Division of Molecular Toxicology

Institute of Environmental Medicine

Karolinska Institutet, Stockholm, Sweden

MOLECULAR MECHANISMS OF THE TRANSCRIPTIONAL CO-ACTIVATOR MASTERMIND-LIKE 1

Magnus Hansson

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ABSTRACT

Gene regulation is a complex process that requires several types of proteins, including chromatin-modifying enzymes, transcription factors, co-activators and co-repressors. We have investigated the molecular mechanisms underlying the action of the co-activator protein MAML1, which was first identified as a transcriptional co-activator for Notch receptors. Recently, MAML1 has been shown to function as a co-activator for other transcription factors, including β-catenin, p53 and MEF2C. We found that the co-activator function of MAML1 can be repressed by two different post-translational modification mechanisms; viz. phosphorylation by GSK3β and SUMOylation. The GSK3β kinase is reported to phosphorylate Notch1 and Notch2, and the GSK3β binding and phosphorylation sites have been mapped to the N-terminus of MAML1. We showed that GSK3 β inhibits MAML1-mediated transcription, and that the inhibition is dependent on active GSK3β. Moreover, immunofluorescence experiments showed that Notch1, MAML1 and GSK3β are co-localized in nuclear bodies.

We found that MAML1 can be SUMOylated at two conserved SUMOylation consensus motifs located in the N-terminus. The SUMO-deficient MAML1 mutant was a much more potent co-activator than the wild type. Moreover, SUMOylation of MAML1 resulted in an increased recruitment of the co-repressor HDAC7. Therefore, we suggest that SUMOylation of MAML1 is a mechanism for suppression of the transcriptional activity of MAML1.

Earlier, we reported that the histone acetyltransferase p300 acetylates MAML1. Here, we describe additional links between the general co-activator p300 and MAML1. First, we show that MAML1 enhances the auto-acetylation of p300 *in vitro* and in cultured cells, which caused increased acetylation of the p300 substrates histone H3/H4 and the transcription factor Egr1. Second, we found that MAML1 and Egr1 physically interact, and synergistically increase the expression of promoters regulated by Egr1, including the p300 promoter.

LIST OF PUBLICATIONS

This Thesis is based on the following publications:

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

IV. **Hansson ML**, Behmer S and Wallberg AE. (2010) MAML1 and Early growth response-1 act cooperatively to increase expression of promoters regulated by EGR1. *Manuscript.*

*These authors contributed equally to this work.

Additional publications not included in the thesis:

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A proline repeat domain in the Notch co-activator MAML1 is important for the p300-mediated acetylation of MAML1. *Biochem J*. 1;404(2):289-98.

Lewis AE, Rusten M, Hoivik EA, Vikse EL, **Hansson ML**, Wallberg AE, BakkeM. (2008) Phosphorylation of steroidogenic factor 1 is mediated by cyclin-dependent kinase 7. *Mol Endocrinol*. 22(1):91-104.

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LIST OF ABBREVIATIONS

ATF-2	Activating transcription factor-2
Brca1	Breast cancer 1
CDK	cyclin dependent kinase
CH3	Cystein-histidine rich region 3
ChIP	Chromatin immunoprecipitation
CtBP	C-terminal binding protein
Egr1	Early growth response 1
Elk-1	Ets like gene 1
FGF2	basic fibroblast growth factor
GNAT	GCN5-related-N-acetyltransferase
GSI	γ-secretase inhibitor
GSK3	Glycogen synthase kinase
GST	Glutathione transferase
HAT	Histone acetyltransferase
HDAC	Histone deacetyltransferase
HEK293	Human embryonic kidney cell line
HES	Hairy and enhancer of split
HP1	Heterochromatin protein 1
MAML	Mastermind-like
MAPK	Mitogen-activated protein kinase
NAD	Nicotineamide adenine dinucleotide
NF-κβ	Nuclear Factor-κβ
NLS	Nuclear localization signal
NuRD	Nucleosome remodeling and deacetylase
PCAF	p300/CBP associated factor
PDGF	Platelet derived growth factor
PIC	Pre initiation complex
PTEN	Phosphatase and tensin homolog
SID	SRC interaction domain
SIM	SUMO-interacting motif
siRNA	small-interfering RNA
SUMO	Small ubiquitin-like modifier
TGF	Transforming growth factor
Topors	Topoisomerase I-binding arginine/serine-rich
TPA	12-O-tetradecanoylphorbol-13-acetate
UBC9	SUMO-conjugating enzyme
U2OS	U2-Osteosarcoma cell line

1. INTRODUCTION

1.1 GENE REGULATION

All human cells contain the same set of 20,000~30,000 genes but the transcriptional activation and repression of these genes controls and defines the differentiated state of hundreds of cell types in humans. Gene expression can be changed by cell-cell interaction or by environmental stimuli through the induction of specific signal transduction pathways. The gene regulation process involves a wide range of proteins, including chromatin-modifying enzymes, transcription factors, co-regulators, histones and RNA polymerases.

Chromatin

DNA in the eukaryotic cell is tightly packed with histones and non-histone proteins into chromatin, which functions as a dynamic scaffold for the regulation of various nuclear processes, such as DNA transcription, repair and replication, as well as chromosome segregation and apoptosis (1). The basic component of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped 1.6 times around an octamer of histones H2A, H2B, H3 and H4 (2). Each of these histones consists of a structured globular domain that is in close contact with DNA, and an N-terminal tail domain. The nucleosomes are separated by a region of "linker" DNA. Histone H1 enables the nucleosome to be compressed into fibers and higher order structures.

Variation of the structure can make chromatin more or less accessible to transcription factors. There are two classes of chromatin-remodeling cofactors: ATP-dependent chromatin modeling complexes and histone-modifying enzymes. The ATP-dependent complex changes the chromatin by disrupting the nucleosomal structure and making the DNA more accessible in a non-covalent manner. The histone-modifying proteins that alter the structure of chromatin by post-translational modifications, mainly on the N-terminal histone tails, include acetylation, ubiquitylation, SUMOylation, methylation and phosphorylation. Different combinations of histone modifications can function as unique docking sites and will determine the type of effector protein that will be recruited; e.g. repressor or activator. For example, heterochromatin protein 1(HP1) binds through its chromodomain to histone H3K9me3, which leads to heterochromatin formation and gene silencing (3-5). The theory that multiple dynamic modifications control gene transcription in an organized and reproducible way is called the histone code (6, 7).

Transcription factors and cofactors

General transcription factors help to position RNA polymerase II correctly and to form a transcription pre-initiation complex (PIC) adjacent to the gene. These proteins are called general because they are necessary for almost all promoters used by RNA polymerase II. The assembly process starts when the transcription factor TFIID binds to the TATA-box (there are other DNA sequences with similar function). The binding of TFIID results in a large alteration in the DNA of the TATA-box, allowing other factors to assemble with RNA polymerase to form an established transcription initiation complex (8).

In addition to the general transcription factors, eukaryotic cells require regulatory transcription factors. These proteins bind to short specific DNA sequences, often located distal from the transcription start site. These sequence-specific transcription factors determine which sub-set of genes will be activated (8). A typical gene has several different regulatory transcription factors, which together adjust the strength of transcription. The eukaryotic regulatory transcription factors are divided into five superclasses, which in turn are divided into numerous classes and subclasses (9, 10). There are approximately 2000 DNA-binding transcription factors and more than 100 transcriptional co-activators and co-repressors that regulate the expression of about 20,000 protein-coding genes in the human genome (11). Among them, there are activators/repressors with a more general role, such as p300 and CtBP, and others with more specific functions (12).

1.2 POST-TRANSLATIONAL MODIFICATIONS

Post-translational modifications are prominent in the way that evolution has increased the range of function of a protein by attaching to it other biochemically functional groups. Proteins can be modified by small molecules (e.g. acetylation, nitrosylation, phosphorylation and methylation) and by larger molecules, including polypeptides or proteins that can be covalently attached to protein (e.g. ubiquitylation, SUMOylation and Neddylation) (13, 14). Post-translational modifications within a cell are, in most cases, enzyme-dependent. The reversible conjugation/deconjugation of these molecules is catalyzed by a set of enzymes that respond to changes of the cellular state and are, therefore, required for dynamic regulation in the cell. Here, we summarize four examples of post-translational modification, SUMOylation, ubiquitylation, acetylation and deacetylation, all of which target and modify lysine residues.

SUMOylation and ubiquitylation of proteins

SUMOylation is involved in many cellular functions, including protein transport, transcriptional regulation, stress response, DNA repair, protein stability, cell-cycle progression and apoptosis (15-17). The importance of SUMOylation in mice has been demonstrated by knockout of the SUMO conjugation enzyme UBC9, which is required

for SUMOylation of proteins. UBC9 knockout mice die at an early embryonic stage owing to defects in chromosomal segregation at mitosis and abnormal nuclear organization (18). Mammals have four SUMO proteins (SUMO1 - SUMO4), each with a mass of approximately 10 kDa. The homology of SUMO-2 and SUMO-3 is higher than that of SUMO-1. Little is known about the newly identified SUMO-4 and no SUMO-4 substrate has been identified. The conjugation of the SUMO protein to its target proteins is a three-step enzymatic process. The conjugation process involves E1, the SUMO-activating enzyme SAE1/SAE2, which binds to the SUMO protein and transfers it to E2, the conjugation enzyme UBC9. Finally, the E3 enzyme attaches SUMO to the target protein (17). A few E3-ligating enzymes have been identified in mammals. Interestingly, the topoisomerase interacting protein topors (topoisomerase I-binding argine/serine-rich) confers both E3 SUMO ligating activity and E3 ubiquitin ligating activity (19, 20). SUMO proteins can be removed from their targets by SUMO-specific proteases (SENPs), which make SUMOylation highly reversible. In addition to the canonical E3 SUMO-ligating enzymes, several noncanonical E3 SUMO proteins have been reported to potentiate the SUMOylation of different proteins. Among them, the tumor suppressor p14Arf and many of the class II HDACs are particularly interesting (21, 22). As mentioned above, SUMOylation plays multiple roles in the cell, depending on the function of the modified protein. Interestingly, histone-modifying enzymes, such as methyltransferases (Clr4), acetyltransferases (GCN5 and p300) and several HDACs, have been reported to be targets for SUMOylation (23-28). However, it is not clear whether SUMO modification affects the catalytic activity of these enzymes directly.

