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GENE REGULATION MECHANISMS BY THE TRANSCRIPTIONAL COREGULATOR MASTERMIND-LIKE 1

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In memory of my grandfather, Osmar Ribeiro

ABSTRACT

MAML1 was first identified as a coactivator for Notch receptors, but later it was also found to function as a coactivator for several other activators, including p53, β-catenin, and MEF2C. MAML1 is critical for Notch signalling and has been shown to act cooperatively with the histone acetyltransferase (HAT) p300 in transcription that is mediated by the Notch intracellular domain (Notch IC). Furthermore, it has been shown that the MAML1 protein is crucial for the p300-mediated acetylation of histones in chromatin templates.

We have investigated the molecular interplay between MAML1 and p300 during Notch-mediated transcription. We reported that the N-terminal domain of MAML1 interacts directly with the C/H3 domain of p300 and with histones, and that the p300-MAML1 complex specifically acetylates the tails of histones H3 and H4. Furthermore, we showed that MAML1 is acetylated by p300, and identified conserved lysines in the MAML1 N-terminus as the target for p300 acetylation. We found that MAML1 contains a proline repeat domain that is important for its activity, for p300-mediated acetylation, and for interaction with p300. Next, we investigated how MAML1 can influence the activity of p300. We found that MAML1 enhances p300 autoacetylation, which requires the HAT and C/H3 domains of p300. MAML1 directly enhances the HAT activity of p300 and this correlates with the translocation of MAML1, p300, and acetylated histones to nuclear bodies.

We found that MAML1 is phosphorylated *in vivo* at serine and threonine residues. We then investigated whether MAML1 could be phosphorylated by GSK3β, which is a kinase known to phosphorylate Notch IC in the nucleus. We found that GSK3β phosphorylates the N-terminus of MAML1, and that inhibition of GSK3β abolishes the GSK3β-dependent phosphorylation of MAML1 *in vitro*. Furthermore, we found that GSK3β interacts with the N-terminus of MAML1 and targets it for downregulation. We also showed that GSK3β must be active in order to repress MAML1. The transcriptional activity of MAML1 and the global levels of histone acetylation are upregulated when GSK3β is inhibited. Finally, MAML1 translocates GSK3β to nuclear bodies, in a process that requires the full-length MAML1 protein.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred by their Roman numerals:

I. Saint Just Ribeiro, M., Hansson, M.L. and Wallberg, A.E. (2007) A proline repeat domain in the Notch co-activator MAML1 is important for p300-mediated acetylation of MAML1. *Biochem. J.*, **404**, 289-298.

II. Hansson, M.L., Popko-Scibor, A., Saint Just Ribeiro, M., Dancy, B.M., Lindberg, M.J., Cole, P.A. and Wallberg, A.E. (2009) The transcriptional Coactivator MAML1 regulates p300 autoacetylation and HAT Activity. *Nucleic acids res.*, in press.

III. Saint Just Ribeiro, M., Hansson, M.L., Lindberg, M.J., Popko-Scibor, A., and Wallberg, A.E. (2009) GSK3β is a negative regulator of the transcriptional coactivator MAML1. *Manuscript.*

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TABLE OF CONTENTS

2 Introduction…………………………………………………………………………..1

1.1 Transcriptional regulation……………………………………………………...1

1.1.1 The general transcription machinery RNA polymerase II …………..…...1

1.1.2 The structure of chromatin ……………………………………………..2

1.1.3 Role of post-transcriptional modifications of the histone tails……………4

1.1.4 HATs and HDACs……………………………………………………...5

1.2 The Notch signalling pathways ………………………………………………...6

1.3 The coactivator Mastermind-like 1……………………………………………...8

1.3.1 MAML1 function in diverse pathways……………………………………9

1.3.2 The MAML coactivator family; biological functions and involvement in diseases. ……………………………………………………………12

1 .4 Post-transcriptional modifications of non-histone proteins…………………....13

1.5 Autoacetylation of PCAF and p300 …………………………………………...14

1.6 Kinases involved in Notch signalling…………………………………………15

1.6.1 CDK8: a kinase recruited by MAML1 to phosphorylate the Notch1 IC...15

1.6.2 Function of the GSK3β kinase in Notch signalling…………………........16

2 Aims of the thesis……………………………………………………………………...18

3 Results and discussion………………………………………………………………19

3.1 Paper I:The molecular interplay of MAML1 and p300 in Notch mediated transcription…..19

3.2 Paper II: Modulation of p300 autoacetylation and HAT activity by MAML1…………………………………………………………………..20

3.3 Paper III: Repression of MAML1 activity by the GSK3β kinase……………...22

4 Concluding remarks and future perspectives……………………………………..24

5 Acknowledgments………………………………………………………………..27

6 References…………………………………………………………………………..28

LIST OF ABBREVIATIONS

CDK	Cyclin dependent kinase
ChIP	Chromatin Immunopreciptation
CTD	C-terminal domain
CSL	<u>*C*</u>BF1 <u>S</u>uppressor of Hairless <u>L</u>ag-1
DNMAML1	Dominant negative Mastermind like protein-1
GADPH	Glyceralaldehyde-3-phosphate dehydrogenase
GSK3	Glycogen synthase kinase 3
GSI	γ-secretase inhibitor
GTF	General transcription factor
HAT	Histone acetyltransferase
HDAC	Histone deacetyltransferase
Hes	Hairy/Enhancer-of-split
HPV	High-risk human papilloma virus
K	Lysine
Notch IC	Notch intracellular domain
NSL	Nuclear localization signal
MAML	Mastermind-like
me	Methylation
MEF2C	MAD box enhancer factor 2C
MSI	Mastermind specific interactors
PIC	Pre initiation complex
Ser	Serine
siRNA	Small interfering RNA
TBP	TATA-box binding protein
TF	Transcription factor
Tyr	Tyrosine

1 INTRODUCTION

1.1 TRANSCRIPTIONAL REGULATION

In a multi-cellular organism, all cells contain the same genes, but the subset of genes that is expressed varies from cell to cell. The phenotype of the tissue formed by a particular cell type depends on the specific combination of proteins that is expressed within that cell, which in turn is determined by the regulation of gene expression. The range of genes that is expressed depends on the biochemical and cellular properties of the cell. Gene expression can be altered by communication with neighbouring cells or in response to different environmental stimuli through the action of specific signal transduction pathways. These include pathways that are critical for development, such as the Notch and Wnt pathways. Signal transduction results in the induction and/or repression of downstream target genes.

In eukaryotic organisms, diverse types of protein are required for the different steps of transcriptional regulation. These include chromatin-modifying complexes, transcription factors, coactivators, and general transcription factors, which include the RNA polymerases. Transcription factors bind to specific sequences within the DNA in response to specific stimuli and regulate gene expression. As our understanding of the complexity of transcriptional regulation has increased, the importance of other molecules, such as transcriptional coregulators (coactivators and corepressors), has been highlighted. Coregulators are proteins that do not bind to DNA directly but form a complex with transcription factors to regulate the expression of specific target genes.

