Networking Notch

regulation of Notch traffic and signaling crosstalk

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To my family and friends

ABSTRACT

During the development of a multicellular organism cells need to communicate in order to decide the prospective nature of each cell. Differentiation, i.e. attaining specific traits that characterize specific cell types, is an essential part of development. The Notch signaling pathway is an important regulator of cell fate decisions. This thesis underlines the importance and effect of Notch receptor and ligand trafficking on stem cell differentiation.

Notch receptor endocytosis is required for activation of the signaling pathway, but the underlying molecular events are unclear. We have identified atypical PKCζ as a regulator of Notch receptor trafficking. aPKCζ mediated phosphorylation of Notch receptors enhances their internalization, but the outcome is dependent of the signaling status of the receptor. Phosphorylation of activated Notch1 receptors potentiates the Notch response. However, phosphorylation of non-activated receptors shifts receptor distribution from the cell surface to intracellular vesicles, without enhancing signaling or receptor degradation. aPKCζ regulates both neuronal and myogenic differentiation through regulation of Notch activity.

In another study we analyzed the ability of astrocytes to regulate neuronal differentiation. Previous work has shown that neurospheres exhibited a contact-dependent increase in differentiation when cultured with astrocytes lacking the cytoskeletal elements glial fibrillary protein (GFAP) and vimentin. We found that the $GFAP^{-/-}Vim^{-/-}$ astrocytes showed reduced Notch signaling, decreased expression and endocytosis of the Notch ligand Jagged1. Furthermore, the increase in neuronal differentiation was counteracted by culturing neurospheres on immobilized Jagged1 ligands. *In vivo* validations showed increased hippocampal neurogenesis in unchallenged mice after injury, in absence of GFAP and vimentin. This project appoints GFAP and vimentin as regulators of Jagged1 in astrocytes and implies that astrocytes regulate neurogenesis through the Notch signaling pathway

The third project also touches upon intermediate filaments as regulators of Notch signaling. We have discovered delayed angiogenesis in $Vim^{-/-}$ mice. At E.12.5 the vasculature shows a reduced number of vessels and sparse branching. *Ex vivo* analyses of aortic rings confirm the reduced branching capacity of $Vim^{-/-}$ endothelial cells. This phenotype is rescued by addition of immobilized Jagged and Dll1, but not Dll4, ligands. When analyzing the signaling properties of vimentin deficient cells we discovered that signal sending ability through Jagged is impaired, despite extensive Jagged expression at the plasma membrane. Furthermore, the signal receiving potential of vimentin deficient cells is enhanced, due to enhanced lysosomal liberation of NICD. This study suggests a previously unreported regulatory mechanism of Notch signaling by intermediate filaments in angiogenesis.

SAMMANFATTNING

Utvecklingen av flercelliga organismer är en mångfacetterad process som kräver kommunikation celler emellan. Under utvecklingen av en organism måste cellerna göra vissa val, som kommer att bestämma riktningen för deras fortsatta utveckling. Utgående från dessa val erhåller cellerna egenskaper som är karaktäristiska för olika celltyper. Notch-signaleringsärckan är en viktig reglerare av valet mellan olika cellöden. Denna avhandling belyser mekanismerna som reglerar omsättningen av såväl Notch-receptorer som -ligander och ökar förståelsen för hur dessa mekanismer påverkar Notch-medierade cellöden i stamceller.

Endocytos av Notch receptorer anses nödvändigt för fullständig aktivering av Notch-signalvägen. De bakomliggande molekylära mekanismerna är dock fortfarande oklara. Vi har upptäckt att atypiskt protein kinas ζ (aPKCζ) reglerar endocytos av Notch-receptorn. aPKCζ fosforylerar Notch, vilket leder till receptorns internalisering, men effekten är beroende av receptorns signaleringsstatus. Fosforylering av ligand-aktiverade receptorer ökar Notch-signalen. Däremot leder fosforylering av overksamma receptorer, som inte deltar i signalering, till deras internalisering, följt av varken ökad signalaktivitet eller degradering. Vår forskning visar att aPKCζ reglerar neuronal- och muskeldifferentiering genom modulering av Notch-signalvägen.

En annan studie fokuserar på astrocyternas förmåga att reglera neuronal differentiering. Differentiering av neurosfärer främjas i närvaro av astrocyter som saknar intermediärfilamenten vimentin och GFAP. Dessa GFAP$^{-/-}$Vim$^{-/-}$-astrocyter uppvisar försämrad förmåga att aktivera Notch-signalvägen, orsakad av minskad expression av Notch-liganden Jagged. Den ökade differentieringen i närvaro av GFAP$^{-/-}$Vim$^{-/-}$-astrocyter motverkas vid tillsats av Notch-ligander. *In vivo*-analyser bekräftade en ökad neurogenes i hippocampus hos GFAP$^{-/-}$Vim$^{-/-}$-möss som följd av nervskada. Våra resultat bevisar att vimentin och GFAP reglerar Jagged-uttrycket i astrocyter samt att astrocyter styr neuronaldifferentiering genom reglering av Notch-signalvägen.

Det sista arbetet för min avhandling analyserar även samspelet mellan Notch-signalering och intermediärfilament. Vi har upptäckt att Vim$^{-/-}$-möss har fördröjd vaskulär utveckling. Vid E12.5 uppvisar dessa möss ett outvecklat vaskulärt nätverk, med ett färre antal blodkärl och förgreningar. Dessa resultat fastställdes med en s.k. "aortic ring"-analys, där *aorta thoracica* avlägsnas från möss och snittas för att undersöka differentiering och avknoppning av nya blodkärl. Snitten från Vim$^{-/-}$-möss påvisade en klart försämrad förmåga till avknoppning. Denna fenotyp motverkades genom tillsats av immobiliserade Jagged- eller Dll1-, men inte Dll4-ligander. En analys av Vim$^{-/-}$-cellers förmåga att motta samt förmedla Notch-signalering visade att Vim$^{-/-}$ celler har nedsatt kapacitet att aktivera Notch i närliggande celler. Däremot besitter de en ökad potential att bli aktiverade av andra celler. Den ökade aktiveringen av Notch i Vim$^{-/-}$-celler är en följd av ökad lysosomal processering av Notch samt ökad frigörelse av NICD. Detta arbete framlägger vimentin som en nyupptäckt reglerare av Notch-signalering under angiogenesen.

Contents

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and a manuscript, which are referred to in the text by their Roman numerals (I-III). In addition, unpublished results are included.

I. Wilhelmsson, U., Faiz, M., de Pablo, Y*., **Sjöqvist, M***., Andersson, D., Widestrand, A., Potokar, M., Stenovec, M., Smith, P.L., Shinjyo, N., Pekny, T., Zorec, R., Ståhlberg, A., Pekna, M., Sahlgren, C. & Pekny, M. Astrocytes negatively regulate neurogenesis through the Jagged1-mediated Notch pathway. Stem Cells 2012; 30:2320-9

II. **Sjöqvist, M***., Antfolk, D*., Ferraris, S., Rraklli, V., Granqvist, C., Mutvei, A., Imanishi, S., Holmberg, J., Jin, S., Eriksson, J. E., Lehndal, U & Sahlgren, C. PKCζ regulates Notch receptor routing and activity in a Notch-signaling dependent manner. Cell Research 2014; 24: 433-450

III. **Sjöqvist, M.,** Antfolk, D*., Isoniemi, K*., Cheng, F., Duran, C., Antila, C., Niemi, R., Landor, S., Bouten, C., Bayless, K., Eriksson, J. E & Sahlgren, C. Vimentin regulates Notch signaling during angiogenesis. Manuscript submitted to Nature Communications.

*equal contribution

ABBREVATIONS

ANK	Ankyrin repeat
aPKC	Atypical protein kinase C
Arf6	ADP-ribosylation factor-6
BSA	Bovine serum albumin
caPKCζ	Constitutively active form of PKCζ
CIE	Clathrin independent endocytosis
CME	Clathrin-mediated endocytosis
CNS	Central nervous system
CSL	DNA binding protein CBF1/RBPKj in mammals, Suppressor of Hairless in *Drosophila*, Lag-1 in *C. elegans*
DAG	Diacylglycerol
DAPI	4´,6-diamidino-2-phenylindole
Dll	Delta like
dnPKCζ	Dominant-negative form of PKCζ
DOS	Delta and OSM-11 domain
DUB	Deubiquitination enzyme
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GSI	γ-secretase inhibitors
Hes	Hairy and enhancer of split
Hey	Hairy/enhancer-of-split related with YRPW motif protein
HUVEC	Human umbilical vein endothelial cells
IF	Intermediate filament
ILV	Intraluminal vesicles
KO	Knock out
LNR	Lin-12-Notch repeats
MEF	Mouse embryonic fibroblast
MHC	Myosin heavy chain
Mib	Mind bomb
mRNA	Messenger RNA
MVB	Multivesicular bodies

NICD	Notch intracellular domain
NECD	Notch extracellular domain
NEXT	Notch extracellular truncation
NLS	Nuclear localization signal peptide
PEST	Conserved peptide of proline-glutamic acid-serine-threonine involved in protein degradation
PBS	Phosphate-buffered saline
PTM	Post translational modification
Rab	Small GTP-binding protein belonging to the Ras superfamily
RAM	RBP-Jκ assosciated molecule
RBP-Jκ	Recombination signal sequence binding protein for Jκ genes
SEM	Standard error of the mean
shRNA	Small hairpin RNA
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Vim	Vimentin
WT	Wild type

INTRODUCTION

The creation of a multicellular organism, such as a mouse or a human being requires a tremendous amount of cell divisions. During the course of development the cells need to form organs, functional units of an organism. This process requires differentiation, the commitment of a cell to become a specialized type of cell with distinct characteristics. The Notch signaling pathway is a signaling system that guides cells in the process of choosing their cell fate.

This thesis shows that Notch signaling is regulated by intermediate filament proteins both during neuronal differentiation and angiogenesis. Intermediate filaments affect ligand expression and intracellular trafficking. In astrocytes lacking GFAP and vimentin Notch signaling is reduced due to decreased expression and endocytosis of the Notch ligand Jagged1. This results in reduced Notch signaling from astrocytes to neural stem cells, followed by increased neuronal differentiation. Vimentin depletion in endothelial cells reduces endothelial sprouting. Vimentin affects Jagged1 localization and trafficking and in its absence Jagged1 is accumulated at the cell surface. Interestingly, vimentin alters Notch activation at two levels. Cells devoid of vimentin are poor signal sending cells but very good Notch signal receivers.

Another part of this work identifies atypical PKCζ as a regulator of Notch receptor trafficking. aPKCζ-mediated phosphorylation of Notch receptors enhances their internalization, but the outcome is dependent on the signaling status of the receptor. Phosphorylation of activated Notch receptors

potentiates the Notch response. However, phosphorylation of non-activated receptors shifts receptor distribution from the cell surface to intracellular vesicles, without enhancing signaling or receptor degradation. aPKCζ regulates both neuronal and myogenic differentiation through regulation of Notch activity. This project led to the identification of a novel aPKCζ specific phosphorylation site in Notch1, critical for Notch activity during stem cell differentiation.

REVIEW OF THE LITERATURE

1. The Notch signaling pathway

The Notch signaling pathway is a signaling system between two contacting cells. The Notch receptor, presented on the surface of one cell, interacts with the ligand on a juxtaposed cell. The receptor-ligand interaction leads to activation of the signaling cascade through proteolytic processing of the receptor. The first cleavage occurs extracellularly followed by an intracellular cleavage event at the cell membrane, releasing the active signaling fragment consisting of the Notch intracellular domain (NICD) (Figure 1). The released NICD translocates to the nucleus and activates transcription of Notch downstream targets.