In transcriptional regulation, SUMOylation has been reported to be involved in both the activation and repression of transcription. Like many other post-translational modifications, SUMOylation can influence gene transcription by modifying histones, transcription factors or co-factors (29, 30). In most cases reported to date, SUMO-dependent regulation of transcription is achieved by either stimulating or disrupting protein-protein or protein-DNA interaction. Histone H4 SUMOylation has been shown to mediate gene silencing through recruitment of histone deacetylases and heterochromatin protein 1 (31).

The mechanistic function of SUMOylation of transcription factors has been demonstrated nicely by the Sharrocks laboratory in their study of the immediate early gene activator Elk-1. Under non-induced conditions, Elk-1 is SUMOylated and associated with HDAC2, leading to the inhibition of target genes. However, activation of the MAPK pathway and ERK-mediated phosphorylation of Elk-1 results in the removal of SUMO and HDAC2 from Elk-1, and the complete activation of target genes. When the pathway is induced by stress, phosphorylation of Elk-1 by p38 kinase does not result in the loss of SUMO or HDAC2, and target genes are only partially activated (26, 32-34). An example of SUMO-dependent gene activation is the SUMO modification of the transcription factor Ikaros, which can repress target genes by recruiting Sin3 and NuRD co-repressor complexes. However, SUMOylation of Ikaros results in displacement of co-repressor complexes and therefore displacement of the repressive function (35).

Ubiquitin, a well known tag for proteosomal proteolysis, contains several lysines that can be modified by sequential addition of new ubiquitin molecules to assemble a polyubiquitin chain. The progressive attachment of a specific lysine residue (K48) marks the polyubiquitylated (polyUb) protein for proteosomal degradation (36). However, polyUb on other lysines or monoUb mark the protein for other purposes, such as DNA repair, endocytosis or nuclear export (37). Ubiquitylation plays important roles in transcriptional regulation. Histone H2B ubiquitylation has been found to be enriched around transcriptionally active sequences, and has been shown to be important to the facilitation of transcriptional elongation (38-40). Histone H2B ubiquitylation of K120 also enables the trimethylation of histone H3K4/K79 and is a good example of trans-tail regulation (41).

Acetylation of proteins

Enzymes that can covalently attach acetyl groups to lysines are often called histone acetyltransferases (HATs) However, because of their ability to acetylate proteins other than histones, they are now called K-acetyltransferases or KATs (42). On the basis of sequence alignment of the AT-domains, mammalian KATs can be divided into four main multi-gene families: GNAT (e.g. PCAF and GCN5); MYST (TIP60, MOZ and MORF), nuclear receptor co-activator (e.g. SRC-1); and the CBP/p300 family (43). The gene families tend to be very conserved in their catalytic domains but these are strictly distinct between the different protein families. The acetyltransferases often function within different multiprotein complexes in which the accessory proteins control the catalytic activity and substrate specificity. For example, histone acetyltransferases GCN5 and PCAF are subunits in at least two distinct multiprotein complexes; ATAC (molecular mass 700 kDa) and STAGA (molecular mass 2 MDa) (44, 45).

The acetylation process is highly reversible and the removal of acetyl groups from lysines is catalyzed by histone deacetylases (HDACs). Similar to the K-acetyltransferases, histones are not the only substrates, as numerous proteins subject to deacetylation have been identified (46). The HDACs exist in high molecular mass complexes with other enzymes, like the NuRD complex (47, 48). So far, 18 mammalian HDACs have been discovered and they are divided into four classes: class I, HDAC1, -2, -3, -8; class II, HDAC4, -5, -6, -7, -9, -10; class III or the sir2 family (sirt 1 - 7); and class IV, HDAC11 (49, 50). Classes I and II, which share significant homology in the deacetylase domain but differ in the N-terminal sequence, are recruited to promoters by direct interaction with co-repressors to deacetylate histones and thereby inhibit transcription. HDAC11 shares some, but not sufficient, homology to classes I and II. Recently, classes I, II and IV knockout studies in mice revealed highly specific functions in embryonic development for individual HDACs (50). The highly conserved class III gene family, also referred to as sirtuins, consists of an NAD-dependent deacetylase domain (51).

1.3 THE p300 ACETYLTRANSFERASE

The closely related transcriptional co-activators p300 and CBP were originally identified as binding partners of the adenovirus protein E1A, and the cAMP-regulated enhancer protein, respectively. p300 and CBP are essential for normal embryonic development; mice completely lacking either p300 or CBP die at an early embryonic stage . Moreover, doubly heterozygous p300+/-/CBP+/- deletion mutants are also embryonic lethal in mice (52, 53). These results indicate that the expression levels of the two proteins are crucial for normal development. Furthermore, several phenotypic differences between p300+/- and CBP+/-knockout mice suggest that they have both overlapping and distinct functions; however, the mechanistic distinction between p300 and CBP is unclear (52). Interestingly, structural differences in the HAT domain have been reported (54). Furthermore, a recent genome-wide study of p300 and CBP analyzed by the ChIP-Seq chromatin immunoprecipitation assay showed an overlapping pattern in synchronized cells. After activation of the cells, however, some differences between the p300 and CBP binding patterns were observed. For example, CBP tends to be more recruited than p300 to genes encoding proteins with a repressor function (55).

More than 400 CBP/p300 binding partners have been identified and the proteins are thought to activate gene expression in four ways: (1) relaxing the chromatin structure by acetylation, through its intrinsic acetyltransferase domain, of histone tails; (2) recruitment of the transcription machinery to the promoter region; (3) function as an adaptor protein; and (4) acetylation of gene regulatory proteins (56).

The p300/CBP family has been reported to catalyze the acetylation of more than 70 proteins. In addition to the acetyltransferase activity of the HAT domain, this family has been shown to have propionyl and butyryltransferase activity (57, 58). The functional importance of those modifications remains to be investigated. Interestingly, the N-terminal domain exhibits E3/E4 ubiquitin ligase activity (59). The p300/CBP family can mediate both acetylation and ubiquitylation of the tumor suppressor p53 but this finding is rather unexpected because acetylation leads to stabilization and activation of p53, whereas ubiquitylation marks p53 for degradation. However, this was shown to be controlled by subcellular localization, where only the cytoplasmic localized p300/CBP possessed E3/E4 ligase activity (60).

Various diseases are linked to aberrant CBP/p300 activity. The Rubinstein-Taybi syndrome is a developmental disorder caused by heterozygous germ-line mutation in the CBP or p300 gene (61). Several types of cancers, including colorectal, breast, ovarian, gastric, lung and pancreatic carcinomas, display loss-of-function mutations in the CBP/p300 genes (62). Furthermore, chromosomal translocations of the CBP/p300 genes have been observed in acute myeloid leukemia (AML) and the MLL gene (63). CBP/p300 has been implicated in other diseases, including Huntington's disease, Alzheimer's disease, cardiac disease and fibrosis (64).

The structure of p300

p300 is a large protein (300 kDa) with several distinct domains with various functions (see figure 1). In particular, the KIX and CH3 domains have been shown to be important for interaction with several transcription factors, including MyoD, c-Myb, p53, p73, BCRA1, c-fos, CREB and c-jun (56, 65-67). Other important protein interaction domains are CH1, Bromo, and SID. Enzymatic studies and the determination of the high-resolution X-ray crystal structure of the HAT domain reveal a unique catalytic mechanism that differs from those of other HATs. The p300 protein first forms a stable complex with the acetyl-CoA cofactor and then the positively charged substrate lysine side chain from the protein substrate transiently binds to the negatively charged P1 pocket, and departs immediately after acetyl transfer. This is called the Theorell-Chance or hit-and-run mechanism and explains the broad range of substrates identified (68, 69).

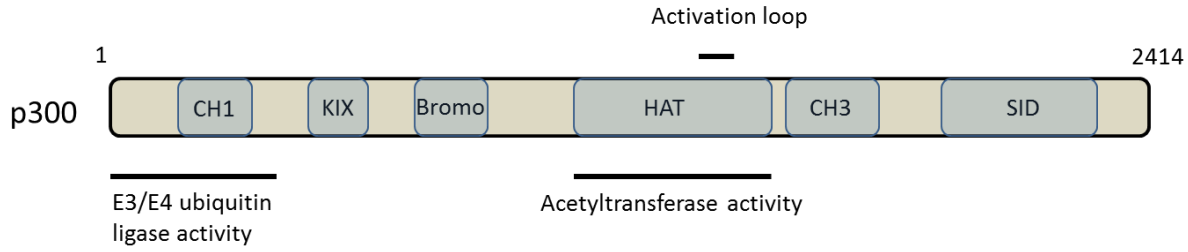

Figure 1. Schematic illustration of the p300 protein and the protein interaction domains.

p300 is regulated in several ways

While much research has focused on the regulation of p300 activity and the functional outcome, relatively little is known about the regulation of the p300 promoter or p300 protein stability. p300 protein levels have been shown to vary during the cell cycle; the levels are low in resting cells with low serum, but the expression of p300 is highly induced upon serum stimulation (70). Interestingly, the p300 and CBP promoters contain 6 and 7 Egr1 (Early growth response 1) binding sites, respectively. Egr1 belongs to the immediate early genes that are activated directly after mitogen stimuli. Serum induces the Egr1 protein, which consequently binds to the CBP/p300 promoters and transactivates the CBP/p300 genes (71, 72).

The level of p300 protein appears to be tightly controlled in various ways. It was shown recently that the BCL6 co-repressor can bind to the p300 promoter and thereby down-regulate the p300 levels in human diffuse large B cell lymphoma (73). Furthermore, p300 has been shown to be regulated by microRNAs. Expression of the CREB-activated miR-132 microRNA is strongly induced after infection by pathogenic viruses, including Kaposi´s sarcoma-associated herpesvirus (KSHV) in lymphatic endothelial cells, and herpes simplex virus-1 and human cytomegalovirus (HCMV) in monocytes. Interestingly, miR-132 regulated the innate antiviral immunity by down-regulating the expression of p300. Targeting the co-activator p300, instead of a signaling protein or transcription

factor, gives miR a wider role in controlling antiviral immunity. It will be interesting to determine whether CREB-mediated activation of miR-132 can regulate the level of p300 in other important biological processes (73, 74).

p300 is a phosphoprotein and can be phosphorylated by a wide range of kinases . The MAP kinase P38 has been shown to phosphorylate p300 in cardiomyocytes treated with doxorubicin, which leads to proteasome-dependent degradation of p300 (75).

p300 autoacetylation

The automodification of different chromatin regulatory proteins has led to great advances in our understanding of the control of gene expression. For example, the histone methyltransferase G9a creates a docking site for the chromodomain of HP1 by automethylation. Auto-ADP-ribosylation abrogates its interaction with chromatin, which releases it from active promoters (76, 77). The autoacetylation of p300 has been proposed to be an intermolecular event and p300 is acetylated all over the protein, including the HAT and CH3 domains (78, 79). Thompson et al. studied the autoacetylation of the HAT domain specifically, and identified many acetylated lysines situated in a proteolytically sensitive loop in the HAT domain. They demonstrated that the loop repressed the catalytic activity of the HAT domain, but a conformational change occurred upon autoacetylation of the p300 loop, which lost its inhibitory properties, resulting in a subsequent increase of HAT activity. The functional role of the loop was further tested in cell culture assays. The full-length loop deletion mutant had greater co-activator activity for the androgen receptor (AR) than wild type p300, and the p300-mediated acetylation of the tumor suppressor p73 was higher for the loop deletion mutant than the wild type p300 (80) . Autoacetylation of the HAT domain has been shown to be important for interaction with the transcription factor ATF2, which has a relatively weak binding affinity for the hypoacetylated p300 HAT domain, whereas when the HAT domain is hyperacetylated the protein interaction was markedly increased when the HAT domain was hyperacetylated. Moreover, ATF2 had a weak inhibitory effect on the HAT activity of hypoacetylated p300-HAT, while hyperacetylated p300-HAT was much more potently inhibited by ATF2 (81).