1.1.1 The general transcription machinery of RNA polymerase II

Gene expression is mediated by the general transcription machinery, which is assembled in a sequential manner on the core promoter. The latter consists of an AT-rich region: the TATA box motif. In the initial step, general transcription factors (GTFs) bind to the core promoter. The GTFs include transcription factor IID (TFIID), which consists of the TATA-box binding protein (TBP) together with TBP-associated

factors (TAFs). TBP recognizes the TATA box of the promoter and causes the DNA to bend. The formation of the DNA/TFIID complex makes it possible for TFIIB to contact DNA sequences that are upstream and downstream of the TATA box. The TFIID/TFIIB complex then interacts with RNA polymerase II and TFIIF. In the following step, RNA polymerase II, together with other GTFs, which include TFIIE and TFIIH, form a larger multiprotein complex termed the pre-initiation complex (PIC). Subsequently, the C-terminal domain (CTD) of RNA polymerase II is phosphorylated, which in turn facilitates promoter clearance and enables transcription elongation to progress(1,2). Modification of the CTD affects its conformation and its ability to associate with factors that are involved in transcriptional elongation. The CTD is phosphorylated mainly at the serine (Ser) residues, Ser2 and Ser5. The phosphorylation of Ser5 peaks during an early stage of the transcription cycle, whereas phosphorylation of Ser2 occurs during elongation(3). Several kinases are involved in the phosphorylation of the CTD, including cyclin-dependent kinase 7 (CDK7) in the TFIIH complex and CDK8, which phosphorylates the CTD at Ser5. In addition, the CTD is phosphorylated at Ser2 by CDK8 and CDK9, which is a component of positive transcription elongation factor b (P-TEFb)(4,5). Transcription is further regulated by the binding of specific factors to distinct regulatory elements, known as upstream activating sequences (UASs), which are located in the promoter or enhancer regions. In turn, these specific factors facilitate the recruitment of additional proteins and protein complexes, which change the chromatin structure and enable communication between the general transcription machinery and the promoter(6).

1.1.2 The structure of chromatin

The eukaryotic genome is assembled into chromatin, which contributes to an additional level of gene regulation. Structural variations in the chromatin, which can vary from a condensed (heterochromatin) to a more open structure (euchromatin), regulate the accessibility of the underlying DNA(7). Compacted chromatin inhibits the binding of regulatory proteins that would activate gene expression. However, the local chromatin structure is sufficiently dynamic to be modulated rapidly by the action of signalling cascades in response to specific stimuli(8-10). The primary unit of chromatin in eukaryotic cells is the nucleosome, which is composed of an octamer of four core histone proteins (two copies each of H2A, H2B, H3, and H4) together with 147 base

pairs of DNA that are wound in two turns around the octamer. Each nucleosome is separated from the next by a region of linker DNA that varies in length. The nucleosomes are further compacted into fibres and higher order structures, and the linker histone H1 is important for this process(11).

Histones are small positively-charged proteins and therefore can bind tightly to DNA, which is highly negatively charged. The assembly of stable nucleosomes depends on the initial heterodimerization of H3 and H4, and the subsequent dimerization of H3 to form the H3/H4 tetramer. The H2A and H2B dimers bind to either side of the H3/H4 tetramer to create a left-handed super-helical octamer, around which the DNA is wrapped(11). The histones consist of globular domains that are important for octamer structure, and tail domains, which are located in the N-terminus of all four core histone proteins and the C-terminus of H2A. The histone tails are very flexible in structure and project away from the histone octamer. They were defined initially by their sensitivity to proteases. The removal of the histone tails does not dramatically alter the properties of single nucleosomes, neither does it affect nucleosome positioning or the correct assembly of nucleosomes *in vitro*(12). The interaction of histone tails with DNA and proteins influences the folding or compaction of the chromatin fibre. The histone tails mediate inter-nucleosomal contacts as the chains of nucleosomes are compacted to form the 30-nm fibre. Furthermore, the histone tails are important for the self-assembly of the condensed 30-nm fibre into higher-order structures(7,11).

Alteration of the chromatin structure can make the DNA more or less accessible to transcription factors. There are two classes of chromatin remodelling coactivators: ATP-dependent chromatin remodelling complexes and histone modifier proteins. The ATP-dependent chromatin remodelling complex changes the structure of chromatin by disrupting the nucleosomal structure, and increases the accessibility of the DNA in a non-covalent manner(13). The histone modifier enzymes alter the chromatin structure by post-translational covalent modification of the histone tails; this includes reversible phosphorylation, ubiquitination, methylation, and acetylation.

1.1.3 Role of post-translational modification of the histone tails

Different post-translational modifications of the histone N-terminal tails constitute the so-called ‘Histone code’, and specific combinations of modifications can indeed promote or repress transcription by altering the chromatin structure. The ‘Histone code’ is not restricted necessarily to a single histone tail, but may involve modifications to several tails in nucleosomes that are co-localized spatially within the chromatin(14). The development of antibodies against specific histone modifications has made it possible to study the post-translational modification of histones in different contexts. The patterns of native chromatin modification are studied mainly by using mass spectrometry and chromatin immunoprecipitation (ChIP) methods. Histone modifications can also be studied at a global level using newer methods that have been developed from ChIP, for example ChIP-ChIP and ChIP-Seq(15). The first well-studied class of histone modification, lysine (K) acetylation, is mediated by the histone acetyltransferases (HATs), which catalyse the transfer of an acetyl group from the donor acetyl-coenzyme A to the ε-amino group of a lysine in the histone protein(16). The acetylation of histones plays an important role in gene regulation in eukaryotic cells by altering the physical properties of the chromatin and recruiting effectors that contain bromodomains. The acetylation of lysine residues introduces a negative charge, which neutralizes the positive charge of the histone tails and thereby decreases the interaction between the histone tail and the negatively-charged phosphate groups in the DNA(17). Furthermore, HATs and other chromatin-associated factors contain a bromodomain, which recognizes and interacts with the acetylated N-terminal histone tails(18). Hyperacetylation of histones, including the acetylation of lysine residues K9/14/18/23 of H3 and K16 of H4, correlates positively with gene transcription(18-20). High levels of H3 and H4 acetylation are often detected at the promoter region and the first residues of an actively-transcribed gene(21-23).

Other post-translational histone modifications include methylation, phosphorylation and ubiquitination. Histones can be mono-, di-, or trimethylated on lysine and arginine (R) residues by the histone methyl transferases (HMTs). The methylation of histones can be correlated with both transcriptional activation and repression; the outcome depends on which lysine residues (or arginine residues) are methylated(7). Di- or trimethylation of lysine 4 on H3 (H3K4me2/3)(19) is associated

with gene activation, whereas H3K9me3, H3K27me3, and H4K20me1 are often correlated with transcriptional repression(24-27). The role of some lysine methylation, including that of H3K36 and H3K79, still remains elusive; these modifications are associated with both gene repression and activation(3). Phosphorylation of serine 10 on H3 (H3S10p) and acetylation of H3K9 is associated with mitogen-stimulated transcription(8,9). The ubiquitination of histone H2A on lysine 119 (H2AK119ub), in combination with the modification H3K27me3, is associated with the silencing of homeotic genes(28,29).

1.1.4 HATs and HDACs.

Histones can be acetylated either when they are synthesized in the cytoplasm or by nuclear HATs whilst they are incorporated into nucleosomes in chromatin. The nuclear HATs can be divided into five different families: GNAT proteins (e.g., GCN5 and PCAF), MYST proteins (e.g., MOF, Tip60, MORF), p300/CREB-binding protein (e.g., p300, CBP), nuclear receptor coactivators (e.g., SRC1, ACTR), and general transcription factors (e.g., TFIIIC)(16). Among these, members of the p300/CBP and GNAT families are the best characterized, and are still studied intensively. This is partly because they are connected to many transcription factors and signalling pathways, but also perhaps because they are more potent acetylases than other HATs, such as SRC1 and TIP60(30). p300/CBP-associated factor (PCAF) was identified initially as a p300/CBP-binding protein, It shares sequence similarity with yeast GCN5 (yGCN5) and can acetylate nucleosomal histones *in vitro* and *in vivo*(31). p300 and CBP, which are homologous proteins, are global transcriptional coactivators that are expressed ubiquitously, and are recruited to chromatin in an activation-dependent manner. p300/CBP also plays a critical role in cell cycle regulation, cellular differentiation, and apoptosis. The deregulation of p300/CBP has been implicated in several types of cancer, cardiac diseases, and inflammatory processes(32-35).