1.1Brief history of Notch

The Notch signaling pathway has obtained its name from the observation of notched wing margins in *Drosophila melanogaster* fruit flies (Dexter, 1914; Morgan and Bridges, 1916; Mohr, 1919) carrying mutations in the Notch gene. In 1983 the Notch locus was described by several research groups (Artavanis-Tsakonas et al., 1983; Kidd et al., 1983) and the protein structure was revealed shortly thereafter (Wharton et al., 1985; Kidd et al., 1989). Homozygous inactivation of Notch turned out to be embryonically lethal, with increased differentiation along the neural lineage and a reduction of epidermal elements (Poulson, 1940). Later on, Notch ligands were identified in *D. melanogaster* (Kopczynski et al., 1988; Fleming et al., 1990; Thomas et al., 1991) and the Notch signaling pathway was recognized to regulate cell fate choices in individual cells. Weinmaster at al. (1991) identified a Notch homolog in

mammals bridging for an ongoing interest in the role of Notch signaling both during development and disease. To date, the Notch signaling pathway is considered a major determinant of cell fate choices. However, there are still many unresolved questions regarding different modifications of both Notch receptors and ligands and how these modifications regulate signaling activity. The cell context dependence of the pathway, i.e. the variable outcome of Notch signaling in different cell types, is also an interesting subject.

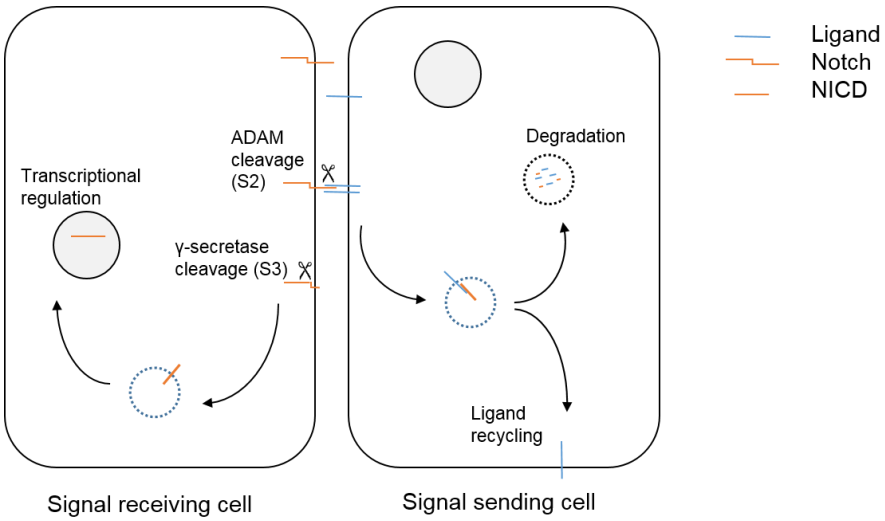

Figure 1. The Notch signaling pathway. Notch signaling is activated by the interaction between Notch receptors, on the signal receiving cell, and Notch ligands, on the signal sending cell. Receptor-ligand interaction activates two proteolytic cleavages of the Notch receptor, conducted by the ADAM metalloprotease (S2) and γ-secretase complexes (S3). Receptor processing leads to generation of the Notch intracellular domain (NICD). NICD translocates to the nucleus and regulates gene expression. The extracellular portion of the Notch receptor is transendocytosed, together with the ligand, into the signal sending cell. In the signal sending cell the receptor fragment is degraded, whereas the ligand may escape degradation and undergo recycling. Modified from D'Souza et al. (2010).

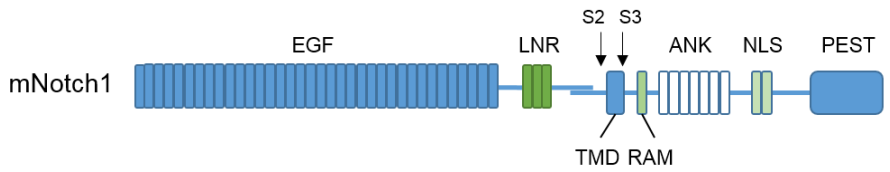

Figure 2. Structure of the Notch receptor. The extracellular portion of the mammalian Notch receptor consist of 36 epidermal growth factor-like- domains (EGF), Lin-12 Notch repeats (LNR) followed by a transmembrane domain (TMD). The intracellular domain is made up of a RBP-Jκ associated molecule domain (RAM), ankyrin repeats (ANK), a nuclear localization sequence (NLS) and a transactivation domain including a proline-glutamic acid-serine-threonine sequence (PEST). The sites for proteolytic processing of the receptor, conducted by the ADAM metalloprotease (S2) and the γ-secretase complex (S3), are indicated by arrows. Modified from Kopan & Ilagan (2009).

1.2 Notch receptors

The Notch receptors are single pass-transmembrane proteins that require ligand binding and proteolytic processing for their activation. Mammals express four different Notch receptors, Notch1-4. The Notch receptor undergoes proteolytic processing at three distinct occasions prior to generation of the active signaling fragment. The first proteolytic cleavage (S1) is executed by a furin-like protease in the trans-Golgi network preceeding insertion to the plasma membrane (Blaumueller et al., 1997; Logeat et al., 1998). The N-terminal domain of the Notch receptor consists of 36 epidermal growth factor-like (EGF)- domains, comprising the ligand interacting EGF 11-12 module, followed by three Lin-12-Notch repeats (LNR) and a hydrophobic region (Figure 2). Together these segments form the extracellular domain of the Notch receptor (NECD). The intracellular domain of the Notch receptor

(NICD) consists of an RBP-Jκ associated molecule (RAM) domain, seven ankyrin repeats (ANK), a nuclear localization signal peptide (NLS) and a transactivation domain including a proline-glutamic acid-serine-threonine (PEST) sequence. In addition, the transmembrane section contains the S3 cleavage site that is processed by γ-secretase following ligand binding and shedding of the NECD. The LNR and hydrophobic region, which mediates heterodimerization, are positioned on the NECD and have been suggested to cover the S2 cleavage site and thereby prevent ligand independent activation of the receptor (Sanchez-Irizarry et al., 2004; Kopan and Ilagan, 2009; Chillakuri et al., 2012). The role of the RAM and ANK domains is to bind CSL and assemble transcription factors mediating Notch driven gene expression (Tamura et al., 1995). The PEST domain is responsible for proteolytic degradation of NICD (Chillakuri et al., 2012).

1.3 Notch ligands

Like the receptors, the Notch ligands are also single pass-transmembrane proteins containing several EGF domains (Figure 3). In mammals, canonical Notch activation relies on the expression of five different DSL (Delta, Serrate, Lag2) ligands. The ligands found in mammals are divided into two groups based on their structural homology with Drosophila ligands. These two groups are the Delta-like ligands (Dll1, Dll3 and Dll4) and the Serrate-like Jagged ligands (Jagged1 and Jagged2) (Bray, 2006). N-terminally, the DSL-domain and the two first EGF repeats are essential for receptor-ligand interaction (Shimizu et al., 1999; Parks et al., 2006). These EGF-repeats are also referred to as the Delta and OSM-11 (DOS) domain (Komatsu et al., 2008). On the

intracellular side the DSL ligands comprise a C-terminal PDZ (PSD-95/Dlg/ZO-1)-motif, through which the ligands may interact with the cytoskeleton (D'Souza et al., 2008). The amino acid structures of these motifs differ between Jagged and Dll and therefore they have different interaction partners (Adam et al., 2013). Delta-like and Jagged ligands also differ in that Jagged ligand consist of several EGF repeats and also have a cysteine-rich domain close to the transmembrane domain (Weinmaster, 1997; Lissemore and Starmer, 1999).

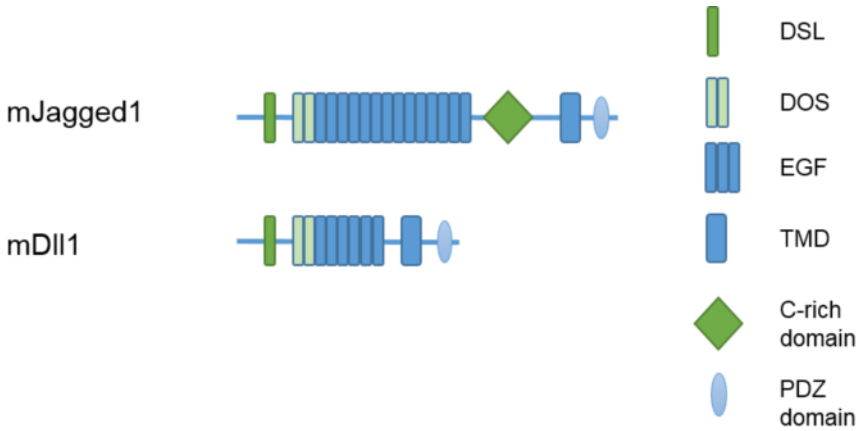

Figure 3. Structure of the Notch ligands. The mammalian Notch ligands Jagged and Delta-like 1 (Dll1) consist of an DSL (Delta, Serrate, Lag2) motif and the Delta and OSM-11 domain (DOS) followed by additional epidermal growth factor-like- repeats (EGF). The DSL and DOS domains are critical for ligand-receptor interaction. Intracellularly the ligands carry a PDZ (PSD-95/Dlg/ZO-1)-motif, through which the ligands may interact with the cytoskeleton. The major difference between Jagged and Dll is the existence of a cysteine-rich domain on Jagged. Modified from Ascano et al. (2003), Kopan & Ilagan (2009).

1.4 Activation of the Notch signaling pathway

The Notch signaling pathway is a cell contact dependent signaling system, requiring direct interaction between two cells for its activation (Artavanis-Tsakonas, 1988). The two participating cells are denoted the signal sending and the signal receiving cell. The signal sending cell presents the ligand on its cell surface activating Notch signaling in the receptor expressing, signal receiving cell (Figure 1). One cell may simultaneously function both as a Notch signal sender and a signal receiver. However, ligands and receptors on the same cell may interact through a mechanism called cis-inhibition. Cis-interactions prevents receptors and ligands from participating in trans-activation of Notch signaling, that is the interaction between two adjacent cells required to activate Notch. Therefore, the quantities of receptors and ligands expressed on the cell surface are important for establishing the sender-receiver phenotype of a specific cell. Futher, the Fringe glycosyltransferases that modify both Notch receptors and ligands are thought to influence both cis-inhibition and trans-activation properties in cells, thereby affecting the signaling status of the cell (del Alamo et al., 2011; LeBon et al., 2014).

Ligand-receptor interaction is thought to induce internalization of the ligand into the signal sending cell. This movement generates a strain on the Notch receptor resulting in a conformational change revealing the S2 cleavage site and facilitating proteolytic processing by a disintegrin and metalloprotease (ADAM) (Rooke et al., 1996; Wen et al., 1997; Parks et al., 2000; Meloty-Kapella et al., 2012). The first extracellular cleavage is followed by intramembranous proteolytic processing of Notch at the S3 cleavage site by the

γ-secretase complex. The γ-secretase complex is a large protein structure consisting of presenilin (PS), and the cofactors nicastrin, Aph-1 and Pen-2. Presenilin is a transmembrane protein found in the endoplasmic reticulum, Golgi and plasma membranes and also in lysosomal membranes (Fortini, 2002; Pasternak et al., 2003; Selkoe and Wolfe, 2007). The cofactors include the integral membrane protein nicastrin, which is shown to join with presenilin at the C-terminus of the Notch receptor (Yu et al., 2000). Furthermore, the cofactors Aph-1 and Pen-2 are also essential for the formation of a functional complex (Francis et al., 2002). The intracellular site of activity for the γ-secretase complex has been unclear. There are evidence of its activity being confined to the plasma membrane and to intracellular vesicles (Kaether et al., 2006a).

Proteolytic processing at the S3 cleavage site releases NICD, the active signaling fragment of the Notch pathway. Activation of one Notch receptor generates one signaling molecule, without further signal amplification. Therefore, the Notch pathway is highly dependent on the cell surface levels of receptors and ligands (Kopan and Ilagan, 2009). NICD translocates to the nucleus where it interacts with the transcriptional regulator CSL ("CBF-1, Suppressor of Hairless, Lag-1," entitled after the mammalian, *Drosophila*, and *C. elegans* orthologues, also called RBP-Jκ in mammals) (Artavanis-Tsakonas et al., 1999; Kopan and Ilagan, 2009). The interaction between NICD and CSL is followed by the assembly of co-activators to induce transcription (Kovall, 2008). The outcome of Notch signal activation varies between different cell types and is considered sensitive to changes in cell context (Miele, 2011).