The autoacetylation of CBP/p300 appears to have two main functions: to regulate the interaction with many binding partners and to regulate its acetyltransferase activity. However, other functions, such as subcellular localization, cannot be ruled out. Several HDACs have been reported to interact with p300, which indicates that autoacetylation is highly reversible in the cell (25, 66, 79, 82). Indeed, treatment of a cell culture with different HDAC inhibitors strongly enhances autoacetylation of p300. Furthermore, p300 is a large and multifunctional protein with several well defined domains and it is likely that several different functional consequences depend on which domain(s) is acetylated. For example, acetylation of the activation loop in the HAT domain leads to increased HAT activity and increased acetylation of interacting substrates, including itself (80). While much has been learned about the biochemical function of HAT autoacetylation

in vitro, several questions remain to be answered. For example, how do the other domains in p300 affect the acetyltransferase activity of the HAT domain and the autoacetylation, and how do the many p300-interacting proteins in the cell affect p300 autoacetylation? The HAT protein PCAF interacts with the CH3 domain of p300 and, although it was shown that the p300-PCAF interaction led to PCAF acetylation, it is not clear whether PCAF can acetylate p300 (83). Likewise, a handful of non-catalytically active proteins have been reported to potentiate or inhibit p300 autoacetylation or HAT activity (84-88). For example, the nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) after apoptosis induced NO signaling resulted in acetylation of GAPDH. The acetylation of nuclear GAPDH was shown to increase p300 autoacetylation and subsequent activation of apoptotic downstream targets of p300, such as p53. Interestingly, p53 has been demonstrated to have a higher binding affinity to autoacetylated p300 (78). Thus, the increased autoacetylation of p300 increased the interaction with p53 and increased the acetylation of p53 (stability) and histones. In contrast to the importance of p300 autoacetylation in the p53-mediated apoptosis pathway, up-regulation of the p300 protein as well as enhanced autoacetylation has been described in oral squamous cell carcinoma (OCC). It was shown that aberrant NO signaling activated nucleophosmin1 (NPM1) and the GAPDH-dependent increase in autoacetylation and hyperacetylation of histones. The use of the HAT inhibitor hydrazinocurcumin (CTK7A) significantly reduced the growth of the xenografted oral tumor in mice (84).

A more general role of p300 autoacetylation in the coordination of the transcriptional pre-initiation complex (PIC) has been demonstrated using the model activator GAL-VP16, which directs the ordered assembly of Mediator, p300 and GTF TFII on immobilized chromatin. Mediator regulates this assembly process by binding to TFIID and p300. Autoacetylation of p300 induces a conformational change that leads to dissociation of p300, and increased TFIID binding and transcription (89).

Several HDACs are reported to interact with CBP/p300 with different molecular consequences. HDAC1 has been shown to interact directly with the CH3 domain of p300, and E1A competes with HDAC1 for binding the CH3 domain. Furthermore, HDAC1 represses the p300 co-activator function of MyoD and p53 (66). SUMOylation of p300 on two conserved lysines potentiated interaction with HDAC6, which repressed p300 transcriptional activity (25). However, it is not clear how p300 autoacetylation was affected by p300 SUMOylation and subsequent recruitment of HDAC6. Among the seven NAD+-dependent class III HDACs, SIRT2 was found to interact with and deacetylate p300. SIRT2 deacetylates lysines in the catalytic HAT domain and restores the binding of p300 to the PIC complex (79).

Autoacetylation of other acetyltransferases

Recently, a growing number of acetyltransferases have been found to be autoacetylated and some of the functional consequences of autoacetylation are summarized here. TIP60, which belongs to the MYST family, can acetylate core histones and a number of non-histone substrates, including p53 and Notch1 (90, 91). TIP60 autoacetylation has been shown to be induced in response to UV damage, which leads to dissociation of the oligomeric inactive TIP60 and facilitates interaction with and acetylation of its substrate, p53. SIRT1 can reverse TIP60 autoacetylation and thereby keep TIP60 in an inactive oligomeric state.

PCAF can be acetylated by p300 and by itself; the autoacetylated PCAF has greater histone acetyltransferase activity compared to the non-acetylated mutant. Furthermore, the acetylated lysines were mapped to the nuclear localization signal (NLS) sequence of PCAF, and it was demonstrated that a PCAF mutant lacking the HAT activity was localized mainly in the cytoplasm. PCAF deacetylation by HDAC3 resulted in cytoplasmic localization of PCAF (83, 92).

Arrest-defective 1 (hARD1) is an acetyltransferase closely linked to cellular growth and human cancers, including breast, prostate, lung and colorectal cancers (93). Autoacetylation of K136 of hARD1 was shown to be important for its enzymatic activity, and for the hARD1-dependent tumor cell growth *in vitro* and tumor xenograft growth *in vivo* (94). Mechanistically, it was shown that hARD1 autoacetylation led to the activation of β-catenin and AP-1 (activator protein-1) transcription factors, which synergistically up-regulate cyclin D1 expression.

1.4 THE NOTCH SIGNALING PATHWAY

The Notch proteins constitute an evolutionarily conserved signaling pathway that is vital for cell fate decision and proper development of the organism. In mammals, Notch signaling has been shown to control hematopoiesis, myogenesis, neurogenesis, vasculogenesis, skin development and other aspects of organogenesis (95-99). The Notch gene, first characterized in *Drosophila melanogaster*, encodes a single-pass heterodimeric transmembrane receptor. The Notch receptor interaction with its ligand (Jagged) present on neighboring cells leads to two proteolytic events. The first cleavage is mediated by ADAM-family metalloproteases called tumor necrosis factor alpha converting enzymes (TACEs). The second cleavage is catalyzed by the γ-secretase complex, which leads to release of the intracellular domain of Notch (ICN) and subsequent transport to the nucleus, where it cooperates with the DNA-binding protein CSL (named after CBF-1, Su(h) and LAG-1) and co-activators to activate target genes (figure 2.) (100, 101).

Transcriptional regulation of Notch target genes

The central part of the activation of Notch target genes is the formation of the DNA-bound complex of Notch IC, CSL and MAML. Genetic, biochemical and structural studies have identified specific regions of Notch IC, CSL and MAML to be important for physical and functional interaction with one another (102-105). The CSL is a highly conserved protein consisting of N-terminal and C-terminal Rel-homology domains and a central β-trefoil domain that binds to DNA in a sequence-specific manner. Under non-signaling conditions (absence of Notch IC), CSL represses expression of Notch target genes by interacting with co-repressors silencing mediator of retinoid and thyroid receptors (SMRT) (106), KyoT2 (107), CBF-1 interacting co-repressor (CIR) (108) and SMRT/HDAC1-associated repressor protein (SHARP) (109). When Notch targets CSL, the co-repressor complex is disrupted and the ICN-CSL-DNA complex forms an interface that is ideal for binding the Mastermind-like proteins, a co-activator family that allows recruitment of the acetyltransferase p300, RNA polymerase II and other unknown proteins. (110-114). This enables transcriptional activation of Notch targets genes including the HES-1 and Hes-5 transcription factors (figure 2).

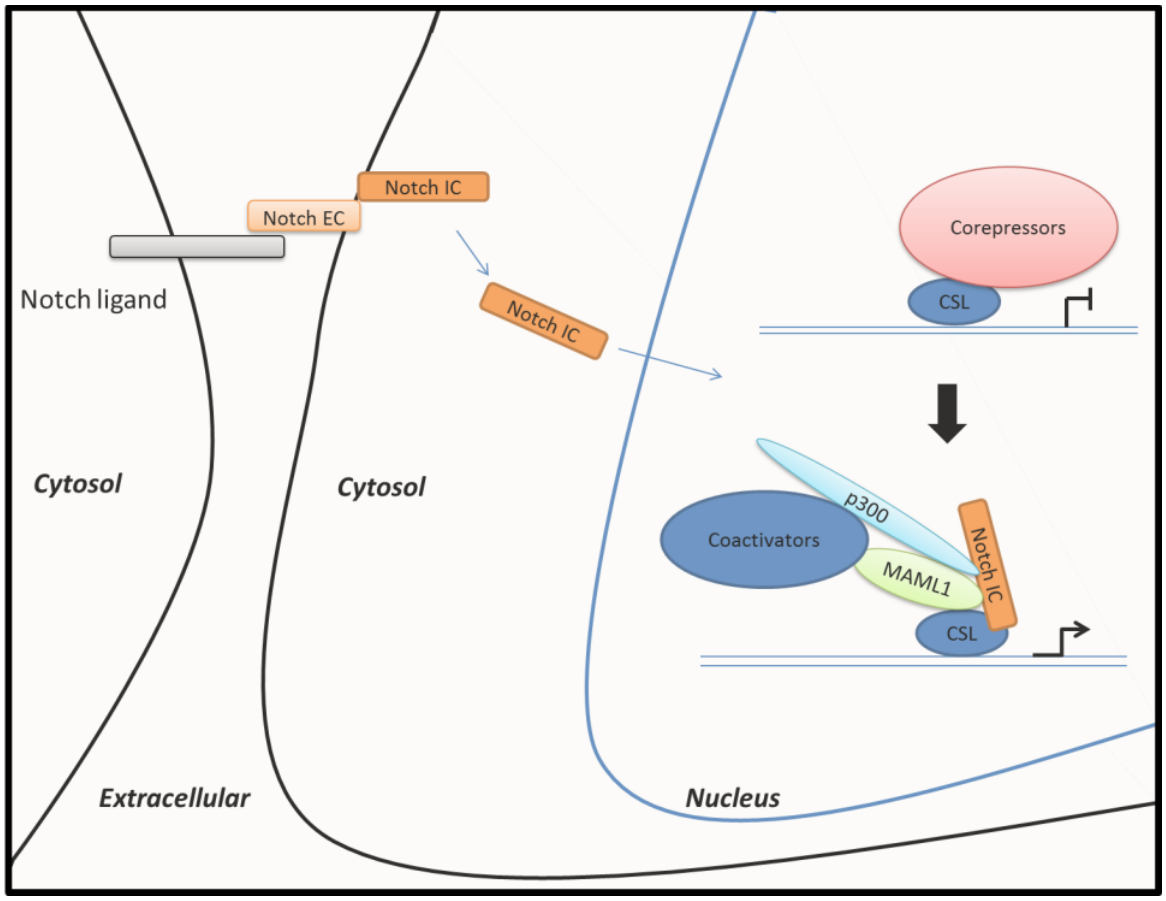

Figure 2. Schematic diagram of the Notch signaling pathway. Interaction between Notch receptor and Notch ligand in neighboring cells leads to release and translocation of Notch IC to the nucleus. Notch IC targets and disrupts the CSL repressor complex and recruits co-activators, including MAML1 and p300.

