SAGA, TFIID and regulation of transcription through chromatin

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ISBN: 978-90-8891-723-3

Cover and invitation: “Oplossend vermogen” by AWS

Printed by: Proefschriftmaken.nl // Uitgeverij BOXPress

Published by: Uitgeverij BOXPress, ‘s-Hertogenbosch

This thesis was (partly) accomplished with financial support
from Greiner Bio-One Frickenhausen, Germany.

SAGA, TFIID and regulation of transcription through chromatin

SAGA, TFIID en de regulatie van transcriptie middels chromatine

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 19 november 2013 des ochtends te 10.30 uur

door

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geboren op 2 december 1984 te Tilburg

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Voor mijn ouders, Arthur en Josephina

Eigen lichaam, eigen lot, eigen leven,
je moet het zelf doen, want niemand komt het geven.

Opgezwolle, Volle Kracht, Eigen Wereld

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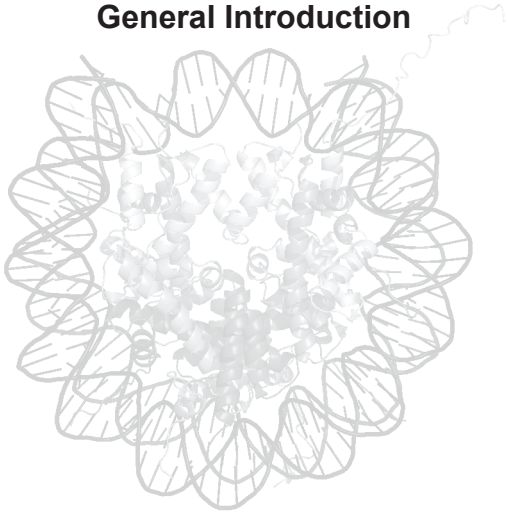
Chapter 1

General Introduction



Gene expression

The flow of genetic information in a biological system is described by the central dogma of molecular biology. This was first formulated by Francis Crick in 1958 and it states that genomic DNA is transcribed into RNA from which proteins are translated [1]. Research into how these apparently simple steps take place is still ongoing. Transcription of genes is a tightly regulated process. In eukaryotes three different RNA polymerases involved in production of RNAs were identified in 1969 [2]. In subsequent analyses using the transcription inhibitor α-amantin, it was discovered that RNA polymerase II (Pol II) is responsible for the production of all messenger RNAs (mRNA). Pol I was found to transcribe ribosomal RNA (rRNA) and Pol III is required for synthesis of transfer RNA (tRNA) [3-6]. Later a fourth and fifth polymerase (Pol IV and V) were identified in plants, which are involved in RNA-mediated gene silencing [7].

Besides RNA polymerases, many more proteins and protein complexes are involved in the process of transcription. An important aspect of this process has to do with how DNA is packed into chromatin and how proteins interact with chromatin. This is the focus of the work described in this thesis. First the three phases of transcription will be described, followed by an introduction into the role of chromatin in transcription.

Transcription initiation

The process of transcription by Pol II can be divided into three major phases: assembly of the pre-initiation complex (PIC), which leads to initiation, elongation and termination. The regulation of each of these phases involves next to a wide variety of proteins, also specific DNA sequences. Here each of the phases and how they are regulated will be introduced.

Officially, initiation is defined as formation of the first phosphoester bond in the nascent RNA. However more loosely, initiation refers to the phase wherein assembly of the pre-initation complex (PIC) on the core promoter occurs. The core promoter is the minimal DNA sequence needed to specify non-regulated or basal transcription. The PIC consists of protein complexes found to be essential for the transcription activity of Pol II [8,9]. These accessory factors are named the general transcription factors (GTFs) of which TFIID is one of the first to bind DNA. TFIID itself is a multi-subunit complex, of which several subunits interact with various core promoter elements and chromatin. Subsequently other GTFs bind to the promoter to enhance binding of TFIID (TFIIA, TFIIB), to recruit and enable Pol II binding to PIC (TFIIE, TFIIF) and to open the double-stranded DNA around the start-site of transcription (TFIIE, TFIIH) (Figure 1, PIC) [2,10-14]. Additionally, it was recently observed that TFIIA not only stabilizes TBP-DNA binding, but also keeps TFIID in its canonical state in the absence of DNA and promotes the required rearranged state of TFIID in the presence of DNA (Figure 1) [15]. Correct assembly of the PIC is essential to recruit Pol II at the appropriate site and start mRNA transcription.

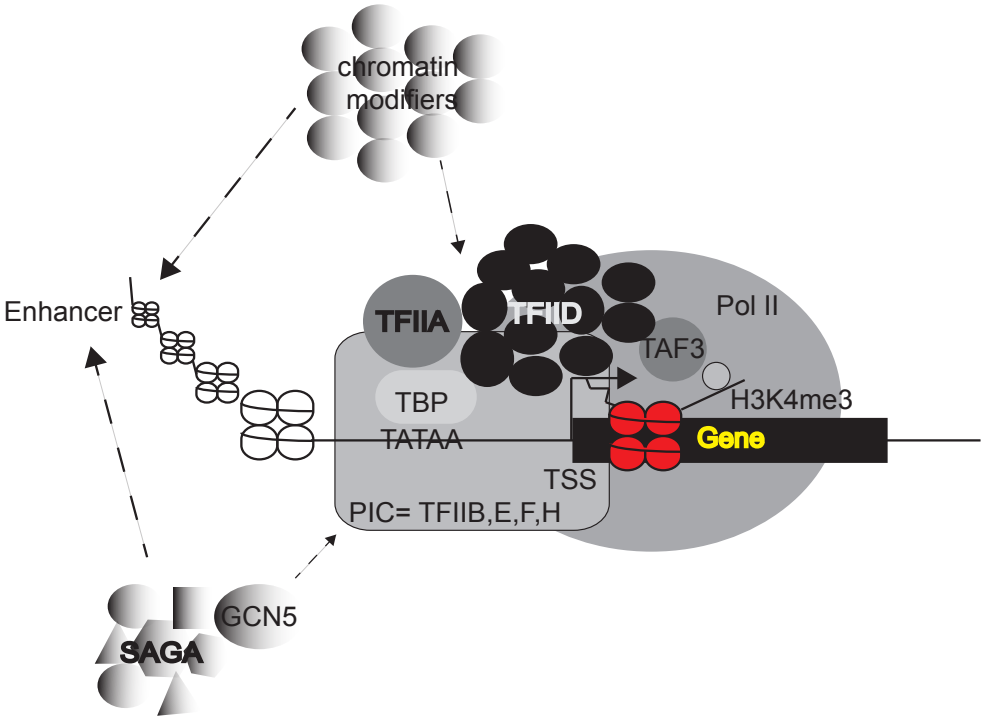


Figure 1: A depiction of a selection of proteins that modify and bind to the promoter region during the process of transcription initiation.

Human TFIID consists of the TATA binding protein (TBP) and 14 TBP associated factors (TAFs). Several TAFs contain a histone fold domain (HFD, further described later), enabling hetero-dimerization of specific TAF pairs. Two of these pairs recognize specific DNA elements found in a substantial percentage of core promoters [16]. The TAF1/2 dimer binds to the initiator element (Inr) [17]. Inr is a DNA element found surrounding the transcription start site (TSS). A second element is found 28 to 35 nucleotides (nt) downstream of the TSS. This downstream promoter element (DPE) is recognized by the TAF6/TAF9 dimer [18,19]. TBP recognizes A/T rich sequences found ~ 25 nt upstream of the transcription start site (TSS), named TATA boxes. The binding of TBP to the TATA box is strengthened by TFIIA and TFIIB binding (Figure 1) [11,15,20].

Element	Consensus	Location	Factor binding
Inr	YYANWYY	- 2 to + 4	TAF1,TAF2
DPE	RGWYV	+ 28 to + 33	TAF6,TAF9
TATA	TATAWAAR	- 31 to - 26	TBP
CpG Islands	Rich in CG	1 kb stretches over TATA less promoters	Various

Table 1: Summary of features of core promoter elements. In the consensus, degenerated nucleotides are represented, wherein Y = C or T (U), A = adenine, N = any base, W = A or T (U), R = A or G, G = guanine, V = A, C or G and T = thymine. Location is relative to the TSS, which is the +1 position. This consensus of core promoter elements is from vertebrates [18,21,22]

Another feature of the DNA sequence found in promoters is stretches of approximately 1 kb that are enriched for the CG dinucleotide, called CpG islands. CpG islands mark 60-70% of mammalian promoters. The cytosine of this dinucleotide can be methylated. Methylated CpG islands in the TSS are associated with gene silencing. Examples include imprinted genes and genes found on the inactive X-chromosome. In contrast, CpG islands found on active TSS are unmethylated [23-29].The methylated cytosine can be deaminated spontaneously or enzymatically, which results in its conversion to a thymine [24]. Overall the genome displays paucity for CG dinucleotides but in promoters that are unmethylated they are highly abundant. This paucity is explained by the conversion to thymine of previously methylated CGs in non-promoter regions. In addition, oxidation of the methylated cytosine results in hydroxymethylcytosine, which is catalyzed by the ten eleven translocation (TET) enzymes. This modification has a role in development and differentiation [30]. Interestingly, CpG island promoters often lack a TATA box and vice versa. CpG promoters are often associated with dispersed transcription, containing 50-100 TSSs. In contrast, TATA promoters have focused on transcription, with single or distinct clusters of TSSs [31-36]. Thus the distinct DNA sequences and modifications found in each promoter have important roles in the initiation of transcription.

The process of transcription activation comprises much more than the assembly of the PIC by GTF binding. Prior to PIC assembly, gene-specific transcription factors can bind to the DNA and influence the subsequent recruitment of GTFs. A characteristic of gene-specific transcription factors is the presence of DNA binding domains, which recognize specific sequences in the promoter region. Furthermore, during the entire process of transcription initiation, co-activator complexes can assist, for example by participating in chromatin remodeling, chromatin modification, promoter recognition and PIC complex stabilization (Figure 1). One such a co-activator complex, the Spt-Ada-GCN5 (SAGA) complex, will be introduced in more detail later.

The recruitment of Pol II and the phosphorylation of its largest subunit (RPB1) at its carboxyl-terminal domain (CTD) directly follow PIC assembly. Concomitant with release of Pol II and the initiation of transcription, phosphorylation occurs at serine 5 (Ser5-P) of the heptapeptide (YSPTSPS) repeats in this CTD (52 repeats in mammals) [37,38]. Together these observations are illustrative for why the intricate interplay of DNA sequences and protein complexes lead-

ing up to the initiation of transcription are the subject of many studies to date.

Transcription: elongation and termination

Following the process of transcription initiation and the release of Pol II, mRNA production occurs during the phase named elongation. During this phase of transcription, the CTD phosphorylation pattern of Pol II on Serine 5, Serine 2, Tyrosine 1 and Threonine 4, play a role in recruiting various complexes [38]. Examples of complexes that get recruited during elongation are polymerase associated factor 1 (PAF1) and negative elongation factor (NELF), which form elongation complexes (ECs) with the moving Pol II. The ECs are required for various steps in the process of elongation, such as capping of mRNA, to protect it from premature degradation and in the quality control of the growing transcript. Moreover ECs can form platforms for other protein complexes, often involved in remodeling and modifying chromatin. The role of these complexes in transcription will be discussed later.

Transcription termination, the final phase of transcription, is the disengagement of Pol II from the DNA template and the release of the mRNA transcript. Here again many complexes are involved in regulating the different steps in this process, also often dictated in part by the phosphorylation status of Pol II. Ser2-P for instance helps in recruiting and stabilizing a polyadenylation factor, required for coupling of transcription and 3' end processing [39,40]. Moreover, the de-phosporylation of Ser2-P has been described to have a role in the recycling of Pol II [41]. Thus specialized complexes, often highly conserved, control single or more steps in each phase and characterize each of the three major phases in transcription.

The complexity of all these processes is magnified by the existence of another important player: chromatin. Chromatin was not discussed so far, but it has a vital role in the process of Pol II transcription. The next section will focus on this role.

Transcription in the context of chromatin

Chromatin

In the cell's nucleus the DNA double helix is packed into chromatin of which the basic building block is the nucleosome. In 1997 the first structure of a nucleosome revealed that it comprises an octamer of histones with 147 bp of DNA wrapped around it (Figure 2A) [42,43]. The discovery of histones dates back to 1884 by Albrecht Kossel, but then they were dismissed as merely being inactive packing material for DNA in the nucleus. It was not until the 1980s that histones were linked to transcriptional repression in yeast and their role in transcriptional regulation was suggested [44-46]. The octamer consists of two copies of histones: H3, H4, H2B and H2A, which have 14 contact points with the DNA that is twisted in a 1.67 times left-handed superhelix [42,43]. A fifth histone, H1, binds both the nucleosome and the entry and

exit points of the DNA, thereby keeping the DNA in place and allowing for higher order structures. All histone proteins are highly conserved from yeast to human. Early work revealed that packaged genomic DNA around an octamer forms a 'beads on a string' configuration, where the nucleosome is being a 'bead' (Figure 2B). This structure can then be further compacted into the 30 nm fiber, which during mitosis forms an even higher order of compaction (Figure 1B) [47,48]. The compaction is required to fit the approximately 2 meters of DNA into the 10 µm diameter of the nucleus.

Two main types of packaging dominate chromatin. The loosely packed or 'open' euchromatin ('beads on a string') is generally associated with active transcription, while the tightly compacted or 'closed' heterochromatin (30 nm fiber) correlates well with transcription repression (Figure 2B). Interestingly, what was once considered a simple scaffolding platform for genomic DNA has now emerged to be a highly important player in the regulation of a wide variation of nuclear processes, also transcription. The regulation of transcription, DNA replication and DNA repair all require access to the chromatin and/or the genetic message encoded by the DNA. Therefore chromatin is constantly subjected to remodeling and modifying activities. Since the state of compactness has such a significant role in the regulation of these processes, the balance between compacting the genomic DNA and making it accessible is under the control of various enzymes that can space and position the nucleosomes in an ATP-dependent manner. These enzymes reside in multi-subunit complexes referred to as chromatin remodelers. Moreover, histones can be post-translationally modified to create a binding platform for protein complexes involved in processes such as transcription. Nucleosome positioning and remodeling will be briefly introduced below, followed by a description of post-translational modifications (PTMs) important for transcription.

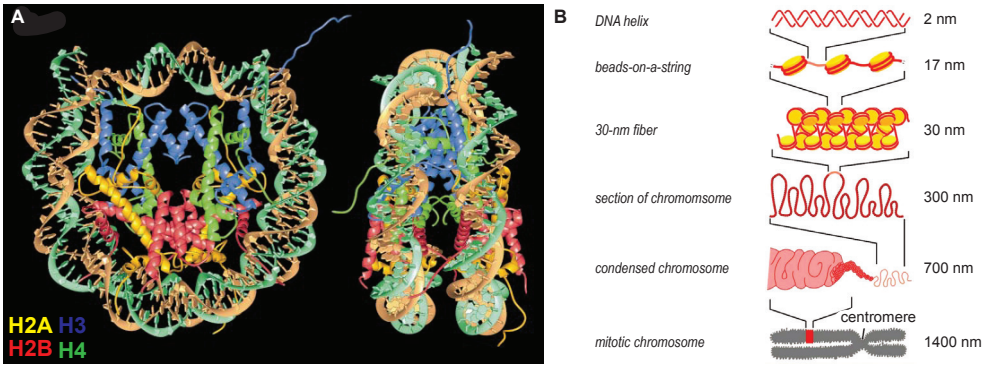


Figure 2: The first crystal structure of the nucleosome core particle at 2.8 Å resolution (A) [42] and a schematic representation of different chromatin packaging structures, adapted from Alberts (B).

Nucleosome positioning

The genome-wide pattern of nucleosomes is determined by the combinatorial interplay of DNA sequence, transcription factors and nucleosome remodelers. Firstly, the DNA

sequence influences the position of the nucleosomes, since homopolymeric sequences poly (dAdT) and poly (dGdC) are structurally stiff and disfavor nucleosome formation [49-53]. Hence many promoters have long poly (dAdT) stretches that provide intrinsic properties for nucleosome depletion, which is required to create the nucleosome free region (NFR). The NFR is found in a promoter and helps to increase transcription factor accessibility [51,54,55]. The first nucleosome, found downstream of the TSS (the +1 nucleosome), is strongly positioned and therefore also the subsequent nucleosomes at the 5' end of genes. The location of the +1 nucleosome differs between species [56,57]. Nucleosomes found in the body of genes are less well localized. However, at the 3' end a NFR and a nucleosome precisely positioned with respect to the gene (comparable to the +1 nucleosome) has been identified in yeast and flies [58-61]. The strong relationship between the TSS and the +1 nucleosome furthermore suggests that the GTF's also have a role in nucleosome positioning [62]. It is speculated that PIC complexes bound to the core promoter form an anchor point and recruit nucleosome remodelers (which will be introduced in the next section) to position the +1 nucleosome [52,63].

Nucleosome remodelers

Besides DNA sequence there is also a group of proteins involved in positioning the nucleosomes at a certain locus: nucleosome remodelers. These remodelers use the energy released by ATP hydrolysis to increase the mobility of nucleosomes and allow positioning at disfavored DNA positions. Remodelers are specifically important for the localization of the +1 nucleosome. This was first observed when purified histones and DNA yielded an *in vivo* nucleosome pattern (including a +1 nucleosome) when crude yeast extract and ATP were added [64]. The DNA binding specificity of remodelers to specific sequences most likely overrides the intrinsic preference of histones to certain DNA sequences. This together with the energy released from ATP hydrolysis establishes re-positioning of the nucleosomes [52,65,66]. Thus, DNA sequences, transcription factors and nucleosome remodeling complexes together establish nucleosome patterns required for transcription at enhancers, promoters and in the coding regions of genes.

