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# ROLE OF POSTTRANSLATIONAL MODIFICATIONS IN REGULATION OF NOTCH AND TRANSCRIPTIONAL COACTIVATOR MASTERMIND-LIKE 1

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# ABSTRACT

The Notch signaling is an evolutionary conserved pathway enabling short range cell-cell interactions, crucial for diverse developmental and physiological processes during embryonic and adult life. The Mastermind-like (MAML) family of transcriptional coactivator proteins has been shown to play an essential role in regulation of Notch-mediated transcription. Formation of DNA-bound ternary CSL-Notch ICD-MAML complex is a crucial event in transcriptional regulation of Notch target genes. More recent studies highlight a broader role of MAML1 by showing that MAML1 coactivates MEF2C, p53, β-catenin and NF-κB. Data presented in this thesis studies demonstrate that MAML1 enhances autoacetylation and HAT activity of p300 acetyltransferase, which coincidences with increased acetylation of histones H3/H4. We further show that p300 acetylates Notch1 ICD, and MAML1 strongly enhances Notch acetylation, presumably by potentiating p300 autoacetylation. MAML1-dependent acetylation of Notch1 ICD by p300 decreases the ubiquitination of Notch1 ICD in cell culture, which might be a mechanism to regulate Notch activity in the nucleus by interfering with ubiquitin dependent pathways. MAML1 has been show to recruit CDK8 kinase, which phosphorylates Notch1 ICD and targets Notch for proteosome-mediated degradation. We found that CDK8 inhibits p300 acetylation of Notch1 ICD and Notch1 ICD-p300 mediated transcription. These findings underscore MAML1 function as coregulator of Notch that, depending on signaling time frame and interacting partner, can modulate the strength of Notch responses in cells.

Considering the importance of MAML1 for Notch and other signaling pathways we investigated the molecular mechanisms of how MAML activity is regulated. Data presented in this thesis reveal that MAML1 transcriptional activity can be modulated by two mechanisms. First, we found that MAML1 is phosphorylated and inhibited by GSK3β kinase. Active and inactive GSK3β interacts with N-terminal MAML1, and GSK3β subcellular localization is changed to nuclear bodies in the presence of MAML1, where they both colocalize. Only active GSK3β is capable of inhibiting MAML1 activity, moreover GSK3 inhibitor SB41 significantly increases the levels of acetylated histones H3 in cells stably expressing MAML1. Although GSK3β interacts and phosphorylates N-terminal MAML1, Notch ICD-MAML1 binding remains unaffected regardless of the phosphorylation status. Second, we found that MAML1 is a target of SUMOylation at two highly conserved lysines residues (K217 and 299), and that MAML1 SUMOylation deficient mutant has significantly higher transcriptional activity. Furthermore, SUMOylation of MAML1 potentiates interaction with HDAC7, which decreases MAML1 activity, and thus might serve as an additional mechanism to control MAML1 function as a coactivator.

# LIST OF PUBLICATIONS

I.  Hansson ML, **Popko-Ścibor AE**\*, Saint Just Ribeiro M\*, Dancy BM, Lindberg MJ, Cole PA and Wallberg AE. (2009). The transcriptional coactivator MAML1 regulates p300 autoacetylation and HAT activity. Nucleic Acids Res. 37(9): 2996-3006.

II. Saint Just Ribeiro M\*, Hansson ML\*, Lindberg MJ, **Popko-Ścibor AE** and Wallberg AE. (2009). GSK3beta is a negative regulator of the transcriptional coactivator MAML1. Nucleic Acids Res. 37(20): 6691-700.

III. Lindberg MJ, **Popko-Ścibor AE**, Hansson ML and Wallberg AE. (2010). SUMO modification regulates the transcriptional activity of MAML1. FASEB J. 24(7): 2396-404.

IV. **Popko-Ścibor AE**, Lindberg MJ, Hansson ML, Holmlund T and Wallberg AE. (2011). Notch1 ubiquitination is regulated by MAML1-mediated p300 acetylation of Notch1. Biochem. Biophys. Res. Commun., in press.

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# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| CDK | Cycline-dependent kinase |
| C/H | Cystein/histidine rich domain |
| ChIP | Chromatin immunoprecipitation |
| DN | Dominant negative |
| CSL | CBF1, Su(H), Lag1 |
| DSL | Delta, Serrate, Lag2 |
| ECD | Extracellular domain |
| GSI | $\gamma$-secretase inhibitor |
| GSK | Glycogen synthase kinase |
| HEK | Human embryonic kidney |
| HES | Hairy enhancer of split |
| HATs (KATs) | Histone acetyltransferases (Lysine acetyltransferases) |
| HDACs (KDACs) | Histone deacetylases (Lysine deacetylases) |
| ICD | Intracellular domain |
| MAML | Mastermind-like |
| MEF2C | MAD box enhancer factor 2C |
| MZB | Marginal zone B-cells |
| NAD | Nicotinamide adenine dinucleotide |
| NF-$\kappa$B | Nuclear factor- $\kappa$ B |
| NLK | Nemo-like kinase |
| NLS | Nuclear localization signal |
| PCAF | p300/CBP associated factor |
| PIAS | Protein inhibitor of activated STAT |
| PIC | Pre-initiation complex |
| PTMs | Posttranslational modifications |
| SIM | SUMO-interacting motif |
| siRNA | small interfering RNA |
| SUMO | Small ubiquitin-like modifier |
| T-ALL | T-cell acute lymphoblastic leukemia |
| TFs | Transcription factors |
| TNF$\alpha$ | Tumor necrosis factor $\alpha$ |
| TSA | Trichostatin A |
| Ub | Ubiquitin |

# 1  INTRODUCTION

## 1.1  REGULATION OF GENE EXPRESSION

Almost every cell in our body carries genetic information encoded in DNA. Every cell, no matter it is a kidney or brain cell, contains the same DNA, but yet may look and function in various ways. This cellular variation and specified function is dictated by differential gene expression. Transcription is one of the fundamental steps in regulation of gene expression and so creation of a cell specific proteome. Knowledge of how signaling pathways, genetic and environmental factors contribute to the gene regulatory network is an important step towards our better understanding of molecular basis of life.

### 1.1.1  Chromatin structure

Chromatin is a highly organized DNA packed into nucleosomes, which are fundamental units consisting of 147 base pairs of DNA wrapped around a protein core with two copies of each H2A, H2B, H3 and H4 histone proteins [1]. While globular histone domains comprise the nucleosome core, histone tails protrude from the core thereby being a subject of multiple posttranslational modifications (PTMs) [2]. Nucleosomes are further packed into chromatin fibers and highly ordered system of looped domains to form a chromosome. This unique organization ensures a very stable DNA-protein complex, but yet highly dynamic chromatin properties under physiological conditions. Based on its state of condensation chromatin is categorized into euchromatin, which is loosely packed and actively transcribed, whereas more densely packed and repressed regions of the genome are called heterochromatin. Heterochromatin can be further classified into facultative, which characterizes silencing of developmental genes and inactive X chromosome in females, and constitutive heterochromatin formed at the telomers and pericentromeric regions [3]. Chromatin modifications vastly contribute to maintenance of different chromatin states, and each state is characterized by a different set of modifications. In mammals, heterochromatin is associated with higher levels of methylation at histones H3K9, H3K27 and H4K20. For instance, pericentromeric heterochromatin is enriched in H3K9me, while H3K27me dominates heterochromatin in inactive X chromosome [2]. Among all of histone modifications, acetylation plays a major role in unfolding of silenced chromatin. It has been shown that acetylation of H4K16 has a negative impact on formation of compact 30-nanometer-like fibers and higher order chromatin interactions [4]. Active euchromatin also contains higher levels of trimethylation at lysines H3K4, H3K36 and H3K79 [2]. However, more recent studies show that both active and repressive marks can coexist, and such bivalent domains have been found highly enriched in key developmental genes encoding important transcription factors in embryonic stem cells. This keeps genes silenced, but poised for activation during differentiation [5].

## 1.1.2 Transcriptional regulation

In eukaryotes, protein expression is a multistep process and chromatin state appears to affect gene transcription at all stages. Transcription is typically initiated by binding of specific transcription factors (TFs) upstream of the core promoter. This leads to recruitment of adaptor complexes, such as SAGA or Mediator, that allow binding of general transcription factors (GTFs) and RNA Pol II at the promoter region of a gene. The core promoter, including the TATA box and transcription initiation site, serves to position RNA Pol II and GTFs to form the pre-initiation complex (PIC). After synthesis of the first 30 bases, RNA Pol II loses its contact with core promoter and GTFs, and enters the elongation step. Termination and RNA processing events lead eventually to production of messenger RNA, translated in the cytoplasm to a protein [6]. Coregulators are essential proteins that modulate chromatin structure, recruited by DNA binding specific transcription factors. There are two main classes of coregulators, ATP-dependent nucleosome remodeling complexes and histone modifying enzymes. ATP-dependent remodeling complexes, such as SWI/SNF or ISWI-based family of complexes, are capable of shifting nucleosomes using the energy of ATP-hydrolysis, regulating access to DNA by exposing or occluding DNA sequences. Histone modifiers work cooperatively with ATP-dependent remodelers by catalyzing the removal or addition of various covalent modifications including acetylation, phosphorylation, ubiquitination, methylation or SUMOylation [7-8].

Most of cycline-dependent kinases (CDKs) play a role in regulation of the cell cycle, however CDK7, 8 and 9 have been shown to regulate gene expression through direct interaction with the transcriptional machinery. CDK8 activity and substrate specificity is regulated by its binding partner cycline C, which together with two other components MED12 and MED13, form a CDK8 complex, which is a subunit of Mediator. The Mediator complex is a major component of the PIC complex and is required for expression of virtually all genes [9-10]. As a part of Mediator, the CDK8 complex has been show to regulate transcription in both negative and positive ways. CDK8 phosphorylates the RNA Pol II C-terminal domain (CTD), which leads to dissociation of Mediator from RNA Pol II [11]. Moreover, CDK8 can phosphorylate CDK7, which inhibits the general transcription initiation factor IIH (TFIIH) and transcription initiation [12]. The positive role of CDK8 in transcription is supported by several studies, including transcriptional coregulation of p53, Wnt/β-catenin, SMADs or thyroid hormone receptor, reviewed in [13].