Development of pharmacologicals targeting the Notch signaling pathway

Aberrant notch signaling has been linked to many diseases, such as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), multiple sclerosis and several types of cancers (115, 116). Perhaps the most prominent and well studied case is the T cell acute lymphoblastic leukemia (T-ALL). Sequencing of the Notch1 gene in T-ALL cancer patients revealed that more than 50% of the patient had activating mutation in the extracellular hetero-dimerization domain and/or in the C-terminal PEST domain of Notch (117). This finding is of great interest for the development of pharmaceuticals that target Notch or components in the Notch signaling pathway. The γ -secretase inhibitors (GSIs), originally developed for treatment of Alzheimer's disease, inhibit Notch signaling in T-ALL lymphoblasts (117, 118). However, clinical development has been hindered by gastrointestinal toxicity and only weak effects in human T-ALL (119). Combinatoral therapies with GSIs and, for example, glucocorticoids are under investigation (120). Moellering et al. have described an alternative and more specific approach that directly targets and disrupts the Notch ternary complex. Using knowledge obtained from the high-resolution structure of the Notch ternary activation complex they synthesized α-helical hydrocarbon stapled (stabilized) peptides that mimic the structure of the extended kinked α-helical N-terminal part of MAML1 (residues 13-74). Several synthesized stapled peptides were tested for binding affinity to the CSL/Notch, a 16 amino acid peptide (corresponding to MAML1 residues 21-36) named SAHM1 was chosen for further evaluation. Treatment of leukemic cells with SAHM1 resulted in a genome-wide repression of Notch target genes and, consequently, suppression of the growth rate. Furthermore, treatment of a Notch1-dependent T-ALL mouse model with SAHM1 caused inhibition of Notch signaling and leukemic progression (121).

Post-translational modifications of Notch IC

Glycogen synthase kinase 3 (GSK3) was first identified as a kinase that phosphorylates and inactivates glycogen synthase. However, as well as its role in glycogen metabolism, it is now evident that GSK3 is a key player in the regulation of different signaling pathways by phosphorylating numerous proteins, including several transcription factors (122). In mammals, GSK3 consists of the highly homologous proteins GSK3α and GSK3β. The centrally located kinase domain has the highest degree of homology (>98%) and the C-terminal and N-terminal domains are less well conserved (122). However, despite their high degree of sequence similarity, they appear to have distinct functions.

GSK3 has a substrate preference for pre-phosphorylated serine four amino acids downstream from the S/T (S/TXXXpS) (123). The GSK3α/β family is unusual, in the sense that it is most often constitutively active in resting cells, whereas it is inactivated via phosphorylation by different kinases on Ser21/Ser9 in GSK3α/β. The phosphorylated residue will function as a primed pseudo-substrate and bind intramolecularly to the catalytic pocket, thereby preventing other substrates from binding and phosphorylation. Reactivation of GSK3 can be achieved by dephosphorylation by protein phosphatase 1

(124). The GSK3, like many other kinases, contains an activation loop that can be activated when phosphorylated at Y216 and Y279 in GSK3ß and GSK3α, respectively. Y216/Y279 phosphorylation is suggested to play a role in forcing open the substrate-binding site but there appears to be no constraint preventing the open conformation in the unphosphorylated state (125). Therefore, tyrosine phosphorylation of the GSK3 loop might facilitate substrate phosphorylation but it is not strictly required for kinase activity (125). It is not fully established which kinases are responsible for the phosphorylation of Y216/279 but the proline-rich tyrosine kinase 2 (PYK2) and autophosphorylation have been proposed to be involved (126, 127).

Shaggy, the GSK3 homolog of Drosophila, interacts genetically with the Drosophila protein Notch (128). In humans, GSK3β binds and phosphorylates Notch2, which leads to repression of the transcription of the Notch target gene Hes-1 (129). Notch stability has been suggested to be regulated by GSK3α/β. However, the functional consequence of GSK3 phosphorylation of Notch1 is divergent; it has been reported to induce degradation as well as stabilization of Notch (130, 131). These conflicting results could be due to different properties of the cells that were used. Given that GSK3 substrates often need to be primed (prephosphorylated), it is possible that several kinases might be responsible for the priming, which could direct the GSK3-mediated phosphorylation to different residues in the Notch sequence with diverse functional outcomes.

Cyclin-dependent kinase 8 (Cdk8) is a highly conserved nuclear kinase that requires cyclin C (CycC) to restore its kinase activity. Cdk8 regulates transcription both negatively and positively by interacting with general and/or specific transcription factors/co-regulators. In mammals, for example, Cdk8 has been shown to phosphorylate the TFIIH subunit Cyclin H and thereby repress the Cdk7-dependent phosphorylation of pol II CTD and the ability of TFIIH to activate transcription (132). Cdk8:CycC can associate with the mediator complex, and thereby provide the catalytically inactive mediator complex with enzymatic properties. MAML1 interacts directly with cdk8, and facilitates phosphorylation of Notch TAD and PEST domains. The phosphorylation of serines in the PEST domain leads to FBW7/SEL10-mediated polyubiquitylation and subsequent proteosomal degradation (133).

The nuclear serine/threonine Nemo-like kinase (NLK) was recently shown to phosphorylate Notch and thereby negatively regulate Notch-dependent transcription by disrupting the formation of the ternary complex. In addition, NLK was found to be able to interact with and to phosphorylate MAML1. However, the functional relevance of this finding was not investigated further (134). Not surprisingly, in addition to phosphorylation, acetylation appears to be an important modification in the regulation of Notch IC activity. It has been shown that UV-induced TIP60 can target and acetylate Notch, which leads to dissociation of the CSL-Notch transcription complex and repression of transcription of the Notch target genes (90) . In addition to the acetylation of Notch, the MAML1 protein is acetylated by p300. The functional relevance of this modification is not clear, although preliminary data suggest a decrease in MAML1 interaction with p300 (135).

1.5 THE MASTERMIND-LIKE PROTEINS

The Mastermind protein was originally identified in *D. melanogaster* as a neurogenic protein genetically linked to the Notch signaling pathway. Three Mastermind-like proteins (MAML1-3) have been identified in humans (136, 137) . All MAML proteins have been shown to physically interact and form a complex with each Notch receptor (NICD 1-4) and CSL, and to potentiate Notch-activated transcription. However, some differences in optimal binding and gene activation were found between the four Notch and three MAML1 subtypes. For example, MAML3 was shown to bind more weakly to the ankyrin repeat domain of Notch1 and to function more efficiently with Notch4 in the Hes1 gene reporter assay (136). Nevertheless, it needs to be emphasized that these effects might be cell type-specific and might not be relevant under physiological conditions.

MAML1 is widely expressed with the highest levels in heart, spleen, pancreas and leukocytes in peripheral blood (138). The MAML proteins consist of a highly conserved N-terminal basic domain and two acidic domains in the middle and the C-terminal region. The N-terminus (residues 1-75) is important for the interaction with the CSL-Notch IC-DNA complex (138, 139). The MAML proteins consist of two transcriptional activation domains, TAD1 and TAD2. TAD1 amino acids 75 and 300 are important for p300 binding; whereas TAD2 is located between amino acids 303 and 1016, which contain glutamine-rich regions and TAD2 is required for transcription in vivo (111, 112).

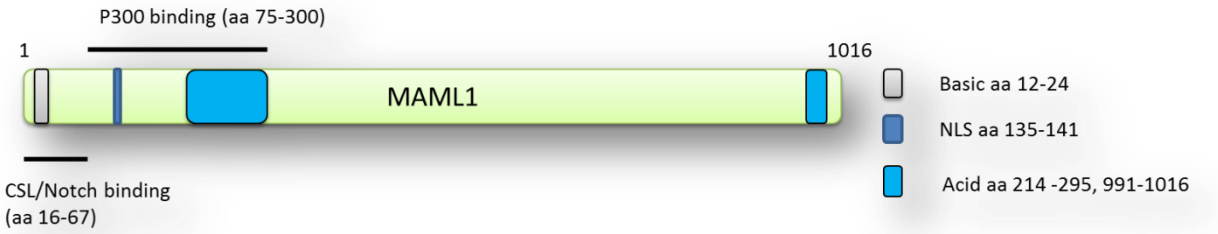

Figure 3. MAML1 structural organization. The MAML1 protein contains one basic domain, two acid domains, and a NLS motif. The amino acids (aa) 16-67 are important for the Notch/CSL interaction, aa 75-300 are known to interact with p300, and aa 300-1016 are important for the MAML1 transcriptional activity with unknown mechanism.

Notch-independent functions of MAML1

The human genome encodes about 2000 transcriptions factors, but only approximately 100 transcriptional cofactors, so it is likely that even the more specific cofactors can bind and activate or repress several transcription factors. Recently, several Notch-independent functions have been reported for MAML1, which are described below. The fact that MAML1 knockout mice obtained a substantially different phenotype compared to the

phenotypes obtained from the Notch (1-4) knockouts gave clues about the possibility of Notch-independent functions of MAML1.

