCH signalling in head and neck cancer: a mini review. Oral Dis. 21, 850–857. https://doi.org/10.1111/odi.12309

Yochem, J., Weston, K., Greenwald, I., 1988. The Caenorhabditis elegans lin-12 gene encodes a transmembrane protein with overall similarity to Drosophila Notch. Nature 335, 547–550. https://doi.org/10.1038/335547a0

Yoon, K., Gaiano, N., 2005. Notch signaling in the mammalian central nervous system: insights from mouse mutants. Nat. Neurosci. 8, 709–715. https://doi.org/10.1038/nn1475

Zerial, M., McBride, H., 2001. Rab proteins as membrane organizers. Nat. Rev. Mol. Cell Biol. 2, 107–117. https://doi.org/10.1038/35052055

Zhang, J., Liu, M., Su, Y., Du, J., Zhu, A.J., 2012. A Targeted *In Vivo* RNAi Screen Reveals Deubiquitinases as New Regulators of Notch Signaling. G3: Genes|Genomes|Genetics 2, 1563–1575. https://doi.org/10.1534/g3.112.003780

Zhang, L.L., Cao, F.F., Wang, Y., Meng, F.L., Zhang, Y., Zhong, D.S., Zhou, Q.H., 2015. The protein kinase C (PKC) inhibitors combined with chemotherapy in the treatment of advanced non-small cell lung cancer: meta-analysis of randomized controlled trials. Clin. Transl. Oncol. 17, 371–377. https://doi.org/10.1007/s12094-014-1241-3

Zhang, Y.U., Xie, Z.Y., Guo, X.T., Xiao, X.H., Xiong, L.X., 2019. Notch and breast cancer metastasis: Current knowledge, new sights and targeted therapy (review). Oncol. Lett. https://doi.org/10.3892/ol.2019.10653

Zheng, L., Saunders, C.A., Sorensen, E.B., Waxmonsky, N.C., Conner, S.D., 2013. Notch signaling from the endosome requires a conserved dileucine motif. Mol. Biol. Cell 24, 297–307. https://doi.org/10.1091/mbc.E12-02-0081

Zhu, H., Zhou, X., Redfield, S., Lewin, J., Miele, L., 2013. Elevated Jagged-1 and Notch-1 expression in high grade and metastatic prostate cancers. Am. J. Transl. Res. 5, 368–78.

Zimber-Strobl, U., Strobl, L.J., 2001. EBNA2 and Notch signalling in Epstein–Barr virus mediated immortalization of B lymphocytes. Semin. Cancer Biol. 11, 423–434. https://doi.org/10.1006/scbi.2001.0409

Daniel Antfolk

Regulation of Notch Signaling by Intracellular Trafficking

The Notch signaling pathway is a critical part of cell-cell communication in all multicellular organisms. During the last few decades, many key aspects of Notch signaling have been described in development and disease. Still, researchers struggle to understand the context-dependent output of Notch. This PhD thesis provides new insights into the regulation of the Notch signaling pathway. In this thesis, a novel phosphorylation site is discovered on the signal-receiving Notch receptor, which regulates Notch signaling activity and influences differentiation of muscle and neuronal cells. Another key finding shows that signal-sending Jagged1 ligands interact with the cytoskeletal intermediate filament vimentin, which affects the activation potential of Jagged1. Furthermore, external addition of Jagged1 ligands can rescue the angiogenic sprouting defects seen from cells lacking vimentin. Finally, an approach to track endocytosis of Jagged1 ligands is developed to identify other new regulators of these Notch ligands in the future. Collectively this thesis presents novel regulators of Notch ligands and receptors, which may help facilitate future efforts to curtail deregulated Notch in disease.

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