The pathway described above is generally termed the canonical Notch pathway to distinguish it from non-canonical Notch signaling, which does not require CSL (Figure 4). Non-canonical Notch signaling may function ligand independently. There are several unanswered questions when it comes to non-canonical Notch signaling, with most focus directed towards Notch regulation of the Wnt signaling pathway (Andersen et al., 2012).

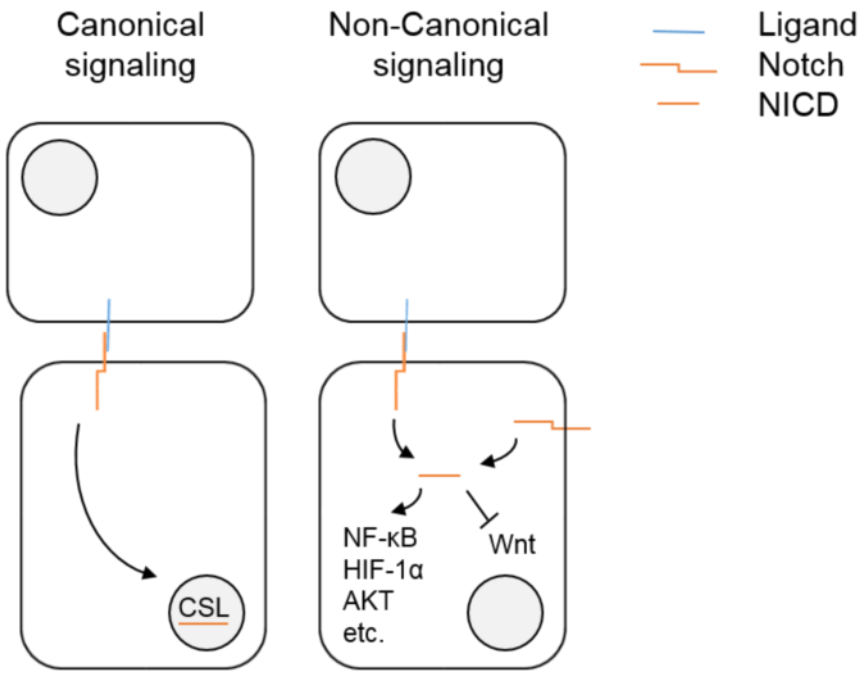

Figure 4. Canonical versus non-canonical Notch signaling. Non-canonical Notch signaling differs from the canonical Notch pathway in that it does not interact with CSL in the nucleus. Instead non-canonical Notch interacts with other signaling pathways such as the Wingless-Int (Wnt), NF-κB, hypoxia inducible factor- 1α (HIF-1α), AKT and c-Jun N-terminal kinase (JNK) pathways to name a few. Non-canonical Notch signaling may become activated ligand independently. Modified from Andersen et al. (2012) and Ayaz and Osborne (2014).

2. Endocytosis of Notch receptors and ligands

2.1 Principles of endocytosis

Endocytosis is defined as the uptake of materia by a cell through invagination of the plasma membrane. Phagocytosis and pinocytosis corresponds to unspecific uptake of particles and fluids by a cell. Receptor-mediated endocytosis is a more specific type of endocytosis, where ligand binding triggers internalization of the receptor-ligand complex. Clathrin is a scaffold protein that interacts with adaptor proteins for endocytosis. Clathrin coated pits form and invaginate periodically from the cell surface. Membrane receptors or ligands may associate with clathrin coated pits and as clathrin coated vesicles are internalized, the transmembrane proteins are taken up simultaneously (Kirchhausen et al., 2014). Examples of receptors internalized by clathrin mediated endocytosis (CME) are: the low-density lipoprotein (LDL) receptor, the transferrin receptor (Conner and Schmid, 2003), receptor tyrosin kinases such as epidermal growth factor receptors (EGFR) (Hanover et al., 1984) and vascular endothelial growth factor receptors (VEGFR) (Lampugnani et al., 2006), G protein coupled receptors (Wolfe and Trejo, 2007), integrins (De Franceschi et al., 2015) and Notch (Windler and Bilder, 2010; Andersson, 2012). However, there are also alternative means of internalization, not linked to clathrin, i.e. clathrin independent endocytosis (CIE). These alternative internalization routes require either caveolin, RhoA, Cdc42 or Arf6 (ADP-ribosylation factor-6) to accomplish the endocytosis of molecules from the cell membrane. Examples of molecules undergoing CIE are: β1 integrin, major histocompatibility protein I (MHC-1), E-cadherin and interleukin-2 receptor (IL-2R) (Mayor and Pagano, 2007).

Endocytosis is way of regulating receptor and ligand levels at the plasma membrane. Different endocytic compartments contain different Rab proteins. Rab proteins are small GTP-binding protein belonging to the Ras superfamily. They are involved in regulation of vesicular trafficking and cargo sorting through recruitment of other effector proteins (Pereira-Leal and Seabra, 2001). Upon endocytosis the vesicles formed merge with early Rab5 containing endosomes. From early endosomes the internalized molecules can move on to the Rab4 marked fast recycling pathway to be reincorporated in the plasma membrane. Another alternative for the cargo is to enter the slow recycling route governed by Rab11 or to remain in the endosomes that will mature into multivesicular bodies (MVBs) and late endosomes. Rab7 is involved in the transition from early to late endosomes. At this point all cargo that is to be recycled is removed and the molecules destined for degradation are incorporated into intraluminal vesicles (ILVs). Finally, the fusion of MVBs and late endosomes with lysosomes results in the degradation of remaining cargo (Zerial and McBride, 2001; Sorkin and von Zastrow, 2009).

This thesis focuses on the internalization of components belonging to the Notch pathway. Endocytosis of Notch receptors and ligands is considered to be involved in either signal regulatory or activating processes. Activation of the Notch signaling cascade requires endocytosis of both the Notch receptor and the Notch ligands (Seugnet et al., 1997; Parks et al., 2000). Notch signal activation is conducted through CME. Both receptors and ligands interact with different clathrin-associated sorting proteins, e.g. epsin and Numb (Windler and Bilder, 2010; Meloty-Kapella et al., 2012; Andersson, 2012). However,

there is evidence of both Notch receptor and ligand internalization occurring independently of the CME route. Okamura and Saga (2008) observed caveolin dependent endocytosis of the Notch receptor in the presomitic mesoderm of mice. In addition, a connection between Notch and caveolin has been observed in neural progenitor cells and this interaction is also proposed to regulate neural differentiation (Campos et al., 2006; Wang et al., 2013). Delta is capable of internalization through dynamin dependent CIE during *Drosophila* oogenesis (Okamura and Saga, 2008; Windler and Bilder, 2010; Banks et al., 2011). The effects of CIE on Notch signaling may be subject to cell type specific variations contributing to the activation of Notch signaling in some cells, whereas only engaging in either protein recycling or degradation in other cell types (Campos et al., 2006; Okamura and Saga, 2008; Windler and Bilder, 2010; Yamamoto et al., 2010; Wang et al., 2013).

Intermediate filaments in regulation of endocytosis

Intermediate filaments (IFs), actin filaments and microtubules form the cytoskeleton of the cell. The IFs form a complex network within the cells providing the cell with mechanical strength and rigidity including ability to endure physical strain (Herrmann et al., 2007). Part of this thesis explores the interplay between Notch signaling and the IFs vimentin and GFAP. Vimentin and GFAP are type III IF proteins expressed by cells of mesenchymal or glial origin respectively. In addition, the type III IFs also consist of desmin, found in muscle tissue, and peripherin, found in the nervous system. The IFs are divided into 6 different groups based on their characteristics. A common

feature of type III IFs is that they can form homopolymers and do not require other IFs in order to polymerize (Goldman et al., 1999; Hyder et al., 2008). IFs are made up of monomers that form dimers and tetramerize, followed by lateral assembly into a protofilament consisting of eight parallel tetramers. Phosphorylation of the IFs is thought to regulate the assembly and disassembly of the filament network (Hyder et al., 2008). The IF network has been proposed to function as a passageway for vesicle movement (Potokar et al., 2007) or to function as an assembly platform for protein complexes within cells (Chang and Goldman, 2004). In addition, vimentin dynamics regulate the recycling of cell surface receptors (Ivaska et al., 2005).

Regulation of endocytosis and trafficking by PKC

Posttranslational modifications (PTM) are highly involved in regulation of endocytosis and sorting of internalized plasma membrane proteins (Donaldson and Segev, 2009). Protein phosphorylation is carried out by protein kinases through attachment of phosphate groups to serine, threonine or tyrosine residues. Protein kinase C (PKC) is a family of serine threonine kinases. This kinase family is divided into three different members: conventional PKCs (cPKC), novel PKCs (nPKC) and atypical PKCs (aPKC). The difference between these groups resides in their mode of activation. cPKCs require phosphatidylserine, calcium (Ca^{2+}) and diacylglycerol (DAG) for their activation, whereas nPKCs require DAG but not Ca^{2+} and aPKCs require neither (Zeng et al., 2012). Alvi et al. (2007) summarizes the importance of PKC as a regulator of endocytosis. Phosphorylation by PKC influences down

regulation or trafficking of G-protein-coupled receptors (GPCR) and epidermal growth factor receptors (EGFR) among other plasma membrane proteins. When it comes to the Notch signaling pathway, there are several indications of interactions between Notch and PKC in *Drosophila* and in mammals (Bayraktar et al., 2006; Smith et al., 2007; Steinhart et al., 2007; Ossipova et al., 2009; Tremmel et al., 2013). The Notch receptor and PKC come into close proximity as PKC requires cell membrane localization in order to become activated (Tremmel et al., 2013). Notch has also been proposed to govern astrogenesis through PKC-mediated mechanisms (Steinhart et al., 2007). In addition, PKC may influence Notch activity by balancing the function of Notch regulatory proteins such as Numb and Mind bomb (Mib) (Smith et al., 2007; Ossipova et al., 2009). Numb is a known and important regulator of endocytosis (Santolini et al., 2000; McGill et al., 2009) whereas Mib regulates endocytosis of Notch ligands (Krahn and Wodarz, 2009).

The role of ubiquitination on endocytosis

Another PTM involved in endocytosis is ubiquitination. Ubiquitination is most commonly related to degradation, but ubiquitin may also function as a recognition tag for proteins to be internalized (Donaldson and Segev, 2009). Ubiquitination has been shown to be important in regulation of both Notch receptor and ligand internalization (Gupta-Rossi et al., 2004; Meloty-Kapella et al., 2012; Heuss et al., 2013) and it is mainly known for its role in targeting proteins for proteosomal degradation (Ciechanover, 2010). Ubiquitination is the post-transcriptional modification of substrate proteins, e.g. Notch

receptors and ligands, by addition of ubiquitin polypeptides to lysines on the substrate through isopeptide linkages. There are various modes of ubiquitin modifications including mono-, multi- and polyubiquitination, each resulting in different outcomes. Ubiquitination involves a series of enzymatic reactions carried out by E1 activating and E2 conjugating ubiquitination enzymes as well as an E3 ubiquitin ligase. Deubiquitination events are also common and mediated by deubiquitination enzymes (DUB) belonging to either cysteine- or metalloproteases. The E3 ubiquitin ligases are substrate specific and have various effects in cells. In addition to regulating internalization of Notch pathway components, ubiquitination is also involved in both signal activation and NICD degradation (Moretti and Brou, 2013).

2.2 Notch receptor endocytosis

Notch receptors are subjected to internalization and trafficking both in the ligand activated state as well as in a non-activated state (Gupta-Rossi et al., 2004; McGill et al., 2009). Since each Notch receptor gives rise to one individual signaling molecule, the output of Notch signaling is especially sensitive to alterations in protein levels (Artavanis-Tsakonas et al., 1999; Andersson et al., 2011). The signaling capacity through the Notch pathway may therefore be regulated by endocytosis and trafficking of receptors to the cell membrane. Internalization can function as a means of reducing Notch receptor expression at the cell surface. Internalized receptors are either recycled back to the cell membrane or degraded (Nichols et al., 2007b; Moretti and Brou, 2013). Dynamin, a protein involved in releasing vesicles from the plasma membrane, is necessary for Notch signal transduction (Seugnet et al., 1997).