Mice with the MAML1 gene deleted remain small and die within 10 days after birth. Furthermore, the MAML1 knockout mice displayed muscular dystrophy defects and increased liver cell death, and were unable to generate mature B cells (marginal zone B cells) (140, 141). The severe muscular dystrophy of mice lacking the MAML1 gene is particularly interesting, because Notch knockout mice show the opposite phenotype (141). This was clarified by the finding that MAML1 can function as a co-activator for MEF2C (MADS box family), a transcription factor that is essential for skeletal muscle development, and Notch activation completely abrogates the myogenic effect of MAML1. The antagonistic effect of Notch was explained biochemically by showing that Notch binding to MAML1 disrupts the MEF2C-MAML1 interaction (141).

The liver phenotype of MAML1 knockouts showed similarities to several knockouts within the NF-κβ signaling pathway (142-144) . Therefore, the potential role of MAML1 in the NF-κβ pathway was explored. It was found that MAML1 can interact with the inhibitor of NF-κβ (Iκβα), and potentiate Iκβα phosphorylation and subsequent degradation of Iκβα, which leads to enhanced activity of nuclear NF-κβ. Furthermore, MAML1 was shown to interact physically with the NF-κβ subunit RelA (p65), suggesting a transcriptional co-activator function of MAML1 for NF-κβ as well (145).

MAML1 was recently shown to function as a co-activator of the β-catenin/TCF transcription factor complex by up-regulating cyclin D1 and c-Myc in colon carcinoma cells (146). Further, MAML1 was able to interact physically with β-catenin cultured cells and *in vitro,* and treatment of colonic carcinoma cells SW480 with siRNA MAML1 (and MAML3) leads to increased cell death. The MAML1 co-activator function of β-catenin-mediated transcription was independent of Notch, because the γ -secretase inhibitor (GSI) that blocks the proteolytic activation of Notch IC did not inhibit the recruitment of MAML1 to β-catenin target gene promoters. Additional evidence of a Notch-independent functional interaction between MAML1 and β-catenin was found in *D. melanogaster* by a genetic screen that identified armadillo (Drosophila β-catenin homolog) as a MAM interactor in both wing and eye development (147). As well as the cell survival function, MAML1 has been reported to play an important role in apoptosis by functioning as a co-activator for the tumor suppressor p53 (148).

Genetic studies of the MAML1 homolog XMAM1 in *Xenopus laevis* found additional evidence of Notch-independent functions. Over-expression of XMAM1 in the Xenopus embryo initiated formation of a pigmented cell mass on the surface and enhanced expression of the RNA-binding protein neuropilin-1 (Nrp-1). This effect was suggested to be Notch-independent, because over-expression of the XMam1 mutant that lacks the Notch-interacting part retained the same phenotype (149).

Mastermind is located in the Drosophila polyetene chromosome and the ecdysone-induced puffs, a region where the co-repressor groucho and the RNA polymerase can be

found. Interestingly, the binding of Mastermind to the polyetene chromosome is lost in mutants of the *Drosophila* TRRAP/Tra1 mutants, a critical component of several HAT complexes (TIP60) (150).

A genetic screen of Drosophila identified 79 Mastermind-specific interactors (MSI) from different functional classes, such as negative regulators, RNA polymerase II-related proteins, factors that negatively regulate metabolism and small GTPase regulator proteins. This suggests a broad role for Mastermind in different processes in Drosophila development. However, it cannot be excluded that some MSI genes interact with components of the Notch pathway that were not included in the screen (147).

Drosophila follicle stem cell (FSC) maintenance in the ovary requires hedgehog (hh) signaling (151-153). A genetic screen identified Mastermind as a modifier of the hh-induced FSC expansion. Loss of Mastermind reduced expression of the hh pathway reporter in FCS but not in wing discs, suggesting a more specific role for Mastermind in hh signaling (154). Furthermore, Notch and other components from the Notch signaling pathway were not required for FCS maintenance, indicating another Notch-independent function for Mastermind (154). Although the molecular mechanism underlying hh-mam signaling is not completely understood, it would be interesting to study the possibility of Mastermind functioning as a co-activator for the hh signaling transcription factor Gli.

Protein	Protein class/function	Binding assay	Proposed function	References
Notch (1-4)	TF	Co-IP, GST-PD	Notch signaling	(138)
CSL	TF	Co-IP, GST-PD	Notch signaling	(138)
P300	Co-activator	Co-IP, GST-PD	Gene activation	(111, 112)
CDK8	Kinase	Co-IP, GST-PD	Notch degradation	(133)
P53	TF	Co-IP, GST-PD	Gene activation Apoptosis	(148)
β-Catenin	TF	Co-IP, GST-PD	Gene activation Proliferation	(146)
GSK3β	Kinase	Co-IP, GST-PD	Repression of MAML1 activity	(146, 155)
HDAC7	Co-repressor	Co-IP, GST-PD	Repression of MAML1 activity	(156)
MEF2C	TF	Mammalian 2-hybrid	Gene activation Myogenesis	(141)
NFκβ (p65)	TF	Co-IP	Gene activation Liver cell survival	(145)
16E6	Oncoprotein	Yeast 2-hybrid	Unknown	(138)
Iκβα	Inhibitor	GST-PD	Iκβα degradation Liver cell survival	(145)
Egr1	TF	Co-IP	Gene-activation Proliferation/apoptosis?	unpublished

Table1. Summary of MAML1 interacting proteins and the proposed function. Abbreviations; transcription factor (TF), Coimmunoprecipitation (Co-IP), GST-pulldown (GST-PD).

1.6 EARLY GROWTH RESPONSE-1

Early growth response-1 (Egr1), also known as krox24, Zif268 and NGFI-A, belongs to the C2H2-type zinc-finger transcription factor family. It is induced by various kinds of extracellular stimulus, such as growth factors, cytokines, and different types of stress, such as radiation, injury or mechanical stress, which leads to activation of different kinase signaling pathways (157). The most commonly used is the MAP kinase pathway. Egr1 consists of a highly conserved DNA-binding domain, an activation domain, a repression domain and a C-terminal P/T/S-rich region. The centrally located DNA-binding domain consists of three zinc fingers that bind to the G+C-rich consensus sequence GCG(G/T)GGGCG. The co-repressors Nab1 and Nab2 (NGF-1A-binding protein) can interact with the repression domain and inhibit its transcriptional activity. The activation domain, located in the N-terminus of Egr1, can interact with the co-activator p300 (158). The Egr1 promoter contains several binding sites for different transcription factors; for example, the promoter contains several serum response elements (SRE), cAMP regulatory elements, the SP1 consensus sequence and an Egr1-binding site (EBS). The levels of the Egr1 protein are normally strictly controlled by several transcriptional negative and positive feedback loops. For example, Egr1 can bind to and activate the promoters of its own co-repressors Nab1 and Nab2, creating a negative feedback loop. In addition, Egr1 regulates the expression of the co-activator paralogs p300 and CBP. The Egr1-mediated up-regulation of p300 and CBP leads to enhanced acetylation of Egr1. Interestingly, acetylated Egr1 appears to have a negative effect on its own promoter and on p300/CBP promoters, whereas it has a positive effect on genes important for cell growth, such as FGF2, PDGF, TGFβ1 and IGF-II (72). Moreover, p300-mediated acetylation of Egr1 appears to increase Egr1 stability.

In contrast to the p300-mediated acetylation of Egr1, stress-induced phosphorylation appears to support cell death by activating tumor suppressor genes. Furthermore, Akt-mediated phosphorylation of Egr1 facilitates SUMOylation, which leads to translocation of Egr1 to the nucleus and activation of specific target genes such as the tumor suppressor PTEN (159). Like many other transcription factors, Egr1 can be ubiquitylated, which leads to rapid degradation by the proteasome machinery (160). However, the E3 ligase responsible for the ubiquitinylation has not been identified. Thus, different post-translational modifications play important roles in the regulation of Egr1 stability, activity and target specificity

Evidence for the function of Egr1 in cancer cells is contradictory. Egr1 has been shown to be an important factor in apoptosis by inducing several tumor suppressors, including p53, p73 and PTEN, and to function as an oncogene in some types of cancers (161). The best known example where Egr1 possesses such an effect is in prostate cancer, where Egr1 is constitutively up-regulated. Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer mortality in men. Several lines of evidence support the role of over-expressed Egr1 in promoting prostate cancer growth. For example, knockdown experiments of Egr1 in prostate cancer cells reduced the cell growth rate *in*

vitro (162, 163), and injection of antisense Egr1 into transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, a transgenic model of prostate cancer, decreases the tumor growth rate (164). Moreover, it has been shown that breeding a transgenic mouse model with prostate cancer and Egr1-knockout delays tumor progression in the offspring (165). At the molecular level, two important questions need to be answered: what is the molecular explanation for the elevated levels of Egr1 and why does over-expression of Egr1 specifically lead to activation of growth-related genes? The oncogenic effect of Egr1 can be explained, in part, by the fact that PTEN and/or p53 are in some way inactivated in most prostate cancers. These defects in the tumor suppressor system are suggested to lead to an uncontrolled activation of TGFβ1 and fibronectin, and subsequent activation of the AKT pathway (161). It is likely also that Egr1 is differentially modified in prostate cancer, which changes the target gene specificity. It has been demonstrated that the prostate cancer cell line PC3 expresses high levels of Egr1, which is highly phosphorylated by casein kinase II (CKII), and it was suggested that this modification would decrease the DNA-binding capacity of Egr1 (166) . However, further studies are needed to confirm the role of CKII in the progression of prostate cancer. Several attempts have been made to understand the mechanism underlying the elevated levels of Egr1 in prostate cancer. The Egr1 target gene and co-repressor Nab2 are down-regulated in primary prostate carcinomas; therefore, the up-regulation of Egr1 and the loss of its co-repressor Nab2 might lead to uncontrolled activation of Egr1 target genes (165, 167). Furthermore, p300 has been reported to be elevated in prostate cancer and, given that Egr1 can be stabilized through p300-mediated acetylation, it has been suggested that this could be the basis for the increased levels of Egr1 (72, 168). In addition, it has been reported that mutated p53 is responsible for the high levels of Egr1, by positive stimulation of the MAPK pathway and subsequent activation of the Egr1 promoter (169). It is clear that further studies are needed to be able to determine the molecular basis of the elevated levels of Egr1 and its oncogenic effect in prostate cancer.