Histone variants

Next to positioning of nucleosomes, remodelers are also involved in the replacement of the canonical histones by their histone variants in certain nucleosomes. For example, for H1, H3 and H2A variants exist. The canonical histones are transcribed from genes clustered in repetitive arrays with transcriptional activity tightly coupled to DNA replication. Genes encoding histone variants are found as single copies. Histone variants differ from the canonical paralogs in primary amino acid sequence and are constitutively expressed. Their functions vary, amongst others, from DNA repair, meiotic recombination, chromosome condensation to transcription. In transcription important roles have been ascribed to the histone variant H3.3 (variant to canonical histone H3.1) and H2A.Z (variant to histone H2A). Histone 3.3 is deposited into transcribed genes, promoters and other gene regulatory elements. Furthermore it is enriched for posttranslational modifications associated with active transcription [67-70].

H2A.Z is found in nucleosomes surrounding the NFR (+1 and -1 nucleosomes) and these nucleosomes enhance efficient Pol II recruitment (Figure 2) [71-73]. Swr1-containing complexes are the remodelers responsible for deposition of H2A.Z-H2B dimers, not only at positions flanking the NFR but also at upstream promoter elements. Interestingly, H2A.Z is also found in heterochromatin where it binds heterochromatin protein 1 (HP1) with a higher affinity than its canonical counterpart H2A [74]. It is thought that the role of H2A.Z in gene regulation has to do with its ability to form more stable nucleosomes than its paralog H2A [70].

Posttranslational modifications on histones

Apart from nucleosome positioning there is another feature of chromatin that is crucial for regulation of many nuclear processes. Similar to several proteins, histones can be posttranslationally modified. Histones are particularly suitable for PTMs since they are rich in basic residues such as lysines and arginines. These residues are found throughout the globular and the N-terminal domains and can be modified with various chemical groups, including ubiquitin, sumo, phospho, methyl and acetyl. In case of methylation, residues can be modified by addition of one, two or three methyl groups (mono-, di- and trimethylation). A review from 2000 has lead to the following popular nomenclature in chromatin biology. To refer to chromatin modifiers, for the proteins that add or remove the modifications the terms ‘writers’ and ‘erasers’ are used. The proteins that bind to the histone marks can be referred to as ‘readers’ [75]. In this thesis, depending on the context, writers/erasers are sometimes referred to as chromatin modifiers and readers are occasionally referred to as chromatin binding proteins. Furthermore, throughout the thesis we will refer to specific histone modifications as follows: H(x)Res(y) mod(z), where H= histone, Res= amino acid residue, mod= covalent modification and x,y,z are numbers. For example H3K9ac refers to acetylation of lysine 9 on histone H3.

Numerous studies have been conducted to analyze the genome-wide distribution patterns of histone modifications and to correlate them to the transcriptional state of genes. For example, silent genes are marked by H3K27me3 and H3K9me2/3 for their entire length (Figure 3). H3K9me2/3 has a role in heterochromatin formation, via recruitment of the HP1 protein [76]. Silent genes are often packed into heterochromatin and H3K9me2/3 correlate well with silent genes and heterochromatin [77]. Also H3K27me3 in the gene body correlates with gene repression, through the inhibition of both initiation of cryptic transcription and elongation [78]. Another mark that anti-correlates with transcription is H4K20me3, which is found on repetitive sequences through the genome [79].

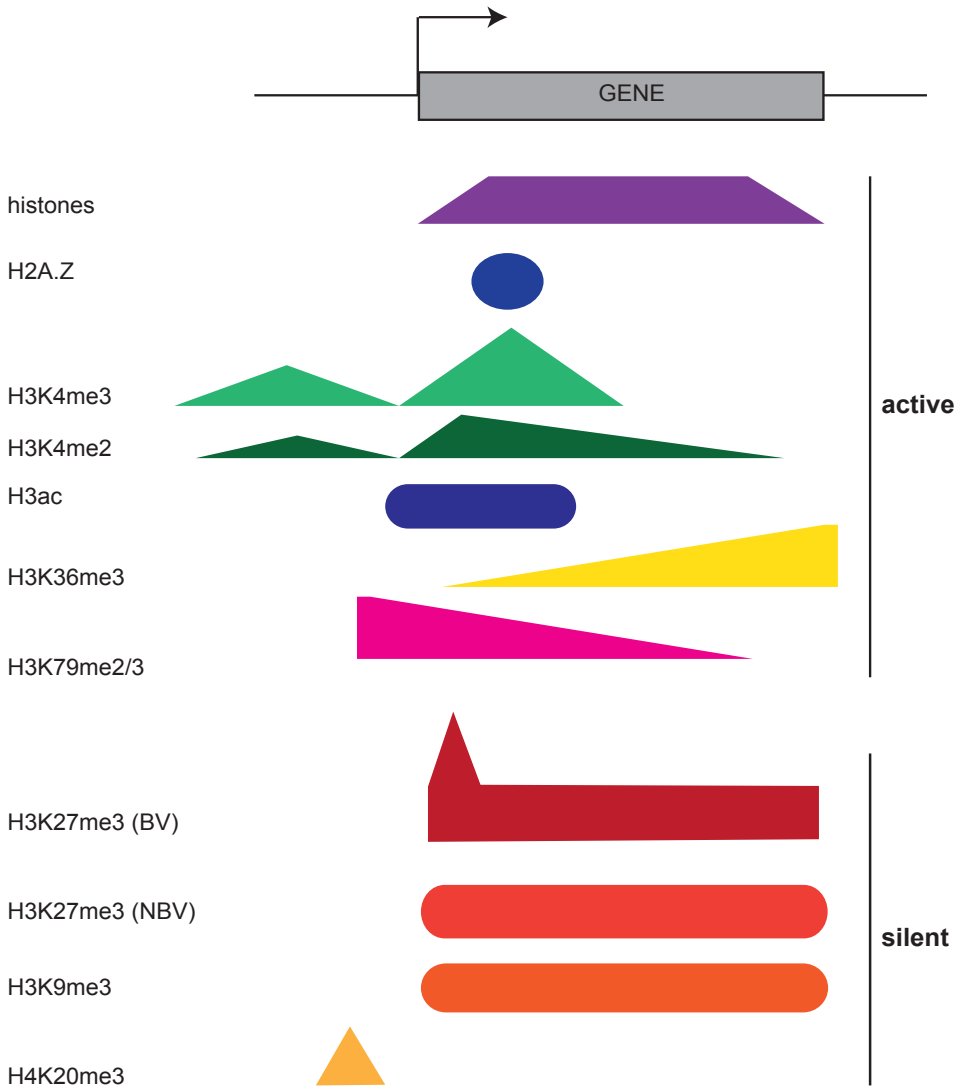


Figure 3: Average distribution patterns for a selection of histone modifications on active and silent mammalian genes. BV= bivalent and NBV = not bivalent, see text.

The chromatin landscape of active genes consists of a different set of histone modifications (Figure 3). Next to the H2A.Z variant found in the +1 and -1 nucleosome, active genes are also marked with H3K4me2/3 and acetylated H3K9 and H3K14, which are discussed below. H3K36me3 marks the coding region of active genes, is associated with elongation and has a strong correlation with expression [77]. Interestingly, the presence of H3K36me3 anti-correlates with the presence of acetylated histones in the coding region. It has been described that H3K36me3 recruits histone de-acetylases

(HDACs), which remove acetyl groups in the wake of elongating Pol II, to prevent aberrant initiation of transcription in the middle of genes (cryptic transcription) [77,79,80]. In humans H3K79me2/3 shows an inverse pattern to H3K36me3, with a peak partly overlapping with H3K4me3 and decreasing towards the 3' end of the gene. H3K79me2/3 shows a weaker correlation to gene expression, but is found on the promoter of active genes [77]. The function of H3K79me2/3 on active promoters as well as the identity of the protein that removes the mark remains to be elucidated. It is tempting to speculate about a correlation with the phosphorylation status of Ser-2 in the Pol II CTD, which displays a similar gradient towards the 3'end of the gene and might be involved in the regulation of methylation of H3K79 in the coding region of active genes. Finally, the nucleosomes found in the coding region of highly expressed genes are enriched for mono-ubiquitinated H2B in humans [81]. The ubiquitination machinery is first recruited to the promoter and subsequently activated by association to the PAF1 complex [82]. The exact role of H2Bub in the coding region remains elusive. Later the role of the co-activator complex SAGA in H2Bub will be discussed in this context [83,84].

Thus the chromatin modification landscape correlates well with the level of gene expression. Furthermore, examples of direct links between modification and transcriptional activity include a recent study wherein the expression of certain genes was shown to be dependent on presence of H3K4me3 [85]. Another study showed that induction of H3K9me3 could silence an artificial gene locus [76]. Together, these kinds of observations indicate that modifications alone or in combination dictate various steps in the transcription process. The next section focuses on two specific PTMs, H3K4me3 and acetylation of histone H3, followed by an introduction to how these modifications are created and removed by chromatin modifiers.

H3K4me3

Trimethylation of lysine 4 on histone H3 is found on the promoters of virtually all actively transcribed genes in many species [79,86,87]. Whilst H3K4me3 forms a sharp peak at the TSS, H3K4me1 and H3K4me2 are not solely found on the promoter and they are thought to have different roles in transcription (Figure 3). H3K4me1 is associated with enhancers and H3K4me2 is found to be more broadly associated with active genes, than just the promoter [88,89]. The enzymes that catalyze the methylation reaction use S-adenosyl-L-methionine (SAM) as the methyl donor. Recently, threonine and SAM metabolism were found to be coupled in mouse ES cells and shown to be required for efficient establishment of H3K4me3, specifically at pluripotent genes [90]. Pluripotent genes are genes involved in maintaining the pluripotent state of ES cells. This further underscores the important role for H3K4me3 in transcription and why its role is the topic of many ongoing investigations.

The function of H3K4me3 in transcription is often thought to be in combination with other PTMs, including ubiquitination. The previously mentioned mono-ubiquitination of H2B at lysine 120 has been shown to be required for methylation of H3K4 and for full transcription activation, from yeast to humans [91-93]. H3K4me3 also cooperates with acetylation of lysines 9 and 14 on histone H3, which will be discussed later. A special combination of H3K4me3, namely with H3K27me3, has been proposed in embryonic stem cells and zebrafish, where they mark the promoters of lineage con-

trol genes. The bi-valency of these genes is thought to maintain them in a 'poised' state, during the pluripotent phase the genes are silenced (H3K27me3). However, immediately upon differentiation the genes are ready for activation (H3K4me3) [94]. Recently, also nucleosomes carrying both H3K4me3 and H3K27me3 on different H3 tails were identified *in vivo* [95]. Research into new combinations of PTMs with H3K4me3 may reveal novel mechanisms important for transcription.

Acetylation of histone H3

Besides H3K4me3, general acetylation of histones H3 and H4 is also associated with active transcription. Its localization does not peak sharply at the +1 nucleosome, but is spread out through the promoter and coding region of genes (Figure 3) [96]. Moreover, acetylation of H3K27 is now considered a hallmark of active enhancer elements, also suggesting a role of histone acetylation in transcription through distal regulatory elements [97-99].

Acetylation is thought to enhance transcription in two ways. First, acetylation of lysines neutralizes their positive charge, thereby reducing the binding ability to the negatively charged DNA. This results in less compacted nucleosomes and looser intra-nucleosomal contacts, providing more access of transcription factors to DNA. This is most prominently illustrated by acetylation of H4K16, the presence of which prevents folding of chromatin into higher order structures [100]. Second, acetylated histones serve as binding platforms for many different complexes involved in transcription. Among the writers for the acetylation mark, the histone acetyl transferases (HATs), a high redundancy is observed. Acetylation of lysines -9 and -14 on histone H3 (H3K9ac and H3K14ac) on promoters and enhancers, correlates with gene activity [101]. Furthermore, the fact that the writer for this mark is a subunit of the co-activator complex SAGA further suggests a correlation between H3K9ac/K14ac and transcription initiation [101-104].

Chromatin modifiers

Methylases/Demethylases

Proteins responsible for methylation of histones are called lysine methyl transferases (KMTs). Two classes of KMTs exist, with the second class, characterized by lack of a SET domain, comprising of only one known member. The first class of KMTs does contain this SET domain, which is a 130 amino acid catalytic domain, first identified in SUV39H1 and highly conserved from yeast to human [105]. Homology search of this SET domain subsequently lead to the identification of many more SET containing KMTs [106,107]. All KMTs display a high degree of specificity towards their target lysine as well as to the number of methylgroups they add (mono, di or tri). This specificity also greatly depends on the context of the complex in which the KMT is found. For instance, KMT2A (MLL1) in a purified form is able to dimethylate H3K4, while when associated to its complex (MLL1 complex) it is responsible for trimethylation of H3K4 [108-112]. In humans the bulk of H3K4me3 is established by one the six complexes of the proteins associated with Set1 (COMPASS) family [113-115]. This family has diverged from yeast, which has only one COMPASS member, Set1, via Drosophila (three members)

to eventually six family members with non-redundant functions in humans [113-117].

Removal of methyl groups from lysines is performed by lysine demethylases (KDMs). The discovery of LSD1/KDM1A as a KDM for H3K4me3 in 2003, terminated the three-decade long discussion on whether these proteins actually existed [118,119]. Demethylation by LSD1 correlates with repression of transcription [118]. KDMs also show a high specificity for target lysines and degree of methylation, which is illustrated by KDM4A and KDM4D that respectively demethylate H3K9me3/2 and H3K36me3/2, but not their monomethylated forms [120-124]. Together, the methylases and demethylases are thought to have an important role in regulation and fine-tuning of the transcriptional process [106].

Histone acetyl transferases/Histone deacetylases

HATs perform the acetylation of lysine residues by addition of an acetyl to the ε-group of the terminal amine of lysine leading to the formation of N-ε-acetyl-L-lysine [125]. Two categories of HATs exist, where the first or A-type HATs are mainly found in the nucleus and the B-types are localized in the cytoplasm. B-type HATs are thought to have a housekeeping role, acetylating newly synthesized histones in the cytoplasm. The A-type HATs have a more specific role in the nucleus, acetylating histones incorporated in the nucleosome and non-histone substrates, such as transcription factors [126,127]. In mammals the nuclear HATs mainly belong to one of three families: GNAT, MYST and p300/CBP. The distinction between families is based on structural homology between members. Other families have been described, such as the TAF250 and steroid receptor families, but their HAT activity has been studied in less detail. HATs of the GNAT and MYST families most often operate in large multi-subunit complexes that determine their specificity and function, similar to KMTs. An exception to this is the p300/CBP family, consisting of human paralogs p300 and CBP, which are not part of a complex, but do associate with a wide range of different proteins. The p300 and CBP proteins show >90% sequence similarity and have highly redundant target sites and function. Therefore they are often referred to as simply p300/CBP [128-130]. The p300/CBP proteins are crucial for normal development, which is demonstrated by the embryonic lethality of double knockout mice [131,132]. The role of p300/CBP in transcription is illustrated by the fact that they were originally identified as co-activators, and only after the discovery of Tetrahymena p55 as a HAT, similar HAT activities were identified in p300/CBP [133,134]. In general, HATs are thought to be transcription activators, also because acetylated histones are mainly correlated with active transcription.

Histone deacetylases (HDACs) are enzymes that catalyze the reverse reaction. Four classes exist of which some are found in the nucleus, while others shuttle between cytoplasm and nucleus. HDACs are generally associated with transcription silencing. Similar to HATs most HDACs do not operate alone but rather in multi-subunit complexes [135]. Notably, an important signal for repression by HDACs comes from the DNA itself, where the methylated CpG islands of silenced genes recruit HDAC complexes, such as NuRD, via their methylation binding subunits (MBD3) [136-138]. As with HATs, targets of HDAC also include non-histone substrates such as p53 and α- tubulin [139,140].

The relation between HDACs and gene repression explains how overexpression of certain HDACs can lead to aberrant gene silencing in various tumor cells [141,142]. A

research field exploiting the role of both chemical and natural compounds that inhibit HDAC activity is emerging and several HDAC inhibitors are already in use or in the phase of clinical trials [143]. HDAC inhibitors such as the hydroxamic acid derivatives usually target two types of HDACs [144]. These are the most studied and potent inhibitors. They include naturally occurring trichostatin A (TSA), which induces cell differentiation and cell cycle arrest in transformed cells. Also vorinostat, a chemical compound, is a potent anti-cancer agent that causes growth inhibition and differentiation in neoplastic cells [145-147]. TSA and vironostat inhibit through binding to the catalytic site pockets of HDACs [148]. Acetylation of histones as a target for cancer therapy is also being explored on the writers and readers side. Although currently problems exist with the administration of HAT inhibitors, their potential as anti-cancer drug is enormous, since the amount of tumors wherein overexpression of HATs is correlated to aberrant cell proliferation is substantial [128]. This potential as anti-cancer drug also exists for the inhibition of reader binding, through bromodomain (BRD) inhibitors, which are also the subject of many current investigations. Together the success of these anti-cancer agents underscores the important role of epigenetics in the development of cancer and the requirement for more functional and molecular analysis of chromatin and its role in gene expression.

Chromatin readers

Recognition of a certain histone modification by a specific reader largely determines the biological outcome of the presence of this modification. These reader proteins often contain domains, which are characterized to bind certain PTMs. Examples include the plant homeo domain (PHD) and the tudor domain, that recognize methylated lysines, as well as bromodomains that bind to acetylated residues [149].

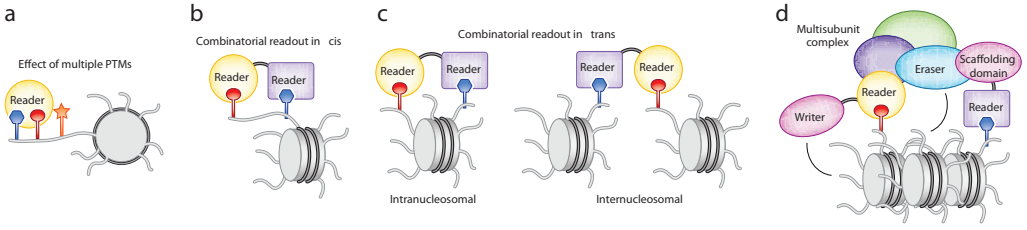


Figure 4: Different forms of histone crosstalk (A-D), adapted from [149].