While the human genome encodes for approximately 25000 genes, almost 5% accounts for TFs. However, genome wide analysis showed that transcription factor DNA binding sites are largely unoccupied and inaccessible in the context of higher chromatin structure. So, how do TFs overcome chromatin barrier to initiate transcription? Nucleosome DNA appears to be to some extent accessible for some TFs prior its decondensation. Cooperative binding of TFs seems to be one of the strategies to bind to DNA, however in some cases specials factors called pioneer TFs can bind first in order to allow other factors to bind. It becomes apparent that silent genes in embryonic stem cells may be bound by such pioneer factors to allow potent activation during differentiation. For instance, the DNA binding domain of FoxA proteins resembles that of linker H1 thereby capable of binding condensed chromatin and open local chromatin, enabling other factors to bind [14].

### 1.1.3 HATs and HDACs

Histone acetyltransferases (HATs) are enzymes catalyzing the transfer of an acetyl group from cofactor acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of substrate lysine residues. Acetylation neutralizes positive charge and generates more accessible chromatin for transcription. The reversible reaction is catalysed by histone deacetyltransferases (HDACs). Both families of enzymes typically function within large protein complexes and quite often cooperate with each other being a part of the same complex. Although initially found to modify histone proteins, subsequent studies indentified a wide range of non-histone protein targets. Thus today they are often referred to as KATs and KDACs (lysine acetyltransferases and deacetylases) [15-16]. The p55 protein, a homolog of the yeast protein Gcn5p in *Tetrahymena*, was the first histone specific HAT that provided a link between histone acetylation and gene transcriptional activation [17]. To date, many KATs have been identified, and major families include Gcn5 N-acetyltransferase (GNATs), MYST (named after protein members Morf, Ybf2, Sas3 and Tip60) and p300/CBP (CREB-binding protein). All are composed of different chromatin binding domains including bromodomains, chromodomains, WD40 repeats, Tudor domains or PHD fingers, which allow for recognition of modified histone tails [18]. How acetyltransferases accommodate such a variety of substrates was neatly demonstrated in structural studies of Gcn5/PCAF requirements for substrate binding, demonstrating that the residues around targeted lysines highly contribute to the substrate affinity [19].

Lysine deacetylases are categorized into four different classes based on their sequence similarity and required cofactor. Class I of HDACs includes HDAC1-3 and HDAC8 proteins, which are related to yeast Rpd3. They are mainly localized in the nucleus and characterized as a class of high deacetylase activity. Class II of HDACs consists of HDAC4-7, HDAC9 and 10, is similar to yeast Hdap1, and are found both in the cytoplasm and cell nucleus [20]. Although they have lower deacetylase activity compared to class I, often possess additional intrinsic activities such as regulation of protein ubiquitination and turn over [21]. The last, IV class of HDACs consists of HDAC11, which localizes predominantly in the nucleus [20, 22]. Class I, II and IV HDACs require $Zn^{2+}$ for deacetylase activity. HDACs class III is related to yeast silent information regulator2 (SIR2) and comprised of SRT1-7 (sirtuins), which require $NAD^+$ as a cofactor. Sirtuins are unrelated to other HDACs, including the mechanisms of substrate deacetylation, thus insensitive to compounds that inhibit class I, II or IV [23]. Aberrant expression of HDACs has been reported in different types of cancer [24], and HDAC inhibition has become a promising strategy in therapeutic treatments. Two HDAC inhibitors, suberoylanilide hydroxamic acid (SAHA, known also as Vorinostat) and romidepsin have been approved for treatment of cutaneous T-cell lymphoma (CTCL) [25-26].

### 1.1.4 Role of p300 acetyltransferase

p300 and CBP are highly conserved, functionally related transcriptional coregulators, originally found to bind E1A and CREB proteins, respectively. Unlike other HATs, p300 and CBP are broader acetyltransferases capable of acetylating all four histones. In addition to histones, a wide range of non-histone substrates have been

identified as p300/CBP targets, including the first shown non-histone substrate, the tumor suppressor p53 [27]. p300 is an essential coregulator in p53-mediated transcription as well as an important regulator of p53 stability [27-29]. Other p300 targets include NF-κB, c-myc, GATA1, MyoD, TFIIE, TFIIF and MAML1 [30-31]. Different domains and interaction regions of p300 define its broad role as a global transcriptional coactivator and its important function in many cellular processes such as differentiation, proliferation, apoptosis and DNA repair [30, 32]. In addition to the centrally located HAT domain, p300 consists of three cystein/histidine rich domains (C/H1-3), CREB binding KIX domain, bromodomain and steroid receptor coactivator interaction domain (SID). Biochemical studies revealed that p300 undergoes autoacetylation in the regulatory loop (1520-1560 aa) of the HAT domain, including acetylation of lysine 1499, which enhances p300 HAT activity [33]. Using an acetyltransferases deficient p300-HAT mutant, it has further been shown that p300 autoacetylation occurs via an intermolecular mechanism [34]. Among other posttranslational modifications, p300 has also been shown to be regulated through SUMOylation, which represses p300 function via recruitment of HDAC6 [35].

Although p300 shares extensive sequence homology with CBP, a number of studies report on their unique functions. p300 knock-outs are embryonic lethal and show defects in neurulation, heart development and cell proliferation. Double p300/CBP heterozygotes and p300 heterozygote both appear to be embryonic lethal, which shows that CBP cannot compensate for the lack of p300 and further demonstrates an overall sensitivity for the levels of p300 [36]. In support of this finding, it has been showed that retinoid acid-mediated transcriptional activation of the cell cycle inhibitor p21Cip1 requires p300 in embryonal carcinoma F9 cells, and reversely p27Kip1 required CBP, leaving p300 dispensable [37]. Furthermore, extensive genome-wide studies using ChIP-seq on cell-cycle synchronized cells revealed heterogenous binding patterns for p300 and CBP, and that AP-2 and SP1 transcription binding sites are enriched in p300-specific targets [38]. p300 is present in limiting concentrations thus competition between different factors for binding to p300 frequently occurs. Arginine methyltransferase CARM1 is an important coactivator controlling the switch between expression of nuclear receptor regulated genes and those that bind CREB. Interestingly, CARM1 mediated methylation of p300 disrupts CREB-p300 interaction, which serves as cofactor methylation-mediated transcriptional switch [39]. A number of studies prove the role of p300 in carcinogenesis. Both p300 and CBP emerge to function as tumor suppressors in thymic lymphoma and histiocytic sarcomas, which was supported by studies of p300 and CBP null chimeric mice, with p300 being absolutely essential for proper hematopoietic differentiation [40]. Analysis of chromosomal translocations in acute myeloid leukemia (MLL) revealed fusion protein MLL-p300 generated by t(11,22) (q23,q13), which caused p300 malfunction in regulation of cell-cycle and differentiation [41]. Somatic mutations in p300 located in the HAT and C/H2 domains, were found in colorectal carcinoma and gastric carcinoma respectively. Truncating mutations causing deletion of important p300 domains and other mutations were also identified in primary tissues and cell lines of breast, ovarian or oral squamous carcinomas [42]. Although the current understanding of p300 function has remarkable potential for development of anticancer drugs, it has so far been a challenge.

## 1.2 POSTTRANSLATIONAL MODIFICATIONS (PTMS)

One important mechanism the cell utilizes to regulate protein function is a wide range of different posttranslational modifications. PTMs are essential in creation of heterogeneity in proteins (Figure 1) and thereby fully functional cell proteome. In general, proteins can be proteolytically cleaved, chemically modified or simply form a complex with an interacting partner. PTMs such as acetylation, phosphorylation, ubiquitination, SUMOylation or methylation, to name a few, have been shown to play important role in normal cells and in human disease. Thus identification of protein PTMs and responsible mechanisms is of high importance in discovery of new pharmacological targets.
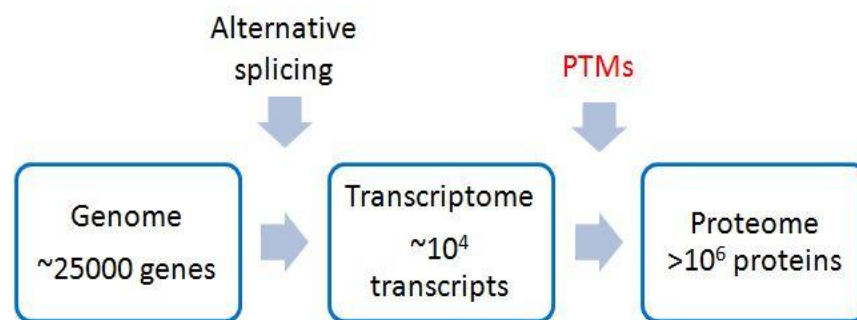


**Figure 1. Posttranslational modifications (PTMs) are important in creation of heterogeneity in proteins.** While human genome encodes for about 25000 genes, the variety in proteins is mainly accomplished by alternative splicing and a wide range of posttranslational modifications.

### 1.2.1 Acetylation

Acetylation is a reversible modification, catalysed by KATs, which neutralizes lysine positive charge with a significant impact on protein function. Acetylation has been shown to regulate protein transcriptional activity, DNA-protein and protein-protein interaction, cellular localization or protein stability [43]. The fact that lysine residues are targets of acetylation and other PTMs, often mutually exclusive, suggests highly dynamic regulatory mechanisms [44]. Direct competition between acetylation and ubiquitination targeting the same lysine residue is one of the mechanisms modulating protein stability. Acetylation can also induce protein degradation via recruitment of third proteins promoting ubiquitination and degradation, or simply lead to complex dissociation, thereby exposing the acetylated protein for degradation [45]. Acetylation has been best characterized for histones, however many non-histone proteins have been identified as targets of acetylation [15].

Emerging studies demonstrate that acetylation is apparently an abundant protein modification, underscoring its importance in many cellular processes. Choudhary and colleagues identified 3600 lysine acetylation sites within 1750 proteins in three different cell lines (MV4-11, A549 and Jurkat) using immunoaffinity purification with acetyl-Lysine specific antibody and high-resolution mass spectroscopy. This study revealed enormous number of new acetylation sites and demonstrated that acetylation

preferentially targets large protein complexes engaged in chromatin remodeling, nuclear transport, DNA replication, cell cycle, splicing or actin nucleation. Among other chromatin remodeling complexes SWI/SNF, NURD or NURF were found to be heavily acetylated, indicating that acetylation is apparently a common mechanism modulating their functions. Interesting, methyltransferases such as MLLs and JARID (Jumonji AT-rich interactive domain) demethylases were extensively modified by acetylation. In addition to nuclear targets, many unique acetylation sites within cytoplasmic and mitochondrial proteins have been identified. This study also provided important information regarding the mechanisms of action of KDACs inhibitors. Treatment of cells with two different inhibitors SAHA and MS-275 increased acetylation of only 10% of proteins, suggesting they are very specific and thus applicable in more sophisticated therapeutic treatments [46].