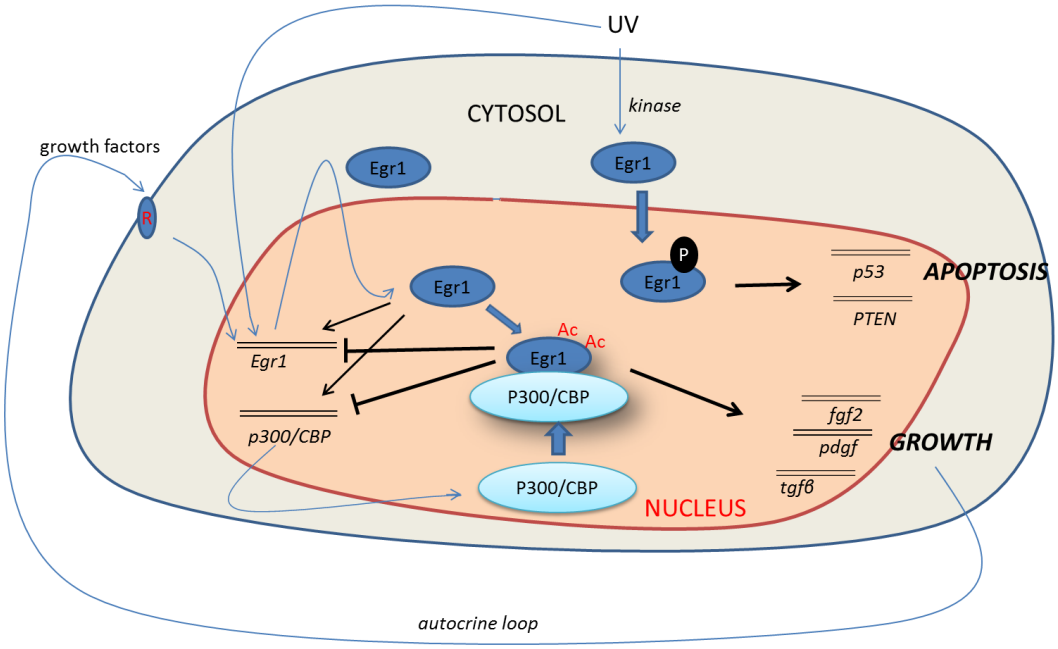

Figure 4. Proposed model of the feedback loops that regulate Egr1 protein levels and how different stimuli result in specific modifications of Egr1, which dictates the activation of specific downstream targets and determine the cell fate [modified from (168)]. See text for detailed description.

2. AIMS OF THE THESIS

- To study the regulatory role of post-translational modifications of MAML1 and to identify the enzymes involved in these modifications.

- To investigate the molecular interplay between p300 and MAML1.

- To elucidate MAML1 functional interaction with the Egr1 transcription factor.

3. RESULTS AND DISCUSSION

3.1 Positive regulation of p300 acetyltransferase activity by MAML1 (Paper I)

In an earlier study, we showed that MAML1 is heavily acetylated by p300, and that a proline repeat motif (PAPAAPAP) located between amino acid residues 83 and 90 in MAML1 is important for the interaction, and subsequent acetylation by p300. Moreover, we showed that MAML1 binds directly to the CH3 domain, close to the HAT domain, in the p300 protein (135). While studying the p300-mediated acetylation of MAML1 we observed, both *in vitro* with purified p300 and MAML1 proteins as well as *in vivo* in cell culture acetylation assays with over-expressed proteins, that p300 itself was more heavily acetylated in the presence of MAML1. We found these observations particularly interesting because the autoacetylation of p300 has been linked to its acetyltransferase activity on histone tails and other substrates (80). First, we showed that MAML1 could not induce acetylation of p300 lacking the HAT domain (p300ΔHAT), which suggests that MAML1 does not enhance p300 acetylation by recruiting other HATs to the p300 protein, or that MAML1 itself has acetyltransferase enzymatic activity.

Autoacetylation of p300 occurs at lysine residues over most of the protein. We used an antibody that specifically recognizes acetylated K1499 in p300. Lysine 1499 is located in the HAT domain in close proximity to the activation loop (amino acid residues 1520-1580), and autoacetylation of K1499 is linked directly to the HAT activity (80). We found that MAML1 enhanced the autoacetylation of lysine 1499 as much as it increased the overall autoacetylation of p300, indicating that MAML1 might potentiate HAT activity of p300 as well. Furthermore, the results of an *in vitro* acetylation assay with purified proteins showed that MAML1 strongly induced the autoacetylation of lysine 1499 in a truncated p300 protein containing only the HAT and the CH3 domains (residues 1195-1810).

Autoacetylation of p300 has been shown to regulate HAT activity, so we used an *in vitro* HAT assay to determine whether MAML1 could affect the p300-mediated acetylation of histones. We found that MAML1 significantly enhanced the p300 acetylation of histone H4 tails. Next, we investigated the connection between MAML1, p300 and acetylated histones in the cell. Immunofluorescence experiments showed that MAML, p300 and acetylated histone H3/H4 were colocalized in MAML1-dependent nuclear bodies. Furthermore, a ChIP assay showed that the acetylation of histone H3 on the Notch target Hes-1 promoter was increased significantly in the presence of full-length MAML1. We used western blot to analyze the global acetylation levels of histones H3 and H4 in a FlagMAML1-HEK293 stable cell line and the control HEK293 cell line, and found that histones H3 and H4 were significantly more acetylated in the MAML1 stable cell line. Conversely, knockdown of endogenous MAML1 in U2OS cells resulted in a decrease of acetylated histones H3 and H4, relative to the U2OS cells treated with control siRNA.

We wanted to determine which region of MAML1 is important for the increased autoacetylation of p300. From our *in vitro* acetylation assay with purified p300 and

MAML1 proteins, we found that only the full-length MAML1 was able to enhance p300 autoacetylation; none of the MAML1 mutants (MAML1 1-300, 309-625, 499-804 and 701-1016) was able to increase autoacetylation of p300. In a cell culture assay, only the full-length MAML1 showed fully enhanced autoacetylation, while MAML1 1-625 showed a moderate increase and MAML1 1-300 had no significant effect on p300 autoacetylation (see figure 5). However, in the presence of the HDAC inhibitor sodium butyrate (NaBu), both of the C-terminal truncated constructs (MAML1 1-300 and MAML1 1-625) showed increased acetylation of p300 similar to that of the full-length MAML1. In this study, we probed for p300-mediated acetylation of full-length MAML1, MAML1 1-625 and MAML1 1-300 with and without treatment with the HDAC inhibitor and we found that acetylation of the MAML1 proteins followed the level of autoacetylated p300 (data not shown). These results showed that full-length MAML1 is not susceptible to deacetylation, whereas MAML1 1-300 is HDAC-sensitive. This might indicate that acetylation of specific lysines in the N-terminal part regulates the autoacetylation of p300, which was reported to be the case for GAPDH, where it was shown that acetylation of a single lysine residue was essential for the GAPDH-induced p300 autoacetylation (86).

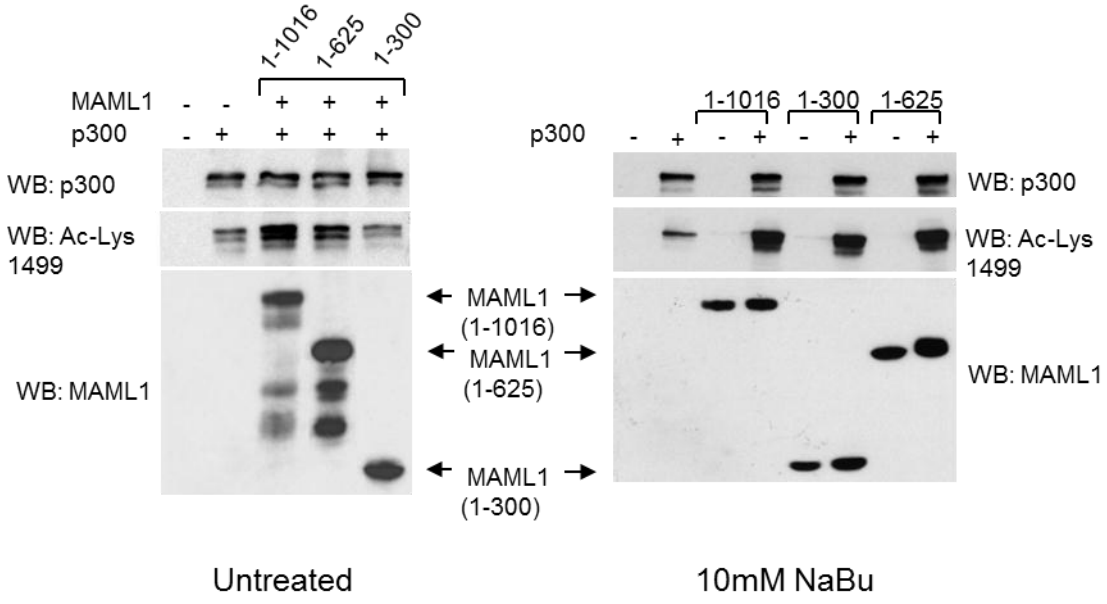

Figure 5. Cell culture acetylation assay. Plasmids expressing p300 and MAML1 1-1016, 1-625 or 1-300 were cotransfected into HEK293 cells with or without treatment of HDAC inhibitor (NaBu). Whole-cell extracts were prepared 48h post-transfection and proteins separated by SDS-PAGE and monitored by western blotting with antibodies recognizing p300, MAML1 or acetylated Lys1499 in p300.

p300 has been reported to contain an autoinhibitory activation loop that regulates p300 autoacetylation and HAT activity. However, we found that MAML1 enhanced the autoacetylation of wild type p300 and the p300Δloop approximately equally well,

indicating that MAML1 does not act directly on the autoinhibitory activation loop in order to enhance p300 autoacetylation. Furthermore, the p300Δloop was shown to be a much more potent co-activator for GAL-MAML1 than the wild type p300.

Earlier, we reported that MAML1 interacts with the CH3 domain of p300, which is located in close proximity to the HAT domain. To determine whether the CH3 domain might be important for the MAML1-dependent increase of p300 autoacetylation, we tested the influence of MAML1 on the autoacetylation of the p300 mutant with the CH3 domain deleted (p300ΔCH3). Interestingly, first we found that p300ΔCH3 was much more autoacetylated than the wild type p300 without MAML1 transfected. This was seen both with and without addition of the HDAC inhibitor. We could not, however, see any further increase in autoacetylation of p300ΔCH3 when co-expressed with MAML1. We know from GST-pulldown experiments that MAML1 can interact directly with the CH3 domain, so we wanted to know if the co-localization between MAML1 and p300ΔCH3 was abolished. Interestingly, wild type p300 and p300ΔCH3 showed different distribution patterns when expressed alone. Both wild type p300 and p300ΔCH3 were localized in the nucleus but wild-type p300 was mainly evenly distributed in the nucleus, whereas p300ΔCH3 was located in small distinct dots. Interestingly, when MAML1 was co-expressed with p300ΔCH3 (or wild type p300), p300ΔCH3 was translocated to colocalize with MAML1, which indicates that there might still be a functional interaction between MAML1 and p300ΔCH3 in living cells.