Ubiquitination has been shown to regulate Notch receptor internalization and several E3 ubiquitin ligases, e.g. Deltex, Itch/AIP4, Cbl and Nedd4, interact with Notch. The effect of ubiquitination on Notch varies from positive regulation of signaling to trafficking and degradation (Diederich et al., 1994; Jehn et al., 2002; Sakata et al., 2004; Chastagner et al., 2008; Yamada et al., 2011; Moretti and Brou, 2013).

The cellular localization for γ-secretase processing of the S2 cleaved Notch fragment to create NICD, the active signaling moiety, is debated. There are indications supporting both intracellular processing as well as cleavage directly at the cell surface (Struhl and Adachi, 2000; Gupta-Rossi et al., 2004; Kaether et al., 2006b; Vaccari et al., 2008; Vaccari et al., 2010). Proteolysis of the extracellularly truncated form of Notch (NEXT) at the cell membrane is considered to promote production of the stable NICD-V molecule, whereas endosomal proteolysis favors creation of the unstable NICD-S variant (Tagami et al., 2008). However, trafficking through both early endosomes and multi vesicular bodies amplify the Notch response (Vaccari et al., 2008; Vaccari et al., 2010). The view of Notch activation in endosomal compartments is corroborated by findings indicating that endosome acidification enhances Notch signaling (Vaccari et al., 2010). γ-secretase is also reported to function efficiently in an acidic environment (Pasternak et al., 2003). Gupta-Rossi et al. (2004) propose that NICD liberation also requires monoubiquitination of membrane bound Notch prior to its release. This due to the fact that presenilin, a component of the γ-secretase complex, interacts with monoubiquitinated Notch (Gupta-Rossi et al., 2004).

2.3 Notch ligand endocytosis

It is established that endocytosis of Notch ligands in the signal sending cell is required for activation of the Notch pathway in the signal receiving cell (Parks et al., 2000; Nichols et al., 2007a) . There is a controversy in the field regarding the existence of a so-called "pulling force" versus ligand maturation through recycling. The pulling force model accentuates the requirement of a mechanical force to initiate Notch receptor proteolysis, whereas the recycling model proposes endocytosis to be critical for Notch ligands to gain affinity for the receptor. Both models underline ligand ubiquitination as an important step in receptor activation (Heuss et al., 2008; Meloty-Kapella et al., 2012) and the reality may actually be a combination of the two proposed models.

The pulling force theory originates in the findings of NECD bound to Delta ligands inside signal sending cells in *Drosophila melanogaster* (Parks et al., 2000). This theory proposes shedding of NECD and exposure of the ADAM cleavage site upon ligand interaction (Nichols et al., 2007a). Meloty–Kapella et al. (2012) determined that ligand bound NECD is endocytosed through the CME pathway. Their findings also established that the ligand-receptor interactions produce a strain that is created through ligand endocytosis and not by movement of the ligand expressing cell away from the receptor. The production of this strain results in ligand ubiquitination and recruitment of epsin. In addition, dynamin and actin polymerization are critical for NECD shedding during ligand interaction. However, epsin and actin are dispensable for endocytosis of soluble NECD, but required during interactions with cell bound or immobilized Notch receptors (Meloty-Kapella et al., 2012). There are

also indications of dynamin dependent but clathrin independent Notch signal activation through endocytosis of Delta in flies (Windler and Bilder, 2010). Recent findings corroborate the presumed force requirement of Notch receptor activation (Wang and Ha, 2013; Gordon et al., 2015). Gordon et al. (2015) show that ligand binding and subsequent receptor trans-endocytosis exposes the S2 cleavage site and enables receptor processing by ADAM metalloproteases. They further state that unveiling of the S2 cleavage site on Notch only requires mechanical force and that ligand binding does not exert a conformational change on the Notch receptor (Gordon et al., 2015).

The recycling model for Notch ligands emphasize the requirement of Notch ligands to recycle in order to mature and gain affinity for the Notch receptor (Wang and Struhl, 2004; Heuss et al., 2008). Whereas ubiquitination is proposed to be important for generation of the pulling force it may also exert its effects on ligand recycling. Heuss et al. (2008) show that ubiquitination is fundamental for recycling and efficient Notch interaction, but not for endocytosis. In absence of ubiquitination Dll1 has low binding affinity for the Notch receptor, indicating that ubiquitination dependent recycling of Dll1 generates signaling competent ligands. Recycling may also direct ligands to certain membrane domains for enhanced engagement in receptor activation (Heuss et al., 2008; Zhang et al., 2011).

3. Notch signaling and cell differentiation

Active Notch signaling is generally considered as inhibitory in terms of differentiation. The differentiation inhibitory role of Notch is confirmed *in vitro* and *in vivo* by many separate studies (Kopan et al., 1994; Nofziger et al., 1999; Koch et al., 2013). However, in some cells types Notch activity promotes differentiation (Baldi et al., 2004). Notch is regarded to function as a regulator of binary cell fate choices, steering differentiation in one direction while suppressing cell fate determination towards other lineages (Bray, 1998).

Lateral inhibition

The Notch signaling pathway controls differentiation through a mechanism referred to as lateral inhibition. Lateral inhibition was first described in *Drosophila*, where it regulates specification between either neural or epidermal cell fates (Artavanis-Tsakonas et al., 1991). During development of the nervous system in the fruit fly some precursor cells commit to a distinct cell fate by expression of the Notch ligand Delta. Delta activates Notch on adjacent cells, inhibiting expression of proneural genes and promoting epidermal cell specification. Expression of Delta is regulated by proneural transcription factors, creating a positive feedback loop where Delta expression is sustained in the absence of active Notch signaling (Artavanis-Tsakonas et al., 1991; Baldi et al., 2004).

Lateral induction

Notch signaling also controls differentiation through another mechanism called lateral induction. In lateral induction ligand expression exerts a positive effect on the neighboring cell, resulting in upregulation of ligand expression. This creates a positive feedback loop where Notch activation regulates the expression of its ligands and thereby also potentiates the Notch response (de Celis and Bray, 1997; Lewis, 1998). In contrast to lateral inhibition, lateral induction induces proximate cells to adopt the same cell fate. Lateral induction is considered as a critical regulatory mechanism during the development of e.g. the inner ear, the eye lens and the artery wall (Saravanamuthu et al., 2009; Hartman et al., 2010; Hoglund and Majesky, 2012; Petrovic et al., 2014).

3.1 Tissue specific consequences of Notch signaling

Notch signaling is essential for normal development and Notch1 mutant mice die early during embryogenesis (Swiatek et al., 1994). Notch signaling regulates the development and differentiation of most, if not all organs. The hematopoetic system relies on Notch signaling during specification of both myeloid and lymphoid lineages and deregulation of Notch signaling is associated with lymphoma and leukemia (Bigas et al., 2010; Bigas and Espinosa, 2012). During development of skin, Notch signaling functions in a promoting manner by stimulating differentiation of epidermal stem cells (Watt et al., 2008). The development of the heart, liver and mammary tissue also depend on functional Notch signaling (Andersson and Lendahl, 2014). This thesis focuses on the effects of Notch signaling during neurogenesis, myogenesis and

angiogenesis. Therefore, the next section briefly summarizes the impact of Notch signaling in the foregoing contexts in mammals.

Nervous system

The development of the mammalian central nervous system (CNS) is governed by Notch-mediated lateral inhibition. Notch counteracts neuronal stem cell fate and maintains cells in an undifferentiated progenitor state (Yoon and Gaiano, 2005). Activation of the Notch signaling system and expression of the downstream target Hairy enhancer of split (Hes) antagonize the effects of proneural genes (Bertrand et al., 2002). Knock-down studies in mice have confirmed that ablation of Notch1 increases neuronal differentiation. Similar results are obtained through silencing of CSL. However, the effects of CSL silencing on neurogenesis are more severe, which suggests that there is functional redundancy among the Notch receptors in the CNS. Thereby, silencing the common nuclear Notch target generates a more pronounced neurogenic phenotype (de la Pompa et al., 1997).

Neurogenesis is followed by glial cell fate specification and Notch signaling has been shown to influence differentiation of glial cells. Activation of the Notch signaling cascade drives expression of the astrocyte differentiation marker glial acidic fibrillary protein (GFAP). However, gene silencing studies indicate that Notch signaling is dispensable for functional astrocyte differentiation (Ge et al., 2002). Notch induced activation of astrogliogenesis follows neurogenesis, during which astrocytic differentiation is prevented (Ge et al., 2002;

Grandbarbe et al., 2003). Grandbarbe et al. (2003) suggest that Notch activity at the early stages of neural stem cell specification promotes astroglial cell fate. Subsequently Notch activation drives astrocytic differentiation but inhibits differentiation of oligodendrocytes (Wang et al., 1998; Morrison et al., 2000; Ge et al., 2002; Grandbarbe et al., 2003).

Notch signaling has also been shown to regulate astrocyte activation upon damage to the nervous system, a process known as reactive gliosis (Widestrand et al., 2007). Reactive gliosis is thought to generate a "glial scar" upon injury to the nervous system. Whether the effects of reactive gliosis are beneficial or not considering neural regeneration are debated. Reactive gliosis is considered to inhibit axonal regeneration, but also to prevent immune-cell invasion into the injured areas (Bovolenta et al., 1991; Shimada et al., 2011; Pekny and Pekna, 2014). Pharmacological inhibition of Notch signaling together with Notch1 silencing is connected to decreased levels of reactive astrocytes at sites of injury. This indicates that Notch signaling regulates induction of reactive astrocytes upon neurotrauma (Shimada et al., 2011). In addition to regulation of neuronal and glial cell fate, Notch signaling has also been associated with synaptic plasticity, generation of memory and learning (Costa et al., 2003; Liu et al., 2014).

The vascular system

Development of the vasculature at embryonic level begins with vasculogenesis, creating the base from which blood vessels begin to form through angiogenesis. Angiogenesis is defined as the development of new blood vessels from preexisting ones. Angiogenesis can proceed either by endothelial sprouting creating new vessels or through division of an existing vessel into two (Patan, 2000). The formation of an endothelial sprout begins as a response to vascular endothelial growth factors (VEGFs) initiating tip cell formation (Gerhardt et al., 2003). VEGF signaling is made up of soluble ligands, consisting of VEGF-A-E and placental growth factor (PlGF), that bind to vascular endothelial growth factor receptors (VEGFR1-3) (Jakobsson et al., 2009).

Notch signaling is involved in angiogenesis through regulation of endothelial branching. The generation of new blood vessels trough sprouting of preceding ones is a complex process, involving crosstalk between many different signaling molecules. The specific role of Notch signaling in this process is currently being unraveled (Hellstrom et al., 2007; Suchting et al., 2007; Benedito et al., 2009; Jakobsson et al., 2010; Benedito et al., 2012; Kangsamaksin et al., 2014; Pedrosa et al., 2015). The effects of Notch signaling on angiogenesis have been explored by gene silencing in mice. Loss of either Notch or Dll4 leads to increased angiogenic sprouting, whereas loss of Jagged1 results in the opposite phenotype with less branching (Xue et al., 1999; Krebs et al., 2000; Suchting et al., 2007). In response to VEGF-A and activation of VEGFR-2 the expression of Dll4 is induced. This in turn activates Notch signaling in adjacent cells creating the leading Dll4 expressing tip cell and the

Notch expressing stalk cells through lateral inhibition (Hellstrom et al., 2007; Suchting et al., 2007). The stalk cells follow the protrusive tip cell making up the frame of the sprout (Siekmann et al., 2013). In this way Dll4 mediated Notch signaling functions in an antiangiogenic manner, which also corresponds to the hypersprouting phenotype observed in $Dll4^{+/-}$ transgenic mice (Hellstrom et al., 2007; Lobov et al., 2007). Activation of Notch by Jagged1 in endothelial cells gives a different outcome. Notch activation by Jagged1 is proangiogenic, promoting angiogenesis and endothelial sprouting (Benedito et al., 2009). It is intriguing how the identity of the Notch activating ligand may direct cell fate choices.