Acetylation is a reversible process, and there are four types of histone deacetylase (HDAC) that remove the acetyl group from histone and non-histone proteins: class I (e.g., HDAC 1, 2, 3, and 8), class II (e.g., HDAC 4, 5, 6, and 7), class III (e.g., Sir2p-related proteins) and the more recently identified class IV (eg.,

HDAC11). The class I and class II HDACs are recruited to promoters by interacting directly with corepressors, whereupon they deacetylate histones and contribute to transcriptional repression. Class III HDACs are dependent on the cofactor NAD+ for their activity(36,37).

1.2 THE NOTCH SIGNALLING PATHWAY

The Notch signalling pathway is an evolutionarily-conserved system for cell-cell communication, and it plays an important role in developmental processes by influencing cellular proliferation, differentiation, and apoptosis. Notch signalling controls the fate of many cell types, including muscle, nerve, and haematopoietic cells(38-40). In *Drosophila*, there is only one Notch receptor, but vertebrates contain four Notch receptors (Notch 1-4). They are single transmembrane-spanning proteins. The Notch ligands are also transmembrane proteins. In both *Drosophila* and vertebrates, they are divided into two classes: Delta and Serrate (Jagged in mammals). Interaction between a Notch ligand and the extracellular part of a Notch receptor in a neighbouring cell triggers the Notch receptor to undergo a series of proteolytic events, which involve ADAM metalloproteases and the γ-secretase complex. Cleavage of the Notch receptor leads to the release of the intracellular portion of Notch from the cell membrane and its translocation to the nucleus. In the cell nucleus, the Notch intracellular domain (Notch IC) associates with the DNA bound transcription factor, CSL, forming a complex with other coregulators. This process activates the transcription of Notch target genes(41).

CBF1 (human), Suppressor of Hairless (*Drosophila*), Lag-1 (*Caenorhabditis elegans*) (CSL), which is also known as RBP-Jk in mouse, is a highly-conserved and ubiquitously-expressed nuclear protein that binds to a specific DNA sequence (CGTGGGAA). When Notch IC is absent from the nucleus, CSL is bound to the Notch target genes and represses their transcription. The transcriptional repression is mediated by the recruitment of HDAC-containing complexes. CSL has been shown to interact with several corepressors, which include the Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor (SMRT) complex(42), KyoT(43), CBF1-

interacting corepressor (CIR)(44), and the SMRT/HDAC-associated repressor protein (SHARP)(45).

The Notch IC cannot bind to DNA directly, but associates with the CSL-DNA complex. When Notch IC interacts with CSL, the corepressor-CSL complex is disrupted and coactivators, such as PCAF, GCN5, p300, and Mastermind-like (MAML) proteins, are recruited to activate expression of the Notch target genes (figure 1). Notch has been reported to interact with the coactivators mentioned above(46-49). Mouse Notch1 IC interacts with mouse PCAF and GCN5 in mammalian cells. Furthermore Notch IC/RBP-Jk mediated transcription is repressed by the two HAT inhibitor proteins E1A and Twist which suggests that HATs, including PCAF and GCN5, play an important role in the RBP-Jk-mediated transactivation(46). The N-terminus is the most conserved region in MAML proteins, and it interacts with the Notch-CSL-DNA complex through the Notch ankyrin repeat domain. It has been shown that amino acids 1–300 and 1–74 of MAML1 can shift a complex of CSL and Notch in an electrophoretic mobility shift assay (EMSA)(50). All MAML proteins appear to form stable DNA-binding complexes with Notch and CSL(47,49).

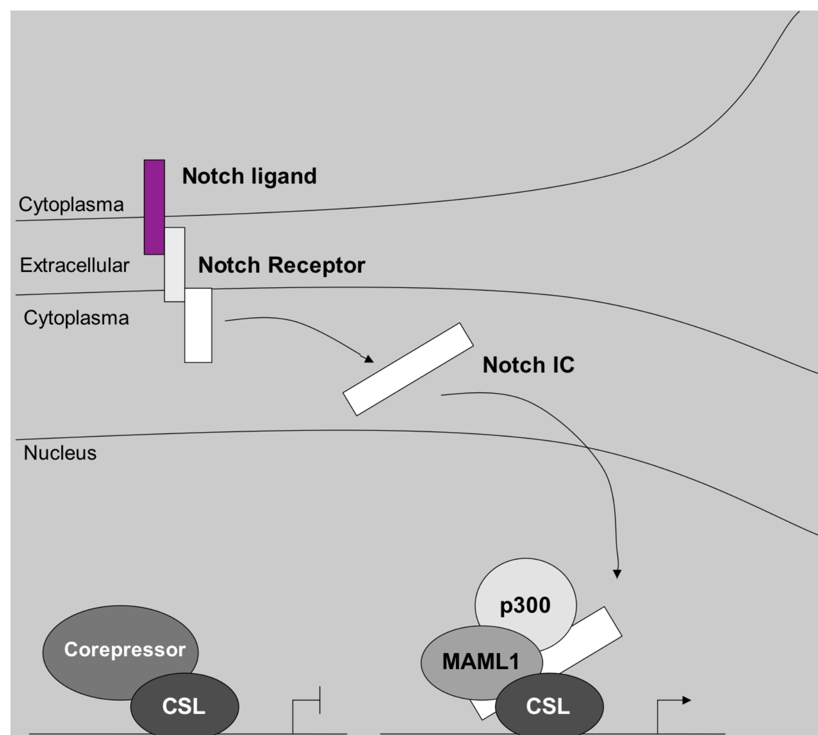

Figure 1) **Schematic of molecular steps in Notch signalling.** Interaction between ligand and Notch receptor in neighboring cells leads to release and translocation, of Notch IC to the nucleus. Notch IC disrupts the corepressor-CSL complex, and form a complex with CSL and coactivators, including MAML1 and p300.

The p300 protein has been shown to interact not only with the Notch IC(48) but also with the coactivator MAML1(50,51). MAML1 potentiates Notch IC-mediated transcription from chromatin templates *in vitro* by recruiting p300 to the DNA(50,51). In addition, p300 can only mediate significant Notch IC-dependent transcription from chromatin templates *in vitro* in the presence of MAML1. Furthermore, PCAF is unable to increase Notch-mediated transcription in the absence of p300, but it does enhance the effect of p300 and MAML1, which indicates that p300 and PCAF act cooperatively to mediate transcription from chromatin(51).

Notch target genes have been identified in various cellular and developmental contexts. The Hairy/Enhancer-of-split (Hes) genes, which were first identified in *Drosophila* as neurogenic genes, encode highly-conserved proteins. Several lines of evidence suggest that Hes1, Hes5 and Hes7, as well as the Hes-related genes Hey1, Hey2, and HeyL, are direct targets of Notch(52). The Hes and Hey proteins are helix-loop-helix transcription factors that function as transcriptional repressors. Hes1, Hes5 and Hey1 are the best-characterized Notch target genes(53,54). In addition, Notch regulates the expression of NFκβ2(55) and genes that are implicated in cancer, such as c-myc(56-58), cyclin D1, and p21/Waf1 (40).

1.3 THE COACTIVATOR MASTERMIND-LIKE 1

MAML1 is a coregulator that was identified originally as a Notch coactivator. It was cloned on the basis of homology to *Drosophila* Mastermind(59), which is a neurogenic gene that is linked genetically to Notch function(60,61). The MAML family contains two other members, MAML2 and MAML3(47,49). The three MAML proteins are widely expressed in adult tissue but have distinct expression patterns during early development in mouse(47,49). All MAML proteins appear to be critical specifically for Notch signalling(47,49). MAML1 and MAML2 strongly enhance ligand-induced transcription of the Notch target gene Hes1, but MAML3 has only a weak effect. However, MAML3 has been shown to work as a potent coactivator for the Notch4 IC(49). More recently, MAML1 has been shown to function as a coactivator for various other signalling pathways that are independent of Notch,

including the MAD box enhancer factor 2C (MEF2C)(62), p53(63), and Wnt pathways(64). MAML proteins may induce Notch signalling and perhaps also other signalling pathways differently depending on their expression levels in various cell types and different activities towards Notch receptors.