In this study, we focused on the role of MAML1 enhancing the autoacetylation of p300. The clear difference between wild type p300 and p300ΔCH3 in autoacetylation and localization in cells is quite exciting and deserves further investigation. The higher level of autoacetylation of p300ΔCH3 compared to wild type p300 is in agreement with published results from in vitro acetylation experiments done with purified full-length p300 and deletion mutants where p300ΔCH3 was found to possess much higher levels of autoacetylation as well as higher HAT activity (170). An interesting question is whether the reason for the difference in autoacetylation and HAT activity between wild type p300 and p300ΔCH3 is purely due to the ability of the CH3 domain to recruit HDACs or some kind of interplay occurs between the neighboring HAT and CH3 domains. The crystal structure for the HAT domain has been reported recently and the structures of the two subdomains of the CH3 domain, i.e. TAZ and ZZ, have been revealed by NMR spectroscopy (69, 171, 172). The structural determination of HAT-CH3 as one molecule should tell if there is any interplay between the two neighboring domains.

So, what is the mechanism behind the MAML1-dependent increase of p300 autoacetylation? Several mechanisms have been shown to control the autoacetylation of p300, including deacetylation by different HDACs, intramolecular control of the catalytic cavity by the autoinhibitory flexible loop and by protein-protein interaction with different non-enzymatic proteins, such as MAML1. One explanation for the MAML1-dependent increase of p300 autoacetylation could be that MAML1 competes with co-repressors, such as HDACs, for interaction with the CH3 domain. However, the fact the MAML1 enhances p300 autoacetylation more strongly when the cells are treated with HDAC

inhibitors (compared to non-treated cells), together with the ability of purified MAML1 to enhance autoacetylation (Lys1499) of recombinant p300 (FlagHAT-CH3, amino acids 1195-1810) affinity-purified from Escherichia coli, suggests that this might not be the case. It is more likely that MAML1 increases the autoacetylation of p300 by inducing a conformational change in the HAT and/or CH3 domain, which might reveal the catalytic region for substrate binding. Further studies are clearly required to understand how MAML1 enhances the autoacetylation of p300.

3.2 MAML1 is negatively regulated by glycogen synthase kinase 3β (Paper II)

Glycogen synthase kinase 3 (GSK3) has been shown to be important in gene regulation. Several transcription factors can be phosphorylated by GSK3 and Notch has been reported to be both positively and negatively regulated by GSK3. These opposite functions might be due to cell-type specificity. In this study, we investigated the influence of GSK3β on the transcriptional co-activator MAML1. In a gene reporter assay with transfected GAL-MAML1, two specific GSK3 inhibitors, SB21 and SB41, increased the transcriptional activity of GAL-MAML1 by >3-fold. Conversely, cotransfection of GAL-MAML1 and GSK3β had approximately 4-fold lower activity compared to when GAL-MAML1 alone was transfected. The results found with the inhibitors suggest that the GSK3β inhibitory effect on MAML1 is kinase-dependent. To confirm this, we performed additional gene reporter assays with active or inactive GSK3β mutants. The constitutively active mutant repressed GAL-MAML1 activity, whereas the kinase-inactive mutant showed activity similar to that of GAL-MAML1 alone.

Several phosphoproteomic studies have identified phosphorylated residues in the MAML1 protein (173). Recently, Nemo-like kinase was shown to interact with and phosphorylate MAML1. (134). We used an *in vitro* phosphorylation assay and found that recombinant GSK3β phosphorylates full-length MAML1 and the N-terminal region of MAML1. We asked whether MAML1 could be phosphorylated by GSK3β in HEK293 and U2OS cells. MAML1 was transfected with or without active GSK3β (S9A) and was left untreated or treated with the GSK3 inhibitor. Highly phosphorylated MAML1 was detected in all samples. However, we were not able to detect any increase in phosphorylated MAML1 in the sample where MAML1 was cotransfected with GSK3β, nor could we see any decrease in phosphorylated MAML1 when the cells were treated with the GSK3-specific inhibitor SB41 (data not shown). These results show clearly that MAML1 is phosphorylated in cell cultures but, owing to the heavily phosphorylated MAML1 we could not detect a GSK3β -specific phosphorylation of MAML1 in cultured cells.

Next, we asked whether MAML1 and GSK3β can physically interact. A GST-pulldown assay with purified proteins as well as co-immunoprecipitation experiments showed that GSK3β could interact with MAML1. The interaction was further mapped to the N-terminal region of MAML1. GSK3β is located mainly in the cytoplasm of cells, whereas

MAML1 localizes in nuclear bodies (at least when it is over-expressed). However, nuclear translocation of GSK3 when binding to transcription factors has been reported. Therefore, we investigated whether MAML1 could affect GSK3β subcellular localization. Indeed, immunofluorescence experiments revealed that GSK3β colocalized with MAML1.

GSK3β has been reported to regulate the activity of Notch proteins, so we sought to investigate how GSK3β affects MAML1-mediated Notch transcription. A gene reporter assay showed that MAML1 strongly enhanced GAL-Notch transcriptional activity, and that GSK3β inhibited GAL-Notch and GAL-Notch cotransfected with MAML1 equally well (5-fold). Moreover, the GSK3 inhibitor SB41 increased the Notch-dependent expression of Hes1 in C33A cells but, when MAML1 was silenced by siRNA, the expression of Hes1 was not significantly affected by treatment with SB41, suggesting that the SB41-dependent enhancement of Hes-1 expression might be reliant on MAML1.

Finally, we asked whether GSK3β-mediated phosphorylation of MAML1 would abrogate the interaction between MAML1 and Notch. However, non-phosphorylated MAML1 and GSK3β phosphorylated MAML1 seem to interact with Notch equally well. Similarly, the constitutively active GSK3β mutant did not alter the colocalization of Notch and MAML1 in COS-7 cells. Actually, all three proteins appeared to colocalize in nuclear bodies. On the basis of these results, we conclude that GSK3β can directly bind, phosphorylate and thereby repress the transcriptional activity of MAML1.

3.3 Suppresion of MAML1 activity by SUMOylation (Paper III)

In this study, we characterized two conserved SUMOylation consensus motifs in the MAML1 sequence. We showed that MAML1 is a target for SUMOylation *in vitro* and in cell culture, and mutational analysis of the predicted SUMOylation sites (K217 and K299) in MAML1 showed that they are important for MAML1 SUMOylation. Furthermore, single mutants (K217R and K299R) suggest that K217 is the major and K299 is the minor SUMOylation site of MAML1. The E2-conjugating enzyme UBC9 was necessary for MAML1 SUMOylation, and the E3-ligating enzyme PIAS1 further enhanced the SUMOylation. Moreover, the SUMO protease SENP1 reversed the SUMOylation of MAML1. We identified HDAC7 as a novel MAML1-binding partner. Interestingly, HDAC7 appeared to have a higher binding affinity for SUMO-modified MAML1 compared to unmodified MAML1. HDAC7 contains several SUMO-interacting motifs (SIMs) and we speculate that they might facilitate the interaction.

Next, we used a gene reporter assay to evaluate the functional importance of MAML1 SUMOylation. HCT116 cells were cotransfected with pG5luc reporter (containing GAL4 binding sites) and plasmids expressing GAL-MAML1 wild type or GAL-MAML1 mutants (K217R, K299R or K217/299R). We found that: (1) GAL-MAML1 K217/299 possesses much higher (40-fold) activity than GAL-MAML1 wild type; (2) the SUMO protease SENP1enhanced the GAL-MAML1 wild type transcriptional activity much more

than that of GAL-MAML1-K217/299R; and (3) knockdown of the E2 enzyme UBC9 strongly increased the activity of GAL-MAML1 wild type, whereas it had little effect on GAL-MAML1 K217/299R. On the basis of these results, we suggest that SUMOylation of MAML1 suppresses the transcriptional activity of MAML1. In addition, plasmid immunoprecipitation (PIP) showed that more SUMO-1 and HDAC7 were recruited to the promoter when cotransfected with GAL-MAML1 wild type compared to the GAL-MAML1-K217/299R mutant. We examined the co-activator function of SUMOylated MAML1 for Notch1 and found that MAML1 K217/299R had a more potent co-activator function for Notch than the wild type MAML1.

Given the strong interplay of acetylation between the p300 and MAML1 proteins, and the common cross-talk between SUMOylation and acetylation, we asked whether the MAML1 SUMOylation could affect MAML1 acetylation or the MAML1-dependent activation of p300 autoacetylation. We found no significant difference in acetylation between SUMOylated MAML1 and non-SUMOylated MAML1. Furthermore, SUMOylated MAML1 increased the p300 autoacetylation equally as well as the SUMO-deficient MAML1 mutant. (see Figure xx).

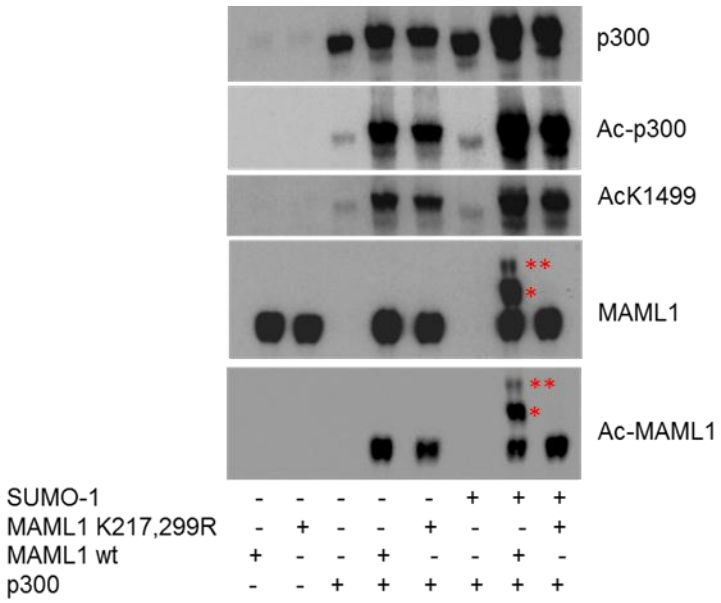

Figure 6. SUMOylation of MAML1 does not interfere with the p300-mediated MAML1 acetylation or MAML1-dependent increase of p300 autoacetylation. Plasmids expressing p300, SUMO-1, MAML1 wt or MAML1 K217/299R were cotransfected into HCT116 cells and whole-cell extracts prepared. Proteins were separated by SDS-PAGE and monitored by western blotting with antibodies recognizing p300, Ac-p300, MAML1 and Ac-MAML1. 1xSUMO-MAML1 is indicated by (*), and 2xSUMO-MAML1 by (**).