Recently it has become evident that the Notch and VEGF signaling pathways conjoin in a mutual regulatory feedback loop (Figure 5). VEGF-A initiates Dll4 expression in prospective tip cells (Suchting et al., 2007). However, recent findings by Benedito et al. (2012) question the requirement of VEGFR2 signaling in this aspect. Stalk cells on the other hand are induced through Notch activity to express VEGFR1. VEGFR1 possesses very low kinase activity and functions more as an antagonist on VEGF signaling. In addition to membrane bound VEGFR1, also soluble forms (sVEGFR1) exist that bind VEGF and inhibit its activity (Shibuya, 2006). Whether Notch activity represses VEGFR-2 expression is debated (Williams et al., 2006; Benedito et al., 2012), but recently VEGFR-3 expression was shown to be under Notch-mediated control (Benedito et al., 2012). Kangsamaksin et al. (2014) found that Jagged1 activated Notch signaling promotes angiogenesis through inhibition of sVEGFR1 expression. Jagged may also favor angiogenesis by enhancing

expression of VEGFR2 and VEGFR3 (Benedito et al., 2009; Pedrosa et al., 2015).

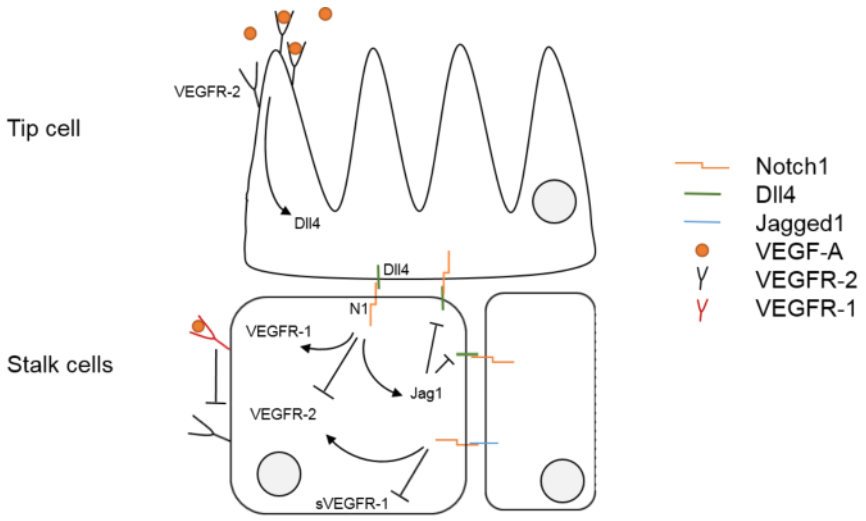

Figure 5. Interplay between Notch and VEGF signaling during angiogenesis. Vascular endothelial growth factor A (VEGF-A) binds to vascular endothelial growth factor receptor-2 (VEGFR-2) on endothelial cells inducing expression of Dll4. Dll4 activates the Notch (N1) signaling pathway in adjacent endothelial cells and promotes tip and stalk cell selection through lateral inhibition. Notch activation in the stalk cell reduces VEGFR-2 expression and induces expression of VEGFR-1, which is catalytically inactive but a highly potent binder of VEGF-A. VEGFR-1 thereby anatagonizes VEGFR-2 activation (Jakobsson et al., 2009; Siekmann et al., 2013). Notch signaling drives expression of Jagged1 in stalk cells (Boareto et al., 2015; Pedrosa et al., 2015) and Jagged1 expression in stalk cells is thought to prevent Dll4 activation of Notch in adjacent cells (Benedito et al., 2009; Pedrosa et al., 2015). In addition, Jagged mediates its proangiogenic function through enhancing VEGFR-2 expression (Pedrosa et al., 2015) and repressing the expression of soluble VEGFR-1 (SVEGFR-1) (Kangsamaksin et al., 2014).

Myogenesis

Notch activity acts in a repressing manner during muscle differentiation. Myoblasts with forced Notch expression show reduced differentiation (Kopan et al., 1994) and perturbation of the Notch signaling pathway also affects myogenesis *in vivo* (Conboy and Rando, 2002; Schuster-Gossler et al., 2007; Vasyutina et al., 2007). Postnatal muscle injury enhances Notch expression and it is proposed that Notch functions by enhancing myoblast proliferation and expansion of muscle stem cells (Conboy and Rando, 2002). Myoblast differentiation on the other hand requires repression of Notch activity (Buas and Kadesch, 2010). Mice expressing only one partially functional Dll1 allele show a significant decrease in muscle development. The underdeveloped skeletal muscle is a result of expeditious and unrestrained differentiation of muscle progenitor cells caused by lack of Notch signaling. The rapid differentiation results in eradication of muscle stem cells in the mutant mice (Schuster-Gossler et al., 2007). A similar outcome has been obtained when silencing CSL (Vasyutina et al., 2007). These findings emphasize the importance of the Notch signaling pathway in sustaining the myogenic stem cell pool (Schuster-Gossler et al., 2007; Vasyutina et al., 2007). The specific mechanisms for the Notch-mediated inhibition of differentiation are debated. There are indications of Notch interfering with transcriptional activation of myogenic genes induced by the transcription factor MyoD (Kopan et al., 1994). Other studies show that Hes1 obstructs interactions between DNA and MyoD (Sasai et al., 1992). In addition, Hairy/enhancer-of-split related with YRPW motif protein-1 (Hey1), a Notch target gene, has also been suggested to conduct promoter specific repression of myogenic genes (Buas et al., 2010).

However, none of the above mentioned mechanisms seem to solely stand for the Notch-mediated regulation of differentiation. It is probable that Notch cooperates with many different signaling pathways, e.g. BMP-4 and HIF-α, in order to repress myogenesis (Dahlqvist et al., 2003; Gustafsson et al., 2005).

OUTLINE AND AIMS OF THE THESIS

This thesis focuses on the role of Notch receptor and ligand trafficking for the differentiation of different stem cells. The general aim of this study was to gain deeper knowledge about factors regulating both Notch receptor and ligand endocytosis. Part of this study focuses on the role of intermediate filaments in controlling Notch ligand trafficking, but it also emphasize the role of posttranslational modifications (PTMs) in efficient Notch signaling.

The specific aims of the study were:

- Analyze the interplay between intermediate filaments and Notch signaling during neuronal differentiation
- Determine the effects of Vimentin ablation on Notch signaling – with focus on angiogenesis
- Determine the relationship between aPKCζ and Notch signaling and the effects on differentiation

EXPERIMENTAL PROCEDURES

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

Cell lines

Name	Publication
Human adrenal carcinoma SW13 cells	III
Human embryonic kidney (HEK293) cells	II
Human embryonic kidney (HEK293FLN) cells stably expressing FLN	I,II,III
Human Hela cervical cancer cells	II
Human umbilical vein endothelial (HUVEC) cells	III
Mouse C2C12 myoblasts	II
Mouse 3T3 fibroblast stably overexpressing Jagged1	III
$Vim^{+/+}$ immortalized mouse embryonic fibroblasts (MEFs)	III
$Vim^{-/-}$ immortalized mouse embryonic fibroblasts (MEFs)	III

Methods

Name	Publication
Angiogenesis assay	III
Aortic ring assay	III
Biotinylation assay	II,III
Cell culture	I,II,III
Densitometry quantification	I,II,III
Fingerprint assay	III
Fluorescence-activated cell sorting	I,II,III
Image analysis	I,II,III
Immunofluorescence	I,II,III
Immunoprecipitation	II
Immunohistochemistry	III
In vitro phosphorylation and kinase activity assay	II

Name	Publication
In vivo transfection	II
Luciferase reporter assay	I,II,III
Microscopy	I,II,III
Neuropshere culture	II
Primary astrocyte culture	I
Quantitative reverse transcription PCR	I,III
Recycling assay	III
SDS-PAGE and westernblotting	I,II,III
Statistical analysis	I,II,III
Time lapse imaging	III
Transient transfections	I,II,III
Ubiquitylation assay	II

Plasmids

Name		Publication
12xCSL luciferase		I,II,III
12xCSL-DsRed		II
CMV-β-galactosidase		I,II,III
CAG-Notch1ΔE-myc-IRES-EGFP		II
caPKCι		II
caPKCζ		II
dnPKCζ		II
GFP-EEA1		II
GFP-FYVE		II
GFP-Rab5	kindly provided by Dr. J. Ivaska	II,III
GFP-Rab7	kindly provided by Dr. J. Ivaska	II,III
Lamp1-GFP	kindly provided by Dr. J. Ivaska	II
Notch1FLN		II
Notch1ΔE		II
Notch1ΔE^{S1791A}		II
Notch1ΔE^{S1791E}		II
Notch1ΔE^{K1749R}		II
Notch1ICD		II

Antibodies

Name	Application	Publication
Anti-Dll4 (Sigma Aldrich)	WB	III
β-actin (Cell Signaling Technology)	WB	I,II,III
β-tubulin (Covance)	IF	I
BrdU (Nordic Biosite)	IF	I
Cleaved Notch1 (Cell Signaling Technology)	IP, WB	II,III
Delta C20 (Santa Cruz Biotechnology)	WB	III
GFP (Clontech)	WB	II
α-GFP (Invitogen)	WB	II
HA1.1 (Covance)	IP	II
Hsc70 (StressGen)	WB	II
Jagged1 (Abcam)	IF	I
Jagged1 28H8 (Cell Signaling Technology)	WB	I,III
Lamp1 (Abcam)	WB	II
Myosin heavy chain (Santa Cruz Biotechnology)	IF, WB	II
NeuN (Chemicon)	IF	I
Notch1 (Sigma Aldrich)	WB	II
Notch C20 (Santa Cruz Biotechnology)	IF, IP, WB	I,II,III
PKCζ (Santa Cruz Biotechnology)	IF, IP, WB	II
Purified Rat anti mouse CD31 (PECAM-1) (BD Pharmingen)	IHC	III
RIP (kind gift from Dr. Hockfield, Yale University)	IF	I
S-100 (Dako)	IF	I
Sox2 (Chemicon)	IF	I
Sox2 (Santa Cruz Biotechnology)	IF	I
Tbr2 (Abcam)	IF	I
Vimentin D21H3 (Cell Signaling Technology)	WB	III

RESULTS AND DISCUSSION

The Notch signaling pathway is a major regulator of developmental cell fate choices. Due to the structure of the pathway Notch signaling may at first seem very simple and straight forward. However, the regulation of the Notch signaling pathway is diverse and includes a variety of cell components. This thesis contributes to the knowledge on Notch signaling regulation by presenting aPKCζ and IFs as novel regulators of the Notch signaling pathway.

1. Intermediate filaments regulate the expression of the Notch ligand Jagged1 (I, III)

1.1 GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes express decreased levels of Jagged1

IFs are most commonly considered as stable and rigid cell structures providing cells with mechanical support. However, IFs have turned out to be highly dynamic cell structures that actively participate in signaling (Pallari and Eriksson, 2006). Widestrand et al. (2007) showed that reactive gliosis, a response to neurotrauma characterized by up regulation of the IFs vimentin (Vim), nestin and GFAP in astrocytes, is attenuated in GFAP$^{-/-}$/Vim$^{-/-}$ mice. Astrocytes regulate the local environment in neurogenic niches both through diffusible and membrane bound factors (Song et al., 2002). Since differentiation of neural progenitor cells along neuronal lineages increases when co-cultured with GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes (Widestrand et al., 2007) and since Notch is an essential regulator of neurogenesis, we sought to determine whether the difference in differentiation could stem from altered Notch signaling. Neural differentiation was analyzed using neurospheres, which are non-adherent cell clusters containing various neural cell types including neural

precursors, neurons and astrocytes. Neurospheres cultures are widely used to study neural differentiation *in vitro*. First we examined whether the increased differentiation of neurosphere cells is dependent on secreted factors or direct cell-cell contact. This was implemented using a co-culture system where neurospheres were grown in direct contact with either WT or GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes and in conditioned media from the opposing cell type (I, Figure 1D). Neuronal differentiation of neurospheres was increased by direct cell-cell contact to GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes as compared to WT (I, Figure 1D). This reinforces the demand of a cell-cell contact mechanism, such as the Notch signaling pathway, to exert the demonstrated effect on neurogenesis. To verify this, WT and GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes were co-cultured with Notch reporter cells transfected with a reporter construct carrying multimerized CSL-binding sites linked to the luciferase gene (12xCSL-luc) (Chapman et al., 2006). The ability of GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes to activate Notch signaling in adjacent cells was reduced compared to WT (I, Figure 2A). Analysis of mRNA and protein levels confirmed a reduction in both Jagged1 mRNA and protein levels in astrocytes devoid of GFAP and vimentin (I, Figure 2B-C). The reduced ligand levels and the impaired ability to activate Notch together with the observed increase in neuronal differentiation associated with GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes supports the hypothesis of perturbed Notch signaling in absence of these IFs. This data represents the first evidence on regulation of Notch ligands by IFs.