### 1.2.2  Ubiquitination

Ubiquitin (Ub) is an abundant 76 amino acids protein tag, attached to lysine residues in a three-step enzymatic reaction. Ubiquitination (also referred to as ubiquitinylation or ubiquitylation) begins with activation of ubiquitin by a ubiquitin-activating enzyme (E1), followed by transfer of activated ubiquitin to an E2-conjugating enzyme. An E3 Ub-ligating enzyme is responsible for substrate recognition and transfer of Ub from E2 to the substrate. Thus E3s are crucial components in ubiquitination, providing specificity through recognition and direct interaction with the substrate. Successive conjugation of Ub to protein targets generates polyUb chains, linked between the C-terminal glycine of one Ub with the ε-amino group of the lysine within preceding Ub. Efficient polyUb requires in some cases an additional conjugating cofactor E4 [47]. Ubiquitination is a reversible modification and deubiquitinases (DUBs) are responsible for removal of ubiquitin from the substrate. In most cases, protein ubiquitination leads to proteosome mediated degradation, however mono- and diubiquitination have been shown to have other consequences on protein function, such as DNA repair, DNA replication, membrane trafficking and transcription [48]. RNA Pol II and a wide range of transcription factors, including p53 and c-myc, have been shown to be regulated via ubiquitination. H2A/B histones are heavily ubiquitinated, which significantly affects chromatin structure, mediates recruitment of protein complexes and acts in a crosstalk with other histone modifications, reviewed in [49]. Over 11000 ubiquitination sites on almost 4300 proteins were recently identified using proteome-wide, quantitative analysis of endogenous ubiquitination in HEK293T and MV4-11 cells. More than 90% of the identified sites were previously unknown, within proteins involved in all major cellular functions. Interestingly, comparison of ubiquitination with known acetylation sites reveled their extensive overlap [50].

### 1.2.3  SUMOylation

Many ubiquitin-like modifiers have so far been identified, among them 100 amino acids polypeptide SUMO (small ubiquitin-related modifier) protein family, consisting of four members SUMO1-4 in mammals. SUMO2 and 3 are highly similar with about 97% sequence identity while being only in 50% similar to SUMO1. Although a SUMO4 gene has been identified it is uncertain whether this isoform can conjugate substrates. The SUMO conjugation reaction is analogues to ubiquitination

but with SUMO specific E1, E2 and E3s. In the first step, SUMO proteins are cleaved at the C-terminus by SUMO specific isopeptidases (Sentric specific proteases (SENPs) with six members in mammals) in order to generate mature SUMO, which is then covalently attached to SUMO E1 activating enzyme consisting of two subunits (SE1/SE2, AOS1-UBA2) in an ATP-dependent manner. The SUMO protein is next transferred to the SUMO E2 enzyme (UBC9), which attaches SUMO to substrates. This step is enhanced by SUMO E3 ligases. Three distinct families of SUMO E3 ligases have so far been identified; protein inhibitor of activated STAT (PIAS), polycomb protein 2 (Pc2) and Ran binding protein (RANBP2) [51-52]. Subsequent studies have demonstrated that class II of HDACs, Topors and p14Arf proteins can also facilitate SUMOylation in some cases [53-55].

The common consensus SUMOylation site includes ΨKXE (Ψ is a large hydrophobic residue, X any amino acid), which is also present in SUMO2/3 thus enabling them formation of polySUMO chains. Unlike SUMO1, which is usually in conjugated form, a significant fraction of SUMO2/3 is present as free in cells, but their conjugation is enhanced upon stress. SUMOylation is a reversible modification; the same enzymes that process nascent SUMO (SENP proteases) are responsible for removing of SUMO from their substrates [51-52]. SUMO can also interact with proteins via non-covalent interactions mediated by SUMO-interacting motifs (SIM) within the targeted protein, for instance SIM within PML is required for nucleation and formation of nuclear bodies [56]. Unlike ubiquitin, which most often targets protein for degradation, SUMO modulates protein function by affecting protein-protein interaction, DNA-protein interaction and subcellular localization. Proteins involved in regulation of gene expression, apoptosis, DNA repair, cell-cycle regulation or proliferation have been found to be SUMOylation target [52]. In many cases, SUMOylation represses transcription, which is often associated with recruitment of HDACs [57]. For instance, SUMOylation of histone H4 leads to transcriptional repression through association with HDAC1 and HP1 [58].

The components of the SUMOylation system have been found to play important roles in tumorgenesis. Increased expression of UBC9 has been found in different types of cancer such as ovarian carcinoma, melanoma and lung adenocarcinoma. Upregulated SUMO E3 PIAS3 has been correlated with cancer of lung, breast, prostate, colon-rectum and brain tumor, while SUMO E1 has been associated with lower survival rates in hepatocellular carcinoma. SENP1 was shown to be upregulated in thyroid and prostate cancers. Other disorders that involve deregulation in SUMOylation of proteins include Alzheimer's, Huntington's, Parkinson's diseases, amyotrophic lateral sclerosis and familial dilated cardiomyopathy, reviewed in [59].

## 1.3   THE NOTCH SIGNALING PATHWAY

The Notch gene was first identified as a notched phenotype, a result of partial gene loss, in the mutant strain of the Drosophila fruit fly by Thomas Morgan in 1917. Notch was cloned and characterized as a gene encoding for a cell surface receptor in the middle of 1980 [60-61]. Notch signaling is an evolutionary conserved system enabling short range cell-cell interactions, crucial for diverse developmental and

physiological processes including stem cell renewal, differentiation, apoptosis or proliferation [62-63].

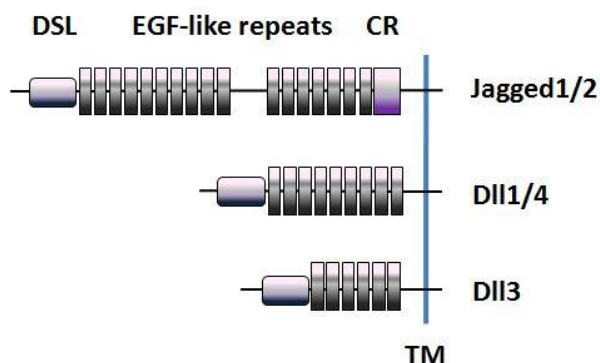## 1.3.1 Notch receptors and ligands

In mammals, there are four Notch receptors Notch1-4 and five canonical DSL (Delta/Serrate/LAG2) ligands, including Delta-like (DLL1, 3 and 4) and Jagged1 and 2 (JAG1, 2) (Figure 2). Notch receptors are type I single-pass transmembrane family proteins consisting of several distinct domains. The extracellular domain (ECD) of Notch consists of 29-36 tandem N-terminal epidermal growth factor (EGF)-like repeats responsible for ligand binding and three LIN12 Notch repeats, which prevent ligand-independent activation. The C-terminal hydrophobic region mediates ECD interaction with the intracellular domain (ICD) of Notch. The intracellular domain contains a RAM domain and seven ankyrin repeats involved in interaction with transcription factor CSL (CBF1/Su(H)/Lag1), nuclear localization signals (NLS) and a C-terminal PEST domain responsible for the overall protein stability [62] (Figure 2A). Notch ligands are also type I transmembrane proteins comprised of distinct structural motifs including N-terminal domain (NT) followed by DSL (Delta/Serrate/Lag2) domain and EGF-like repeats. Unlike DLL ligands, JAG1 and 2 contain an additional cystein rich domain (CR) (Figure 2B). The intracellular region contains no obvious structural homology, however multiple lysines important for ligand signaling activity and C-terminal PDZ (SD-95/Dlg/ZO-1) essential for interaction with the cytoskeleton, are distinct motifs common for most of DSL ligands. Noncanonical ligands have also been described to activate Notch in some context, including F3/Contactin, Delta/Notch-like EGF-related receptor (DNER) or microfibril-associated glycoprotein family MAGP1 and MAGP-2 [64].

A
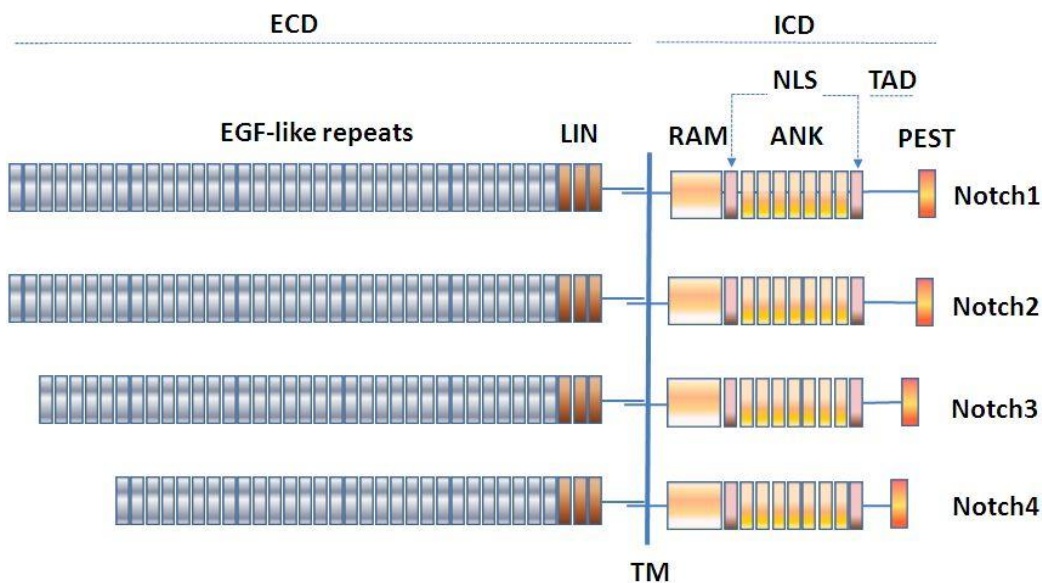
**B**



**Figure 2. Domain organization of Notch receptors and ligands. (A)** Diagram presenting four human Notch receptors, Notch1-4. Notch comprises of two main domains, the extracellular domain (ECD) and the intracellular domain (ICD). Notch ECD consist of EGF-like repeats, LIN repeats followed by the heterodimerization domain (HD). Notch ICD consists of the RAM domain, seven ankyrin repeats (ANK), two nuclear localization signals NLS, transactivation domain (TAD) and PEST domain. **(B)** Classical Notch ligands include Jagged1/2 and Delta-like ligands Dll1/3/4, which consist of DSL domain and EGF-like repeats. Jagged ligands also contain cystein rich domain (CR).