PTMs also display histone crosstalk, providing combinatorial read-out of multiple PTMs (Figure 4). Different forms of histone crosstalk exist, such as the presence of one modification adjacent to another, enhancing or inhibiting recognition by a reader protein (Figure 4A). The latter is the case for phospho-methyl switches where phosphorylation of a residue adjacent to a methylated residue prevents the binding of a reader complex. For example, binding of the TFIID complex to H3K4me3 during mitosis is prevented by the presence of adjacent phosphorylated H3T3 [150]. The transient nature of phosphorylations can contribute to rapidly switching between different outcomes. Other forms of

histone crosstalk include the recognition of multiple modifications on one tail (cis) or on multiple tails (trans) by one protein containing multiple reading domains (Figure 4B and C). This has been described for the TAF1 protein, which contains two bromodomains that both recognize one acetyl group of the di-acetylated H4 tail [151]. The most complex form of crosstalk is the multivalent engagement of a protein complex, which harbors multiple subunits with PTM binding domains. In addition to recognizing multiple PTMs, these complexes may also contain supplementary writer and/or eraser proteins that can subsequently modify chromatin (Figure 4D), such as the HBO1 complex that can bind H3K4me3 and acetylate histone H3 and H4 [152]. In conclusion, combinations of PTMs can have a crucial role in a wide variety of processes, amongst others transcription.

Previously a “histone code theory” was used to refer to the extra layer of information on top of the information found in the DNA sequence [75]. Herein it was stated that a histone code consisting of (or combinations of) PTMs could dictate a certain biological outcome through the interactions of proteins to certain histone PTMs. However, more and more evidence indicates that the outcome of a certain modification is context dependent. For example, the presence of H3K4me3 near a TSS dictates active gene expression, while in the context of DNA damage, H3K4me3 recruits, via the PHD finger of inhibitor of growth 2 (ING2), the repressing complex Sin3A-HDAC1 [153]. Therefore a term gaining popularity is chromatin signaling [154]. In the following section we will introduce two important reader complexes involved in chromatin signaling of transcription: the basal transcription factor TFIID and the co-activator Spt-GCN5-Ada (SAGA) complex.

The TFIID complex

Structure

During the process of PIC assembly to initiate transcription, TFIID is one of the first proteins to bind the promoter. The structure of this complex has recently been elucidated by electron microscopy. This study confirmed what other studies had already suggested [155,156] namely that the core of human TFIID is composed of two copies of TAF4, TAF5, TAF6, TAF9 and TAF12. These TAFs form the following (hetero)dimers through their histone fold domains (HFD): TAF5-TAF5, TAF4-TAF12 and TAF6-TAF9. The symmetric core complex forms a holo basket-like structure, with the N-terminal of TAF4 on both ends forming earlobe-like structures (Figure 5A). The homodimer TAF5-TAF5 forms the basis of this basket-like structure and its N-terminal WD40 repeats serve as a scaffold. The TAF4-TAF12 and TAF6-TAF9 pairs are positioned such that the DNA interacting domains point outwards facilitating DNA binding (Figure 5A) [157]. The other TAFs are suggested to be present in one copy, except TAF10, which also form heterodimers with TAF8. These two pairs are crucial for incorporation of the other peripheral TAFs (TAF1, TAF2, TAF3, TAF7, TAF11, TAF13) and the formation of the clamp-like TFIID holo-enzyme (Figure 5B) [157-159]. The stoichiometry of the endogenous human TFIID remains to be determined.

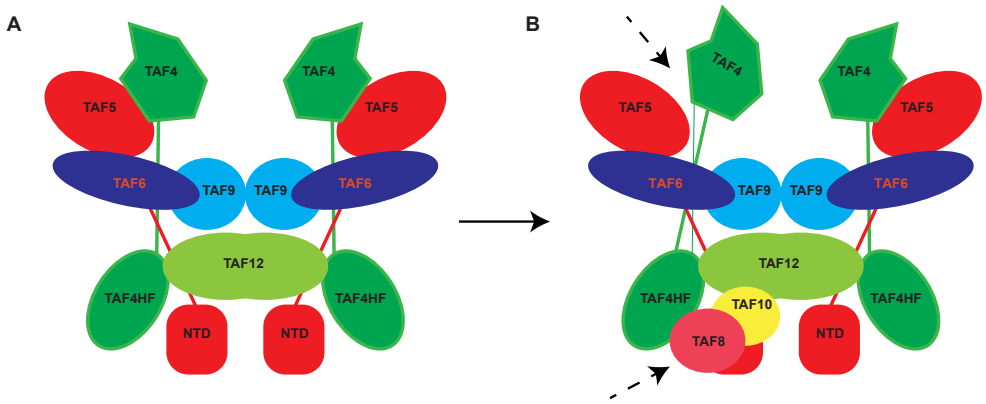


Figure 5: Schematic representation of the symmetric TFIID core structure (A) and of the asymmetric core plus the TAF8-TAF10 dimer (B). Arrows indicate new binding surfaces where the remaining TAFs and TBP assemble to form the TFIID holo-complex. Adapted from [157].

TBP and TAF variants

Specialized functions of TFIID in a tissue specific manner are suggested by the existence of paralogs (i.e., related proteins coded by different genes belonging to the same family) of both TBP and multiple TAFs. TBP related factors (TRFs) either show similar affinity to the TATA sequence as TBP, for instance vertebrate TRF3 protein. However, the composition of their associated TFIID complex might differ through altered affinity for PIC members TFIIA and TFIIB. Moreover they are also regularly differentially expressed in tissues [160]. These characteristics can determine their specificity for certain genes. In contrast, the TRF2 protein has reduced affinity for the TATA sequence but binding to TFIIA and TFIIC is enhanced, explaining its affinity to certain promoters.

TAF paralogs are predominantly expressed in gonads and germ cells and often required for fertility [161-163]. However, general TAF variants also exist, such as TAF9B, which is ubiquitously expressed in cells and shows 83% sequence similarity to TAF9. Most importantly, TAF9B is present also in TFIID, but knockdown of TAF9B versus TAF9 showed a subset of genes specifically regulated by TAF9B [164]. In addition, during muscle differentiation it was found that a TAF3/TRF3 complex replaces the canonical TFIID complex. In the transition from myoblasts to myotubes, expression levels of TFIID holo-enzyme diminish while TAF3-TRF3 becomes dominant and is required for MyoD dependent activation of the myogenin gene [165,166]. In contrast, TAF10 has no known variants and knockdown of this subunit during keratinocyte differentiation merely changed the expression of a subset of genes [167]. Furthermore, a role in lineage commitment of mouse embryonic stem cells (mESC) was also proposed for TAF3 [168]. A specialized function of TFIID is also found in the mESC where knockdown of core subunits TAF5 and TAF6 leads to premature differentiation of the stem cells. This indicates that TFIID has a role in pluripotency and stem cells maintenance [169]. This and other data together strongly suggest the existence of distinct structural and functional TFIID subcomplexes.

TFIID in the context of chromatin

The TFIID interaction with DNA sequences in the promoter region is complemented by several TAF subunits binding to specific promoter histone modifications (Figure 1). The TAF3 PHD finger has high affinity for H3K4me3 and is considered the most important reader for this mark in mammals [170]. Furthermore, the TAF1 subunit was shown to interact with double acetylated histone H4 [151,171]. The binding of TFIID to synthetic histone peptides is greatly enhanced, when in addition to H3K4me3, also K9ac and K14ac are present. The double bromo-domain of TAF1 is perhaps also responsible for this binding [170]. Recently it was observed that the TAF3-H3K4me3 interaction is especially important for selective gene activation. Here it was also shown that this interaction can facilitate PIC assembly (Figure 1) on promoters that lack a consensus TATA sequence, indicating the interaction of TFIID with chromatin can supplement and replace DNA interaction at specific loci [85]. TFIID is a good example of multivalent engagement with the promoter, because it harbors both subunits interacting with the DNA, as well as subunits interacting with the chromatin. These synergistic interactions are the subject of many investigations, including the ones described in this thesis.

The SAGA complex

The co-activator complex SAGA is a mega-dalton complex that is highly conserved from yeast to mammals [172]. During evolution the yeast SAGA complex has diverged into two related complexes, SAGA and ATAC, with distinct roles in transcription and histone modifications [172,173]. Here we will discuss the structure and function of the SAGA complex, as this is the most relevant for the work described in this thesis.

Structure

The yeast SAGA complex shares a number of TAF proteins with the TFIID complex: Taf5, Taf6, Taf9, Taf10 and Taf12 [174,175]. It was observed by electron microscopy that yeast Taf6 and Taf9 heterodimerize through their HFD and form a core structure together with a Taf5-Taf5 homodimer. Within SAGA these heterodimers were observed to form a similar core structure as they form within TFIID (Figure 6). In humans Taf5 and Taf6 are replaced by highly homologous TAF5L and TAF6L that have the same role in the structure of the SAGA core [176]. Besides the Tafs, the other yeast SAGA subunits, Ada1 and Spt7, also contain a HFD allowing heterodimerization with Taf10 and Taf12 (Figure 6) [175,177,178]. Analysis of recombinant Ada1-Taf12 and Taf6-Taf9 heterodimers revealed the formation of an octameric complex (Figure 6) [179].

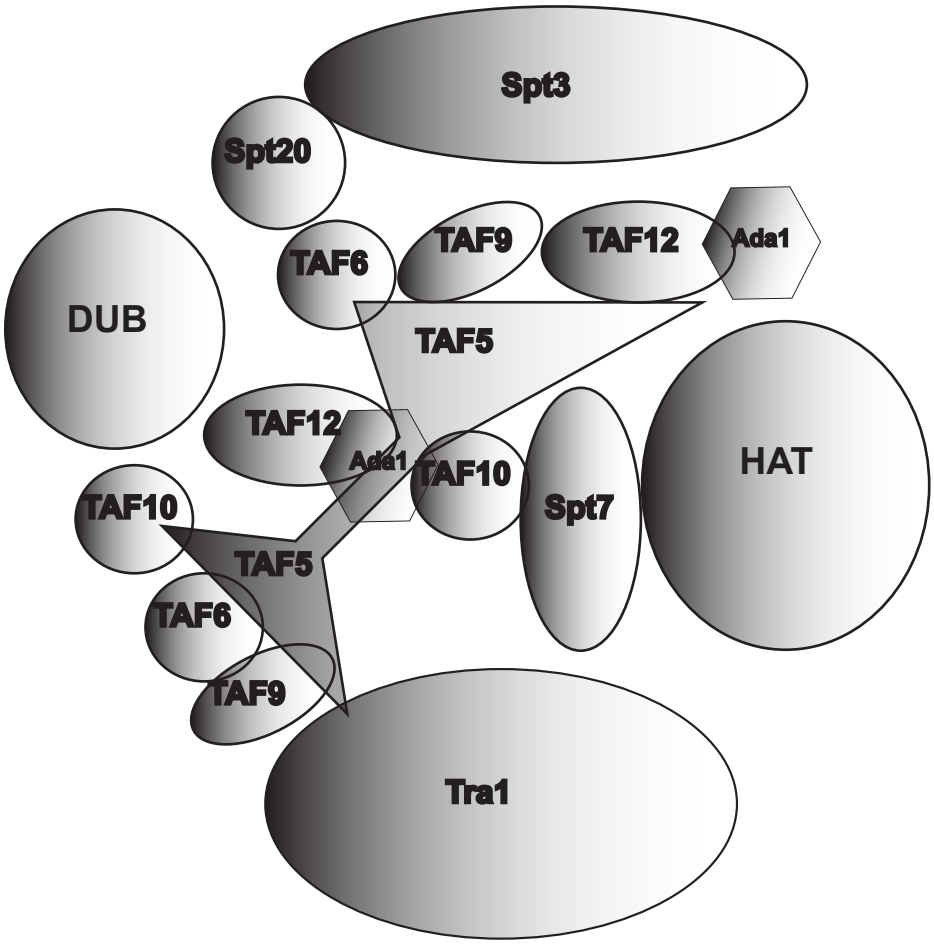


Figure 6: Schematic representation of the yeast SAGA, adapted from [175].

Besides Ada1 and Spt7, Spt20 is part of the SAGA core and important for structural integrity (Figure 6) [103,180-182]. Spt20 is localized on top of the Tafs and away from the center [175]. Tra1 completes the SAGA core and is found under the previously mentioned Taf dimers (Figure 4) [175]. Tra1 has been shown to interact with several gene-specific transcription factors *in vitro*, suggesting it is required for SAGA recruitment to certain promoters [183]. Homologs for Ada1 (TADA1), Spt7 (SUPT7L), Spt20 (SPT20) and Tra1 (TRAPP) were identified in human SAGA [184-187]. These proteins display high sequence and domain similarity (HFDs), suggesting that SAGA's core is identical in humans. Indeed the three-dimensional structure of human SAGA (formally known as TFTC or STAGA) is highly similar to yeast SAGA [185,188]

Peripheral subunits of SAGA include Spt3 and Spt8 that regulate TBP-TATA binding to promoters in yeast (Figure 6) [189-191]. SUPT3H was identified in human SAGA as an Spt3 homolog. The two proteins share 30% overall identity that defines three conserved regions and the function of SUPT3H in humans seems to be conserved [192]. No ortholog of the SPT8 gene is present in humans [172].

The remaining subunits in the SAGA complex form two distinct modules with different enzymatic activities: the de-ubiquitination (DUB) module and the HAT module. Both modules contain highly conserved subunits [172] and are found left (DUB) and right (HAT) of the core complex (Figure 6) [175,185]. The yeast DUB module consists of Ubp8 (de-ubiquitination enzyme), Sgf73, Sgf11 and Sus1. Sgf73 is important for incorporation of the module into the complex and Usp8 is only active when correctly assembled in the module [173,193]. Human orthologs of these proteins are ATXN7 (Sgf73), ATXN7L3 (Sgf11), ENY2 (Sus1) and USP22 (Ubp8). All have been shown to be structurally and functionally similar to their yeast counterparts [83].

The DUB activity of the SAGA complex has been linked to different processes in the nucleus. The DUB activity can be directed against H2B, which is important for the previously mentioned crosstalk between mono-ubiquitinated H2B and H3K4me3. This will be further introduced in the next section. In addition, the yeast Sus1p subunit has also been described to have a function in mRNA export, since it is a shared subunit with the transcription export (TREX) complex. Further experiments suggest a functional link between transcription and mRNA export [194]. It is likely that this function is conserved in humans, although the ENY2 proteins are less well studied [195]

Both ATXN7 and ATXN7L3 DUB subunits contain an atypical zinc finger, named spinocerebellar ataxia type 7 (SCA7), which in case of ATXN7L3 allows for interaction with nucleosomes [172,196]. Polyglutamine expansions in the N-terminal region of ATXN7 are found associated with spinocerebellar ataxia type 7, which is characterized by motor coordination deficiencies (ataxia) and retinal effects [197]. Together, these observations indicate an important role of the DUB module of SAGA in epigenetic regulation of this disease.

In yeast the HAT module consists of Ada2, Ada3, Sgf29 and Gcn5, the latter being the active enzyme. In humans the GCN5 gene has been duplicated and diverged to generate the p300/CBP associated factor (PCAF) gene. PCAF shows 73% sequence similarity to GCN5 and assembles into SAGA in a mutually exclusive manner [198]. In yeast it was shown that the presence of Ada2 potentiates the catalytic activity of Gcn5, while Ada3 facilitates nucleosomal acetylation and expands Gcn5 lysine specificity [182,199]. Thus Ada2 and Ada3 are both required for Gcn5 HAT activity within the yeast SAGA complex. In mammals the ADA2 gene has been duplicated into an ADA2A and ADA2B gene. This is where the difference between SAGA and ATAC complexes originates, ADA2B is exclusively found in SAGA and ADA2A in ATAC. The rest of the HAT module of ATAC is similar [187,200]. Outside the HAT module, ATAC is comprised of other subunits than SAGA and functional analyses have shown distinct, but important roles for this complex in transcription, histone modification, signaling pathways and cell-cycle regulation [172,187,201,202].

The function of ADA2B and ADA3 is again conserved in humans. The two subunits are important for the HAT activity of GCN5 on nucleosomal templates [203]. The fourth subunit of the HAT module SGF29, also conserved from yeast to human, is described to bind to H3K4me3 via its double tudor domain and is responsible for the recruitment of SAGA to this modification [204-206].

Thus the function of the HAT module is to support the acetylation activities of GCN5, which alone is not able to acetylate histones incorporated into the nucleosome [103]. The acetylation of nucleosomes by the SAGA complex is an important part of its role in the regulation of transcription. The following section will introduce the role of SAGA as a chromatin modifier.

SAGA in the context of chromatin

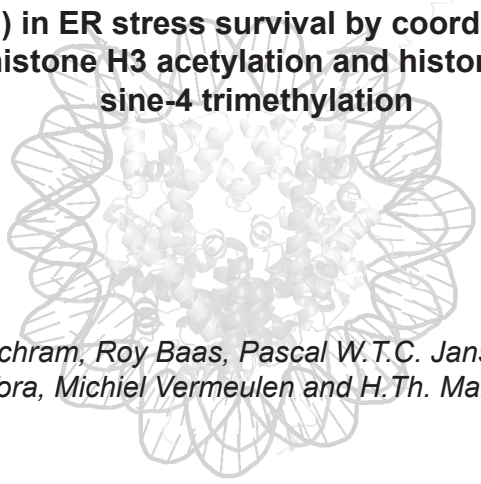
The roles of SAGA and TFIID have been shown to be redundant for global transcription [207]. SAGA is a co-activator. In yeast, between 10% and 20% of the genes is predominantly occupied by SAGA versus 80-90% by TFIID. These 10% are mostly stress-induced genes [208,209]. Interestingly, this role is not conserved to humans, where a genome-wide analysis of SAGA showed binding also to the promoter of housekeeping and tissue specific genes [210]. Specific roles for SAGA in transcription can partly be found in its role as a chromatin modifier (Figure 1). In yeast, de-ubiquitination by Ubp8 was shown to be required for optimal gene expression through regulation of H3K4me3 and H3K36me3 levels [93,211]. Furthermore, it was demonstrated that USP22 de-ubiquitinates H2A and H2B *in vitro* [212-214]. *In vivo* analyses determined SAGA's DUB activity to be directed against H2B. This is required to fully activate SAGA-regulated inducible genes [83]. In mammals it was described that the endoplasmic reticulum stress target genes are amongst the SAGA target genes [186]. An interesting form of crosstalk involving SAGA was found in yeast. It was shown that H3K9ac and transcriptional activation of the promoter of the GAL1 gene were dependent on the presence of Sgf29. This implies that binding of SAGA to H3K4me3 is required for acetylation and subsequent transcriptional activation [204]. Since the Sgf29 protein is also conserved to humans, it might serve a similar function to establish crosstalk between H3K4me3 and H3K9ac and/or H3K14ac. Furthermore acetylating activities by SAGA's GCN5 might also be directed at non-histone proteins, maybe even subunits of TFIID, since the composition of TFIID can also be determined by post-translational modifications on its subunits [215]. Therefore the role of SAGA in chromatin signaling remains open for further investigation.