1.2 Jagged1 accumulates at the cell surface in vimentin deficient cells

The intriguing relationship between Notch and IFs in astrocytes made us further explore the connection. Lack of vimentin is connected to defects in the modulation of vascular tuning and integrity, endothelial sprouting, and flow-induced arterial remodeling (Schiffers et al., 2000; Kwak et al., 2012). Similar phenotypes have been linked to aberrant Jagged1 signaling. Mice lacking or expressing non-functional forms of Jagged1 die early during embryogenesis due to hemorrhages (Xue et al., 1999; Hansson et al., 2010). Deregulation of Jagged ligands is associated with vascular injuries and linked to disturbances in vascular remodeling in patients with pulmonary arterial hypertension (PAH) and in animals with experimental pulmonary hypertension (Lindner et al., 2001; Yamamura et al., 2014). Analysis of WT and $Vim^{-/-}$ mouse embryonic fibroblasts (MEFs) revealed elevated Jagged1 mRNA levels and accumulation of Jagged1 at the cell membrane in absence of vimentin (III, Figure 6A-C). When relating signal activation to the expression levels of Jagged1 at the cell surface, it is evident that only a fraction of the surface localized ligands in $Vim^{-/-}$ cells are engaged in signaling. In regard to this, the signal sending potential of $Vim^{-/-}$ MEFs is reduced (III, Figure 6E).

2. Trafficking of Notch receptors and ligands affects signaling (I, II, III)

2.1 Jagged1 trafficking is altered both in vimentin deficient and GFAP$^{-/-}$/Vim$^{-/-}$ cells

Microtubules and actin filaments have an established role during vesicular movement in cells (Wacker et al., 1997; DePina and Langford, 1999). The IFs are also involved in vesicle mobility in different cell types. In fibroblasts, vimentin is involved in integrin recycling, a process regulated through vimentin phosphorylation by PKCε (Ivaska et al., 2005). IFs have also been shown to regulate vesicle mobility and directional movement in astrocytes (Potokar et al., 2007). In addition, there are some indications of IFs functioning as physical barriers in cells, directing vesicle movement (Potokar et al., 2007).

Reduced internalization of Jagged1 in GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes

As ligand endocytosis and trafficking are regarded as essential steps in Notch activation we analyzed general endocytosis and internalization of recombinant NECD in GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes. General endocytosis, represented by internalization of dextran coated beads, as well as ligand-mediated internalization of recombinant NECD, was reduced in GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes compared to WT when analyzed by FACS. In addition, a reduction in Jagged1 positive vesicles was observed in the GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes (I, Figure 2D-F). This is in line with the reduced Jagged1 expression in these cells and could account for the reduced Notch signaling potential of the GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes described previously.

Ablation of vimentin alters endocytic routing of Jagged1

Similar experiments on ligand-mediated internalization of recombinant NECD in regard to vimentin depletion revealed enhanced Jagged1 internalization in absence of vimentin. This corresponds to the enhanced surface levels of Jagged1 observed in $Vim^{-/-}$ cells. We also analyzed the ligand-mediated pulling force, by attaching protein A beads to recombinant NECD peptides (NECD-PrtA), and measuring internalization. When comparing the internalization of NECD-PrtA in WT and $Vim^{-/-}$ MEFs no net differences were detected. However, if the pulling force is related to the levels of Jagged at the membrane it reveals that the pulling force in $Vim^{-/-}$ MEFs is significantly reduced (III, Figure 6F). These results are in accordance with the reduced signal sending potential observed of $Vim^{-/-}$ cells and indicates a role for vimentin in the creation of the pulling force. The endocytic factors required for creation of the pulling force and Dll-mediated activation of Notch are being unraveled. Actin, dynamin and epsin have been identified as critical elements for Dll-mediated Notch activation (Meloty-Kapella et al., 2012). Whether Jagged mediated trans-endocytosis and subsequent Notch activation relies on the same or different endocytic factors are currently unknown. To further investigate intracellular routing and movement of Jagged1 ligands we tracked the movement of endocytosed NECD in Jagged1 overexpressing WT and $Vim^{-/-}$ MEFs. In WT cells the average linearity of the vesicle tracks was significantly reduced as compared to $Vim^{-/-}$ cells, implying that vimentin decreases directional mobility of Jagged1-positive vesicles (III, Figure 5A-C). No significant differences were detected in movement speeds of the vesicles (III, Figure 5D). According to the recycling model, Notch ligand recycling to and

from the cell membrane is required for the ability of the ligands to activate Notch signaling (Wang and Struhl, 2004; Heuss et al., 2008). Therefore, we analyzed recycling of Jagged1 in the presence and absence of vimentin using a biotin cell surface labeling and stripping protocol. The result indicates that fairly small amounts of Jagged1 are recycled in WT and $Vim^{-/-}$ MEFs. But the initial recycling of Jagged1 appears faster in $Vim^{-/-}$ MEFs (III, Supplementary figure 5) In addition, we looked at the occurrence of Jagged1 in different endosomal compartments. Jagged1 colocalization with Rab4 or Rab11 linked recycling endosomes is reduced in $Vim^{-/-}$ MEFs compared to WT. Rab4 and Rab11 are known markers for fast and slow endosomal recycling routes, respectively (Jones et al., 2006). Our results suggest that Jagged1 is found in both types of recycling endosomes and that vimentin-deficiency reduces the overall amount of Jagged1 in these endocytic compartments. Taken together, this could indicate that in the absence of vimentin Jagged1 is rapidly routed back to the cell membrane spending less time in endocytic vesicles. This is supported by the fact that vimentin is known to reduce intracellular movement of vesicles (Guo et al., 2013). Whether a direct interaction between Jagged1 and vimentin exists is still unclear. The C-terminal PDZ-motif on Jagged constitutes a possible link between the ligand and vimentin. Afadin, a cell junction protein, is presently the only identified PDZ-interacting molecule known to interact with Jagged. Afadin and Jagged interact at adherens junctions (Popovic et al., 2011), which in addition to actin is also known to connect with the vimentin network (Kowalczyk et al., 1998; Shasby et al., 2002). However, vimentin could also affect Jagged by perturbing the function of actin, since actin is known to be essential for the creation of the pulling force (Meloty-

Kapella et al., 2012). Vimentin and actin may interact directly or with the aid of auxiliary proteins (Esue et al., 2006; Jiu et al., 2015; Huber et al., 2015). In addition, the actin microfilament network is altered in cells devoid of vimentin (Eckes et al., 1998), which supports this hypothesis.

2.2 Phosphorylation by PKCζ affects Notch signaling and localization depending on the signaling state

Trafficking of Notch receptors is equally important to Notch signal activation as is trafficking of ligands. However, the effects of Notch receptor endocytosis are debated. There are indications of endocytosis both promoting Notch activation as well as counteracting it (Gupta-Rossi et al., 2004; Nichols et al., 2007b; Sorensen and Conner, 2010). We have identified a novel atypical PKCζ (aPKCζ) specific phosphorylation site at serine (S) 1791 on the intracellular domain of the Notch receptor (Figure 6). Atypical PKCζ is a serine threonine kinase involved in survival and differentiation processes as well as in regulation of intracellular trafficking in cells (Nishimura and Kaibuchi, 2007; McCaffrey and Macara, 2009; de Thonel et al., 2010). aPKCζ showed interaction with both the FLN receptor and Notch1ΔE, a membranetethered activated form of Notch1 (II, Figure 2A-C). Despite evidence of interaction between NICD and other members of the PKC-family (Zhu et al., 2013), we did not detect an interaction between aPKCζ and NICD (II, Figure 2D). Another PKC isoform, PKCε, has been shown to enhance Notch1 expression (Steinhart et al., 2007), which corroborates our data. The interaction between aPKCζ and both FLN and Notch1ΔE was enhanced in the presence of γ-secretase inhibitors (GSI) (II, Figure 2B-C). Phosphorylation by aPKCζ enhances ligand-mediated Notch

signaling and receptor internalization, but the final outcome of phosphorylation is dependent on the signaling status of the receptor.

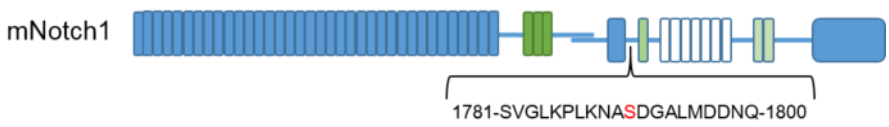

Figure 6. Notch1 is a substrate for PKCζ phosphorylation. Mass spectrometry analyses identified S1791 on murine Notch1 as the predominant phosphorylation site by PKCζ.

PKCζ promotes recycling of Notch receptors

Non-activated Notch receptors are internalized upon PKCζ phosphorylation, shifting receptor distribution from the cell membrane to intracellular vesicles (II, Figure 7A). In the presence of a constitutively active form of PKCζ (caPKCζ) we detected enhanced ubiquitylation of Notch when blocking lysosomal maturation using chloroquine. In addition, caPKCζ facilitated increased interaction between Notch1 and the ubiquitin binding endosomal sorting protein Hrs (II, Figure 7F). However, the internalization of FLN receptors in response to PKCζ-mediated phosphorylation was not connected to increased lysosomal degradation but tentatively associated with recycling and reinsertion in the cell membrane. These results are in line with previous findings by others showing that FLN receptors are constitutively recycled from the cell membrane (McGill et al., 2009).

PKCζ enhances Notch signaling

aPKCζ phosphorylation exerted a positive effect on Notch signaling in the activated state. Expression of caPKCζ resulted in increased levels of NICD and shifted the localization from intracellular vesicles to the nucleus (II, Figure 3E, H). Since there are evidence of kinase-independent interactions between Notch and members of the PKC-family (Kim et al., 2012) we wanted to verify whether the kinase-activity of PKCζ is essential for the detected effects on Notch signaling. We adopted a mutant form of PKCζ (dnPKCζ), which is catalytically impaired but has intact substrate binding ability. The dnPKCζ mutant showed opposite effects on NICD production and localization when compared to caPKCζ (II, Figure 3F, I). This indicates that functional kinase activity of aPKCζ is required for the observed effects on Notch signaling. These findings were verified by employing PKCζ specific phosphorylation-deficient and phosphomimetic forms of Notch1ΔE, Notch1ΔE^{S1791A} and Notch1ΔE^{S1791E}, respectively. In accordance with our previous results, Notch signal activation was enhanced when expressing the phosphomimetic form of Notch1ΔE and reduced by the phosphorylation-deficient forms of Notch1ΔE (II, Figure 4B-D). In addition, the intracellular localization of these constructs mimicked the localization of Notch observed when expressing caPKCζ or dnPKCζ. Notch1ΔE^{S1791E} displayed enhanced nuclear localization whereas Notch1ΔE^{S1791A} was mainly detected in the cytoplasm (II, Figure 5A). Regarding intracellular localization, both phosphomimetic and phosphodeficient Notch1ΔE mutants were found in early and late endosomes. However, the phosphomimetic Notch1ΔE showed decreased interaction with late endosomal marker Rab7 (II, Figure 5D-E). This could indicate that

phosphorylation by PKCζ enhances release of NICD from late endosomes. Despite the prevailing view of productive NICD generation occurring at the plasma membrane or in early endosomes, γ-secretase may function at multiple stages of endocytosis (Fortini and Bilder, 2009; Pasternak et al., 2003). The phosphorylation deficient form of Notch1ΔE also showed increased interaction with lysosomal associated membrane protein 1 (Lamp1) (II, Figure 5F) and enhanced lysosomal degradation of Notch1ΔE^{S1791A} when compared to Notch1ΔE^{S1791E} or Notch1ΔE (II, Supplementary figure 8). Therefore, one could conclude that phosphorylation by PKCζ is essential for increased nuclear entry of NICD and protection against lysosomal degradation in the active state. In accordance, we introduce aPKCζ as a novel regulator of Notch activation, endocytosis and intracellular trafficking.