## 1.3.2 Ligand-induced activation of Notch

Notch receptors are synthesized as precursor proteins and cleaved at the site S1 by furin-like proteases in Golgi before being transported to the plasma membrane. Notch signaling is activated by binding of the ligand, which induces a conformational change within the extracellular domain allowing a second cleavage S2 by the metalloproteases of the ADAM10 or TACE (TNF-α converting enzyme, also known as ADAM17) family to occur. Subsequent cleavage S3 mediated by the γ-secretase complex, allows the ICD to translocate to the nucleus. In the absence of Notch ICD, Notch target genes are repressed by CSL and recruited corepressors, including SMRT/NCoR, SKIP, CIR and HDACs [65], as well as SHARP [66] and CtIP/CtBP [67]. When Notch ICD enters the nucleus, the corepressor complex is displaced and Notch ICD associates with CSL, and coactivators such as PCAF, GCN5, p300 and Mastermind-like (MAML) are recruited to activate expression of target genes [68-72] (Figure 3). Interestingly, recent studies show that multimerization of Notch ICD is a prerequisite for formation of the active Notch complex on DNA [73]. Among the main targets of Notch are basic helix-loop-helix (bHLH) family proteins, including the hairy enhancer of split (HES) and hairy related transcription factor (HEY), both known as transcriptional repressors [74]. Other Notch targets include c-myc, cyclin D1, p21/Waf1, GATA3, NF-kB2, Bcl-2, E2A or HoxA5, 9 and 10, reviewed in [75]. Cooperative assembly of the higher-order Notch complexes on promoters containing "sequence paired" binding sites (SPSs) ensures tight regulation of Notch-mediated responses. Such a cooperative formation of the Notch dimmers, dependent on both CSL and MAML1, occurs at the HES promoters [76-77]. C-terminal PEST domain plays a crucial role in regulation of Notch ICD stability, and cycline dependent kinase 8 (CDK8) has been shown to phosphorylate the C-terminal region of Notch ICD and

target it for proteosomal degradation mediated by SEL10/Fbw7 [78-79]. This leads to disassembly of the Notch ternary complex so that the cell may act upon the next round of activation.
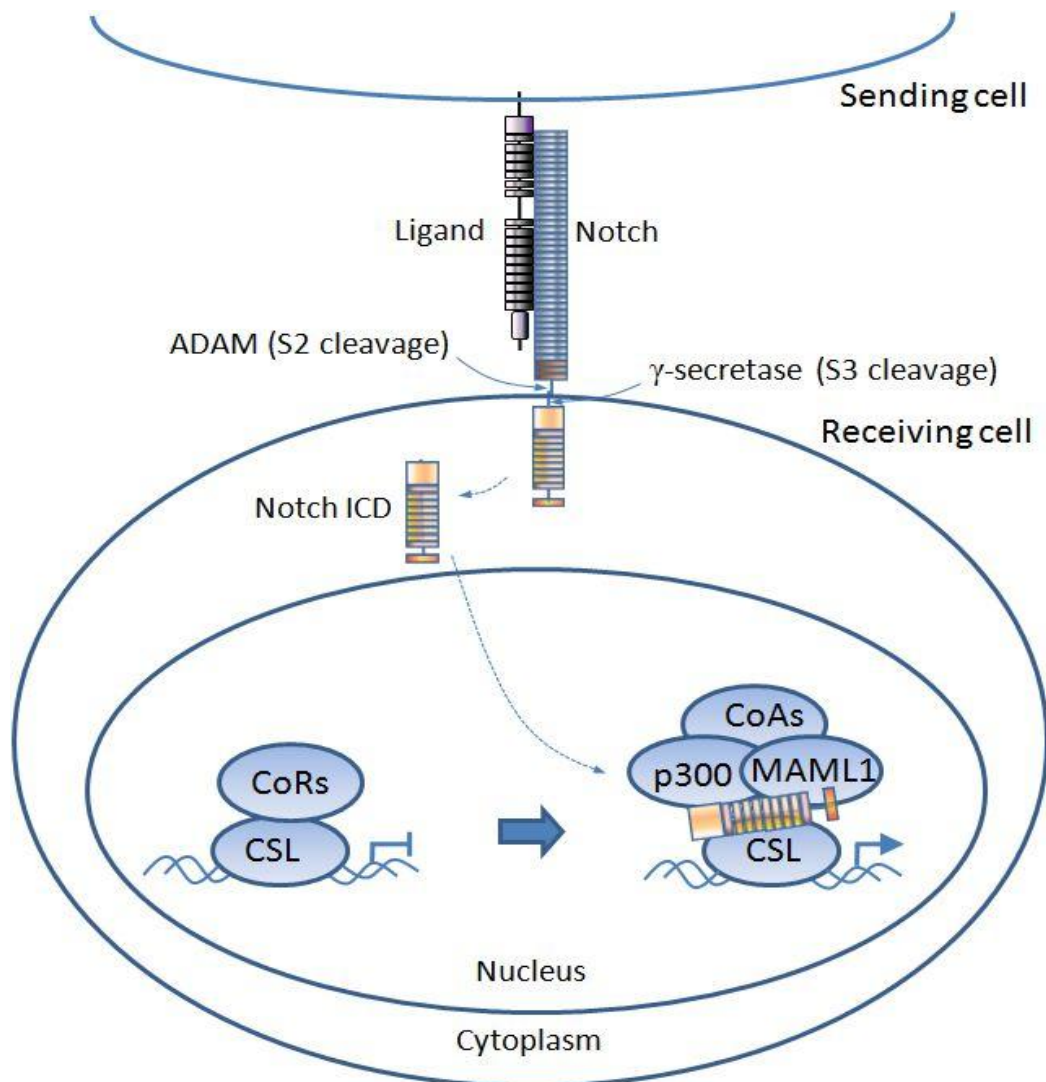


**Figure 3. The Notch signaling pathway.** Upon ligand binding, Notch receptor is cleaved at site S2 and S3 by ADAM metalloproteases and γ-secretase, respectively. This leads to release of the Notch ICD into the cytoplasm. In the absence of Notch ICD, Notch target genes are repressed by CSL and corepressors (CoRs). When Notch ICD is translocated to the nucleus, it associates with CSL, p300, MAML1 and other coactivators (CoAs) to activate the expression of Notch target genes.

While Notch trans-interactions with the ligands through cell-cell interaction lead to Notch activation, an inhibitory cis-interaction between Notch and ligands within the same cell has also been reported [80-83]. Structural studies revealed that trans- and cis-interactions seem to be mutually exclusive since they require the same surfaces of receptor and ligand [84]. Interestingly, it has recently been shown that cis-interactions force cells to expand into receiving or sending cells without apparent transcription mediated feedback, providing new insight into developmental patterning processes regulated by Notch [85].

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### 1.3.3 Notch posttranslational modifications

One of the unique characteristics of the Notch signaling pathway is lack of signaling cascade upon activation by the ligand. There are no kinases or other secondary messengers involved, thereby the intensity of Notch signaling mediated responses depends highly on different cellular regulatory mechanisms. Thus identification of Notch posttranslational modifications is an important step in our better understanding of how Notch is modulated and how it can be treated when deregulated.

Notch EGF repeats have been shown to be targets of *glycosylation*. Notch glycosylation mediated by O-fucosyl transferase is essential for folding and transport of Notch to the cell membrane [86]. Moreover, it has been reported that glycosylation by other glycosyl transferases such as Fringe can affect Notch affinity for different ligands, so that Notch is preferentially activated by Delta and relatively resistant to Serrate/Jagged ligands [87]. Importance of *ubiquitination* in regulation of Notch has been demonstrated by several studies. It has been reported that monoubiquitination and endocytosis of Notch are prerequisite for γ-secretase mediated cleavage and that mutation of lysine 1749 abolished Notch monoubiquitination, endocytosis and accumulation of Notch ICD in the nucleus [88]. However, an alternative explanation for this finding came with the later studies, showing that diversity in S3 cleavage can generate two types of Notch ICD with different stabilities and thus different signaling strength. Mutations of residues around the S3 site, including lysine 1749, can drastically shift the precision of S3 cleavage and result in production of a highly unstable Notch ICD [89]. Notch ICD has been shown to be ubiquitinated by different E3 ligase families. SEL10/Fbw7 (F-box/WD40 repeat containing protein 7 in mammals; SEL10 in *C. Elegans*) can ubiquitinate Notch ICD and promote Notch proteosome-mediated degradation [90-93]. SEL10/Fbw7 regulation of Notch ICD requires the PEST domain and phosphorylation of Notch ICD is a prerequisite for SEL10/Fbw7 binding [91, 93]. Mammalian Itch/AIP4 and Drosophila Nedd4, HECT domain containing E3 ligases have been shown to ubiquitinate the ICD of the membrane tethered Notch1 and regulate its sorting and lysosomal degradation [94-95]. Two members of RING fingers E3 ligases, Deltex (dx) and c-Cbl, have also been reported to ubiquitinate and regulate Notch sorting. While C-Cbl promotes degradation of unactivated Notch, studies in *Drosophila* showed that increased expression of Deltex positively regulates Notch signaling in endocytic vesicles [96-97]. Thus, ubiquitination seems to play important role in regulation of available Notch and thereby Notch signaling activity.

Numerous studies demonstrated that following cleavage Notch undergoes *phosphorylation* at multiple sites [98-101]. Ubiquitination has been shown tightly linked with protein phosphorylation. Notch1 ICD phosphorylation by serine threonine kinases such as glycogen synthase kinase 3 (GSK3), cycline-dependent kinase 8 (CDK8), integrin-linked kinase (ILK) or serum- and glucocorticoid inducible kinase (SGK1) has been linked with regulation of ICD turnover [79, 102-104]. GSK3 protein family is a multifunctional serine/threonine kinase, comprised of two members in mammals, GSK3α and GSKβ. Studies in mice showed that although GSKα and β are structurally similar, they execute different functions [105]. GSK3β-null mice are embryonic lethal due to extensive hepatocyte apoptosis and massive liver degeneration.