Outline of this thesis

Chromatin has an important role in eukaryotic transcription. Research into this role is ongoing and genome-wide analysis has correlated various histone modifications to multiple elements in active and silent genes, such as enhancers, promoters and coding regions. Modifications often serve to recruit complexes involved in the process of transcription, such as TFIID to H3K4me3 via the TAF3 subunit. Chromatin modifying complexes include the co-activator complex SAGA, which acetylates lysine-9 and -14 on histone H3. The work described in this thesis was aimed at exploring multiple aspects of chromatin signaling. Both the writing and the reading of several histone modifications were investigated, to better understand the role of chromatin in transcription. In **chapter 2** we set out to study crosstalk of H3K4me3 and acetylation of histone H3 via SAGA's subunit SGF29 in mammalian cells. SGF29 is required to recruit SAGA to H3K4me3. We focused on the endoplasmic reticulum (ER) stress target genes, which were shown to recruit SAGA upon ER stress induction. Strikingly, we discovered that on these genes not only H3K14ac decreases in SGF29 knockdown cells, but also that this knockdown results in a decrease of the H3K4me3 modification. Furthermore, both H3K4me3 and SGF29 were present prior to ER stress, suggesting a 'poised' state for the ER stress target genes. We hypothesize that at least on ER stress target genes, SAGA's role is two-fold. Prior to ER stress, SAGA recruitment is involved in maintenance of the H3K4me3, possibly via the recruitment of MLL-complexes. Upon induction SAGA is required for acetylation of the promoter and transcriptional induction. In an **addendum** to this chapter the development of a quantitative mass spectrometric-based method to study histone modifications in an unbiased and antibody independent manner is described. The global histone acetylation levels in SGF29 knockdown cells were determined and showed a reduction in H3K9acK14ac. This is in line for what was found in chapter 2 on the ER stress genes, thereby showing that this method is useful to study histone modifications. In **chapter 3** work aimed at identifying the HAT responsible for H3K4 acetylation in mammalian cells is presented. A series of knockdown and overexpression experiments revealed a role for HBO1 and the HBO1 complex. **Chapter 4** describes an investigation of the multivalent engagement of the general transcription factor TFIID to promoters, both *in vitro* and *in vivo*. Synergistic binding of TFIID to both DNA and histone modifications on promoter nucleosomes is revealed. These results accentuate the co-operative role of DNA and histones in the process of transcription initiation. Finally, **chapter 5** discusses concepts and implications for future studies emerging from the work presented in this thesis.

Chapter 2

A dual role for SAGA-associated factor 29 (SGF29) in ER stress survival by coordination of both histone H3 acetylation and histone H3 lysine-4 trimethylation



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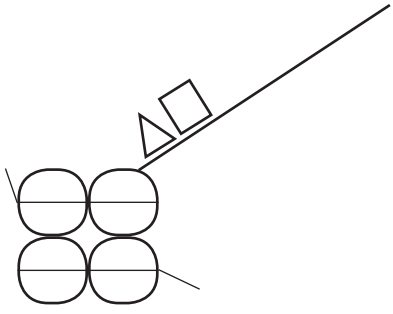
PLoS One. 2013 Jul 23;8(7):e70035



Abstract

The SGF29 protein binds to tri-methylated lysine-4 of histone H3 (H3K4me3), which is a histone modification associated with active promoters. Human SGF29 is a subunit of the histone acetyltransferase module of the SAGA (Spt-Ada-Gcn5 acetyltransferase) and ATAC (Ada-Two-A-containing 2A) co-activator complexes. Previous work revealed that the SAGA complex is recruited to endoplasmic reticulum (ER) stress target genes and required for their induction. Here, we report the involvement of SGF29 in the survival of human cells from ER stress. SGF29 knockdown results in impaired transcription of the ER stress genes *GRP78* and *CHOP*. Besides histone H3K14 acetylation, we find that SGF29 is also required for the maintenance of H3K4me3 at these genes, which is already present prior to ER stress. Reduced levels of H3K4me3 in the absence of SGF29 correlate with a decreased association of ASH2L, which is a core component of the SET1/MLL complexes, to *GRP78* and *CHOP.* In conclusion, our results suggest that the H3K4me3-binding protein SGF29 plays a central and dual role in the ER stress response. Prior to ER stress, the protein coordinates H3K4me3 levels, thereby maintaining a 'poised' chromatin state on ER stress target gene promoters. Following ER stress induction, SGF29 is required for increased H3K14 acetylation on these genes, which then results in full transcriptional activation, thereby promoting cell survival.

Keywords: SAGA; chromatin; transcription regulation; ER stress response; histone methylation; histone acetylation

Background

Eukaryotic transcription is a tightly regulated process, which is controlled by a wide variety of proteins including gene-specific transcription factors, co-regulators and the basal RNA polymerase transcription machinery. Important control mechanisms are exerted at the level of chromatin, of which nucleosomes comprise the basic building block. Post-translational modifications (PTMs) of the histone tails protruding from nucleosomes play a major role in the regulation of transcription and gene expression. The link between PTMs and activation of transcription was stressed by the discovery of the co-activator GCN5 as a histone acetyltransferase (HAT) [133]. Genome-wide localization studies revealed that acetylation of the H3 tail at lysine-9 and -14 (H3K9ac and H3K14ac) are linked with transcriptionally active genes [101,102,104]. Besides histone acetylation, methylation is also important for gene activity [216-218]. Tri-methylation of lysine-4 on histone H3 (H3K4me3) is strongly associated with the promoters of actively transcribed genes [79,86,87]. Subsequent studies have shown that both histone acetylations and methylations can serve as recognition sites for chromatin and transcription regulatory complexes. H3K4me3 is recognized by a number of different binding or “reader” domains within proteins such as the Chromo, PHD and double Tudor (Td) domains [219-221]. Well-studied examples of H3K4me3 binders are the PHD finger-containing proteins BPTF, a member of the chromatin remodeling NURF complex [222], and TAF3 [170], a subunit of the basal transcription factor TFIID [223]. TAF3 binds to H3K4me3 with high affinity and can act as a transcriptional co-activator in a PHD finger dependent manner [85,150,170]. Thus, TAF3 forms the molecular link between the active chromatin state of a promoter and the basal transcription machinery.

Biochemical purifications revealed that in higher eukaryotes GCN5 is part of the evolutionary-conserved SAGA and ATAC co-activator complexes [103,172]. GCN5 is central in their HAT modules, which is consists of the ADA2B, ADA3 and SGF29 proteins in SAGA. ADA2A replaces ADA2B in the HAT module of ATAC [201]. The SAGA complex is composed of ~20 subunits, which are organized into distinct modules. The core of SAGA is formed by SPT20, SPT7, ADA1 and supplemented by several TAF (-like) proteins [224]. Yeast and human SPT20 are highly homologous proteins and they are essential for the structural integrity of the SAGA complex [103,182,186]. Besides the core and HAT modules, SAGA contains modules involved in transcription activation and in de-ubiquitination of histone H2B. Several subunits of SAGA harbor domains capable of interacting with modified chromatin. SPT7 and GCN5 harbor Bromo domains, which can recognize acetylated lysines, and SGF29 contains a double Tudor domain capable of binding H3K4me3 peptides. Indeed, SGF29 is required for binding of the SAGA complex to this mark [205]. Deletion of yeast SGF29 does not affect SAGA integrity nor composition of the HAT module indicating that SGF29 is a peripheral subunit in this complex [225,226]. Furthermore, deletion or knockdown of SGF29 leads to decreased global levels of H3K9, K14 and K23 acetylation in yeast and human cells

Yeast SAGA is particularly important for stress-induced transcription [204,208] and this function seems conserved during evolution and extended to both SAGA and ATAC [172]. Genome-localization studies showed that SAGA mostly localizes to gene promoters, whereas ATAC has a preference for gene enhancers [210]. In the human

stress response the functions of the SAGA and ATAC complexes also seem to have diverged [172]. For example, SAGA can be recruited to the promoters of ER stress target genes [186] and not to immediate early (IE) genes, where ATAC is found upon TPA induction [201]. Many proteins are involved in mediating and recovering from ER stress. The function of the GRP78 protein lies in the detection of mis-folded proteins that cause ER stress. GRP78 activates the PERK and ATF6 pathways, which in turn induce transcription of CHOP and GRP78 via the ATF4 and ATF6 transcription factors. CHOP has further downstream functions in the ER stress response [227]. Interestingly, *in vitro* promoter binding studies show that ATF6α, an important transcription factor in the ER stress response, can bind and recruit both the SAGA and ATAC complexes to immobilized DNA templates [201]. When ER stress is induced *in vivo* by thapsigargin treatment, SAGA is recruited to the TSS of *GRP78* and *CHOP* and functional intact SAGA is required for proper transcription of these genes [228].

Here we examined the role of SGF29 in the recovery of human osteosarcoma cells from ER stress. We observed that the survival of cells after ER stress is reduced in SPT20 and SGF29 knockdown cells. This lower ER stress resistance correlates well with lower transcription induction of *GRP78* and *CHOP* and a lower H3K14 acetylation of their promoters. Interestingly also H3K4me3 levels of the *GRP78* and *CHOP* promoters are greatly decreased in SGF29 KD cells. The importance of H3K4me3 in the ER stress response is further stressed by the finding that knockdown of the H3K4me3-reader of TFIID, TAF3, results in similar, yet less pronounced, effects on gene induction and cell survival after ER stress. Reduced levels of H3K4me3 are concomitant with a reduced association of the SET1/MLL core subunit, ASH2L. We hypothesize that the concerted action of a number of transcriptional activators including TFIID, MLL and SAGA is required for maintaining stress response genes in a 'poised' chromatin state and facilitate their rapid, full activation upon stress induction.

Materials and Methods

Cell culture, knockdown, lentivirus infection and ER stress treatment:

Human osteosarcoma cell line U2OS (#HTB-96), HeLaS3 (CCL-2.2) obtained at ATCC and HeLa FRT [229] cells were maintained as monolayers in dishes (Greiner Bio-One, Frickenhausen, Germany) in normal glucose Dulbecco's modified Eagle's medium (DMEM, InVitrogen), containing 10% fetal bovine calf serum (FBS, Lonza) and 1% penicillin/streptavidin and L-glutamine (Lonza). pLKO-puro constructs (Sgf29 #1-TRCN0000141325, Sgf29 #2-TRCN0000144091, Taf3 #5.2 TRCN0000016608 and Taf3#5.3 TRCN0000016609) expressing shRNAs that target SGF29 or TAF3 mRNAs were obtained from Open Biosystems. As a control for the knockdown experiments, a non-targeting shRNA with the following sequence 5'- CCGGGCGAACAAGAAGAAGGACAAACTCGAGTTTGTCCTTCTTCTTGTTCGCTTTTT-3' was used. Viral production was performed in Cos7 cells by transfection of 2 µg each of pRSV-rev, pMDLg/pRRE, pMD2.G, and pLKO-shRNA using Fugene 6 (Roche). Lentivirus containing medium was collected 48 h after transfection, filtered and concentrated by ultracentrifugation (2.5 hour, 76,000 g, 18°C). $2.5*10^6$ cells (U20S and HeLa FRT) were used for infection and puromy-

cin selection was started two days later. U2OS were plated at low density for monoclonal outgrowth and colonies picked after 14 days of culturing. Stable GFP-tagged SGF29, ASH2L and RBBP5 cell lines were created by cloning of the ORFs into pCDNA.5/FRT/TO (InVitrogen) and subsequent recombination into Hela FRT cells carrying the Tet repressor for inducible expression [186]. Endoplasmic reticulum (ER) stress was induced by the addition of 5 µg/µl tunicamycin (Sigma-Alldrich) to the growth medium for indicated times.

Chromatin immunoprecipitation

Cells were cross-linked at 80-90% confluency (15 cm plates, (Greiner Bio-One, Frickenhausen, Germany)) using 1% paraformaldehyde in PBS for 10 min at room temperature. Reactions were quenched by addition of 125 mM glycine for 5 minutes on ice. After a cold PBS wash cells were scraped and collected by centrifugation (5 min, 400 g, 4^{o}C). Pelleted cells were resuspended in ChIP lysis buffer (1% SDS, 10 m1M EDTA, 50mM Tris-HCl pH 7.9, 1 mM DTT, 5 µM sodium butyrate (Merck) and complete protease inhibitors (Roche)) and disrupted by sonication (Bioruptor, Diagenode: seven cycles, 30 sec on/off, high setting) to produce an average DNA fragment size of ~ 400 bp. Samples were centrifuged (5 min, 200 g, 4^{o}C) and supernatant collected. For immunoprecipitation, chromatin was diluted in IP buffer (0.5% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 7.9, 150 mM NaCl, 1 mM DTT, 5 µM sodium butyrate and complete protease inhibitors (Roche)), 1-5 µg antibody was added and rotated overnight at 4^{o}C. Immunocomplexes were collected for 4 hrs at 4^{o}C on protein A/G PLUS-agarose beads (Santa-Cruz), after o/n blocking in 1.5% fish gelatin and washing. Subsequently beads were washed four times at 4^{o}C with wash buffer (0.25% NP-40, 0.05% SDS, 2 mM EDTA, 20 mM Tris-HCl pH 7.9, 250 mM NaCl, 5 µM sodium butyrate and complete protease inhibitors) and once with TE (10 mM Tris-HCl pH 6.8, 1 mM EDTA). Cross-links of protein-DNA were reversed by overnight incubation at 65^{o}C and eluted in 100 µl elution buffer (100 mM NaHCO3, 1% SDS). Samples were treated with 1 mg/ml proteinase K (Roche) and 1 mg/ml RNase A for 2 hours at 37^{o}C. DNA was purified using PCR purification kit (Qiagen) and amplified in a 25 µl reaction mixture (iQ SYBR green supermix (Biorad)) in a real-time PCR machine (CFX96, Biorad). Primer sequences are available upon request.

Fluorescent-activated cell sorting (FACS) analysis

To measure apoptosis 2*10^{5} wildtype or knockdown U2OS cells were seeded in 6-well plates (Greiner Bio-One, Frickenhausen, Germany). After tunicamycin treatment, cells were washed twice with PBS and recovered overnight in normal DMEM. All cells were collected, centrifuged (10 min, 600 g, RT) and resuspended in PBS with 5 mg/ml propidium iodide and in some experiments Annexin V (InVitrogen). Cells were incubated 5 min on ice before analysis on a Becton Dickinson FACS Calibur.

mRNA expression analysis

Total RNA was isolated using RNeasy kit (Qiagen) and cDNA was synthesized using the First-strand cDNA synthesis kit (Qiagen) both according to the manufacturers manual. Subsequently the cDNA was amplified in a 25 µl reaction mixture (iQ SYBR green supermix (Biorad)) in a real-time PCR machine (CFX96, Biorad).

XBP1 mRNA was measured by RT-PCR. Samples were loaded on a 2% agarose gel and stained with ethidium bromide. Primer sequences are available upon request.

SDS-PAGE, immunoblot analysis and antibodies

Whole cell lysates were analyzed by immunoblotting according to standard procedures. In short, cells were lysed and scraped in sample buffer and boiled at 95°C for 5 min. Reactions were run on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane. Molecular mass markers were obtained from New England Biolabs. Antibodies used were anti-SGF29 [230], anti-tubulin (Calbiochem CP06), anti-H3 (Abcam Ab1791), anti-H3K4me3 (Diagenode pAB-003-050), anti-RBBP5 (Bethyl BL766), anti-ASH2L (gift from Winship Herr), anti-WDR5 (a gift from Winship Herr) and anti-GFP (a gift from Geert Kops). Additional antibodies used for ChIP are anti-H3K14ac [201], anti-H3K4me3 (Millipore 05-745R) and anti-H3K18ac (Abcam Ab1191).

Results

SAGA and SGF29 are involved in cell survival of U2OS cells after ER stress.

Previous studies of SAGA and ER stress involved thapsigargin treatment of Hela cells, which is known to cause irreversible damage to cells [231]. Tunicamycin is a milder treatment to induce ER stress and in contrast to thapsigargin many human cell lines can recover from tunicamycin treatment using moderate doses [232]. We first tested the toxicity of tunicamycin on a human osteosarcoma cell line (U2OS). Following treatment of cells in increasing amounts, FACS analysis was performed to determine the survival rate of cells. The cells were treated for 4 or 8 hours with tunicamycin, or with DMSO as a control. Non-fixed cells were then treated with propidium iodide (PI), which stains the DNA of cells with disrupted membranes such as necrotic or apoptotic cells. Increasing the amount of tunicamycin resulted in a mild increase in PI positive cells after 8 hours of treatment (Fig.1A). This indicates that the amount of apoptotic cells is negligible at all tested tunicamycin concentrations. We also investigated cell recovery after tunicamycin treatment by monitoring restoration of unspliced XBP1 mRNA. XBP1 splicing by IREp is an early step in the ER stress response, leading to increased amounts of the (smaller) spliced variant [233]. Upon recovery from ER stress the amount of the unspliced variant should be restored. U2OS WT cells were treated for the indicated times with 5 µ/ul tunicamycin and XBP1 mRNA was measured by RT-PCR (Fig. 1B). The unspliced XBP1 isoform starts being restored after 8 hours of treatment and full restoration is observed after 24 hours of tunicamycin treatment. These results indicate that U2OS cells are capable of responding to this dose of tunicamycin.