The MAML proteins are nuclear proteins, and all MAMLs have been shown to form nuclear dots when overexpressed in mammalian cells(47,49). The N-terminus of MAML shows the highest homology between MAML proteins(47,49), and amino acids 1−75 of MAML1 have been shown to interact with the Notch-CSL-DNA complex(50). The N-terminus of MAML1 is also crucial for interaction with the coactivator p300(50), as well as proteins in other signalling pathways, including p53 and MEF2C(62,63). The MAML1 protein also contains a nuclear localization sequence (NLS), PGHKKTR, in the N-terminal domain (amino acids 135−141), which is important for the nuclear localization of MAML1(59). The C-terminus of MAML1 is important for MAML1 transcriptional activity *in vivo*. It is believed that the C-terminal domains of MAML1 are important for the recruitment of additional factors, but at present only a few proteins have been shown to interact with this portion of MAML1(50) (figure 2).

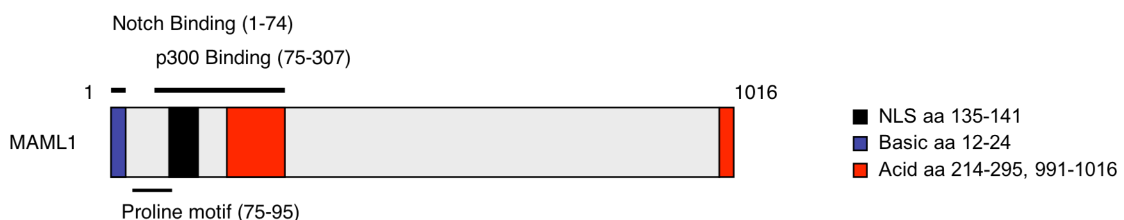

Figure 2) **Schematic of domains in the MAML1 protein.** The MAML1 protein comprises one basic domain, two acid domains, and a nuclear localization sequence (NLS). Amino acids (aa) 1-74 are known to interact with Notch IC, and amino acids 75-305 are important for the interaction with p300. The C-terminal, 305-1016, contains uncharacterized regions.

1.3.1 MAML1 functions in diverse pathways

MAML1 has been shown to act in many different signalling pathways, including those of MEF2C, Wnt, and p53. MEF2C is a member of the Mef2 family of

transcription factors. The Mef2c gene is necessary for mouse embryonic development, during which it controls cardiac morphogenesis and myogenesis. The finding that MAML1-deficient mice display severe muscular dystrophy led to the identification of MAML1 as a coactivator for MEF2C(62). In cell culture, the level of MAML1 expression directly affects the ability of MEF2C to induce myogenesis. Increased expression of MAML1 leads to the enhanced formation of myotubes and increased expression of muscle-specific target genes, whereas reduced expression of MAML1 impairs muscle cell differentiation. It appears that MEF2C and MAML1 work together in a complex to activate the transcription of several other genes that are required for muscle development and function(62). However, MEF2C interacts with the N-terminus of MAML1, as does Notch IC, and MAML1 serves as a coactivator for MEF2C only when the Notch receptors are inactive. Upon Notch activation, Notch IC competes MAML1 away from MEF2C, which leads to the down-regulation of muscle-specific target genes and up-regulation of Notch target genes(62).

The Wnt/β-catenin signalling pathway affects cellular decisions by modulating distinct processes, such as differentiation and proliferation. MAML1 is recruited by β-catenin to the promoters of cyclin D1 and c-Myc *in vivo*, and knockdown of the MAML proteins in colonic carcinoma cells affects the ß-catenin-induced expression of cyclin D1 and c-Myc, which results in cell death(64). Furthermore, MAML1 has been shown to function as a coactivator for β-catenin in the Wnt/β-catenin pathway, independently of Notch signalling, by using γ-secretase inhibitor (GSI), which is a potent inhibitor of the Notch pathway(64). GSI prevents the cleavage of Notch and its release from the membrane, and subsequently blocks the transactivation of Notch target genes. In presence of GSI inhibitor the MAML1 and Notch combination were not able to stimulate a CSL-reporter. In contrast GSI does not affect MAML1-induced activation of the TCF/ β-catenin target gene cyclin D1(64).

The interaction of MAML1 with p53 involves the N-terminal region of MAML1 and the DNA-binding domain of p53(63). It has been shown by ChIP that MAML1 is part of the activator complex that binds to native p53-response elements within the promoters of the p53 target genes, p21, Bax, and GADD45(63). Furthermore, expression of MAML1 enhances p53-dependent gene induction in mammalian cells independently of Notch, and knockdown of MAML1 reduces p53-

dependent gene expression(63). Moreover, MAML1 increases the half-life of p53 protein and enhances its phosphorylation and acetylation upon DNA damage of cells(63). These recent findings suggest that MAML1 functions as a coactivator in diverse cellular processes and can be viewed as a mediator of crosstalk between different signalling pathways (figure 3).

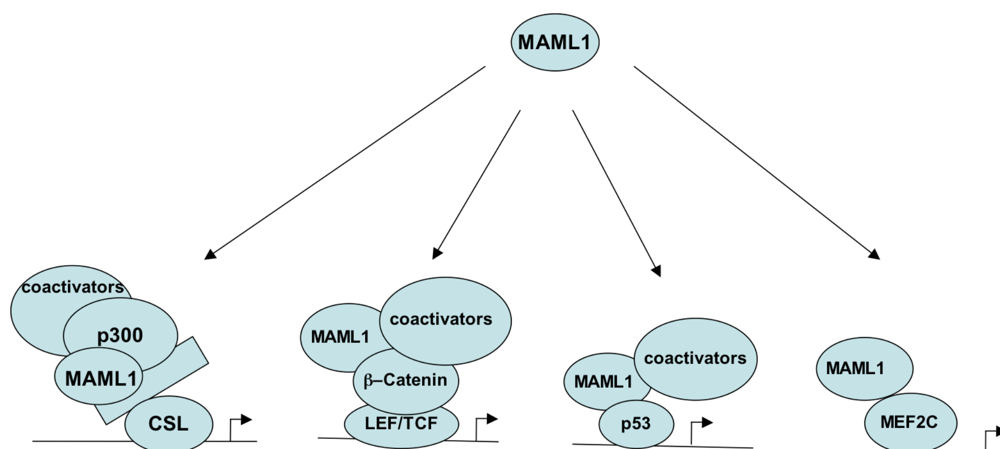

Figure 3) **The MAML1 protein acts as a coregulator for diverse transcription factors.** A schematic picture illustrating that MAML1 can be recruited by various activators to regulate different signalling pathways.

Evidence that MAML proteins have functions that are independent of Notch has also been established using the model organisms *Xenopus* and *Drosophila*. A study in *Xenopus* has shown that XMam1 can induce cells to differentiate into the neurogenic lineage in a Notch-independent manner(65). Furthermore, overexpression of XMam1, which lacks the N-terminus that is necessary for interaction with Notch, in *Xenopus* embryos leads to the expression of Neuropilin-1 (NRP-1), an RNA-binding protein that is expressed in the developing nervous system(65). Recently, several Mastermind-specific interactors (MSIs) were identified in a genetic screen in *Drosophila,* using the Exelixis collection of insertion mutations, for genes that dominantly modified the *Drosophila* mutant (C96-MamN) wing-marginal phenotype. The MSI genes failed to interact with the Notch pathway and were classified into

several functional categories, which included negative regulators, factors with RNA polymerase II transcriptional activity, factors with small GTPase regulator activity, and factors that negatively regulated metabolism(66). One of the identified MSI genes is the *Drosophila* β-catenin homologue Armadillo. Although it is possible that some of the MSI genes interact genetically with components of the Notch pathway that were not included in the screen, the diverse MSIs that were identified suggest a broad role for Mastermind in various developmental processes(66).