Phosporylation can up- or down-regulate the SUMOylation of different substrates. For example, a specific motif (ψKxExxSP) of the SUMOylation target protein that requires serine phosphorylation for optimal SUMOylation. We have seen that MAML1 is strongly phosphorylated in the cells, and have shown that GSK3β is able to phosphorylate MAML1. Interestingly, one of the SUMOylation motifs in MAML1 contains an SP motif in close proximity (IKTEFSP). Therefore, we asked whether GSK3β or the SP motif is important for SUMOylation of MAML1in HCT116 cells. We did not detect any difference in the SUMOylation of MAML1 in the HCT116 cells.

We conclude from this study that two highly conserved lysines (K217 and K299) are important for repressing the activity of MAML1, by being a target for SUMO modification, which facilitates recruitment of the co-repressor HDAC7 (see Figure 7). However, it remains to be determined whether the HDAC7-mediated inhibition of MAML1 is due to recruitment of additional co-repressors and/or deacetylase-dependent.

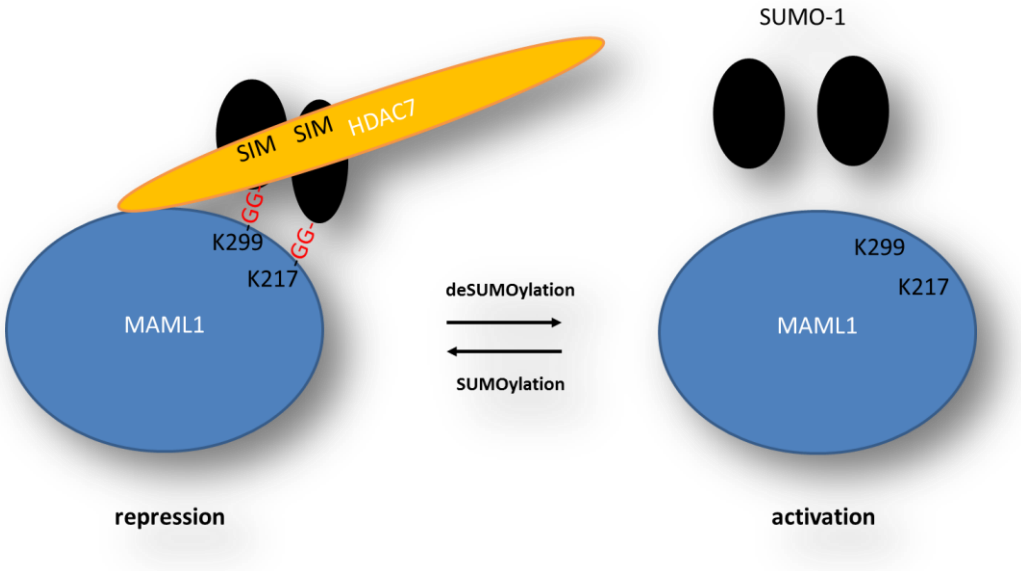

Figure 7. Proposed model of SUMO/HDAC7-mediated repression of MAML1 transcriptional activity.

3.4 MAML1 up-regulates Egr1 and co-activates Egr1-mediated transcription (Paper IV)

In this study, we investigated the role of MAML1 in the early growth response-1 pathway. We fou nd that the HEK293-FlagMAML1 stable cell line increased the TPA-induced level of the Egr1 protein compared to that of the HEK293 control cells, and siRNA knockdown of MAML1 resulted in a weaker TPA-mediated induction of Egr1 compared to that in cells treated with scrambled siRNA. Furthermore, a gene reporter assay revealed that treatment with TPA induced the Egr1 promoter, and the induction was greater in the cell line stably expressing Flag-MAML1 compared to the HEK293 control

cells. This result indicates that MAML1 might enhance levels of protein Egr1 by functioning as a transcriptional co-activator on the promoter of Egr1.

Moreover, we found that endogenous MAML1 interacts with Egr1, and that MAML1 functions synergistically with EGR1 to increase the expression of promoters containing four EGR1 binding sites. A region located between residues 75 and 124 and the C-terminal transcriptional activation domain (TAD2) were shown to be important for MAML1-dependent activation.

Because it has been reported that the p300 promoter contains several Egr1 binding sites and is regulated by Egr1, we wanted to see whether MAML1 can potentiate the Egr1-mediated activation of the p300 promoter. We found that Egr1 potentiates the p300 promoter reporter in the presence of TPA, and that Egr1 and MAML1 synergistically activate the p300 reporter in the absence and in the presence of TPA. However, the effect was much stronger with the addition of TPA. Egr1 has been shown to be a target for p300-mediated acetylation and, because we showed in paper I that MAML1 potentiates p300 autoacetylation and subsequent histone acetylation, we asked whether MAML1 affects the p300-dependent acetylation of Egr1. We found that p300 acetylates Egr1, and that MAML1 enhances the p300-dependent acetylation of Egr1, which has been shown to result in increased stability of Egr1. Therefore, we did a stability test in which HEK293-FlagMAML1 and HEK293 control cells were treated with TPA for 2 h to induce Egr1 and then treated with cyclohexamide to block protein synthesis. We did not detect any difference in the degradation rate of Egr1 between the two cell lines (data not shown).

4. CONCLUSIONS AND PERSPECTIVES

We conclude from papers II and III that the transcriptional co-activator MAML1 is subjected to SUMOylation and GSK3β-mediated phosphorylation, which both result in repression of the transcriptional activity of MAML1. While we suggest that the mechanism behind repressive effect of the SUMO-modified MAML1 is due to recruitment of the co-repressor HDAC7, the molecular basis underlying the GSK3β inhibition of MAML1 activity needs further study. We have investigated the possibility of cross-talk between GSK3β-mediated phosphorylation of MAML1 and SUMOylation. For example, GSK3β phosphorylation of MAML1 could cause increased SUMOylation following recruitment of HDAC7. However, we have not seen any indication of interplay between GSK3β and SUMOylation in their effects on MAML1. It will be important to identify the phosphorylation sites to gain deeper insight into how GSK3β inhibits the co-activator function of MAML1.

In papers I and IV we showed that MAML1 can stimulate acetylation of other proteins through up-regulation of p300 autoacetylation, which is followed by an increased acetylation of histones (paper I) or Egr1 (paper IV). Furthermore, in paper IV we demonstrated that MAML1 positively regulates the p300 promoter by functioning as a

novel co-activator for Egr1. The finding that MAML1 and Egr1 act cooperatively to activate Egr1 target genes is particularly interesting given the dual roles of Egr1 as a tumor suppressor and an oncogene. Egr1, like MAML1, is a protein that is greatly modified by many different enzymes and it is likely that the different modifications influence the activity as well as the target specificity of Egr1, by controlling the interaction with different co-activators or co-repressors. Therefore, it will be interesting to determine on which occasions MAML1 functions as a co-activator for Egr1; e.g. which are the upstream signals and modifications that induce the Egr1/MAML1 activation complex and which target genes are activated? The interplay between Egr1 and p300 is complex, and involves tightly controlled negative and positive feedback loops. It would be interesting to study the role of MAML1 in the Egr1/p300 transcription complex and to determine how p300 autoacetylation is regulated. Use of the AcK1499-p300 antibody in ChIP assays could be a valuable tool for these studies.

MAML1 and its two paralogs MAML2 and MAML3 have all been shown to function as co-activators in the Notch-mediated transcription (136, 137). So far, only additional co-activator functions have been explored for MAML1 and future studies will show the Notch-independent roles of MAML2 and MAML3. Gene knockout studies of MAML2 and MAML3 in mice would give information about this; furthermore, it would be interesting to test if MAML2 and MAML3 can increase the autoacetylation and HAT activity of p300, and thereby increase the acetylation of p300 target proteins

Given the wider role of MAML1 as a co-activator it would be interesting to test how SUMOylation or GSK3β-mediated phosphorylation of MAML1 affects its co-activator function for other transcription factors, such as p53, β-catenin and MEF2C, and to elucidate the importance of the interplay between MAML1 and p300 on the p53, β-catenin or MEF2C target genes. It is important to investigate the conditions under which MAML1 is SUMOylated to gain a better understanding of the physiological role of MAML1 SUMOylation.

We and others have shown the importance of the N-terminal domain of MAML1 for protein-protein interaction. Almost all proteins reported to interact with MAML1 bind within amino acids 1 and 300. The N-terminal (1-300) part is also a target for various kinds of post-translational modifications, such as acetylation, phosphorylation and SUMOylation, which suggests that these modifications could be of importance for the regulation of the protein-protein interaction. The central and C-terminal parts consist of the TAD2 domain, which is essential for the transcriptional activity of MAML1. However, the mechanism and the interacting proteins responsible for the TAD2 activity are unknown and warrant further study.

The main results presented here are based on findings obtained from studies done in human cell lines. It would be interesting to evaluate the *in vivo* function of our findings by using different animal models. For example, the role of MAML1 SUMOylation in early development or in neurogenic/muscle differentiation could be investigated in Xenopus oocytes or zebrafish (*Danio rerio*) as model systems. *In vivo* studies of the role

of GSK3β-dependent inhibition of MAML1 is more challenging because we have not identified the phosphorylation sites. An important question is how does GSK3β inhibit MAML1? The use of an *in vitro* transcription system with purified proteins and chromatin template could help to define a more precise role for GSK3 in its repression of MAML1 transcriptional activity, and to exclude potential cross-regulatory effects.

One of our goals is that our findings should be useful for development of novel pharmaceuticals. Egr1 has been shown to be constitutively active and up-regulated in prostate cancer. We want to elucidate the role of MAML1 in prostate cancer; e.g. does MAML1 participate in the up-regulation of Egr1, and can MAML1 function as a co-activator for Egr1? The oncogenic role of MAML1 in prostate cancer can be explored further by using mice tumor xenografts with MAML1 knockdowns. Furthermore, how is MAML1 modified in different diseases, such as various cancers? The agonistic role of MAML1 on p300 activity might be of clinical interest. For example, high p300 protein levels as well as enhanced p300 autoacetylation in oral squamous cell carcinoma have been described recently (84).

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