SGF29 is responsible for binding SAGA to the H3K4me3 modification on ER stress target gene promoters, thus we decided to investigate the involvement of SGF29 in cell survival and gene activation after ER stress induced by tunicamycin. To first examine ER stress dependence on SAGA in this cell system, SPT20 kd cells were created by lentiviral transduction of two independent short hairpin (sh)RNAs targeting SPT20 or as a control, a non-targeting hairpin (Fig. 1C). A FACS based assay was used to determine the survival rate of cells from tunicamycin. The cells were treated for 8 hours with tunicamycin, or with

the DMSO carrier only, and allowed to recover overnight. Non-fixed cells were treated with PI, FACS analysis reveals a higher percentage PI-positive cells in SPT20 knockdown (kd) cells (24% and 16%) than the control kd cells (5%) after tunicamycin treatment and recovery from ER stress (Fig. 1D). This observation correlates well with the reduced ER stress gene induction in HeLa cells [234] and indicates that SPT20 (and most likely the SAGA complex) plays a general role in the ER stress response pathway of human cells.

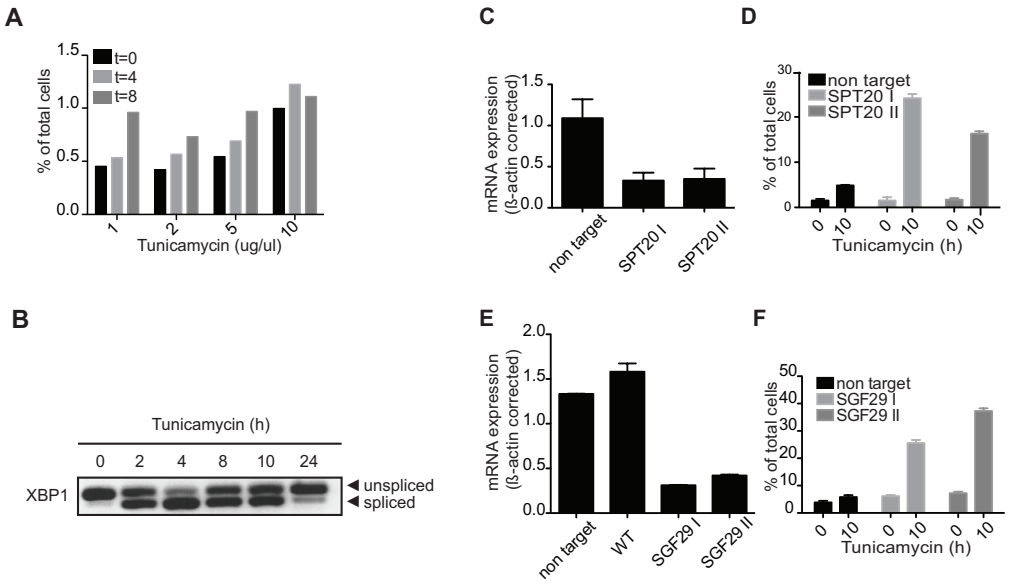


Figure 1: U2OS cells were transduced with lentiviruses targeting SPT20 or SGF29 (two different shRNAs) or a non-target control shRNA. A. Different amounts of tunicamycin were tested for toxicity in U2OS WT cells. Schematic representation of FACS results of propidium (PI) positive cells. B. Recovery of unspliced XPB1 mRNA in U2OS wt cells after tunicamycin treatment (5ug/ul) for indicated times. Amount of XPB1 mRNA is measured by RT-PCR. C. and E. Analysis of mRNA expression levels of SPT20 and SGF29 by quantitative RT-PCR, corrected for β-ACTIN. Standard deviations represent technical triplicates. D. and F. Schematic representation of FACS results, increase in PI positive cells upon SPT20 knockdown (KD) and increase of PI- and annexin V-positive cells upon SGF29 KD. Samples were measured after 8 hours treatment with tunicamycin or DMSO followed by an o/n recovery. Standard deviations represent technical duplicates. Similar results were observed in two independent experiments.

Having established the U2OS system to study ER stress induction by tunicamycin, we focused on the SGF29 protein, which is a subunit of both the SAGA and ATAC complex. An important feature of both SAGA and ATAC is the ability to bind to chromatin and more specifically, to the H3K4me3 modification. This interaction is mediated by SGF29 [204,205]. To investigate a potential role for SGF29 in the ER stress response, a U2OS cell line with a stable knockdown of SGF29 was created (Fig.1E). SGF29 or control KD cells were assayed for survival from ER stress by FACS analysis for PI- and annexin V-staining after tunicamycin treatment. As was observed in SPT20 kd cells, SGF29 kd cells showed a higher staining both for PI and for annexin V (25% and 37 % vs. 6%) detecting apoptotic cells (Fig. 1F), suggesting not only SAGA activity, but also specific recruitment to H3K4me3 is required for its role in ER stress survival.

SGF29 protein is required for induction and H3 acetylation of ER stress genes.

Given the fact that reduction of SGF29 and SAGA yielded similar effects on ER stress survival and SPT20 binds to the promoters of ER stress target genes GRP78 and CHOP [186], we further investigated the role of SGF29 in this process. ER stress was induced by tunicamycin and mRNA levels of *GRP78* and *CHOP* were determined. SGF29 KD resulted in lower gene activation upon ER stress (Fig. 2A).

To facilitate chromatin immunoprecipitation (ChIP) using a GFP-antibody, a doxycycline-inducible cell system for GFP-SGF29 expression was created (Fig. 2B). Multiple primer pairs were designed to detect GFP-SGF29 binding to ER stress target genes (Fig. 2C). After GFP-SGF29 expression was established by the addition of doxycycline, ER stress was induced by a 4-hour tunicamycin treatment. GFP-SGF29 binding was detected close to the transcription start sites (TSS) of GRP78 and CHOP both prior to and after tunicamycin treatment (Fig. 2D). GFP-SGF29 was not detected with primer pairs distal to the TSS, nor to a non-coding control region. Upon tunicamycin treatment (t=4), GFP-SGF29 binding to the TSS is increased about two-fold (Fig. 2D). Previously, recruitment of the SAGA subunits SPT20, SPT3 and ATXN7L3 was reported, as was a functional requirement of SPT20 for transcriptional induction. Together, these and previous results suggest that SGF29 and the SAGA complex bind to these genomic loci.

SGF29 could be important for the recruitment of SAGA to the ER stress target genes and subsequent induction. In this scenario, SGF29 depletion may result in lower levels of H3 acetylation on the *GRP78* and *CHOP* promoters. Indeed, we observed reduced induction of H3K14ac in the absence of SGF29 (Fig. 2E), which is in agreement with data obtained in yeast [204]. Together these results reveal a central role for SGF29 in the ER stress survival, ER stress target gene acetylation and induction.

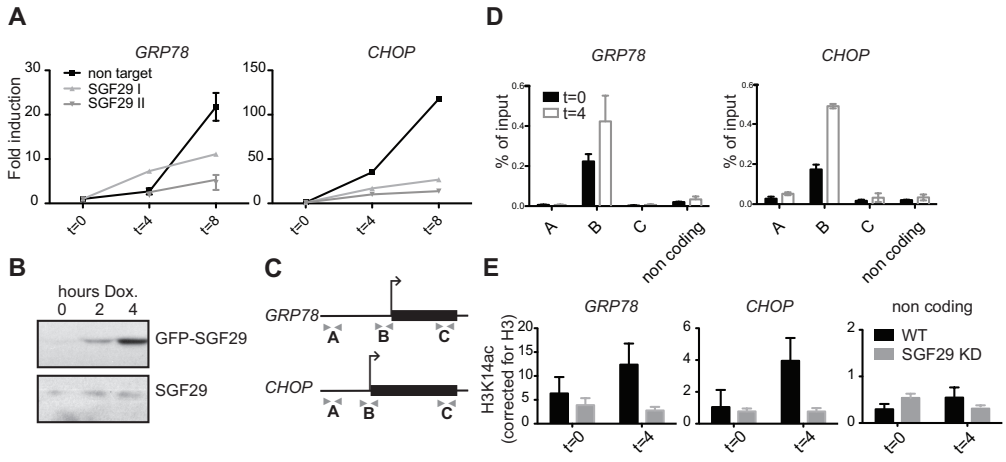


Figure 2: A. Analysis of mRNA expression levels of *GRP78* and *CHOP* by quantitative RT-PCR. Levels were normalized to β-ACTIN and are presented as change compared to a control DMSO-treated sample. Samples were analyzed 4 and 8 h after tunicamycin treatment. B. Immunoblot analysis for doxycycline-inducible GFP-SGF29 and endogenous SGF29. C. Localization of the primer pairs used for ChIP. D. ChIP analysis of GFP-SGF29. On the x-axis are indicated amplicons for the *GRP78* and *CHOP* genes or a non-coding control region. Cells were not treated (t=0, black histograms) or treated for 4 h with tunicamycin (t=4, white bars). Standard deviations represent technical triplicates and similar results were observed in at least three independent experiments. E. H3K14ac ChIP (percentage of input relative to H3 ChIP) at the transcription start site of ER stress target genes and a non-coding control region for 0 and 4 hours treatment with tunicamycin. Standard deviations represent technical triplicates and similar results were observed in at least two independent experiments.

H3K4me3 on ER stress target genes is SGF29-dependent and TAF3 is also required for proper gene induction and cell survival.

H3K4me3 is already present at the *GRP78* and *CHOP* promoters before (t=0) tunicamycin induction. These results indicate that the H3K4me3 mark is established independent of ER stress and prior to transcriptional induction. Recently it was shown that TAF3, the TFIID subunit that binds to H3K4me3 [170] regulates specific sets of target genes upon DNA-damage stress [85].

We therefore hypothesized that ER stress target genes could display a similar dependence on TAF3 and H3K4me3. To test a potential requirement for TAF3 in ER stress, stable TAF3 KD cell lines were created through transduction of two independent shRNA constructs targeting TAF3 (Fig. 3C). Indeed, *GRP78* and *CHOP* induction after tunicamycin treatment was lower after TAF3 knockdown (Fig. 3D). FACS analysis of PI stained cells showed that TAF3 knockdown increased the percentage of apoptotic cells after tunicamycin treatment compared to the control cells (Fig. 3E).

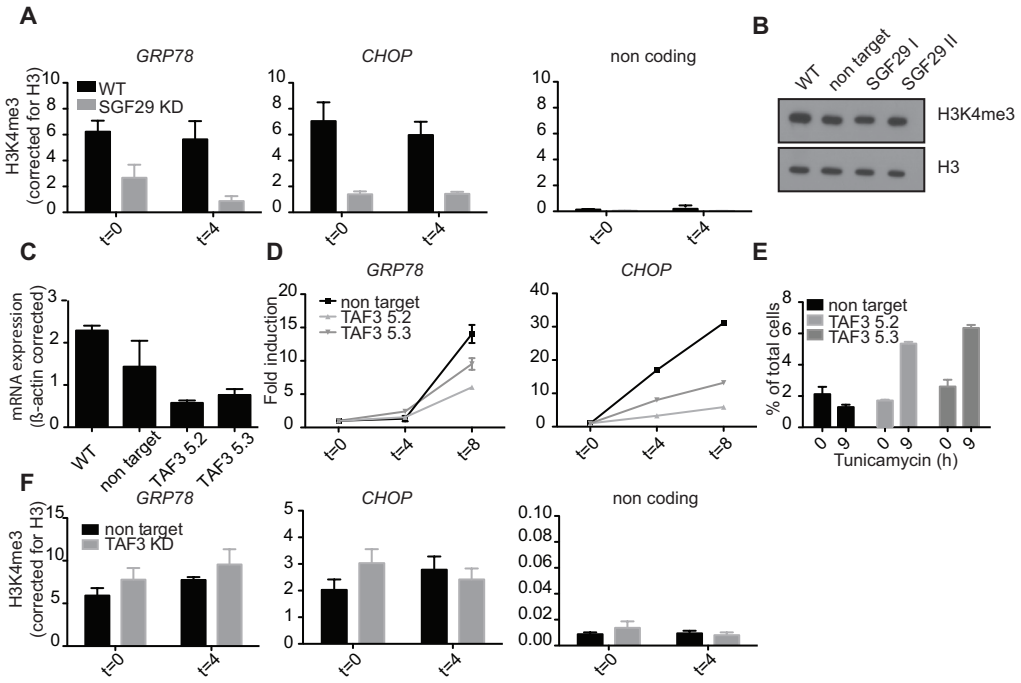


Figure 3: ChIP of H3K4me3 and U2OS cells transduced with lentiviruses carrying TAF3 or non-target control shRNAs. A. H3K4me3 ChIP (percentage of input relative to H3 ChIP) at the transcription start site of ER stress target genes and a non-coding control region for 0 and 4 hours treatment with tunicamycin. Standard deviations represent technical triplicates and similar results were observed in at least three independent experiments. B. Immunoblot analysis of proteins from SGF29 KD cells for global levels of H3K4me3. C. Analysis of mRNA expression of TAF3 by quantitative RT-PCR, corrected for β-ACTIN and standard deviations represent technical triplicates. D. Analysis of mRNA expression levels of *GRP78* and *CHOP* by quantitative RT-PCR. Levels were normalized to β-ACTIN and are presented as change compared to a control DMSO-treated sample. Samples were analyzed 4 and 8 h after tunicamycin treatment. E. Schematic representation of FACS results, increase in propidium (PI) positive cells in TAF3 kd cells after tunicamycin treatment. Samples were measured after 8 hours treatment with tunicamycin or DMSO and o/n recovery. Standard deviations represent technical duplicates. Similar results were observed in two independent experiments. F. H3K4me3 ChIP (percentage of input relative to H3 ChIP) at the transcription start site of ER stress target genes and a non-coding control region for 0 and 4 hours treatment with tunicamycin. Standard deviations represent technical triplicates and similar results were observed in at least two independent experiments.

Interestingly, however, H3K4me3 levels are not affected in TAF3 KD cells and do not significantly change upon ER stress induction (Fig. 3F). These findings suggest that the TAF3-H3K4me3 interaction is not essential for maintaining ER stress response genes in a 'poised' state, but rather has a role in the full transcriptional activation upon ER stress and subsequent cell survival.

SGF29 and the recruitment of H3K4 methyltransferase complexes.

The ChIP results indicate involvement of SGF29 in maintenance of H3K4me3 at ER

stress target genes. The reduction in H3K4me3 could be due to either reduced H3K4 methylation or increased turnover. To explore this further we examined involvement of the SET1/MLL methyltransferase complexes, responsible for the bulk H3K4me3 in mammalian cells [235], at the ER stress target genes. Global levels of ASH2L, RBBP5 and WDR5, core subunits of these methyltransferase complexes [85] showed no decrease in SGF29 KD cells (Fig. 4A). Next, we employed the doxycycline-inducible system to create GFP-ASH2L or GFP-RBBP5 cell lines for ChIP analysis (Fig. 4B). ChIP analysis indicated that ASH2L and RBBP5 associate to the TSS of GRP78 and CHOP (Fig. 4C) at the same location as SGF29 (Fig. 2D). To test the effect of SGF29 KD on the localization of MLL complex on ER stress genes, we knocked down SGF29 in GFP-ASH2L cells (Fig. 4D). SGF29 KD does not affect the levels of doxycycline-induced GFP-ASH2L expression (Fig. 4E). Strikingly, we observed a significant decrease of GFP-ASH2L at the GRP78 and CHOP promoter in the absence of SGF29 (Fig. 4F). These results suggest involvement of SGF29 and SET1/MLL complexes for maintenance of H3K4me3 levels prior to ER stress, thereby prompting rapid transcriptional induction in an ER stress situation.

Discussion

In this study we have uncovered an important role for SGF29, subunit of HAT coactivator complexes SAGA and ATAC, in the human ER stress response. We find that SAGA is important for cell survival after ER stress in multiple cell systems, since SPT20 knockdown leads to lower survival rates (Fig. 1A). SGF29 KD cells also display a decreased resistance to ER stress (Fig. 1D), while SGF29 might be central in recruiting a HAT complex to the H3K4me3 on the stress gene promoters. It is important to mention that SGF29 is part of both SAGA and ATAC. The composition of the HAT module of ATAC is highly similar to the SAGA HAT module and the main difference is that ADA2B is replaced by ADA2A [114,216,236]. SAGA seems to have a preference for promoters, while ATAC is more found on enhancers [210]. Interestingly, ATAC subunits were not identified to bind to H3K4me3 peptides in human cell extracts [237]. However, ATAC was found to bind H3K4me3 peptides in different mouse tissue extracts [205] and *in vitro* ATF6 mediates recruitment of both SAGA and ATAC to the immobilized GRP78 promoter [238]. Based on these studies it is possible that (part of) the effects we observe in SGF29 KD cells are not only dependent on the recruitment of SAGA, but may also involve the ATAC complex. Future experiments including specific subunit analysis of both SAGA and ATAC at multiple ER stress genes will resolve this issue. It has been shown previously that SPT20 recruitment to ER stress gene promoters is clearly linked to the transcriptional induction of these genes [227].