1.3.2 The MAML coactivator family: biological functions and involvement in diseases

The MAML proteins play an essential role in the regulation of Notch signalling, and it is not surprising that they have been linked to cancer. MAML1 was cloned originally as a protein that bound to the high-risk human papillomavirus (HPV) type 16E6 oncoprotein in a yeast two-hybrid screen. Transformation by HPV is mediated partly through its ability to induce the ubiquitination and degradation of the tumour suppressor p53, but some forms of HVP are unable to degrade p53 and additional mechanisms are involved. 16E6 associates with MAM/Notch IC/CSL in the nucleus and the MAML-16E6 interaction affects the ability of MAML1 to induce expression of the Notch target gene Hes1. It has been proposed that the interaction of MAML with E6 contributes to its ability to inhibit epithelial cell differentiation through the inhibition of Notch signalling(40). Notch2 IC has been shown to control the invasive properties of gliomastoma cells by inducing the expression of Tenascin-C. Overexpression of dominant-negative MAML1 (DNMAML1) or knockdown of RBP-JK in LN319 cells reduced cell migration and decreased the expression of Tenascin-C(67).

As part of a fusion protein, MAML2-MECT1, MAML2 has been shown to be involved in diverse forms of cancer, including mucoepidermoid carcinoma and cervical cancer. MAML2-MECT1 is able to induce Notch targets, independently of Notch, via the interaction of MECT1 with CREB(68). Other MAML2 fusion proteins that have been implicated in disease are TORC1-MAML2 in skin tumours (69) and MLL-MAML2 in secondary acute myelogenous leukaemia and myelodysplastic syndrome(70,71).

The DNMAML1 mutant has been used in many studies and reveals the essential role that MAML1 has in Notch signalling. DNMAML1 inhibits all of the MAML proteins by competing with the wild-type proteins to form a complex with Notch IC and CSL(72). It can be used to inhibit Notch signalling. MAML1 may be a good target for the modulation of other signalling pathways, including those of Wnt, p53, and MEF2C. Both the use of DNMAML1 and knockout studies of MAML1 have shown that MAML1, together with the Notch2 receptor, are important for the formation of Marginal zone B (MZB) cells in the spleen(73). Future knockdown experiments using small interfering RNAs (siRNAs) against MAML will lead to a better understanding of the function of MAML proteins in cells. It has been shown using siRNA techniques that MAML1 can have an anti-apoptotic role in colon cancer by acting as an oncogene(64). Future studies will be aimed at understanding the MAML1-mediated crosstalk between different signalling pathways and how the role of MAML1 in diseases may vary depending on the pathways that are involved.

1.4 POST-TRANSLATIONAL MODIFICATIONS OF NON-HISTONE PROTEINS

After the discovery of HATs, such as PCAF and p300/CBP, a number of non-histone proteins were identified that are substrates for HATs. Factor acetyltransferases (FATs) are HATs that have also been reported to acetylate transcriptional activators, coactivators, basal factors, chromosomal proteins, and other non-histone proteins with a substrate specificity that is similar to that for histone acetylation. So far, no acetylases that do not act on histones have been discovered, but many non-histone proteins are bona fide substrates for HATs. It remains to be shown whether the major targets of some of the HATs with low HAT activity are non-histone proteins(30). The acetylation of non-histone proteins has various functions, which include increased or decreased DNA binding, together with effects on stability, localization, and protein-protein interaction(34,74). PCAF acetylates non-histone proteins such as E2F1(75,76), Ku70(77), NFκβ(78), and p53(79,80). p300/CBP also has many non-histone target proteins, including p53(81), p73(82), NFκβ(83), STATs(84), c-myc(85), and GATA-1 and -3(86-88).

1.5 AUTOACETYLATION OF PCAF AND P300

It is known that some acetyltransferases, such as PCAF and p300, can be autoacetylated(89-93). Recently, the mechanism and function of autoacetylation has been studied. PCAF has been shown to be both acetylated by p300 and autoacetylated, via intermolecular and intramolecular mechanisms, respectively(91). Autoacetylation of PCAF leads to an increase in the HAT activity of PCAF *in vitro*. Moreover, autoacetylation of PCAF occurs in the NLS, and is important for the nuclear localization of the protein(91). The acetylation of different proteins by p300 plays a critical role in the regulation of gene expression, and the p300 protein is itself regulated by autoacetylation. This autoacetylation is believed to occur primarily by an intermolecular mechanism because a catalytically-defective p300 HAT domain can be acetylated efficiently by active p300 (94). However, it is still possible that autoacetylation of p300 may be an intramolecular event. The biological significance of the modification of proteins *in trans* is that regulation will depend on the local concentrations of the partners that are involved. Given that p300 and CBP are not abundant cellular proteins and that deacetylase enzymes are also present, the basal state of acetylation is unlikely to be high. However, it is possible that multiple p300 molecules are recruited to transcriptional promoters, which would enhance autoacetylation(94). The p300 protein contains a conserved proteolytically-sensitive loop region (residues 1520–1560) in the HAT domain, which serves as an autoinhibitory loop. The p300 loop can be modified by autoacetylation of several lysine residues within the loop, which enhances the HAT activity of the protein(93). A mutant of p300 protein, p300Δloop, in which the loop has been deleted**,** has been shown to be a more potent coactivator for the androgen receptor as compared to wild type p300. Autoacetylation of p300, including residue 1499, has been shown to regulate the HAT activity of p300(93).

Autoacetylation induces structural changes in the p300 HAT domain(95). It has been suggested that the conformational change of p300 by autoacetylation after its recruitment to the preinitiation complex leads to its dissociation and the enhanced binding of the general transcription factor TFIID(96). The autoacetylation activation loop and its mechanism of action resemble the autophosphorylation/upstream kinase activation loops in kinases. There have been several reports of factors that regulate

p300 autoacetylation by binding directly to p300; these include subunits APC5 and APC7 of the Anaphase-promoting complex/cyclone (APC)(97), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)(98).

1.6 KINASES INVOLVED IN NOTCH SIGNALLING

Post-translational modifications provide key regulatory switches for cell signalling pathways and the regulation of gene expression. Protein phosphorylation is the most widespread type of post-translational modification used in signal transduction. Proteins can be phosphorylated at serine, threonine, and tyrosine residues. Phosphorylation of a protein can modulate various protein functions, for example subcellular localization, protein-protein interactions, protein-DNA binding, ubiquitination, and enzymatic activity. The Notch receptor is phosphorylated at several residues and the phosphorylation of different residues has been shown to correlate with the stimulation or inhibition of Notch activity, nuclear translocation, and cellular transformation(99-103).

1.6.1 CDK8: a kinase recruited by MAML1 to phosphorylate the Notch IC

CDK8 is involved in both the positive and negative regulation of transcription, and CDK8 activity is regulated by binding to cyclin C. CDK8 and cyclin C are components of the RNA polymerase II holoenzyme complex, which phosphorylates the CTD of the largest subunit of RNA polymerase II. CDK8 has been shown to regulate transcription by targeting the CDK7/cyclin H subunits of TFIIH, and thus provides a link between the 'Mediator-like' protein complexes, which activated transcription and also contain CDK8/cyclin C, and the basal transcription machinery. MAML1 interacts directly with CDK8, which then mediates the phosphorylation of serine residues in the PEST domain of Notch IC, and targets Notch for proteosomal degradation(104). The PEST-dependent proteolysis of Notch IC in the nucleus is mediated by the Fbw7/Sel10 ubiquitin ligase(105).