What is certainly unusual about this kinase is the fact that it is constitutively active in unstimulated cells, which is reflected by the wide range of phosphorylated substrates. Phosphorylation of GSK3β at tyrosine 216 (Tyr279 in GSK3α), located within the kinase activation loop (T-loop) facilitates substrate phosphorylation. In contrast, upon various stimuli, GSK3β is phosphorylated at serine 9 (Ser21 in GSK3α), which inhibits its function due to pseudo-substrate interaction between phosphorylated Ser9 and a substrate docking motif. Structural studies further demonstrated that GSK3 has a preference for pre-phosphorylated substrates with phosphorylation prerequisite site located at the C-terminus and GSK3 target residue at the N-terminus of consensus site (Ser/Thr-X-X-X-Ser/Thr-P). Although initial genetic studies of the Drosophila GSK3β homolog (Shaggy) indicated that this kinase might positively regulate Notch function, later reports showed opposing role of GSK3 in regulation of Notch. GSK3β interacted with and phosphorylated Notch1 ICD, and Notch signaling was significantly decreased in GSK3β null fibroblasts, due to reduced Notch1 ICD stability [102]. By contrast, a later study showed that Notch2 ICD is negatively regulated by GSK3β. GSK3β directly interacted and phosphorylated residues within Ser/Thr rich (STR) region of Notch2 ICD, which inhibited transcriptional activation of Notch target genes [106]. Another group has reported that GSK3α/β act as negative regulators of Notch1 ICD and that treatment with LiCl or dominant negative GSK3α/β increase Notch1 levels [107]. This discrepancy in results may be partially explained by the crosstalk of both GSK3β and Notch with various signaling pathways and different cellular context.

To date, numerous other kinases have been identified to target Notch. It has been reported that constitutively active AKT phosphorylates Notch1 ICD and down-regulates Notch-mediated transcription via regulation of Notch1 ICD nuclear localization [108]. The Down syndrome-associated kinase (DYRK1A) showed overlapping expression patterns with Notch in various tissues during development. DYRK1A directly interacts with and phosphorylates Notch1 ICD at multiple sites within the ankyrin domain, leading to attenuation of Notch signaling in neuroblastoma cells. The exact mechanism by which DYRK1A-mediated phosphorylation of Notch1 ICD inhibits Notch signaling awaits further investigation, however it does not involve regulation of Notch1 ICD stability [109]. The nemo-like kinase (NLK) was identified in a genetic screen as a modifier of activated Notch [110]. More recent studies demonstrated that NLK-mediated multiple site phosphorylation of Notch1 ICD negatively regulates Notch signaling by influencing formation of ternary complex. The NLK-mediated inhibitory effect on Notch promotes zebrafish neurogenesis, as NLK knock out enhances transcription of Notch target genes, repressing neuronal differentiation. Interestingly, MAML1 was also found to be a target of phosphorylation by NLK, however the NLK-dependent effect on Notch complex formation was mediated by phosphorylation of Notch1 ICD and not MAML1 [111]. In addition, Notch1 ICD has been shown to be phosphorylated by casein kinase 2 (CK2). Phosphorylation of Notch1 ICD at serine 1901 within ankyrin domain by CK2 leads to subsequent phosphorylation at threonine 1898, which causes dissociation of CSL-Notch-MAML1 complex from DNA [112].

Although Notch has been shown to interact with different HATs [68, 70], only recent studies demonstrate that Notch is a target of *acetylation*. UV induced TIP60 acetylates Notch1 ICD and suppresses Notch transcriptional activity through

dissociation of CSL-Notch1 ICD complex. Interestingly, TIP60 association with Notch1 ICD occurs only when Notch is bound to CSL, however it does not require formation of trimeric complex. The zinc finger and acetyl coenzyme A domain of TIP60 interact with Notch1 ICD ankyrin domain, where four lysine residues K2019, K2039, K2044 and K2068 are suggested as major TIP60 acetylation sites [113]. Guarani et al 2011 recently reported that Notch1 ICD is a target of acetylation by p300 and PCAF, and that SIRT1 acts as Notch1 ICD deacetylase, regulating Notch1 ICD protein turnover in endothelial cells. Using *in vivo* mice and zebrafish models the authors demonstrate that upon inactivation of SIRT1 the development of vascular branching and density is impaired due to enhanced Notch signaling [114]. Since SIRT1 deacetylase depends on cellular levels of $NAD^+$, any change in redox and metabolic state in the cells will have consequences on its function and the levels of acetylated Notch. Thus this finding provides an important link between Notch-mediated regulation of vascular growth and metabolic homeostasis in endothelial cells.

### 1.3.4 Notch in disease

The role of Notch1 in cancer was initially demonstrated by analysis of a rare chromosomal translocation t(7,9) in T-cell acute lymphoblastic leukemia (T-ALL), which resulted in expression of activated Notch1 [115]. In subsequent years, it has been discovered that activating mutations in Notch1 are present in over 50% of all T-ALL cases. Most of these mutations were found within the heterodimerization domain (HD) that cause ligand independent activation of Notch, and PEST domain that render Notch ICD protein more stable [116]. Cell cycle progress and inhibition of apoptosis were described as key cellular processes that drive Notch-mediated transformation in T-ALL [117]. Activating mutations were also found in chronic lymphocytic leukemia (CLL) [118-119], and Notch was found to play an important role in lung cancer (NSCLC) [120]. Many studies have demonstrated oncogenic properties of Notch, however it has also been shown that Notch can act as a tumor suppressor [121]. Examples of Notch role in suppression of tumors include small-lung cancer, prostatic epithelium, hepatocellular carcionoma and skin cancer, reviewed in [122]. More recently, novel somatic inactivating mutations in Notch were found in patients with chronic myelomonocytic leukemia (CMML) [123]. High-throughput analysis of head and neck squamous cell carcinoma (HNSCC) revealed numerous loss of function mutations in Notch1, which was the most frequently mutated gene in HNSCC (15% of patients) next to p53. Unlike in case of mutations described in haematological cancers, most Notch1 mutations observed in HNSCC were located within the N-terminal EGF region, and nearly half were predicted to truncate Notch1. This finding strongly implies Notch role as a tumor suppressor in HNSCC [124-125]. In some cancers, the role of Notch in carcionogenesis is far more complex, depending on the stage of the tumor as it is in the case of cervical cancer. Although many studies reported that Notch expression is upregulated in cervical cancer, subsequent findings showed that Notch was downregulated in the later stages of HPV-induced tumors and that downregulation of Notch was required for sustained expression of HPV-E6/E7 and malignant transformation [126]. Aberrant expression of the ligand can also contribute in Notch-mediated tumorgenesis, as showed for prostate or glioblastoma cancer [127-128].

To date, different approaches in inhibition of Notch have been established. Notch interaction with the ligand can be blocked by soluble Notch decoys consisting of EGF11 and 12 repeats [129]. γ-secretase inhibitors (GSI) have been shown to be useful in blocking of Notch receptor activation and is currently used in clinical trials for treatment of T-ALL, glioblastoma, melanoma, pancreatic and breast cancer [130]. However it is important to note that it blocks all four Notch receptors and many other receptors regulated by the γ-secretase complex, which is the cause of severe intestinal toxicity. Considering Notch cross-talk with other signaling pathways, combined use of inhibitors will certainly aid in development of more effective therapeutic strategies. Inhibitors with potential for treatment of aberrant Notch signaling include proteosome inhibitors [131-132]. Direct inhibition of the Notch transactivation complex was achieved by using synthetic hydrocarbon-stapled peptide SAHM1, which prevented assembly of the Notch complex. Treatment of T-ALL cells with SAHM1 led to genome-wide inhibition of Notch target genes with anti-proliferative effects in cultured cells and T-ALL mouse models [130]. A more specific approach was recently provided through development of therapeutic antibodies that target Notch receptors individually [133].

## 1.4   MASTERMIND-LIKE (MAML) PROTEIN FAMILY

The mastermind gene was identified in multiple genetic screens for novel modifiers of Notch in Drosophila as a "neurogenic" loci [134-135]. Similar to Notch, loss of function for mastermind resulted in excessive neural cells. In mammals, the MAML family consists of three members MAML1-3. All MAML proteins are glutamine-rich nuclear proteins, widely expressed in human adult tissues, but with different expression patterns in mouse early spinal cord development [72, 136]. MAML proteins are comprised of the basic domain (BD) within the N-terminus, and two acidic domains located in the middle and C-terminal region [71] (Figure 4). MAML1 contains two transcriptional activation domains TAD1 and TAD2. TAD1 (75-300 aa) contains a p300/CBP binding site important for p300/CBP-mediated nucleosome acetylation at the Notch enhancer, and transcription activation in vitro, however TAD2 (303-1016 aa) is required for transcription in vivo [78]. MAML1 C-terminus contains a CDK8 binding site important for Notch1 ICD phosphorylation and subsequent proteosome-mediated degradation [79]. Biochemical studies showed that MAML1-3 interact with all Notch receptors and CSL, however MAML3 efficiently coactivates only Notch4 ICD [72]. However, it remains to be further investigated whether this variation is caused by structural differences within binding interfaces, recruitment of different sets of additional coregulators or perhaps different posttranslational modifications. The short N-terminal region of MAML1 is crucial for interaction with Notch and CSL [71, 137-138]. Subsequent structural studies of the multiprotein complex consisting of CSL, Notch ICD ankyrin repeats and N-terminal MAML1 polypeptide on cognate DNA, demonstrated that ANK and both the C-terminal and N-terminal domains (CTD and NTD) of CSL are important in creating of binding surface for recruitment of MAML1 [139-140]. The Notch RAM domain induces allosteric change within the NTD of CSL, which contributes in recruitment of MAML1 [141]. Dominant negative MAML1 (DN-MAML1, a pan-Notch inhibitor), comprising of N-terminal peptide capable of binding Notch ICD and interfering with endogenous MAML proteins, has consequently been used in many studies to show functional importance of MAML1 in regulation of Notch.