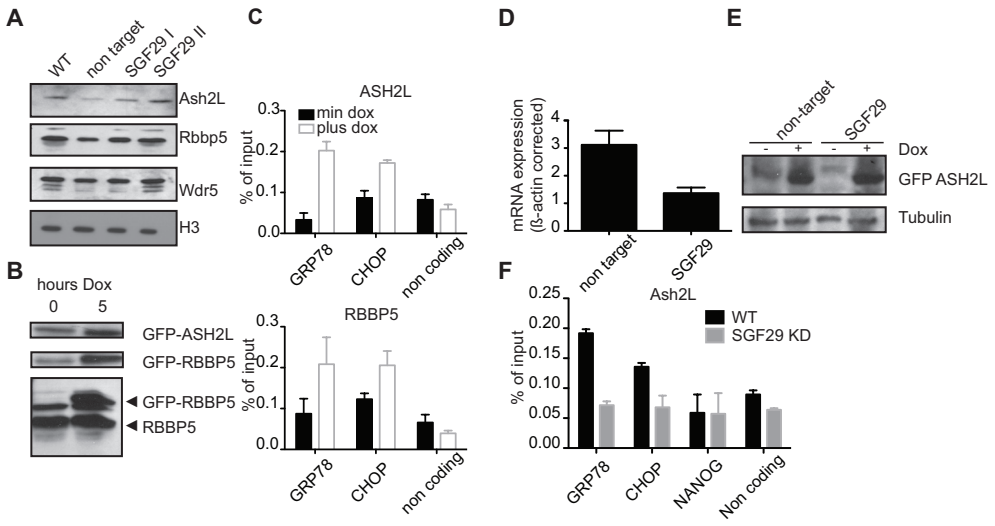


Figure 4: ChIP of ASH2L and RBBP5 at the transcription start site of the *GRP78* and *CHOP* genes. A. Immunoblot analysis of proteins from SGF29 KD cells for global levels of SET1/MLL subunits ASH2L, RBBP5 and WDR5. B. Immunoblot analysis of protein levels of inducible GFP-ASH2L and GFP-RBBP5 and endogenous RBBP5. C. ChIP analysis of GFP-ASH2L and GFP-RBBP5. Standard deviations represent technical triplicates and similar results were observed in at least three independent experiments. D. Analysis of mRNA expression of SGF29 by qPCR, corrected for β-ACTIN and standard deviations represent technical triplicates. E. Immunoblot analysis of GFP-ASH2L protein levels in GFP-ASH2L cell line infected with non target (lane 1 and 2) and SGF29 hairpin (lane 3 and 4). F. ChIP analysis of GFP-ASH2L in control and SGF29 KD lines. Standard deviations represent technical triplicates and similar results were observed in at least three independent experiments.

Their impaired induction upon SPT20 or SGF29 knockdown would affect recovery from ER stress and decrease cell survival. Since SGF29 is responsible for binding of SAGA to H3K4me3 [186], the fact that SGF29 loss results in similar effects as SPT20 knockdown (our results and [204,205]) suggests that SAGA binding to H3K4me3 via its SGF29 subunit has a central role in the function of HAT complexes in the ER stress response. The effects observed on the H3K14ac of the *GRP78* and *CHOP* promoters in SGF29 KD cells underscores this role.

SGF29 forms a mechanistic basis for crosstalk between histone modifications like H3K4 methylation and H3 acetylation and how this is linked to active transcription of ER stress genes. It is interesting to note that the activation of DNA damage genes requires a different HAT complex, but employs a similar mechanism. For these genes the PHD finger-containing ING4 subunit of the HBO1 HAT complex links H3K4me3 and H3 acetylation with transcriptional activation of these genes [239]. H3K4me3 is already present before ER stress treatment on the *GRP78* and *CHOP* promoters and its levels do not increase during transcriptional induction in human U2OS osteosarcoma cells (Fig. 4A). These results mirror previous findings of a constitutive H3K4 methylation of the CHOP locus in human HepG2 hepatoma cells [240]. Similar to the ER stress gene promoters the DNA-damage inducible SMC4 promoter also carries the H3K4me3 mark prior to activation [239]. This implies that binding of SAGA to a promoter is not solely dependent

on SGF29. This is not surprising since SAGA was previously shown to be recruited to promoters by transcription factors such as ATF6 [227]. Furthermore, the TRRAP subunit of SAGA can directly interact with c-myc and other activator proteins [183,241].

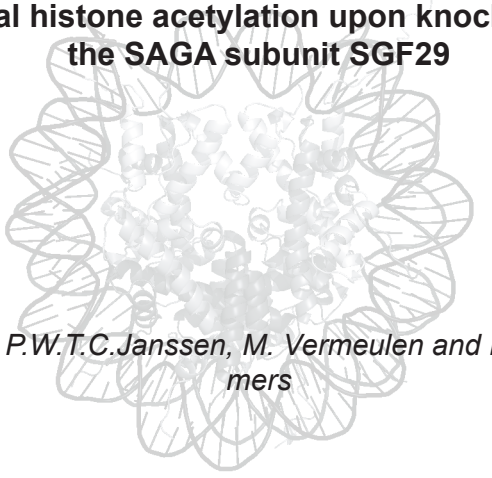
The role of SGF29 in human cells is more complicated than simply anchoring SAGA to H3K4me3-modified nucleosomes as was proposed for yeast SGF29 [204]. Depletion of human SGF29 also results in a reduction of H3K4me3 from ER stress gene promoters. This decrease of H3K4me3 was unexpected since SGF29 is mainly regarded as a downstream effector for this mark. What could be the mechanistic basis for this? Our ChIP analyses indicate involvement of the SET1/MLL methyltransferase complexes as ASH2L and RBBP5 associate to the *GRP78* and *CHOP* promoters also prior to ER stress. The expression of ASH2L and RBBP5 is not reduced in SGF29 KD cells but the association of these SET1/MLL core subunits to the GRP78 and CHOP promoters is affected. Future experiments should be aimed at identifying the specific SET1/MLL complexes involved in ER stress gene transcription and how SGF29 and SAGA are involved in their recruitment. In summary, our results reveal a sophisticated and fine-tuned interplay between distinct chromatin modifying enzymes and the basal transcription machinery, which is required for the prompt activation of target genes in response to stress.

Acknowledgements

We thank Ineke Braakman for useful suggestions on ER stress treatments; Mathijs Vleugel and Astrid Eijkelenboom for critical reading of the manuscript and members of Timmers and Vermeulen laboratories for discussion and suggestions.

Addendum

Quantitative mass spectrometry-based analysis of global histone acetylation upon knockdown of the SAGA subunit SGF29



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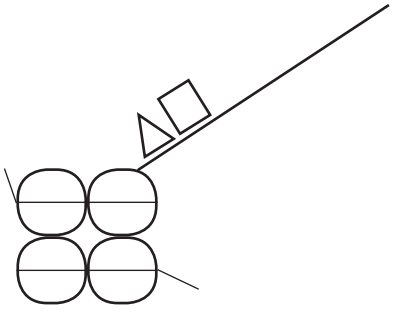

Abstract

Posttranslational modifications on histone tails have important roles in regulating transcription. These modifications are often investigated using antibodies. Major drawbacks of methods involving antibodies are specificity of the antibody and the limited scope of experiments, whereby only one modification can be investigated at a time. Here we set out to determine global histone acetylation levels in U2OS cells by implementing an unbiased approach using state-of-the-art quantitative mass spectrometry. As a proof-of-principle, wildtype cells were compared to cells with knockdown of the histone reader SGF29. SGF29 is part of the co-activator SAGA (Spt-Ada-GCN5) complex and is required for binding of SAGA to trimethylated histone H3 lysine 4 (H3K4me3), a histone modification found on the promoter of active genes. SAGA acetylates lysines-9 and -14 on histone H3 (H3K9ac and H3K14ac). In yeast global histone H3 acetylation has been shown to be dependent on SGF29 binding to H3K4me3 using conventional antibody based methods. In SGF29 knockdown cells we observe a three-fold reduction in the abundance of peptides containing H3K9acK14ac. Evidence for such histone crosstalk between H3K4me3 and H3 acetylation via SGF29 has also been found on ER stress target genes. The unbiased quantitative mass spectrometry approach presented here confirms this crosstalk and forms a useful tool for analysis of chromatin modifications in general.

INTRODUCTION

Eukaryotic transcription is a complex process regulated by a large number of proteins and also the chromatin plays an important role. The basic building block of chromatin is the nucleosome, which is comprised of an octamer of histones wrapped around with 147 bp of DNA. Histones can be post-translationally modified with with various chemical groups, such as methyl and acetyl. The identification of the first histone acetyl transferase (HAT), p55 in tetrahymena, launched a research field wherein posttranslational modifications (PTMs) of histones are investigated in the context of various nuclear processes including transcription [133]. An important modification for transcription is trimethylation of histone H3 (H3K4me3), which is enriched at the core promoters of virtually all actively transcribed genes [79,86,87]. PTMs on histones can function as a binding platform for protein complexes involved in transcription. These proteins are often referred to as reader proteins [75,242]. Important readers for H3K4me3 are the TAF3 subunit of the general transcription factor TFIID and the SGF29 subunit of the co-activator Spt-Ada-GCN5 (SAGA) complex [170,204,205]. SAGA furthermore contains a histone acetyltransferase; GCN5 [103]. Acetylation targets of SAGA are lysine residues -9 and -14 on histone H3 (H3K9ac and H3K14ac), both modifications that correlate with active transcription [101,102,104]. Interestingly, in another study, crosstalk between H3K4me3 and H3K9/K14ac was discovered, with peptides carrying both modifications showing increased binding of TFIID [170]. The link between H3K4me3 and H3K9ac was also found in yeast, where SAGA binding to H3K4me3 via Sgf29 was shown to be required for global H3 acetylation and proper transcriptional induction of the GAL1 promoter [204].

Many investigations concerning histone PTMs make use of antibodies. Major drawbacks of antibody-based approaches are the dependency on specific and sensitive antibodies. Many cases have been reported wherein both aspects fail in one antibody, making it difficult to obtain reproducible and interpretable results. Furthermore, usage of antibodies implies a biased approach, studying preselected PTMs. The aim of this study was two-fold. Primarily we set out to develop a method to investigate histone PTMs in a more unbiased manner. To do so we developed a quantitative mass spectrometry-based method to study levels of histone modifications. The use of stable isotope labeling of cells in culture (SILAC), followed by mass spectrometric analysis provides a quantitative filter and at the same time avoids the obstacles raised by using antibodies. Secondly, we aimed to determine global acetylation levels in SGF29 knockdown cells, also as a proof-of-principle. Interestingly, mass spectrometric analysis of SGF29 knockdown cells revealed a three-fold lower abundance of the peptide carrying both H3K9ac and H3K14ac compared to wildtype cells. This observation shows the power of using this unbiased SILAC mass spectrometric approach to investigate global PTM levels in comparison to antibody based approaches.

MATERIAL AND METHODS

Histone acid extraction and mass spectrometric analysis

For histone acid extraction and nucleosome preparation, aproximately $5*10^6$ U2OS

wildtype and SGF29 knockdown cells were grown in monolayers in plates (Greiner Bio-One, Frickenhausen, Germany) and harvested [245]. Nuclei were prepared using hypotonic lysis [243]. Before extraction, non-labeled and labeled SILAC cells [170] were mixed in equal amounts. Nuclear pellets were resuspended in 2 M HCl and extracted overnight at 4°C. Samples were centrifuged (30 min, 4000 g, 4°C), supernatant was collected and for the pellets acid extraction was repeated for 4 hours at 4°C. Harvested histones were pooled and precipitated with acidified acetone. After centrifugation (30 min, 4000 g, 4°C) histones were resuspended in 25 µl 5.8 M urea, 16% glycerol, 4.8% ß-mercaptoethanol. Tryptic peptides were prepared by filter aided sample preparation (FASP) [244]. Next peptides were separated on an EASY nLC-system (Thermo Scientific, Germany), using a shallow 220 min, 7-32% acetonitrile gradient followed by a sharp increase to 98% acetonitrile. Mass spectra were recorded on a LTQ-Orbitrap-Velos mass spectrometer (Thermo Scientific, Germany), selecting the 10 most intense precursor ions of every full scan for fragmentation using higher-energy collisional dissociation (HCD). Raw data was analyzed using Maxquant software (version 1.2.2.5) and searched against the human IPI database (version 3.68) using the Andromeda search engine [230] with standard settings. N-terminal acetylation and acetylation of lysines were selected as variable modifications during the database search. The maximum number of mis-cleavages was set to 5.

RESULTS AND DISCUSSION

SGF29 is important for global histone H3 acetylation.

In order to develop an unbiased method to investigate histone modifications we determined the global histone acetylation levels in wildtype and SGF29 knockdown cells using SILAC-based quantitative proteomics. Wildtype and SGF29 knockdown cells [245] were labelled 'light' and 'heavy' using $^{12}C^{14}N$-lysine/$^{12}C^{14}N$- arginine and $^{13}C^{15}N$-lysine/ $^{13}C^{15}N$–arginine, respectively. Equal amounts of light SGF29 knockdown and heavy control cells were mixed after which histones were extracted (forward experiment). As a control, a label swap experiment was performed (reverse experiment) (Figure 1A). In these experiments (modified) histone peptides that are not affected by SGF29 knockdown will have a 1:1 ratio, whereas (modified) histone peptides that are altered by SGF29 knockdown deviate from this (Figure 1A). As shown in Figure 1B, the peptide corresponding to histone H3 acetylated on both lysine-9 and -14 (KacSTGGKacAPR) has a ratio of 2.7 in the forward and 0.3 in the reverse experiment. These results indicate an approximately three times lower abundance of this peptide in SGF29 knockdown cells compared to wildtype control. In contrast, an unmodified histone H3 peptide (aa 73-84) as well as a doubly acetylated H2B peptide, (KacAVTKacAQK; aa 16-23) showed an approximately 1:1 ratio in both experiments (Figure 1B). For completeness we also show the corresponding MS spectra, from which the ratios for the unmodified H3.3. peptides and H3K9ac/K14ac were derived (Figure 2).

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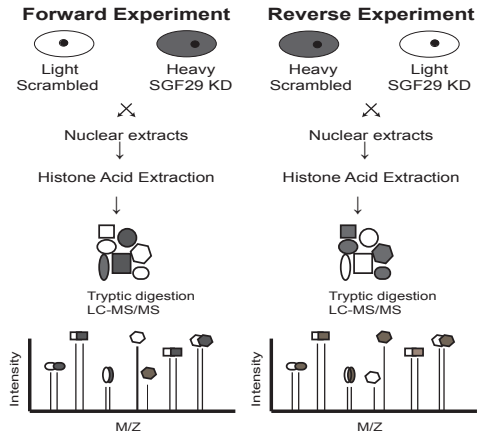


B

label	protein	peptide	H/L
Forward	H3.3.	EIAQDFKTDLR	1.0
	H3K9,K14ac	KacSTGGKacAPR	0.3
	H2BK17ac,K21ac	KacAVTKacAQK	1.1
Reverse	H3.3.	EIAQDFKTDLR	0.7
	H3K9,K14ac	KacSTGGKacAPR	2.7
	H2BK17ac,K21ac	KacAVTKacAQK	0.8

Figure 1: Global acetylation levels of histone H3K9,K14ac are decreased in SGF29 knockdown cells. SGF29 knockdown cells were analyzed for differences in chromatin composition. A. Schematic representation of the SILAC-based experiment to determine histone modifications. B. Ratios of detected histone peptides of wildtype and SGF29 knockdown cells. Experiments were done in duplicate with a label swap between wildtype and SGF29 knockdown cells. Forward depicts the ratio of SGF29 knockdown over wildtype control and reverse is vice versa.

The results indicate that similar to yeast [204], human SGF29 is involved in maintaining global acetylation levels of histone H3K9 and K14. This is in line with our previous observations at the individual gene level, since SGF29 knockdown decreased H3K14ac at the promoter of ER stress target genes [245]. Taken together, these results indicate that acetylation of specific histone H3 residues both at a global level and at the ER stress loci is affected by depletion of SGF29, the reader for H3K4me3. This shows a conserved function of SGF29 from yeast to human in crosstalk of H3K4me3 and acetylation of H3 [204]. Furthermore we show that this method can be a useful tool to investigate PTMs in an unbiased manner. The analysis can easily be extended towards other modifications such as ubiquitin for which the data will also be available from the same experiment. The approach is suitable for measuring which PTMs change at a global level during general perturbations, for instance upon stress treatment or during other biological processes such as differentiation. The use of SILAC allows for a highly quantitative filter. The approach therefore has wide-ranging potential for research into chromatin and its role in cellular processes.

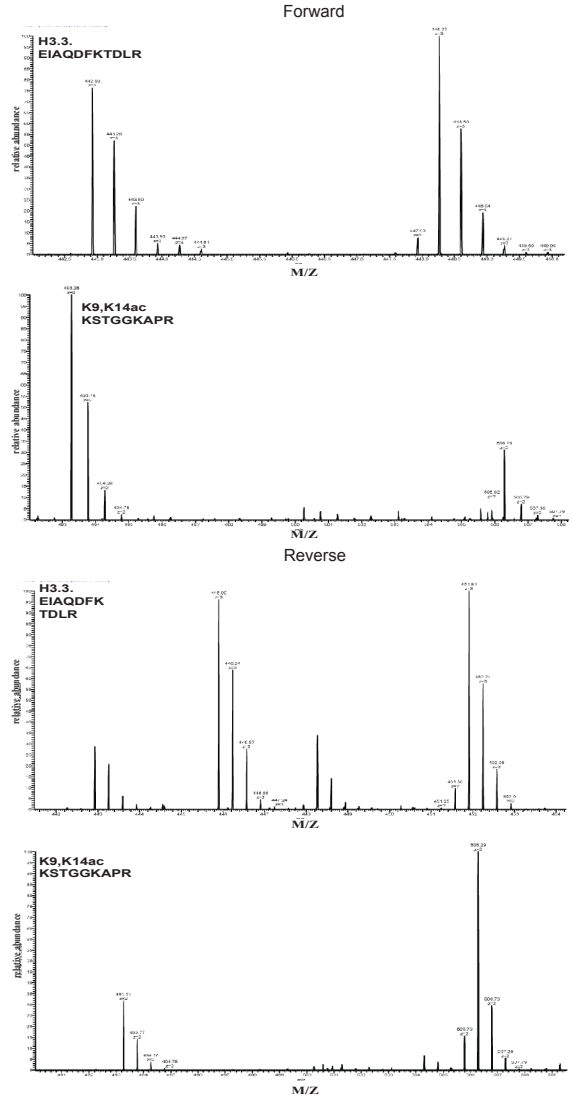
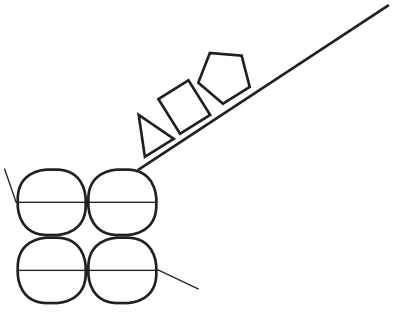


Figure 2: Mass spectra. Representative mass spectra of SILAC pairs of indicated histone peptides in forward (upper two panels) and reverse (lower two panels) experiments from wildtype control and SGF29 knockdown cells.