1.6.2 Function of glycogen synthase kinase 3β in Notch signalling

Glycogen synthase kinase 3 (GSK3) is a proline-directed serine/threonine kinase that was identified initially as a kinase that phosphorylates and inactivates glycogen synthase, a key enzyme in glycogen metabolism. There are two closely-related isoforms of GSK3, GSK3α and GSK3β. GSK3 has been shown to phosphorylate a number of different cellular proteins, which include p53, c-Myc, c-Jun, heat shock factor 1 (HSF1), cyclin D1, and Notch(106,107) (fig 4). GSK3 has been implicated in fundamental cell processes such as cell fate determination, metabolism, transcriptional control, and oncogenesis due to its involvement in the regulation of protein synthesis, cell proliferation, cell differentiation, cell mobility (microtubule assembly/disassembly), and apoptosis(106,107).

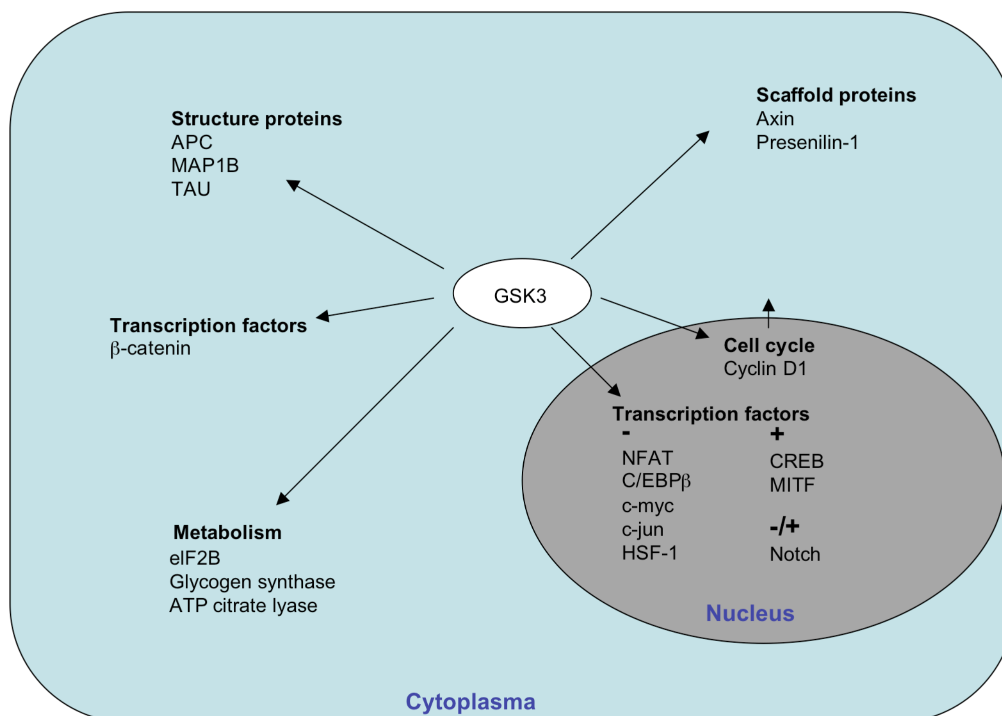

Figure 4) **Substrates of GSK3β and their function.** GSK3β phosphorylates various proteins with diverse cellular function, including proteins involved in the metabolism, structure proteins, scaffold proteins, components of the cell cycle machinery and transcription factors.

Many protein kinases have flexible regions known as activation loops that are phosphorylated by upstream kinases or autophosphorylation. Activation of GSK3β is dependent upon the phosphorylation of Tyr216 (Tyr279 in GSK3α)(107).

No kinase for Tyr216 has been identified *in vivo* and many studies have suggested that the phosphorylation of this residue occurs by autophosphorylation. Consistent with this hypothesis, Tyr216 residue in GSK3β is also phosphorylated when the protein is expressed in *Escherichia coli*(108). The activity of GSK3β has been shown to be downregulated by the phosphorylation of Ser9 (Ser11 in GSK3α). Ser9 is phosphorylated by the downstream kinases AKT, protein kinase A (PKA), and protein kinase C (PKC)(109-111), which results in the downregulation of the activity and function of GSK3β. The activity of GSK3β has also been shown to be downregulated by the kinase p38(112). The substrate specificity of GSK3β is unusual in that the kinase prefers substrates that have already been phosphorylated. The consensus phosphorylation site for GSK3β is Ser/Thr-X-X-X-Ser/Thr-P, and GSK3β phosphorylates the first Ser/Thr. This phosphorylation is much more efficient when the last Ser/Thr has been phosphorylated previously by another kinase (primed phosphorylation)(113).

Shaggy, which is the *Drosophila* homologue of GSK3β, has been reported to be a positive modulator of Notch(114,115). In mammals, phosphorylation of Notch by GSK3β has been shown to either up- or down-regulate the Notch IC by affecting Notch stability(100,116). Notch needs to be processed prior to phosphorylation by GSK3β, and only the Notch IC is phosphorylated by GSK3β(100). By comparing Notch expression and stability in GSK3β null and wild type MEF fibroblasts, it was shown that the GSK3β-mediated phosphorylation of Notch1 IC protects the latter from proteosomal degradation(116). Other studies have shown that phosphorylation by GSK3β leads to the inhibition of Notch signalling, mainly by negatively affecting the stability of Notch2 IC (100).

2 AIMS OF THE THESIS

To investigate the interplay between MAML1 and p300 and to identify the role of MAML1 acetylation by p300 in Notch-mediated transcription: Paper I.

To study how MAML1 affects the coactivator function of p300: Paper II.

To investigate the function of GSK3β-dependent phosphorylation in the transcriptional downregulation of MAML1: Paper III.

3 RESULTS AND DISCUSSION

3.1 PAPER I: THE MOLECULAR INTERPLAY OF MAML1 AND P300 IN NOTCH MEDIATED TRANSCRIPTION

In this study, we investigated the interplay between MAML1 and p300 during Notch-mediated transcription. It was shown previously that amino acids 75–305 of MAML1 interact with p300 in HeLa nuclear extract(50). To confirm that amino acids 1–300 of MAML1 (MAML 1–300) interact directly with p300 we first performed a protein-protein interaction assay *in vitro*. We found that FLAG-tagged p300, which had been purified from Sf9 insect cells, interacted strongly with full-length GST-tagged MAML1 and GST-MAML1 1–300. To study the interplay between MAML1 1–300 and p300 during Notch-mediated transcription from chromatin templates, we utilized a cell-free system with highly-purified transcription factors (RBP-Jk and Notch), and cofactors (MAML1 and p300). We found that the N-terminus of MAML1 (MAML1 1–300) was sufficient for the stimulation of p300-dependent transcriptional activation by CSL and Notch. p300 has been reported previously to acetylate chromatin templates that contain Notch IC, CSL, and MAML1(50). In the current study, we showed that full-length MAML1 and MAML1 1–300 could stimulate the p300-dependent acetylation of chromatin. To analyse the acetylation of recombinant chromatin further, the CSL template was reconstituted with recombinant intact core histones or histones that lacked tails. The combinations of the latter that were used included tailless H3 + tailless H4, tailless H2A + tailless H2B, and totally tailless core histones. We showed that the p300-MAML1 complex specifically acetylated histones H3 and H4, and that the removal of the H3 and H4 tails, but not the H2A and H2B tails, completely abolished the p300-dependent acetylation of histones by the p300-MAML1 complex. Given that the acetylation of the H3 and H4 tails by p300 was dependent on MAML, we speculated that MAML1 might interact directly with histones. We performed a protein-protein interaction assay and showed that MAML1 interacted directly with GST-tagged histone H3 and histone H4. We further mapped the binding of MAML1 to histone H3 using various MAML1 domains. We showed that full-length MAML1 and MAML1 1–300 interacted with acetylated H3. We also detected a weaker interaction between acetylated H3 and amino acids 701–1016 at the C-terminus of MAML1.