2.3 Vimentin affects endosomal processing of NICD

Until this point, we have focused on the effects of IFs on Notch signaling from the ligand point of view. Cells devoid of vimentin have enhanced Jagged expression but a compromised ability to activate Notch signaling (Figure 7). Recently a positive feedback loop has been identified between Notch and Jagged (Manderfield et al., 2012; Boareto et al., 2015), indicating that Notch activation drives Jagged expression. Surprisingly, we found that Vim$^{-/-}$ cells have enhanced signal receiving capacity compared to WT (III, Figure 2 C-E). However, we could not detect any differences between Vim$^{-/-}$ and WT cells regarding Notch receptor surface expression or NICD stability. Intriguingly, vimentin has been shown to regulate endo-lysosomal sorting (Styers et al., 2004) and our previous findings indicate that NICD could be released from the

late endosomes/lysosomes during active signaling (II). Inhibition of lysosomal maturation using chloroquine revealed a direct interaction between Lamp-1 and Notch. Chloroquine treatment also enhanced the ΔENotch/NICD expression levels in Vim$^{-/-}$ MEFs (III, Supplementary figure 2). Confocal microscopy showed differently distributed Lamp-1 positive vesicles in WT and Vim$^{-/-}$ cells. Further, analysis of co-localization indicated enhanced co-localization between Lamp-1 and Notch in absence of vimentin (III, Supplementary figure 2). Therefore, the enhanced Notch activity in absence of vimentin could be a result of disturbed lysosomal processing of Notch upon activation.

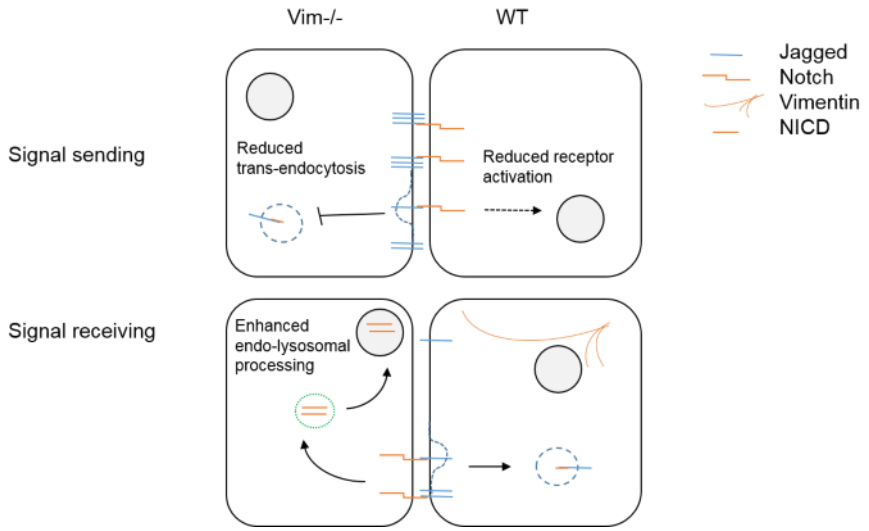

Figure 7. Vimentin regulates both Notch signal sending and signal receiving capacity. The signal sending capacity of vimentin deficient cells (Vim$^{-/-}$) is weakened due to compromised receptor trans-endocytosis. Activation of the Notch signaling pathway is enhanced in Vim$^{-/-}$ cells as a result of enhanced endo-lysosomal processing of NICD.

3. Notch signaling is critical for normal cell differentiation (I, II, III)

The regulatory function of Notch signaling during differentiation of various tissues is well established. Notch may undertake both a positive regulatory role but more commonly Notch activity inhibits differentiation and promotes stem cell fate. Notch also guides cells in the decision between two different cell fates (Kopan et al., 1994; Apelqvist et al., 1999; Hansson et al., 2004; Nobta et al., 2005). In some cases, the identity of the ligand activating Notch determines the direction of differentiation and commits the cell to a distinct cell fate. Thereby Jagged and Dll-ligands, even though activating the same signaling cascade, can generate different outcomes (Amsen et al., 2004; Benedito et al., 2009).

3.1 Astrocytes regulate neurogenesis through the Notch pathway

The previous findings by Widestrand et al. (2007) demonstrated amplified neuronal differentiation and enhanced survival of neural grafts in the absence of major IFs. Our discovery of decreased Notch signaling, reduced Jagged expression and ligand-mediated endocytosis in GFAP$^{-/-}$/Vim$^{-/-}$ astrocytes led us to investigate the impact of the Notch signaling during neuronal differentiation in this context. Notch signaling regulates progenitor cell commitment along neuronal versus glial lineages. Active Notch signaling inhibits neuronal differentiation, but promotes glial cell fate (Louvi and Artavanis-Tsakonas, 2006). To specifically pinpoint the Notch derived effect on neuronal differentiation we analyzed Notch activation in neural stem cells when co-cultured with GFAP$^{-/-}$/Vim$^{-/-}$ and WT astrocytes. Notch activity in neural stem cells was significantly reduced in presence of GFAP$^{-/-}$/Vim$^{-/-}$

astrocytes when compared to WT astrocytes (I, Figure 2G). We adopted neurospheres from GFAP$^{-/-}$/Vim$^{-/-}$ and WT mice in order to analyze neuronal differentiation in the presence of immobilized recombinant Jagged1 ligands. The differentiation of WT neurospheres was not affected by addition of external Jagged1 ligands, whereas Jagged1 reduced the differentiation of GFAP$^{-/-}$/Vim$^{-/-}$ neurospheres to the level of WT neurospheres (I, Figure 3A). Addition of GSI counteracted the effects of recombinant Jagged1 on differentiation of GFAP$^{-/-}$/Vim$^{-/-}$ neurospheres (I, Figure 3B). This fortifies the involvement of the Notch signaling pathway in astrocyte-mediated regulation of neurogenesis.

Further, in vivo analysis of WT and GFAP$^{-/-}$/Vim$^{-/-}$ mice showed that the difference in Jagged induced Notch signaling between these mice did not affect neural stem cell pool maintenance or proliferation. However, when analyzing the differentiation of newly born astrocytes and neurons using prolonged BrdU treatments, GFAP$^{-/-}$/Vim$^{-/-}$ mice showed a significant increase in the survival of new cells and enhanced formation of new neurons compared to WT (I, Figure 4D). In addition, the neurogenic response to hippocampal injury in terms of cell proliferation was decreased, but the cell fate of the dividing cells was predominantly shifted toward neuronal cell fate in GFAP$^{-/-}$/Vim$^{-/-}$ mice (I, Figure 4H). Ablation of GFAP and Vimentin enhances neuronal differentiation as a direct consequence of decreased Notch signaling due to reduced ligand levels. The effects are however not as severe as in mice with induced Notch inhibition, where deletion of CSL resulted in exaggerated neuronal differentiation and stem cell depletion (Imayoshi et al., 2010). Notch

signaling is very sensitive to signaling dosage (Grandbarbe et al., 2003), and small imbalances in receptor or ligand levels may affect the signaling outcome. Jagged is expressed in both the SVZ and SGZ, but detection of Dll1 has only been weak or undetectable (Stump et al., 2002; Givogri et al., 2006). This would indicate that Delta-like is not compensating for the perturbed Jagged1 signaling in absence of GFAP and Vimentin, which could be the case if Dll was present and differently regulated, i.e. not affected by these IFs. Notch has previously been shown to regulate gliotic responses upon injury to the brain (Givogri et al., 2006) and our results imply that vimentin and GFAP are important players in the astrocyte-mediated regulation of neuronal differentiation. In addition, astrocytes expressing Notch ligands are also involved in disease, as metastatic tumor cells activate astrocytes in the brain. Upon activation the astrocytes show elevated Jagged levels, activating Notch signaling in adjacent cancer stem cell-like cells and promoting their self-renewal. Administration of Notch inhibitors has proven to restrain brain metastases *in vivo* (Xing et al., 2013). With this in mind, Jagged is a highly potential therapeutic target not only in cancer, but also for improving neurogenesis after trauma or with regard to stem cell therapeutic applications in the brain.

3.2 Angiogenesis is delayed in vimentin deficient mice due to unbalanced Notch signaling

Notch signaling is essential in the regulation of angiogenesis. Dll4 versus Jagged1 activation of Notch regulates formation of the leading tip cell and creation of new sprouts (Benedito et al., 2009). The antiangiogenic effects of

Dll4-Notch signaling are widely accepted (Gale et al., 2004; Hellstrom et al., 2007; Suchting et al., 2007; Trindade et al., 2008), but the promoting role of Jagged in angiogenesis is still being deciphered (Benedito et al., 2009; Pedrosa et al., 2015). The previously observed dysfunctions in Notch signaling in the absence of vimentin led us to analyze vessel formation and angiogenesis in $Vim^{-/-}$ mice. Despite having a mild phenotype vimentin deficient mice display altered characteristics linked to the vasculature. Arterial remodeling in response to changes in blood flow is altered in mice lacking vimentin (Schiffers et al., 2000) and vimentin is also required for endothelial cell invasion and angiogenic sprouting (Kwak et al., 2012). Analysis of vascularization in $Vim^{-/-}$ embryos at E11.5 revealed a sparsely branched network with smaller blood vessels compared to WT (III, Figure 1B). To follow this up, we analyzed endothelial branching *ex vivo* in aortic rings from WT and $Vim^{-/-}$ mice. Aortic rings from $Vim^{-/-}$ mice produced fewer and shorter sprouts compared to WT (III, Figure 1C). Considering our impression of $Vim^{-/-}$ cells as poor signal senders but good signal receivers, we analyzed the effects of external immobilized ligands on endothelial sprouting from aortic rings. In accordance with the prevailing view of Notch ligands in angiogenesis, Jagged1 and Dll1 enhanced sprouting whereas Dll4 did not (III, Figure 3A, D). During angiogenesis Dll1 is expressed by adjacent tissues and promotes sprout formation and vessel branching (Napp et al., 2012). Similar result were obtained in a 3D angiogenesis assay with human umbilical vein endothelial cells (HUVEC) when silencing vimentin (III, Figure 3B). Analysis of Notch pathway components demonstrated enhanced expression of Jagged and NICD in endothelial cells lacking vimentin, corresponding to our earlier findings in

MEFs (III, Figure 2A). These results confirm the previously stated aberrations of Notch signaling in absence of vimentin. Ablation of vimentin enhances antiangiogenic Notch activation by Dll4 at the expense of proangiogenic activation by Jagged.

PKC-family proteins have been linked to angiogenesis (Xu et al., 2008; Nakayama et al., 2013) and PKC mediated phosphorylation of vimentin regulates recycling of cell surface proteins (Ivaska et al., 2005). A plausible hypothesis is that PKC phosphorylation of vimentin, which regulates assembly and disassembly of vimentin (Inagaki et al., 1997), regulates the interaction between vimentin and Jagged. We amployed phorbol-12-myristate-13-acetate (PMA), a PKC activator, and bis-indolylmaleimide I (BIM), a PKC inhibitor, and analysed their effects on Jagged expression. Expression of Jagged1 was enhanced or reduced in the prescence of PMA or BIM, respectively (III, Figure 7A). Analysis of Notch signal activation revealed that pretreatment with PMA prior to co-culture with Notch reporter cells enhanced signaling activity (III, Figure 7B). Similar results were obtained when when adopting vimentin mutants, with mutated PKC phosphorylation sites (III, Figure 7D). It remains to be seen if an actual interaction between vimentin and Jagged is obtained and whether the interaction is dependent on the phosphorylation status of vimentin. The insoluble nature of vimentin may be the reason why we have been unable to detect a direct interaction with Jagged. Other proteins are known to mainly interact with the small soluble pool of vimentin (Inagaki et al., 1997; Ivaska et al., 2005; Perlson et al., 2005; Hyder et al., 2011). Our finding of Jagged co-localizing with phosphomimetic forms of vimentin in

intracellular vesicles indicates that Jagged would interact with the phosphorylated pool of viementin (III, Figure 7C, Supplementary figure 5). However, these fidings require validation using more specific methods e.g. proximity ligation assays.