Chapter 3

siRNA screening reveals the HBO1 complex as an acetyltransferase for histone H3 lysine four

A.W.Schram, F.M.A. van Schaik and H.Th.M. Timmers



Abstract

Recently, it was shown that lysine four of histone H3 (H3K4), in addition to being a target for methylation, can also be acetylated. Here we set out to identify the histone acetyltransferase (HAT) responsible for the acetylation of histone H3 lysine- 4 (H3K4ac) in human cells. A siRNA screen involving all known (human) HATs and histone deacetylases (HDACs) identified HBO1, a component of the HBO1 complex, as a possible HAT for H3K4. Interestingly, multiple PHD fingers present in the HBO complex subunits JADE1 and ING4/5 link the complex to trimethylated lysine- 4 and lysine- 36 on histone H3 (H3K4me3 and H3K36me3). Additional knockdown experiments using different siRNAs targeting HBO1 and its complex subunits showed reduced H3K4ac upon HBO1 and JADE1 knockdown. Overexpression of HBO1 and JADE1 had the opposite effect. Although a purified HBO1/JADE1 complex failed to acetylate H3K4 on pre-acetylated histone peptide tails *in vitro*, our results nevertheless suggest an important role for the HBO1 complex in acetylation of H3K4.

Background

Eukaryotic transcription is a complex process regulated by many different factors including chromatin. The basic building block of chromatin is the nucleosome, consisting of 147 bp DNA wrapped around an octamer of histone proteins. The N-terminal tails of these histones are subjected to post-translational modifications [246] and the first direct link between chromatin and transcription was found through the identification of transcriptional cofactor GCN5 as a histone acetyl transferase (HAT) [133]. Hereafter the functional relationship between histone acetylation and transcription was further established. Histones cannot only be acetylated on their N-terminal tails, but also in their globular domain [247,248]. Addition of an acetyl group to the ε-group of the terminal amine of lysine creates N-ε-acetyl-L-lysine and neutralizes the positive charge of lysine [125]. The role of acetylation in transcription is two-fold. Firstly by the neutralization, the negatively charged DNA is less tightly bound around the nucleosome leading to weaker intra-nucleosomal interactions. This increases the accessibility to the DNA for other nuclear factors, such as transcription factors [249]. Secondly a family of reader proteins called the bromodomain (BRD) family recognizes acetylated histones. The BRD domain was first described in the early 1990s and occurs in at least humans, Drosophila and yeast [250]. Currently 56 unique BRD proteins have been identified in humans, often as part of multisubunit complexes and many mediate a role in transcription [250,251]. This includes TAF1, a subunit of the basal transcription factor TFIID, which contains a bromodomain that binds to diacetylated histone H4 tails found on active promoters [156], illustrating the role of acetylated histones as scaffolds for transcription.

Genome-wide localization studies have linked acetylation of lysine-9 and -14 (H3K9ac and H3K14ac) of histone H3 to actively transcribed genes [101,102,104]. Another histone modification linked to transcription activation is trimethylation of histone H3 at lysine 4 (H3K4me3). This modification is enriched around the transcription start site (TSS) of active genes and was shown to recruit TFIID via its TAF3 subunit [77,85,170]. Next to their co-occurrence on active genes, H3K4me3 and H3K9/K14ac were also shown to cooperatively improve the binding of both TFIID and the co-activator complex SAGA [170,205]. Such co-operative binding to two modifications is one of the forms of histone crosstalk. The crosstalk of H3K4me3 and histone H3 acetylation has been shown to have a role in activation of transcription [149,204,245].

Several studies have also investigated acetylation of the lysine-4 residue on histone H3 (H3K4ac). In the fission yeast Schizosaccharomyces pombe (*S. pombe)* this modification was found to be important for formation of heterochromatin and the ortholog of HIV tat-interacting protein 60 (Tip60) was shown to be the HAT [252]. In contrast in mammalian systems and in the yeast Saccharomyces cerevisiae (*S. cerevisiae*) H3K4ac was localized to actively transcribed genes both on promoters and in their coding regions [253,254]. More specifically ChIP-chip analysis revealed that on the promoter, H3K4ac is present just upstream of H3K4me3 [253,254]. This suggested a role for H3K4ac in transcription. In *S. cerevisiae* Gcn5 and Rtt109 were identified to be the HATs for H3K4ac. Interestingly single and double deletion strains of these HATs did not alter global levels of H3K4me3, suggesting that acetylation of H3K4 is more than a simple competition for the substrate [253].

We set out to determine the HAT responsible for H3K4ac in humans to further explore the role in regulation of transcription. In mammals a total of 16 HATs have been identified, divided over several families [249]. The distinction between families is based on structural homology between members [255]. Most HATs operate both alone and in multisubunit complexes. These complexes often also contain subunits binding to acetylated or otherwise modified residues on the histone tails.

Here we have performed a screen, using a small siRNA library containing all known human HATs and HDACs (histone deacetylases) to identify the HAT responsible for *in vivo* acetylation of H3K4. Knockdown of HAT bound to ORC1 (HBO1) and of TIP60 decreased the global levels of H3K4ac. Validation by knockdown of TIP60 and HBO1 in a different cell line suggested, together with a previously described role in transcription, that HBO1 was the most likely candidate to acetylate H3K4 [253]. Subsequent knockdown and overexpression experiments strongly suggest that HBO1 in a histone acetyltransferase for H3K4 *in vivo.*

Material and methods

Plasmids.

Coding sequences for HBO1 and JADE1S and JADE1L were PCR amplified from existing plasmids using Phusion polymerase (Finnenzymes) and cloned into the Gateway Entry System (InVitrogen) modified pMT2_HA for N-terminal fusion of the HA tag or into pBABE_FLAG_His for N-terminal fusion of the FLAG and 6XHis tag. The human HBO1 cDNA was a kind gift from Masayoshi Iizuka [256]. cDNA clones for JADE1S and JADE1L were obtained from Imagene (clone numbers SC111825 and SC111826).

Cell culture, siRNA mediated knockdown and overexpression.

U2OS and MCF7 cells were maintained as monolayers in plates (Greiner Bio-One, Frickenhausen, Germany) in normal glucose Dulbecco's modified Eagle's medium (DMEM, InVitrogen), containing 10% fetal bovine calf serum (FBS, Lonza) and 1% penicillin/streptavidin and L-glutamine (Lonza). 293T cells were maintained as monolayers and subsequently adapted for suspension growth by culturing in Minimum Essential Medium (MEM, InVitrogen) plus 10% fetal bovine serum (Lonza), 1% pen/strep, 1% L-glutamine, 1% non essential amino acids, 1% sodiumpyruvate (Lonza).

siRNA mediated knockdown was performed by Fugene 6 (Roche) transfection with siRNA smartpools and/or individual siRNAs (Thermo Scientific) against proteins defined in the results section in MCF7 cells. As controls, cells were transfected with siRNA targeting GAPDH or with a non-target control siRNA. 48 hours after transfection cells were washed with PBS and harvested by sample buffer (50 mM Tris HCl pH 6.8, 10% glycerol, 2.5 % SDS, 1% BFB and 5% 2-mercaptoethanol) lysis. Lysates were boiled 5 minutes at 95°C, centrifuged (5 min, 16000 g, 4°C) and supernatants were transferred to a new eppendorf tube.

Transient overexpression experiments were performed by Fugene 6 (Roche) trans-

fection of HBO1 and JADE1 expression constructs into U2OS cells. After 48 hours of transfection, cells were washed once with PBS and harvested by sample buffer lysis.

SDS-PAGE, immunoblot analysis and antibodies.

Whole cell lysates were analyzed by immunoblotting according to standard procedures. Lysates were run on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Molecular mass markers were obtained from New England Biolabs. Antibodies used were anti-H3 (Abcam Ab1791), anti-H3K4ac (Millipore #07-539), anti-acetyl-Histone H4 (Millipore #06-866), anti-HBO1 (Santa-Cruz sc-13283), anti-GAPDH (Gift from Dr. Bos), anti-HA (tissue culture sup) and anti-FLAG (Sigma F-3165).

HBO1/JADE1S affinity purification.

Extraction of $2*10^6$ asynchronous 293T cells transfected with pMT2-HA HBO1 and pBABE_FLAG_His JADE1S was done by hypotonic lysis [257]. The nuclei were extracted overnight at 4°C and subsequently centrifuged at 168000 g for 45 minutes at 4°C. A 2 mL Nickel-Nta column was equilibrated with buffer C300 (300mM NaCl, 20 mM HEPES-KOH pH 7.9, 2 mM MgCl2, 0.2 mM EDTA 0.1% NP40, 0.5 mM DTT and complete protease inhibitors (Roche)). The sample was loaded, washed with 9 column volumes buffer C300 and subsequently with 9 column volumes HAT buffer (50 mM Tris-HCl pH 8.0, 10% glycerol, 0.1 mM EDTA, 1 mM DTT and complete protease inhibitors (Roche)) with 10 mM Imidazol. The complex was eluted into 20 fractions by HAT buffer containing 300 mM Imidazol. Washes and elution were all performed at 4°C.

In vitro HAT assays.

Affinity purified JADE1S-HBO1 complex was incubated with 2.5 µM histone peptide tails bound to 20 µl streptavadin coated beads (Dynal, InVitrogen) and 5 µl [3H]AcetylCoA (Perkin-Elmer) in 25 µl HAT buffer for 30 minutes at 37°C. Reactions were stopped by addition of 1% SDS and heating the reaction mixes for 5 minutes at 65°C. Reactions were washed three times with 1 ml HAT buffer. Subsequently reactions were dissolved in 5 ml scintillation fluid (Ecolite+, MP Biomedicals) and measured on a Packard 1600TR liquid scintillation analyzer.

Results

siRNA screen in MCF7 cells to identify a HAT for H3K4.

Experiments in yeast have indicated that acetylation of H3K4 might play a role in transcription initiation [253]. To investigate the role of H3K4ac in mammalian cells, we set out to identify the HAT. First the H3K4ac antibody was characterized to determine specificity for recognition of H3K4ac. Histone peptide tails carrying different modifications and a biotin label were bound to streptavidin, run on a gel and transferred to a membrane. The blot was subsequently probed with the H3K4ac antibody. The H3K4ac antibody recognized H3K4ac (Figure 1A, lane 1). Some weak cross reactivity towards H3K9ac was detected (Figure 1A, lane 2), indicating that the antibody might also recognize H3K9ac,

although to a lesser extent than H3K4ac. This antibody does not recognize H3K14ac, other acetylated lysines or unmodified and methylated residues (Figure 1A). Interestingly phosphorylation of the neighboring threonine, H3T3, decreased the H4K4ac recognition (lane 9), suggesting steric hindrance of this modification for the binding of the antibody.

Having established the specificity of the antibody, MCF7 cells were transfected with a siRNA library targeting all known human HATs and 18 HDACs. After 48 hours of transfection, cells were harvested by whole cell lysis and analyzed by immunoblotting. As a control for knockdown efficiency MCF7 cells were transfected with siRNA targeting GAPDH, which left H3K4ac levels unaltered, but decreased GAPDH protein levels (Figure 1B). Also when a non-targeting siRNA was transfected, to control for off target effects caused by siRNA knockdown, H3K4ac levels were unaffected (Figure 1B). Knockdown of individual HDACs did not increase the level of H3K4ac, likely due to redundancy [258]. Interestingly knockdown of either TIP60 or HBO1 reduced global H3K4ac levels (Figure 1B). TIP60 and HBO1 are members of the Moz, YBF2, Sas2p, Tip (MYST) HAT family, all members being highly conserved and associated with a variety of nuclear processes [259]. Strikingly, the primary sequence of HBO1 is highly homologous to TIP60, which was identified in S. pombe as the HAT for H3K4 [252]. In mammalian cells however HBO1 is the only member that was shown to acetylate histone H3 [260]. TIP60 was described to acetylate histone H4 and H2A, also on enhancer elements [259,261,262]. To test the generality of the finding that knockdown of both TIP60 and HBO1 decreased H3K4ac, the experiment was repeated in U2OS cells also as biological duplicates. In these cells only knockdown of HBO1 and not of TIP60 was found to decrease H3K4ac (Figure 1C). Taken together these results suggest that HBO1 is a good candidate to acetylate H3K4, thus we focused on HBO1 in the following experiments.

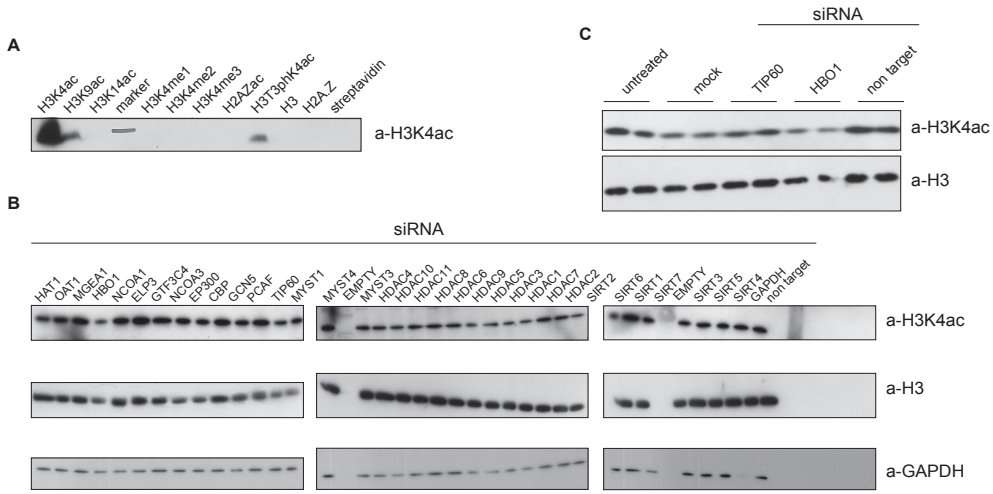


Figure 1: HAT siRNA library screen in MCF7 cells. A. Immunoblot analysis of H3K4ac antibody specificity for different histone peptide tails: H3 (1-17) and H2A.Z (1-17). Modified residues are indicated above the blot. Amounts of peptide tails used were calculated. B. Immunoblot analysis of whole cell extracts from MCF7 cells transfected with siRNAs targeting all known human HATs, HDACs, GAPDH and non-targeting control. C. Immunoblot analysis of whole cell extracts from U2OS cells transfected with siRNAs against the indicated mRNAs.

HBO1 is a HAT for H3K4 in vivo.

The siRNA screen and subsequent knockdown study suggested HBO1 as a candidate to acetylate H3K4 *in vivo*. In the original screen a smartpool containing four different siRNAs targeting HBO1 was used. In the following experiment two independent siRNAs and also the original smartpool were transfected separately into MCF7 as well as in U2OS cells. Both the smartpool as well as the independent siRNAs mediated efficient knockdown of HBO1 (Figure 2A). Furthermore both in MCF7 and U2OS cells HBO1 knockdown resulted in decreased levels of H3K4ac (Figure 2A). These results indicate that HBO1 has a role in the acetylation histone H3K4 *in vivo.*

HBO1 is found in two slightly different HBO1 complexes. A smaller complex comprised of only HBO1 and JADE1S and a larger complex containing a different splice variant JADE1L together with HBO1, EAF6 and ING4 or ING5 [152]. It was therefore determined whether the other subunits of these HBO1 complexes are involved in the acetylation of H3K4. We found that knockdown of JADE1 or EAF6 decreased H3K4ac levels. In contrast ING4 and/or ING5 knockdown did not reduce H3K4ac levels (Figure 2B). This is in line with the previously described acetylation activity of HBO1 on histone H4, which is both *in vivo* and *in vitro*, enhanced by the presence of JADE1 [260].

The observation that HBO1 and JADE1 knockdown results in decreased levels of H3K4ac led us to determine whether HBO1 and JADE1 overexpression would have the opposite effect. Therefore U2OS cells were transfected with expression constructs for HBO1 wildtype and a catalytic mutant (E508Q) [260]. JADE1S and JADE1L were co-transfected to enhance HBO1 HAT activity. Immunoblot analyses of cell lysates showed that expression of HBO1 WT and not the catalytic mutant increased the levels of H3K4ac (Figure 2C). Additionally, overexpression of JADE1S or JADE1L further boosted H3K4ac levels in combination with HBO1 overexpression (Figure 2C). JADE1S shows a slightly higher increase of H3K4ac than JADE1L. Acetylation of H4 was measured by an antibody, which recognizes four acetylated lysines (5,8,12,16) on H4. With this sensitive antibody only a small increase of acetylation was detected when HBO1, JADE1S and JADE1L alone or in combination were overexpressed (Figure 2C). Thus overexpression of HBO1 alone and in combination with either JADE1S or JADE1L increases acetylation of H3K4.

Purified JADE1S/HBO1 acetylates H3 peptide tails.

The *in vivo* experiments indicated a direct or indirect role for HBO1 in acetylation of H3K4. Next *in vitro* HAT activity of purified JADE1S/HBO1 complex on histone peptide tails was tested.Figure 3A depicts the workflow for purification of the complex. Human 293T cells, adapted to suspension growth were transfected with expression constructs for HBO1 WT or catalytic mutant tagged by 6xHis and FLAG.