Expression of DN-MAML1 (13-74 aa) in bone marrow impaired development of T-cell and marginal zone B-cell (MZB). DN-MAML1 knock-in mouse models showed MAML importance in development of skin, vascular smooth muscle as well as impaired cardiovascular development, reviewed in [142]. MAML1 knock-out mice remained small in size, died within the perinatal period and showed muscular dystrophy, ongoing hepathocyte death, and impaired development of MZB cells [143-145]. Although MAML1 knock out had no clear effect on development of T-cells, MAML1 was required for generation of MZB cells. This phenotype closely resembles Notch2 deficiency, suggesting MAML1 specifically coactivates Notch2 in MZB cells [145]. MAML3 knock-out mice had no apparent defects, however double MAML1/3 knock-out mice died in midgestation and resembled the phenotype characteristic for deficiency in Notch signaling, as in case of pan-Notch inhibitors. Expression of genes under strict control of Notch was not detectable in MAML1/3 double null mice, which was not observed in single null mice embryos. This finding indicates that both MAML1 and MAML3 are crucial for Notch signaling in vivo [146].
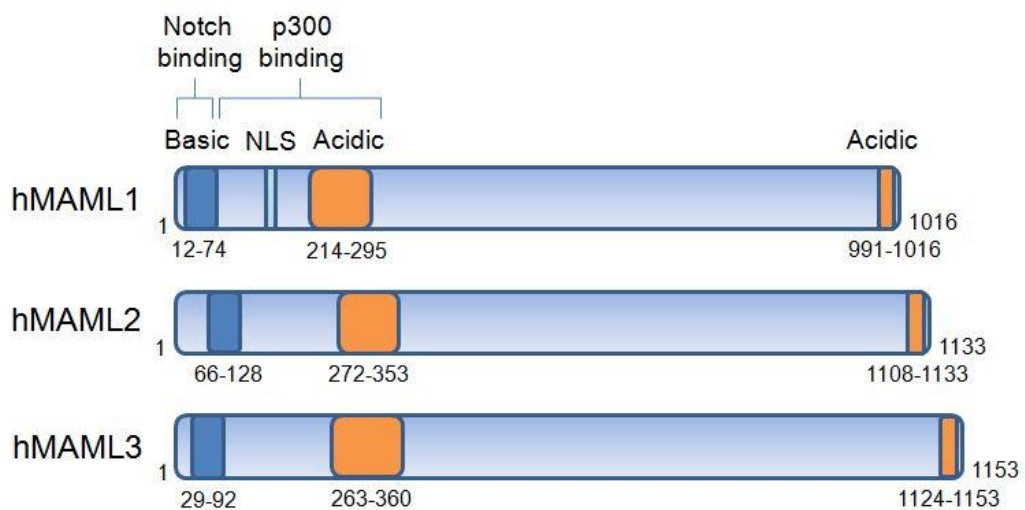


**Figure 4. Domain organization of MAML proteins.** MAML proteins are comprised of N-terminal basic domain and two acidic domains. MAML1 basic N-terminal region interacts with various proteins including Notch and CSL and region between 75-300 aa interacts with p300. MAML1 C-terminal region remains less characterized, the region between 300-1016 aa is important for the interaction with CDK8.

## 1.4.1  Notch-independent role of MAML1

More recent studies demonstrate that in addition to Notch, MAML1 is apparently important for other signaling pathways including coactivation of MEF2C [143], p53 [147], β-catenin [148] and NF-κB [149]. Studies of MAML1 knock-out mice revealed that MAML1 plays an important role in myogenesis owing to severe skeletal muscle defects typical for muscular dystrophy.  MAML1 deficient embryonic fibroblast failed to undergo MyoD-induced myogenic differentiation, while exogenous expression of MAML1 rescued myotube formation. MAML1 interacted with MEF2C and specifically coactivated MEF2C-mediated transcription. Interestingly, upon activation of Notch MAML1 enhanced myogenesis was significantly impaired, as a

result of competitive recruitment of MAML1 by Notch. This study revealed a unique Notch independent role of MAML1, and MAML1 dependent cross-talk between Notch and MEF2C in myogenic differentiation [143]. MAML1 was then identified as a specific coactivator of β-catenin, an essential component of Wnt signaling pathway. MAML1 binds β-catenin at the cyclin D1 and c-myc promoters, independently of the Notch pathway. In colon cell carcionoma, MAML1 siRNA caused cell death due to decreased cyclin D and c-myc expression. This study demonstrated that MAML1 is essential for survival of colon cancer cells via regulation of β-catenin transcriptional activity [148]. In subsequent years, MAML1 was found to be present at the native p53 response elements within promoters of p53 target genes. MAML1 increased p53 half-life and enhanced p53 phosphorylation and acetylation upon DNA damage. MAML coactivated p53, which was important for p53-mediated germ cell apoptosis [147]. The phenotype of MAML1 knock-out mice suggested yet another transcription factor, which could be coregulated by MAML1. The liver phenotype with multiple regions of cell death in MAML1 deficient mice closely resembled knock out models with defective NF-κB signaling. This led to discovery that MAML1 enhances NF-κB via two different mechanisms, coactivation of RelA(p65)-mediated transcription and second, increased degradation of the NF-κB inhibitor, IκBα. Moreover, MAML1-deficient mouse embryonic fibroblasts displayed impaired TNFα stimulation of NF-κB and increased TNFα-dependent cellular cytotoxicity [149]. Studies in Xenopus embryo also support MAML1 Notch-independent function in induction of neurogenic lineage, overexpression of XMAML1 resulted in appearance of pigmented cell mass on the embryo surface and stimulated expression of RNA-binding protein nrp-1 [150]. Furthermore, a genetic screen for Mastermind modifiers in Drosophila identified 79 Notch-independent Mastermind specific interactors (MSI). Among them were genes with RNA Pol II transcription factory and GTP-ase regulatory activity, negative regulators of transcription and metabolism [151].

## 1.4.2 MAML in disease

MAML1 was originally cloned as a binding partner for high-risk HPV type 16 E6 in a yeast two-hybrid screening, and MAML1-E6 interaction might interfere with Notch signaling at the early stages of cervical cancer [71]. Mucoepidermoid carcinoma (MEC) is the most common type of salivary gland malignancy, characterized by t(11,19) (q21, p13) chromosomal translocation. This rearrangement fuses mucoepidermoid carcinoma translocated 1 (MECT1) with MAML2, generating MECT1-MAML2 fusion protein capable of HES-1 induction independently of ligand-activated Notch signaling. MECT1-MAML2 induced foci formation in RK3E epithelial cells, which demonstrated its transformation potential [152]. MECT1-MAML2 fusion transcript serves a diagnostic and prognostic marker for low and intermediate stages of MEC, particularly in the lung [153]. MLL was identified as a second fusion partner of MAML2 in secondary acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) with inv(11)(q21q23). MLL-MAML2 suppressed both basal and Notch1 ICD induced activation of HES-1, however its precise role in carcinogenesis still remains to be better investigated [154]. Many studies demonstrated MAML contribution in tumorgenesis by using DN-MAML1. Continued growth and survival of Notch induced T-ALL requires MAML1 as shown by transduction of lymphoid cell lines with DN-MAML1 [155]. Aberrant Notch signaling was observed in glioblastoma (GBM), the

most common brain tumor, and expression of DN-MAML1 resulted in significant reduction of GBM growth in vitro and in vivo [156]. Moreover, activated Notch signaling pathway was detected in a wide panel of rhabdomyosarcoma (RMS) tumors and cell lines, and DN-MAML1 significantly decreased mobility and invasiveness of RMS in vitro [157]. Aberrant expression of MAML2 significantly contributes to dysregulated Notch signaling in several B-cell derived lymphomas, including classical Hodgkin lymphoma cells [158]. MAML1 was identified as a potential biomarker for Huntington's disease [159]. Moreover, expression of MAML1 was recently introduced as a marker of advanced tumor in esophageal squamous cell carcinoma (ESCC) [160].

# 2  AIMS OF THE THESIS

The overall aim of this thesis was to study the role of posttranslational modifications in regulation of Notch and MAML1 protein function.

The specific aims were as follows:

Paper I and IV - to investigate the role of MAML1 coactivator in p300 autoacetylation and p300-mediated acetylation of Notch1.

Paper II and III - to investigate how GSK3β kinase and SUMOylation regulate MAML1 coactivator function.

# 3   RESULTS AND DISCUSSION

## 3.1   PAPER I. MAML1 INCREASES P300 AUTOACETYLATION AND HAT ACTIVITY.

It has previously been shown that MAML1 potentiates Notch ICD-mediated transcription, by recruiting p300 acetyltransferase [78, 161]. Subsequent studies demonstrated that MAML1 and p300 co-occupy the HES-1 promoter in cell culture [78]. Moreover, MAML1 itself is a target of acetylation by p300, and MAML1-p300 complex specifically acetylates chromatin H3 and H4 histone tails [31]. Data presented in this paper demonstrate the molecular mechanism of how MAML1 modulates p300 function. Using in cell culture and *in vitro* acetylation assays we demonstrated that MAML1 enhances p300 autoacetylation. Previous studies demonstrated that p300 histone acetyltransferase (HAT) domain contains a highly conserved autoinhibitory activation loop region, which includes catalytically important acetylation sites. In order to render p300 catalytically active this loop has to undergo autoacetylation, and acetylation of lysine 1499 located adjacent to this region, has been established as a hallmark of p300 HAT activity [162]. We found that MAML1 significantly enhanced acetylation of lysine 1499, suggesting MAML1 may play a role in regulation of p300 activity. The MAML1-mediated effect did not depend on the autoinhibitory activation loop, since we observed that MAML1 could potentiate autoacetylation of both p300 wild type and p300Δloop proteins in similar ways. To assess whether MAML1 is capable of increasing p300 HAT activity directly, we performed *in vitro* HAT assay. MAML1 concentration-dependent increase in p300 HAT activity significantly enhanced acetylation of H4 histone tails.

MAML1 has previously been reported to localize in nuclear bodies [71] and to relocate there Notch and p300 [71, 78]. We therefore asked if MAML1 affects p300 and histones acetylation pattern in cell culture. Immunostaining experiments showed that MAML1 indeed localized to nuclear bodies. While in the absence of MAML1 acetylation of p300 at lysine 1499 was barely detectable, and p300 diffusely spread throughout the nucleus, MAML1 coexpression led to p300 relocalization and significant increase of acetylated lysine 1499 signal in nuclear bodies. Moreover, in the presence of MAML1 acetylated histones H3/H4 were found to localize in nuclear bodies. It has been shown that nuclear bodies might be the sites of active transcription; however it remains to be further investigated whether this is the case for MAML. In order to further verify that MAML1 influences acetylation status of histones at the promoter region we performed ChIP assay with primers that encompassed HES-1. We found that the presence of MAML1 was critical for detection of acetylated histone H3. Using western blotting, we also observed significantly higher levels of acetylated histone H3/H4 in cell line stably expressing MAML1, and reduced levels in cells treated with siRNA MAML1.