Given that p300 is known to acetylate not only histones, but also non-histone proteins, and that MAML1 interacts strongly with p300, we tested whether p300 might acetylate proteins in the Notch signalling pathway. By employing an *in vitro* acetylation assay, we found that full-length MAML1 and MAML1 1−300 were acetylated by p300 *in vitro*. We mapped the acetylation site to conserved double lysine residues in the N-terminus of MAML1. In addition, we found that a proline repeat motif in MAML1 was important for the p300-mediated acetylation of MAML1, and the interaction between MAML1 and p300. Firstly, we showed, by an *in vitro* acetylation assay, that a proline repeat peptide, which corresponded to amino acids 73−95 of MAML1, negatively affected the p300-mediated acetylation of MAML1, but not the acetylation of histones by p300. Secondly, we showed, using an immunoprecipitation assay, that wild-type MAML1, but not a MAML1 proline mutant in which amino acids 81−87 had been deleted, interacted with p300 *in vivo*. Furthermore, we showed that MAML1 1−300, but not the MAML1 proline mutant, was able to increase the transcriptional activation of a reporter gene by Notch. Using a GST-pulldown assay with GST-MAML1, FLAG-tagged p300, and an increased salt concentration, we showed that MAML1 interacted more weakly with p300 in the presence of acetyl-CoA as compared to the absence of acetyl-CoA. We also showed that MAML1 was acetylated *in vivo* by p300. After co-transfecting plasmids that expressed MAML1 and p300, or just the plasmid for MAML1, into HEK293 cells, we immunoprecipitated MAML1 with an antibody against MAML1 and analysed the levels of MAML1 protein and acetylation by immunoblotting. MAML1 acetylation was detected only when the construct for MAML1 was co-transfected with that for p300.

3.2 PAPERS I AND II: MODULATION OF P300 AUTOACETYLATION AND HAT ACTIVITY BY MAML1

In this study, we investigated how MAML1 regulates the activity of p300. In the previous study, we found that p300 acetylated MAML1 and noticed that the autoacetylation of p300 was enhanced in the presence of MAML1 in our *in vitro* acetylation assay. To study further whether MAML1 affected p300 autoacetylation in cell culture, we transfected plasmids that expressed MAML1, wild type p300 or p300ΔHAT (in which the HAT domain has been deleted) into HEK293 cells. We then immunoprecipitated the p300 proteins from whole cell extracts using an antibody

against p300. Immunoblotting with antibodies against MAML1, p300 or acetylated lysines in p300 showed that p300 acetylation increased in the presence of MAML1 but was undetectable when p300 lacked the HAT domain. An increase in p300 autoacetylation was also detected when an antibody against K1499 of p300 was used. This antibody has previously been used to shown that autoacetylation of K1499 increase p300 HAT activity(93). When Cos-7 cells were transfected with plasmids that expressed p300 and MAML1, we observed by immunostaining that MAML1, p300, and acetylated histones co-localized in nuclear dots. When the construct for p300 was transfected alone, p300 and the histones co-localized in a diffuse distribution in the nucleus.

The p300 protein contains several well-defined protein interaction domains that regulate transcription by forming a bridge between the basal transcriptional machinery and transcription factors that bind DNA with sequence specificity(117). In paper I, we showed, using an *in vitro* binding assay, that MAML1 interacts directly with the p300 C/H3 domain. The C/H3 domain is situated close to the HAT domain and, given that MAML1 interacts with the C/H3 domain, we speculated that this domain could be involved in the increase in p300 autoacetylation in the presence of MAML1 (figure 5).

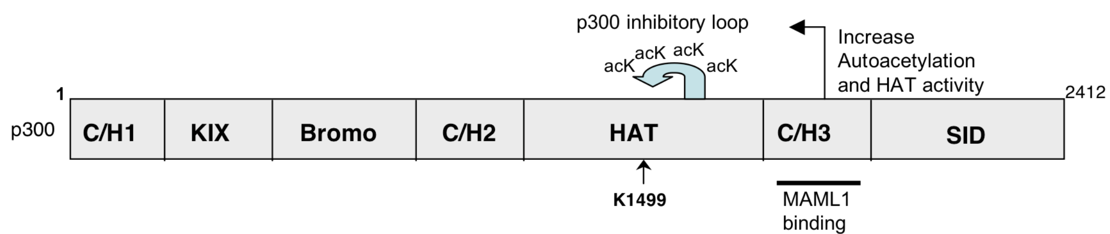

Figure 5) **MAML1 enhances p300 autoacetylation and HAT activity.** Schematic of p300 and MAML1 domains. The p300 protein contains several domains including three cystein-and-histidine-rich domains (C/H1, C/H2 and C/H3). MAML1 interacts with the p300 C/H3 domain located next to the HAT domain. MAML1 can modulate autoacetylation of lysines in the inhibitory loop, including K1499 and increase the p300 HAT activity.

As expected, we found that the MAML1-dependent autoacetylation of p300 required the C/H3 domain. Unexpectedly, the deletion mutant of p300 that lacked the C/H3 domain (p300ΔC/H3) was distributed in small nuclear dots when transfected alone, and co-localized with MAML1 in larger nuclear dots when the constructs for

MAML1 and p300ΔC/H3 were co-transfected. Our data indicate that other factors or domains within the two proteins may be important for the co-localization of MAML1 and p300 *in vivo*. In addition, we observed that MAML1 did not require the p300 activation loop to enhance the autoacetylation of the p300 HAT domain. The p300Δloop protein is a more effective coactivator for GAL4-MAML1 than the full-length protein in a reporter gene assay. We speculate that the binding of MAML1 to C/H3 either changes the structure of the p300 HAT domain or prevents the binding of corepressors to the C/H3 domain.

ChIP assays were performed to examine whether histone acetylation occurs at the promoter region of the Notch target gene Hes1 in response to MAML1. Our data showed that the acetylation of histones H3 and H4 was enhanced significantly in the presence of MAML1. Furthermore, the global acetylation of histones H3 and H4 was significantly higher in a cell line that expressed MAML1 stably as compared to control cells. We also used a siRNA against MAML1 to knock-out MAML1 in U2OS cells, and showed that the levels of acetylated histone H3 were decreased significantly in cells in which MAML1 was downregulated as compared to cells transfected with a control siRNA. The protein levels of the histones and GADPH were not affected by the MAML1 siRNA. Given that MAML1 increased the autoacetylation and HAT activity of p300, we performed reporter gene assays to investigate whether the transcriptional activity of p300 was affected by MAML1. We showed that the transcriptional activity of a GAL4-p300 fusion was enhanced 20-fold when p300 was co-transfected with MAML1.

3.3 PAPER III: REPRESSION OF MAML1 ACTIVITY BY THE GSK3Β KINASE

GSK3β has been shown previously to phosphorylate the Notch IC and affect Notch signalling(100,116). In this study, we investigated whether GSK3β regulates the activity of the coactivator MAML1. By performing a reporter gene assay with GAL4-MAML1 and GSK3β in U2OS cells, we showed that GSK3β directly affected the activity of MAML1. The transcription of the reporter gene decreased approximately 4-folds when GSK3β was co-expressed together with MAML1, compared to the transcriptional activity of GAL4-reporter and MAML1. In the

presence of the GSK3β inhibitors SB41 and SB21, the transcriptional activity of MAML1 was increased significantly. To investigate how the coactivator function of MAML1 is affected by GSK3β, reporter gene assays were performed with GAL4-Notch, MAML1, and GSK3β. The expression of GSK3β totally abolished transactivation by Notch, whereas transcription was enhanced more than 15-fold when GAL4-Notch and MAML1 were co-transfected. Furthermore, when GAL4-Notch, MAML1, and GSK3β were co-expressed, Notch activity was downregulated by five-fold. The addition of SB41 to either GAL4-Notch or GAL4-Notch and MAML1 led to a two-fold upregulation in expression of the reporter gene. Given that, in a previous study, we showed that MAML1 increases the global acetylation levels of histone H3, we further investigated whether GSK3β could affect the level of histone acetylation by treating 293 and 293-MAML1 cells with SB41. We found that the inhibition of GSK3β with SB41 increased global H3 acetylation significantly in 293 cells that contained FLAG-tagged MAML1, whereas the level of tubulin or GADPH was unaffected.