Recent findings emphasize the role of Fringe glycosaminyltransferases during angiogenesis (Benedito et al., 2009; Boareto et al., 2015). Glykosylation of Notch receptors by Fringes are known to affect both the transactivating and cis-inhibiting properties of ligands (LeBon et al., 2014). Mice lacking Lunatic Fringe ($Lfng^{-/-}$) display enhanced sprouting of the retinal vasculature (Benedito et al., 2009). Our preliminary data reveal elevated protein levels of Manic Fringe in endothelial cells when silencing vimentin. In addition, we observed a 3-fold increase in Lunatic Fringe mRNA expression in $Vim^{-/-}$ MEFs. Both Manic- and Lunatic Fringe are known to enhance Dll-Notch signaling at the expense of Jagged-Notch signaling (Yang et al., 2005). However, Fringe suppression of Jagged induced signaling is not a result of reduced affinity for the ligand. On the contrary, Fringe modifications enhances Notch1 interaction with both Dll1 and Jagged1 (Taylor et al., 2014). The precise mechanism for Fringe-mediated repression of Jagged induced Notch signaling is still unknown. Whether ablation of vimentin somehow is linked to Fringe expression is unclear and requires further investigation, but this data certainly adds to the complexity on how vimentin and the Notch signaling pathway interact during angiogenesis.

In addition to being proangiogenic and promoting tumor vascularization, Jagged has been linked to many aspects of tumorigenesis (Li et al., 2014). Vimentin is also linked to many steps of oncogenic progression (Hyder et al., 2011; Ivaska, 2011; Vuoriluoto et al., 2011). Analysis of tissue expression data using the GeneSapiens database (Kilpinen et al., 2008) reveal that Jagged expression positively correlates with vimentin expression in many different tissues and in different types of cancer (III, Supplemetary figure 4). Vimentin is also implicated in tumor angiogenesis (van Beijnum et al., 2006; Bayless and Johnson, 2011) and targeting vimentin using antibodies is shown to inhibit both angiogenesis and tumor growth (van Beijnum et al., 2006). Our findings allow us to speculate the possibility of targeting vimentin in order to perturb Jagged1 function and thereby obstructing tumor angiogenesis.

3.3 Phosphorylation of Notch by PKCζ regulates neuronal and myogenic differentiation in vivo and in vitro

To assess the physiological effect of Notch phosphorylation by aPKCζ we analyzed the development of the chick central nervous system (CNS) (Holmberg et al., 2008) under conditions of forced Notch1ΔE expression together with pharmacological inhibition of PKCζ. PKCζ was inhibited using a pseudosubstrate peptide (PS; Myr-SIYRRGARRWRKL) that binds to the PS region of PKC and functions by blocking kinase activity (Zeng et al., 2012). CAG-Notch1ΔE-Myc-IRES-EGFP was electroporated into the neural tube of HH stage 10 chick embryos to induce Notch1 expression. Notch1ΔE expression was restricted to one side of the neural tube, with the other side remaining untransfected and functioning as a control. Neuronal

differentiation was detected by staining against the neuronal marker Tuj1. In the presence of elevated Notch1ΔE expression neuronal differentiation was inhibited (II, Figure 1D) in a previously described manner (Holmberg et al., 2008). However, when PKCζ inhibitor was added Notch1ΔE expressing cells also showed expression of Tuj1, indicating that these cells undergo neuronal differentiation despite the enhanced Notch1 levels (II, Figure 1E-F). In addition, PKCζ inhibition seemed to reduce nuclear Notch and shifted localization to the cytoplasm, when comparing to the control (II, Figure 1G-I). These results confirm the previously stated effects of PKCζ on Notch signaling. The outcome of PKCζ inhibition resembles the findings obtained with the phosphorylation deficient Notch1ΔE mutant, with reduced Notch signaling and enhanced cytoplasmic localization of NICD. PKC mediated regulation of Noch signaling has also been indicated in other tissues. Zhu et al. (2013) showed that inhibition of PKCδ reduces Notch-mediated osteoblastogenesis of human mesenchymal stem cells.

To further assess the role of aPKCζ specific Notch1 phosphorylation on differentiation, we analyzed myotube formation during forced expression of the phosphorylation-deficient form of Notch1ΔE in C2C12 cells. Myogenic differentiation is negatively regulated by Notch signaling (Kopan et al., 1994; Nofziger et al., 1999). Immunoreactivity for the myogenic differentiation marker myosin heavy chain (MHC) was significantly enhanced in cells expressing Notch1ΔE^{S1791A} compared to Notch1ΔE at 72 h after induction of differentiation (II, Figure 6E). In addition, MCH levels increased upon PKC silencing using siRNA (II, Figure 6C). Similarly MHC expression was delayed

when caPKCζ was over expressed in differentiating C2C12 cells (II, Figure 6D). Similar observations have been found when analyzing astrocyte differentiation, where Notch exerts a differentiation promoting role. In that study a phosphorylation deficient form of PKCε blocked Notch induced astrocytic differentiation (Steinhart et al., 2007).

Many PKC isoforms highly expressed and involved in cancer progression (do Carmo et al., 2013). Given our findings, one could assume that PKC also could regulate Notch activity in cancer. In breast cancer, PKCα expression correlates positively with expression of Notch-4 (Yun et al., 2013) whereas PKCθ is a downstream target of Notch-3 in T-cell lymphoma (Felli et al., 2005). Enhanced expression of Notch pathway components in cancer is often linked with poor prognosis (Reedijk et al., 2005; Du et al., 2014), but the elevated expression is not always caused by Notch mutations. PKC medited activation of Notch could account for some of the observed Notch overexpression in cancer that does not stem from Notch linked mutations. When pharmacologically targeting PKC, one should also bear in mind that it may affect Notch signaling.

CONCLUDING REMARKS

This thesis highlights aPKCζ and IFs as novel regulators of Notch signaling. The importance of cytoskeletal elements like actin and tubulin are confirmed when considering Notch receptor and ligand internalization. However, there are few indications of intermediate filament involvement in Notch signaling. My thesis reveals two different contexts of Notch signaling regulation by intermediate filament proteins. In astrocytes intermediate filaments regulate Jagged expression and promote differentiation towards neuronal cell fate. Ablation of vimentin in endothelial cells compromise their ability to form angiogenic sprouts. Vimentin affects both the signal sending and signal receiving capacity of cells. Ligands accumulate at the plasma membrane in absence of vimentin, but have a reduced capability to activate signaling. On the other hand, signal activation is potentiated in the absence of vimentin presumptively due to altered lysosomal processing of Notch. Our finding of Notch phosphorylation by PKCζ also shows corresponding results. Phosphorylated receptors are endocytosed upon ligand activation and yield a higher Notch activation response, with increased nuclear localization of NICD. Ligand activated unphosphorylated receptors localize to the lysosomes and are eventually degraded. PKCζ also regulates receptor levels at the cell membrane through internalization and trafficking of receptors that are not engaged in signaling. Figure 8 summarizes the novel findings of this thesis together with other established regulators of the Notch signaling pathway.

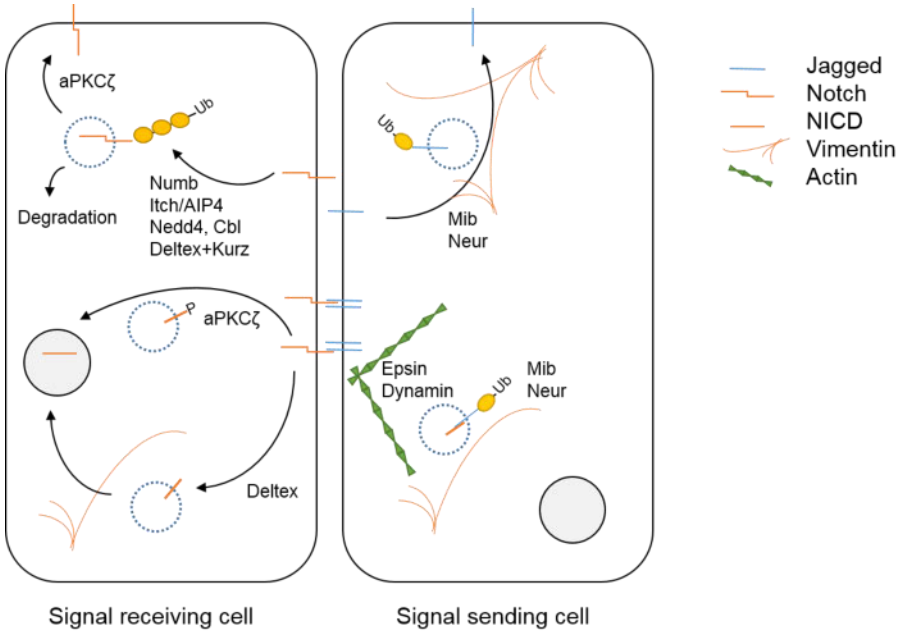

Figure 8. Simplified diagram of the Notch signalling pathway and associated regulatory proteins. In the signal sending cell, ligand recycling require ubiquitination (Ub) by E3 ubiquitin ligases Mindbomb (Mib) or Neuralized (Neur). Vimentin regulates recycling of Jagged and recycling is faster in the absence of vimentin. Receptor activation and trans-endocytosis of ligand bound NECD also require ligand ubiquitination by Mib or Neur. Epsin, dynamin and actin participate in trans-endocytosis. Our findings indicate that vimentin is necessary for efficient trans-endocytosis and creation of the pulling force. In the signal receiving cell, ubiquitination by E3 ubiquitin ligases (Numb, Itch/AIP4, Nedd4, Cbl, Deltex+Kurz) promotes Notch receptor degradation. aPKCζ mediated phosphorylation of non-activated Notch receptors facilitates receptor internalization and ubiquitination, but not receptor degradation. aPKCζ may instead promote receptor recycling. During active signaling aPKCζ promotes endocytosis and NICD translocation from intracellular vesicles to the nucleus. In addition, our findings show a role for vimentin in regulation of receptor processing and NICD release from late endosomal compartments. Adapted from Sigismund (2012) and Moretti and Brou (2013).

Regarding future prospects, there are still many unanswered questions within these projects. Considering the interplay between intermediate filaments and Notch signaling, my impression is that we are only beginning to understand their interaction. Whether a direct interaction between Jagged and vimentin exists is unknown and so far we have been unsuccessful in detecting such a connection. However, some of the Notch ligands, including Jagged1, carry a PDZ motif on their intracellular domain. Different Notch ligands carry distinct PDZ motifs, indicating that the motifs could form ligand specific regulatory domains. Since vimentin is known to interact with membrane tethered proteins through PDZ interactions, this motif represents a possible link between the IFs and the Notch signalling pathway. Vimentin deficient cells have an intriguing profile when it comes to Notch signaling. Signal sending is compromised but activation in absence of vimentin leads to an elevated Notch response. How this affects the interplay between different cell types during differentiation, e.g. endothelial cells and vascular smooth muscle cells during angiogenesis, would be an interesting avenue to follow. Preliminary data also indicate higher levels of Notch glycosylating Fringe proteins in vimentin devoid cells. It would be interesting to unravel the reason for this enhanced expression and also whether it actually affects Notch signaling in these cells and how.

The perception that Notch processing at the cell surface generates more stable and potent NICD fragments is somewhat contradictory to our findings. Our findings clearly indicate that phosphorylation by PKCζ takes place prior to γ-secretase processing in endosomes. My theory is, that the phosphorylation by

PKCζ could function in guiding the γ-secretase complex, to yield stable and signaling efficient NICD even from late endosomal compartments. To fully understand the physiological importance of Notch phosphorylation by PKCζ, transgenic mice carrying mutations of Notch at this specific site could be generated to analyze their development. In conclusion, this thesis presents novel insight into the regulation of the Notch signaling pathway and how this regulation affects differentiation of different tissue specific stem cells.

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This thesis focuses on the role of Notch signaling in regulation of stem cell differentiation. Recycling of both Notch receptors and ligands is required for activation of the signaling pathway. The thesis describes how Notch receptor trafficking and signal activation is regulated through phosphorylation and how this phosphorylation guides both neuronal and muscle development. In addition, the thesis presents intermediate filaments as important regulators of Notch signaling, by regulation of ligand expression and trafficking, during neuronal differentiation and angiogenesis.

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