Different domains and interaction regions of p300 define its broad role as a global transcriptional coactivator and its important function in many cellular processes. In addition to the centrally located HAT domain, p300 consists of three cystein/histidine rich domains (C/H1-3), CREB binding KIX domain, bromodomain

and steroid receptor coactivator interaction domain (SID). We were particularly interested in C/H3 domain since our previous studies showed that MAML1 interacts with this domain [31]. To demonstrate the relevance of C/H3 domain in MAML1 dependent regulation of p300 autoacetylation we performed in cell acetylation assay using p300 construct with C/H3 domain deleted. We observed a robust MAML1 independent increase in overall autoacetylation and acetylated lysine 1499 of p300ΔC/H3 compared with p300 wild type. Moreover, unlike in case of wild type p300, addition of HDAC inhibitor sodium butyrate did not affect significantly the autoacetylation status of p300ΔC/H3. The rationality behind this is possible interaction of HDAC(s) with the C/H3 domain, which may be counteracted in the presence of MAML1. The fact that C/H3 is closely located to the HAT domain may also suggest their possible regulatory interaction. Alternatively, MAML1 binding itself could induce a conformational change. We have also investigated MAML1 domains in regulation of p300 autoacetylation in vitro and in cell culture assays, however we observed that only full length MAML1 could efficiently enhance p300 autoacetylation. The levels of p300 acetylation at lysine 1499 were next tested in a presence of MAML1 full length, MAML1 1-625 and 1-300 domains in cell culture. We detected significant p300 increased acetylation by full length and to some extent by MAML 1-625. Furthermore, using cell immunostaining and gene reporter assays we demonstrated that MAML1 full length, but not C-terminally truncated MAML1, could efficiently increase histone acetylation in nuclear bodies and Gal4-p300 activity, respectively. All together, this data imply that MAML1 full length is required in order to fully enhance p300 activity.

## 3.2 PAPER II. MAML1 ACTIVITY IS NEGATIVELY REGULATED BY GSK3B.

GSK3β has been shown play an important role in various signaling pathways and cellular processes such as proliferation, apoptosis or differentiation [105]. To date, a number of cytoplasmic and nuclear GSK3β targets have been identified including p53, β-catenin, NF-κB [163] and Notch [102, 106]. In this study we investigated the role of GSK3β kinase in regulation of MAML1 activity. We first performed the reporter gene assay and showed that GAL4-MAML1 activity was significantly decreased when coexpressed with GSK3β, whereas treatment with GSK3 inhibitor SB41 greatly enhanced MAML1 activity in cell culture. MAML1 and GSK3β can interact in cells, and this interaction is independent and direct as we further demonstrated in *in vitro* interaction assay with recombinant proteins. Moreover, our data show that recombinant GSK3β strongly phosphorylates MAML1 in vitro, which is significantly suppressed in the presence of SB41 inhibitor. We have previously reported that MAML1 can enhance acetylation of histones in cell culture. Thus, we treated the cells stably expressing MAML1 and control cells with SB41 to see if the levels of acetylated-histone H3 are affected. Inhibition of GSK3 significantly increased the levels of acetylated histones in MAML1 expressing cells. We further investigated which MAML1 domain(s) are important for interaction with GSK3β, and found MAML1 N-terminal region to be crucial for GSK3β binding. Moreover, using in vitro kinase assay we demonstrated that this region may also contain potential GSK3β phosphorylation sites. Thus our findings imply that N-terminal MAML1 is an important protein interaction region, crucial in executing MAML1 regulatory function as a coactivator.

To better characterize the biological context of GSK3β-mediated phosphorylation of MAML1 we evaluated if the status of GSK3β is important in regulation of MAML1. In order to test it we used GSK3β mutants, constitutively active (S9A) and kinase deficient (K85R) GSK3β. While all, wild type, constitutively active and inactive forms of GSK3β interacted equally well with MAML1, only wild type and GSK3β-S9A were capable of inhibiting GAL4-MAML1 activity. It has previously been shown that MAML1 directs Notch, p300 or MEF2C to nuclear bodies [71, 78, 143]. In order to test if MAML1 is also capable of relocating GSK3β we performed cell immunostaining. We could detect GSK3β in the cytoplasm and to some extent in the nucleus, however coexpressed MAML1 changed GSK3β subcellular localization to nuclear bodies. This was independent of GSK3β activation status since both S9A and K85R mutant proteins were similarly relocated. The precise role of MAML1 in regulation of GSK3β subcellular localization awaits further investigation.

Previous studies demonstrated that GSK3 modulates Notch activity [102, 106-107]. Thus, we evaluated GSK3β role in regulation of MAML1 coactivator in the context of Notch. We wanted to know if GSK3β affects the Notch1 ICD-MAML1 interaction and first performed cell immunostaining experiments. We could see that all proteins GSK3βS9A, Notch and MAML1 colocalized with each other, suggesting their interactions are not mutually exclusive. We also performed *in vitro* interaction assay in the absence or presence of ATP. We could see that regardless of the protein's phosphorylation status, MAML1 interacted with Notch equally well. In addition, we showed that GAL4-Notch activity is reduced to the same extend by GSK3β in the presence and absence of MAML1. GSK3β-mediated repression of MAML1 had consequences on expression of endogenous Notch target HES-1, as we could see significantly higher levels of HES-1 in the presence of both, MAML1 and SB41 inhibitor.

## 3.3 PAPER III. SUMOYLATION INHIBITS MAML1 ACTIVITY.

Data presented in this study demonstrate that SUMOylation is yet another mechanism to regulate MAML1 transcriptional activity. We found that MAML1 is SUMOylated at two evolutionary conserved sites, lysine K217 and K299 in cell culture. Lysine 217 appeared to be a major SUMOylation site, however only mutation of both (K217/299R) completely abolished MAML1 SUMOylation. Since it has previously been shown that SUMOylation can affect protein subcellular localization, we performed cell immunostaining analysis. However, this was not that case for MAML1 as we could see that both wild type and SUMO deficient mutant localized in the nucleus in the similar way. We next investigated if SUMOylation affects MAML1 transcriptional activity. We could see that the activity of SUMOylation deficient GAL4-MAML1217/299R was increased by 40-folds, when compared to wild type. Significantly higher activity of SUMOylation mutant was also observed using Notch target HES-1 as a reporter gene. We further confirmed that SUMOylation may regulate MAML1 transcriptional activity at the promoter level using plasmid immunoprecipitation assay. We could detect more SUMOylation in the presence of MAML1 wild type, comparing with the mutant protein.

In order to investigate if E2 conjugating enzyme UBC9 is required for MAML1 SUMOylation to occur we used siRNA. We showed that UBC9 siRNA significantly decreased MAML1 SUMOylation, and that GAL4-MAML1 activity was enhanced in UBC9 siRNA treated cells. SUMOylation of MAML1 was further evaluated in *in vitro* SUMOylation assays. We demonstrated that E1 ligase SAE1/SAE2, UBC9 and SUMO1 are required for SUMOylation of MAML1, moreover PIAS1 E3 potentiated MAML1 SUMOylation in vitro. In many cases, SUMO1 and SUMO2/3 have been shown to target different substrates. MAML1 was strongly SUMOylated by SUMO1; however we could also detect MAML1 SUMOylation by SUMO3 at the same lysine residues. Similar to SUMO1, PIAS1 significantly enhanced SUMOylation of MAML1 by SUMO3. Whether SUMO1 and SUMO3 compete for binding to MAML1 or perhaps target MAML1 under different cellular conditions awaits further investigation. Since SUMOylation is a reversible posttranslational modification, we also tested if SENP1 protease affects MAML1 SUMOylation levels in cells. We showed that SUMOylation of MAML1 was significantly diminished in the presence of SENP1. Furthermore coexpression of SENP1 greatly potentiated GAL4-MAML1 activity, which suggested than SENP1 can efficiently deSUMOylate MAML1 in cell culture.

It has previously been shown that SUMOylation-mediated repression is often associated with recruitment of HDACs [57]. Thus we investigated if HDACs play any role in regulation of MAML1. We found that among other HDACs tested, HDAC7 significantly decreased GAL4-MAML1 activity; moreover HDAC7 preferentially interacted with SUMOylated MAML1 in cell culture. Using plasmid immunoprecipitation we also detected higher levels of HDAC7 at the promoter region in the presence of wild type MAML1, comparing to SUMOylation deficient mutant. Interestingly, HDAC7 contains several SIM consensus motifs, which may explain its preference for SUMOylated MAML1. We found that MAML1 directly interacts with C-terminal HDAC7, and some of these SIM sites can be found in this region. It remains to be further investigated if SUMOylated MAML1 recruits HDAC7 to deacetylate histones as well as if additional corepressor proteins are required in this event.

## 3.4  PAPER IV. MAML1 ENHANCES ACETYLATION OF NOTCH1 BY P300.

Since it has previously been reported that Notch1 can interact with different acetyltransferases such us PCAF, GCN5 and p300, we investigated if p300 can acetylate Notch. In order to test it, we performed *in vitro* acetylation assay with recombinant proteins and found that Notch1 ICD is acetylated by p300 *in vitro*. To confirm this finding, we performed in cell acetylation assay using p300 wild type and p300 lacking the HAT domain. We found that Notch1 ICD is acetylated by p300 in cell culture, and that the p300 HAT domain is required for Notch acetylation to occur. We also tested whether GCN5 and PCAF were capable of acetylating Notch. Under our assay condition PCAF and GCN5 did not acetylate Notch1 ICD, however both PCAF and p300 strongly acetylated Notch3 ICD in cell culture (unpublished observation). In order to confirm that endogenous Notch is a target of acetylation, we used T-ALL cell lines RPMI-8402 and CCRF-CEM. Our data indicate that Notch1 may be a target of acetylation in T-ALL.