Next, we investigated whether GSK3β needs to be active to affect the transcriptional activity of MAML1 using two different GSK3β mutants, GSK3β S9A and GSK3β K85A. The phosphorylation of S9 has been shown to inactivate GSK3β and the residue K85 is essential for kinase activity(109-111,118). We found that GSK3β does need to be active to downregulate MAML1, because only wild type GSK3β and GSK3β S9A, and not the GSK3β K85A mutant, affected MAML1 activity. To elucidate whether MAML1 interacted equally well with the GSK3β mutants, we co-expressed MAML1 with wild type GSK3β and the mutant proteins. We then immunoprecipitated MAML, probed for GSK3β by western blotting, and found that all the GSK3β proteins interacted to the same degree with MAML1. We also showed that FLAG-MAML1 associates with endogenous GSK3β in cells, and that recombinant GSK3β interacts directly with affinity-purified MAML1. Furthermore, we mapped the site of interaction with GSK3β to the N-terminus of MAML1, amino acids 1–300. Using an *in vitro* phosphorylation assay, we found that GSK3β phosphorylates full-length MAML1 and MAML 1–300. In addition, we performed an *in vitro* kinase assay in the presence or absence of the GSK3β inhibitor SB41 and concluded that SB41 inhibited the GSK3β-mediated phosphorylation of MAML1. Using different domains of MAML1, we mapped the phosphorylation site for GSK3β to the N-terminus of MAML1. We then investigated whether GSK3β targets the N-terminus of MAML1

and downregulates its activity in cellular assays. We showed that GSK3β significantly downregulated the activity of MAML1 1–1016 and 1–300 and that the level of decreased transcription was similar for both proteins. Given that MAML1 has been shown previously to target various proteins to nuclear dots, we investigated whether MAML1 affects the localization of GSK3β in the cell. By immunostaining Cos-7 cells that had been transfected with the constructs for GSK3β and MAML1, we found that MAML1 translocates GSK3β to nuclear dots. We also showed that MAML1 translocates endogenous GSK3β to nuclear dots.

4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

We conclude from our studies that the N-terminus of MAML1 plays an essential role in the coactivator function of this protein. The N-terminus has previously been shown to interact with activators, such as Notch and p53(59,63). We have focused our studies on the interplay between MAML1 and p300 or GSK3, which also interact with the N-terminus of MAML1. Whereas the interplay between MAML1 and p300 results in increased transcriptional activity of both proteins, GSK3β inhibits the coactivator function of MAML1. The observation that the MAML1 N-terminus interacts with both a coactivator and a potential corepressor raises certain questions: how are the recruitment of coactivators and corepressors regulated in cells, and can the balance between these proteins be regulated by competition for binding. Future studies might focus on whether, and how, competition for binding to the MAML1 N-terminus can affect the interplay between MAML1, p300, GSK3β and other proteins. One interesting question is whether the interaction of Notch or p53 with MAML1 can affect the interaction between MAML1 and GSK3β. The roles of MAML1 post-translational modifications were examined in Paper I (acetylation) and Paper III (phosphorylation), and in Paper II we focused on the MAML1-modulated post-translational modification of p300 (autoacetylation). We conclude that acetylation and phosphorylation of the N-terminus of MAML1 play an important role in regulating the activity of this protein.

We have identified lysine residues in MAML1 that are acetylated by p300, which may help to elucidate the function of p300-dependent acetylation of

MAML1. Our study suggested that the acetylation of MAML1 residues decreases the interaction between MAML1 and p300, and therefore is important for the dissociation of the MAML1/p300 complex. In paper II, we also speculated that autoacetylation of p300 might decrease the MAML1-p300 interaction. We would like to address these questions in future studies, probably by using MAML1 lysine mutants in different assays. However, it is still necessary to investigate which modifications occur in the MAML1 C-terminus and the roles that these modifications play.

We have shown that GSK3β phosphorylates MAML1 *in vitro* and that the kinase activity of GSK3β is required to inhibit MAML1 transcriptional activity *in vivo*. Furthermore, SB41 and SB21, which are inhibitors of GSK3β, are capable of enhancing MAML1 activity. Our current data indicate that MAML1 is phosphorylated by several kinases *in vivo*. It will be necessary to identify the residues in MAML1 that are phosphorylated by GSK3β in order to study the role of MAML1 phosphorylation further, to dissect the role of GSK3β-mediated phosphorylation of MAML, and to investigate whether priming occurs prior to phosphorylation by GSK3β. It has been reported previously that GSK3β does not bind to the promoter of the Notch target gene Hes1 *in vivo*(104). However, it is possible that GSK3β is present at the promoter of other genes that are regulated by MAML1 because the kinase affects MAML1 transcriptional activity. Furthermore, we showed that MAML1 translocates GSK3β to nuclear dots, but the mechanism and role of this translocation still needs to be clarified. Interestingly, we also found that the inhibition of GSK3β by SB41 enhances the acetylation of histone H3.

It has been reported previously that MAML1 induces the phosphorylation of p300 and the translocation of p300 to nuclear dots(50). In paper II, we showed that MAML1 modulates the HAT activity of p300 by enhancing p300 autoacetylation. The MAML1-dependent modulation of p300 autoacetylation is dependent on the C/H3 domain of p300. The p300 C/H3 domain is situated next to the HAT domain, and C/H3 is known to interact with both transcription factors and the repressor HDAC1(117,119). It is also the domain that interacts with MAML1 (shown in paper I). It remains to be investigated whether the binding of MAML1 to the C/H3 domain changes the conformation of the p300 HAT domain or whether MAML1 enhances p300 autoacetylation by preventing the binding of p300 repressors, including

HDAC1. The p300 loop region has been shown previously to regulate p300 autoacetylation and HAT activity, and a p300 mutant that lacks the loop region is more active(93). We have shown that the enhancement of p300 autoacetylation by MAML1 is stronger if p300 lacks the autoinhibitory loop, and that p300Δloop functions as a better coactivator for MAML1. On the basis of our data, we conclude that the MAML1-dependent modulation of p300 acetylation does not require the loop region.

In paper I, we showed that MAML1 enhances the acetylation of histone H3 and H4 on chromatin templates in a p300-dependent manner. Furthermore, we concluded that the interaction of MAML1 with the histones could explain, in part, the mechanism of MAML1-dependent acetylation of histones in chromatin by p300. In paper II, we showed that MAML1 is important for increasing histone acetylation at the promoter of the Notch target gene Hes1, and also that it enhanced the global levels of acetylation of histones H3 and H4. The mechanism by which MAML1 increases histone H3 and H4 acetylation could involve the autoacetylation of p300, by increasing p300 HAT activity and its acetylation of the H3 and H4 tails. We identified amino acids 1–300 at the N-terminus of MAML1 and amino acids 701–1016 at the C-terminus as important for interaction with histone H3 in paper I. However, it remains to be investigated whether, and how, the interaction of MAML1 with histones plays a role in increasing histone acetylation and/or the formation of the MAML1/p300/histone complex.

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