Considering that MAML1 is an essential coactivator for Notch signaling, we investigated if MAML1 contributes to p300-mediated acetylation of Notch1 ICD. We therefore performed in cell acetylation assays in the presence of MAML1. MAML1 significantly enhanced p300-mediated acetylation of Notch1, and we showed that MAML1-dependent increase in p300 autoacetylation may be one mechanism through which MAML1 regulates acetylation of Notch1 ICD by p300. We also investigated the levels of acetylated Notch in MAML1 siRNA treated cells, and found that acetylation of Notch1 ICD was considerably lower in MAML1 depleted cells. Next, we evaluated which MAML1 domain(s) are important for Notch acetylation by p300. MAML1 truncation proteins included MAML1 1-300 domain containing both Notch and p300 binding sites, a dominant negative MAML1 1-74, and MAML1 75-1016 incapable of binding to Notch, but capable of binding to p300. We observed that while MAML1 full length (1-1016) protein significantly increased acetylation of Notch1 ICD, we detected only modest increase with MAML1 1-300 and no increase with MAML1 75-1016. The levels of acetylated Notch1 ICD were significantly reduced in the presence of dominant negative MAML1, presumable due to competition with endogenous MAML1. Using ChIP assay, we further showed higher levels of p300 acetylated at lysine 1499 and MAML1 at the HES-1 promoter upon induction of Notch signaling.

Notch ICD consists of several well defined motifs and domains, including RAM and ankyrin repeats domains involved in interaction with CSL and MAML1, two nuclear localization signals (NLS), TAD and PEST domain important for overall Notch stability (Figure 1). As CSL is an important component of the Notch transactivation complex we investigated if Notch acetylation is affected in the presence of co-expressed CSL in cell culture. Our data show that CSL enhances Notch acetylation by p300, which relied on the presence of MAML1. We also found that mutation of Notch WFP motif, previously shown to be crucial for Notch binding to CSL, impaired significantly acetylation of Notch1 ICD. To identify which lysine residues may be important p300 targets, we performed site-directed mutagenesis and mutated lysines chosen based on sequence conservation and preliminary mass spectrometry results. We found that mutation of 10 lysines across NLS2 significantly impaired overall acetylation of Notch. However, we could not detect any considerable changes in the levels of acetylation in the case of other Notch mutants, NLS1 or triple lysines mutant (K1780/81/82, located closely to WFP motif). More recently, it has been reported that TIP60 interacts and acetylates Notch1 within the ankyrin repeats domain. Interestingly, Notch1 ICD interacts strongly with TIP60 in the presence of CSL, but not with ternary complex. TIP60-mediated acetylation appears to inhibit Notch signaling, leading to dissociation of Notch-CSL complex [113].

Considering that acetylation often servers as a mechanism to regulate protein ubiquitination and stability, we performed in cell ubiquitination assays to investigate the levels of ubiquitinated Notch1 ICD in the presence of p300 and MAML1. We could see that Notch was significantly less ubiquitinated and more acetylated when p300 and MAML1 were coexpressed. This decrease in ubiquitination depended greatly on p300 HAT domain since in the presence of coexpressed p300ΔHAT, Notch1 ICD was more ubiquitinated and consequently less acetylated. We therefore speculate that MAML1-dependent acetylation of Notch1 ICD by p300 may prevent Notch1 ICD ubiquitination, and might serve as a mechanism to regulate Notch stability. MAML1 has previously

been shown to recruit CDK8 kinase to phosphorylate and degrade Notch1 ICD [79]. We found that in cells treated with CDK8 siRNA Notch1 ICD is more acetylated and that CDK8 inhibited p300-mediated Notch1 ICD transcription of a reporter gene. Fryer at al 2004 showed that MAML1 recruits CDK8, which phosphorylates Notch and targets Notch for proteosome-mediated degradation [79]. It seems that MAML1 may serve a dual role in regulation of Notch1 activity in the nucleus in the way that, depending on interacting partner and signaling time frame, it may either trigger Notch for degradation or prevent it.

Although we have not thoroughly investigated the role of HDACs in regulation of Notch, we found that HDACs class I and II might modulate Notch activity. We observed almost 8 fold decrease in GAL4-Notch activity in the presence of HDAC4, and minor but consistent decrease with HDAC5 and HDAC7 (Figure 5). To our surprise coexpression of HDAC3 led to a robust increase in Notch1 activity. Whether these HDACs are capable of directly interacting with Notch1 ICD and the biological significance of this interaction remains to be further investigated.
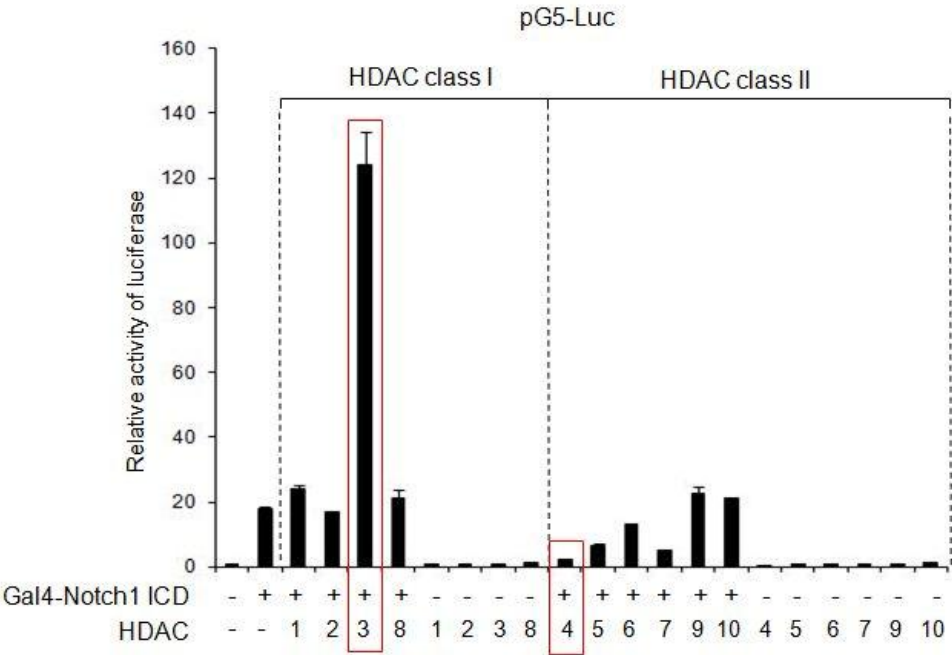


**Figure 5. Regulation of Notch1 ICD activity by HDACs.** HEK-293 cells were cotransfected with vectors expressing pG5-Luc reporter, GAL4-Notch1 ICD and HDACs. Data are presented as means ± SD.

# 4 CONCLUDING REMARKS

The data presented in this thesis is an extension of our current understanding of how MAML1 and p300 function as transcriptional coregulators of Notch and how posttranslational modifications modulate their function. From study I and IV we conclude that MAML1 plays an important regulatory role in p300 and Notch protein activity. MAML1 significantly enhances p300 autoacetylation and HAT activity and consequently affects p300-mediated acetylation of histones. Data presented in paper IV provide evidence that MAML1 strongly enhances p300-mediated acetylation of Notch1 ICD. Likewise, Notch3 ICD is acetylated by p300 and MAML1 potentiates Notch3 ICD acetylation (unpublished observation). Thus it seems plausible to conclude that MAML1 might modulate acetylation of many p300 targets in a similar way. MAML1 not only enhanced Notch1 ICD acetylation, but simultaneously decreased Notch1 ICD ubiquitination, suggesting it as a possible mechanism to regulate Notch1 ICD stability in the nucleus. Since MAML1 has also been shown to recruit CDK8 kinase, which leads to Notch phosphorylation and subsequent degradation [79], it seems that the MAML1-mediated effect depends on co-interacting coregulator partner and signaling time frame upon induction. Moreover, our data indicate T-ALL oncogenic Notch may be acetylated. It would be interesting to see if acetylation of Notch contributes to Notch-driven oncogenesis. Nevertheless, it still remains to be evaluated if endogenous Notch is acetylated in p300-MAML1-dependent manner in T-ALL. It also remains to be investigated if other Mastermind family members, MAML2 and MAML3 could potentiate p300 activity and thus enhance Notch acetylation and likely acetylation of other p300 targets. Interestingly, Guarani et al (2011) recently reported that Notch1 ICD can be reversely acetylated by p300 and PCAF, suggesting it as a mechanism to regulate Notch responses in endothelial cells. This study revealed that SIRT1 is apparently an important negative modulator of Notch by controlling its acetylation levels and turn over. Inactivation of SIRT1 in zebrafish and mice models impaired vascular branching and density due to enhanced Notch signaling [114]. Since SIRT1 deacetylase depends on cellular levels of $NAD^+$, any change in redox and metabolic state in the cells has important consequences on its function. Thus, this study reveals a biologically significant link between Notch-mediated regulation of vascular growth and metabolic homeostasis in cells. In view of the apparent role of Notch in human disease, combinatorial drug intervention that includes HDACs or proteosome inhibitors has certainly a great therapeutic potential in types of cancer where Notch plays a role of a tumor suppressor.

Data presented in paper II and III demonstrate the molecular mechanisms of MAML1 regulation by different posttranslational modifications. In addition to the previously published finding that MAML1 is acetylated by p300 [31], we show now that active GSK3β kinase is capable of phosphorylating MAML1, which has negative consequences on MAML1 coactivator function. We demonstrated that the GSK3 inhibitor SB41 significantly upregulated MAML1 activity, and consequently the expression of Notch target genes, such as HES-1. We further showed that MAML1 is yet a target of SUMOylation at two highly conserved lysine residues (K217/299). SUMOylation inhibited MAML1 transcriptional activity, which was associated with

recruitment of HDAC7. This had significant consequences on Notch-mediated transcription, as we could see that MAML1 SUMOylation-deficient mutant was a more potent Notch coactivator. Whether SUMOylation of MAML1 affects histone modification remains to be further investigated. It will be interesting to see if GSK3β and SUMOylation also affect responses mediated by other signaling pathways through direct regulation of MAML1. More research is needed to fully understand the biological context under which these MAML1 modifications occur, including triggering stimuli and other coregulators involved. Since SUMOylation sites identified in MAML1 are highly conserved among all MAML protein family members, it will be interesting to see if MAML2 and 3 are also targets of SUMOylation. Finally, more physiological in vivo models with relevant MAML1 mutants will certainly aid into future clarification of their biological relevance. Considering the fact that MAML1 is likely involved in different biological processes via coactivation of various signaling pathways, as well as its increasing role in cancer, it will be also interesting to see the future studies of the regulatory mechanisms underlying MAML1 gene expression.

# 5  ACKNOWLEDGEMENTS

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