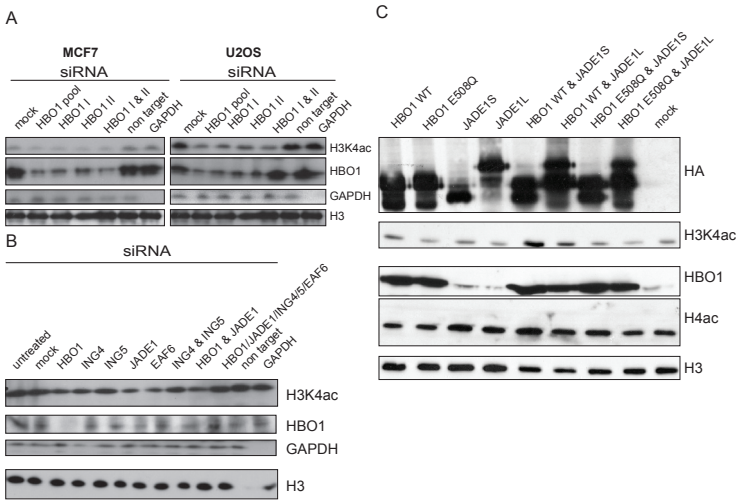


Figure 2: Validation of HBO1 as HAT for H3K4. A. Immunoblot analysis of whole cell extracts from MCF7 and U2OS cells transfected with siRNA pool (4x individual, used in figure 1B) individual siRNAs targeting HBO1, control GAPDH siRNA or non-target siRNA. B. Immunoblot analysis of whole cell extracts from U2OS cells transfected with siRNA targeting the individual HBO1-complex subunits and as a control GAPDH and non-target siRNA. Note that on blot probed with H3, last lane was shifted one slot. C. Immunoblot analysis of whole cell extracts from 293T cells transfected with various HBO1 and JADE1 expression plasmids.

In addition, HA-tagged JADE1S was co-transfected. After transfection cells were transferred to a bioreactor and cultured in 1-liter suspension for two days (Figure 3A). Subsequently the His-HBO1/HA-JADE1S complex was purified on a nickel agarose column and eluted into fractions that contained both HBO1 (a-FLAG) and JADE1S (a-HA) (Figure 3B). The peak fractions were then used in an *in vitro* HAT assay, using synthetic histone peptide tails as substrate. As expected [260] a strong HAT activity could be detected using H4 (1-17) peptide with the WT and not the mutant JADE1S/HBO1 complex (Figure 3C). There was some residual acetylation in case of the catalytic mutant, likely due to contaminating HAT activity in the elution. As a control we used a pre-acetylated H4 peptide (H4K5ac/K8ac/K16ac) and the pre-acetylation strongly reduced HAT activity on the histone tail (Figure 3C). The results indicate that we have purified an active HBO1 complex that acetylates the H4 tails as expected [260].

Next HAT activity of the purified complexes on synthetic H3 peptide tails (1-17) was tested. Here it was found that the wildtype HBO1/JADE1S complex, but not the catalytic mutant displays HAT activity on the H3 tail (Figure 3D). The HAT activity on H3 peptides was lower than on H4 peptides (Figure 3D and C). It seems that all three lysine residues can be acetylated by the JADE1S/HBO1 complex when no or single pre-acetylated peptides are used as substrate (compare lane 1-4). However on double pre-acetylated tails, only H3K14 can be acetylated by JADE1S/HBO1 (compare lane 5 to 6/7), although this activity is 50% reduced. This indicates that *in vitro* presence of H3K9acK14ac inhibits acetylation of H3K4 by JADE1S/HBO1 and the presence of H3K4ac/K14ac inhibits acetylation of H3K9. Acetylation of H3K14 is only partially inhibited by the presence of both H3K9ac and H3K4ac (lane 5). On a

completely pre-acetylated tail no HAT activity is detected (lane 8). Thus *in vitro* the direct or indirect targets of JADE1S/HBO1 are not entirely clear, but include more than H3K4 alone. As is discussed below, the *in vivo* observed activity of JADE1S/HBO1 towards H3K4 might therefore also involve other subunits or complexes.

Discussion

In human cells and yeast, ChIP studies revealed that H3K4ac is mainly found around the TSS of actively transcribed genes and localizes just upstream of H3K4me3 [253,254]. Our siRNA-based screen of MCF7 cells suggests HBO1 as a potential HAT for lysine-4 on histone H3. Subsequent validation by knockdown using different siRNAs targeting HBO1 or overexpression using HBO1 and/or JADE1 validate that HBO1 and JADE1 are involved in H3K4ac *in vivo*, but an indirect role cannot be ruled out. The acetylation of H4 or non-histone substrates by HBO1 might be required for acetylation of H3K4. Furthermore additional HATs could be involved, perhaps also other MYST family members. Finally more experiments are required to elucidate the exact role of the other subunits of HBO1 complexes in the acetylation of H3K4.

HBO1 belongs to the MYST family, defined by a highly conserved HAT domain named MYST. This domain contains an acetyl CoA binding domain and an atypical zinc finger domain [249]. In mammals the family consists of five members HBO1, TIP60, MYST1, MYST3 and MYST4 [263]. MYST family proteins have a role in the regulation of many different cellular processes varying from apoptosis, cell cycle, DNA replication to transcription [261]. The HAT activity of most MYST family members is directed towards histone H4 [261]. Interestingly, HBO1 was reported to expand its HAT activity towards histone H3K14 [259,264,265]. This HAT protein is found in two related HBO1 complexes [260]. Splice isoforms of the JADE1 protein determine the composition of the complex.Presence of JADE1L protein specifies assembly of ING4 or 5 and EAF6 into the complex, while JADE1S solely co-purifies with HBO1 [152]. JADE1 itself was previously identified to be a transcriptional activator, and its activity is dependent on its PHD fingers [260,266]. The JADE1L/HBO1 complex contains three PHD fingers; two in JADE1 and one in ING4/5, the JADE1S/HBO1 complex consists of two PHD fingers both in JADE1. The second PHD finger of both JADE1S and JADE1L is required for general binding to the chromatin, both *in vivo* and *in vitro*. The first PHD finger in JADE1 and the PHD finger of ING4 and ING5 respectively, direct the binding of their complexes towards H3K36me3 and H3K4me3 [152].

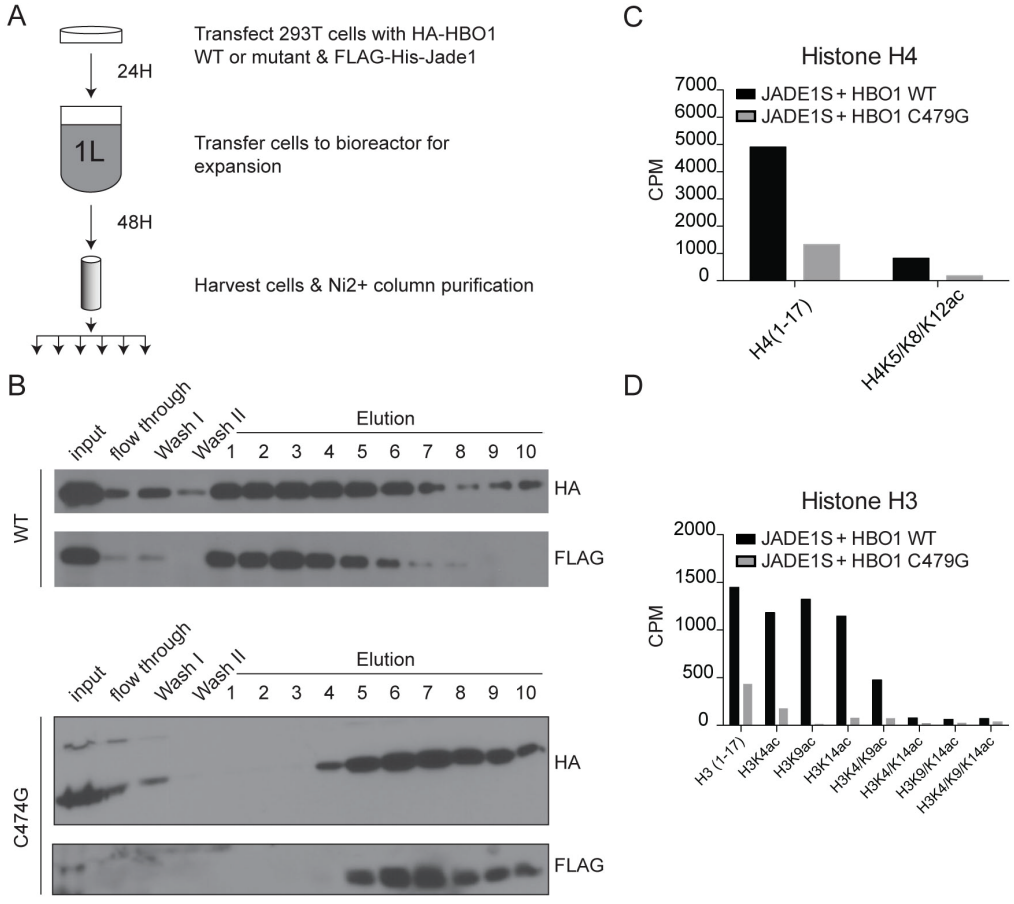


Figure 3: Analysis of *in vitro* HAT activity of HBO1 using histone H3 peptide tails as substrates. A. Schematic representation of HA-HBO1/FLAG-JADE1 complex affinity purification. B. Immunoblot analysis of purified HA-HBO1/FLAG-JADE1 complex. C. Bar graph representation of *in vitro* HAT assay with purified HA-HBO1/FLAG-JADE1 complex on various histone H4 peptide tails. D. Bar graph representation of *in vitro* HAT assay with purified HA-HBO1/FLAG-JADE1 complex on various histone H3 peptide tails.

As a consequence the HAT activity of JADE1L/HBO1 is H3K4me3 dependent, while JADE1S/HBO1 probably has a role in H3K36me3 dependent acetylation of histone H3 *in vivo* [152]. H3K36me3 is found in the coding regions of active genes [77]. Genome-wide localization of tagged HBO1 showed its presence in both the coding regions as well as around the TSSs, which is concomitant to the modification dependent binding specificities of both HBO1 complexes [152]. At this point genome-wide localization of JADE1S versus JADE1L has not been investigated. However since biochemical data indicate that ING4/5 directs HBO1 towards the TSS, this suggests that JADE1L will also localize there. HBO1's role in transcription regulation is also indicated by the fact that together with JADE1, it can enhance transcription in a luciferase assay [260,266]. Furthermore, it has been reported that HBO1 by binding to the progesterone receptor can induce transcription [267]. Together with studies in yeast and mammalian cells,

which showed that H3K4ac is present just upstream of H3K4me3 [253] we hypothesize that JADE1L/HBO1 binds to H3K4me3 via ING4/5 and subsequently acetylates H3K4 in the promoter region to initiate transcription. This might also explain the somewhat confusing results in the transient overexpression experiment, where JADE1S/HBO1 showed a higher increase in H3K4ac than JADE1L/HBO1. The abundance of nucleosomes with H3K36me3 in the genome exceeds the amount of nucleosomes with H3K4me3 [268,269]. Therefore when global H3K4ac is monitored, as was done in this experiment, a higher increase is expected when JADE1S is overexpressed in combination with HBO1. Locally the larger JADE1L/HBO1 complex might increase H3K4ac more that the smaller JADE1S/HBO1 complex, but ChIP experiments after overexpression of both complexes should be performed to confirm this. In our subunit knockdown experiment JADE1 knockdown also decreased H3K4ac levels, the siRNA used however affects both JADE1S and JADE1L expression.

Interestingly the lack of H3K4 acetylation in the *in vitro* HAT assay on pre-acetylated tails, suggests that acetylation of other residues on the H3 tails influences the HAT activity of at least the smaller JADE1S/HBO1 complex. The purified complex does have strong HAT activity towards histone H4. JADE1S/HBO1 acetylated H3K14 in our *in vitro* assay. This is in line for what was found in embryonic stem cells, where HBO1 is responsible for the bulk H3K14 acetylation and for the expression of a wide range of developmental genes [270]. Also overexpression of JADE1 increased H3K14ac on a promoter in HeLa cells and a purified mixture of HBO1/JADE1S/L complexes acetylated H3K14ac on free histones *in vitro* [152,265]. Our *in vitro* results however are not conclusive on whether H3K4 is also a direct target of the JADE1S/HBO1 complex. The presence of H3K9ac in combination with H3K14ac seems to prevent H3K4 acetylation. We did not test the effect of HBO1 knockdown on H3K9ac or H3K14ac levels *in vivo.* The localization of H3K9ac and H3K14ac overlaps more with H3K4me3 compared to H3K4ac [101,253], therefore the inhibiting effect of H3K9ac/K14ac on the acetylation of H3K4 might serve to prevent this at locations where H3K4 should be trimethylated.

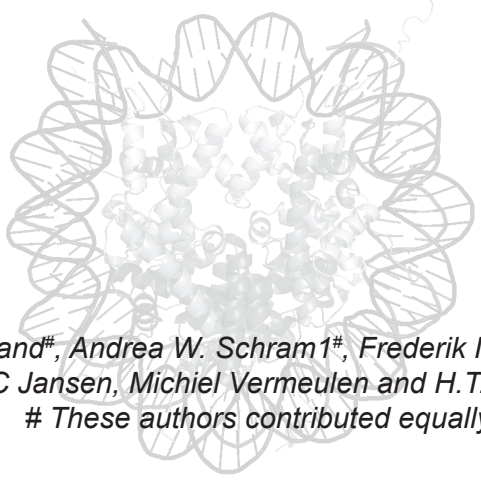
Thus we hypothesize that the role of HBO1 in transcription can be linked to H3K4 promoter acetylation by the larger JADE1L/HBO1 complex binding to H3K4me3. Further experiments however are required to elucidate the exact role of both HBO1 and H3K4ac in transcription.

Acknowledgements

We wish to thank the Timmers and Vermeulen laboratories for useful discussions and suggestions. We thank Klaas Mulder for reagents and discussions and Maria Koster for critical reading of the manuscript.

Chapter 4

Multivalent engagement of TFIID to nucleosomes



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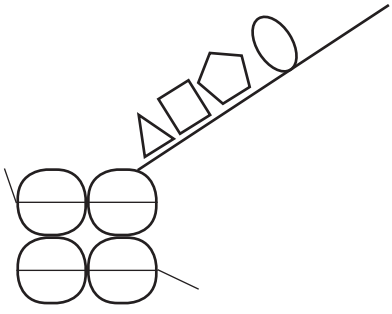
PLoS One. 2013 Sep 11;8(9):e73495.



Abstract

The process of eukaryotic transcription initiation involves the assembly of basal transcription factor complexes on the gene promoter. The recruitment of TFIID is an early and important step in this process. Gene promoters contain distinct DNA sequence elements and are marked by the presence of post-translationally modified nucleosomes. The contributions of these individual features for TFIID recruitment remain to be elucidated. Here, we use immobilized reconstituted promoter nucleosomes, conventional biochemistry and quantitative mass spectrometry to investigate the influence of distinct histone modifications and functional DNA-elements on the binding of TFIID. Our data reveal synergistic effects of H3K4me3, H3K14ac and a TATA box sequence on TFIID binding *in vitro*. Stoichiometry analyses of affinity purified human TFIID identified the presence of a stable dimeric core. Several peripheral TAFs, including those interacting with distinct promoter features, are substoichiometric yet present in substantial amounts. Finally, we find that the TAF3 subunit of TFIID binds to poised promoters in an H3K4me3-dependent manner. Moreover, the PHD-finger of TAF3 is important for rapid induction of target genes. Thus, fine-tuning of TFIID engagement on promoters is driven by synergistic contacts with both DNA-elements and histone modifications, eventually resulting in a high affinity interaction and activation of transcription.

Introduction

RNA polymerase II (pol II) mediates the transcription of all protein coding genes in eukaryotic cells. Activation of transcription by sequence-specific DNA-binding transcription factors leads to recruitment of basal transcription factors to core promoters that together establish the pre-initiation complex (PIC) [271]. PIC assembly is initiated by core promoter association of the TFIID complex, followed by the sequential binding of other basal factors and recruitment of pol II [272]. TFIID is a large complex and contains ~13 TBP associated factors (TAFs) and the TATA binding protein (TBP) [206]. 9 of the 13 TAFs contain a histone fold dimerization domain that allows multiple pairwise interactions within the complex [177,273]. TFIID adopts a clamp-like shape that features a symmetrical core. The TFIID complex has been studied extensively in yeast using multistep affinity purified complexes. Coomassie staining based analysis of these complexes revealed that a subset of TAFs (TAF4, TAF5, TAF6, TAF9, TAF10, TAF11 and TAF12) are present in more than one copy [156]. Recent work on reconstituted human TFIID confirmed these results and showed that upon addition of the TAF8/TAF10 dimer, a new surface is created that allows the assembly of single copies of the other TAFs to form a full TFIID complex [157]. Structural heterogeneity has been observed in TFIID preparations isolated from human cells and this was linked to a sub-stoichiometric TAF2 presence [274]. Additionally, the binding of TFIID to DNA induces a structural rearrangement within the complex [15]. The mechanism for this remains to be elucidated and it might be influenced by changes in TFIID composition.

Several subunits within TFIID can bind to specific DNA-elements found at promoters. TBP interacts with the TATA element, which is found upstream of the transcription start site (TSS). In yeast as well as in mammals, only a subset of genes contains a high affinity TATA box sequence [275,276]. Surprisingly, in yeast, TFIID association with promoters is inversely correlated with the presence of a consensus TATA sequence [63]. TBP association with TFIID and the TATA sequence is stabilized by binding of the TFIIA complex [277,278]. Additionally, TAFs1/2 interact with the initiator element (INR) [17] and TAFs6/9 can bind to a downstream promoter element (DPE) [279].

Chromatin has an important role in the regulation of transcription. The basic building block of chromatin is the nucleosome, comprised of an octamer of histone proteins. Post-translational modifications on the protruding tails of histones contribute to transcription regulation. Effector proteins that contain specific binding modules can recognize these chemical modifications and get recruited to genomic loci [242,280,281]. Tri-methylation of lysine 4 on histone H3 (H3K4me3) is associated with virtually all active and poised promoters both in yeast and in mammals [77,282]. Several H3K4me3 binding proteins have been identified, including the chromatin remodeler BPTF and the TFIID subunit TAF3. Binding of these proteins to H3K4me3 occurs through their plant homeodomain (PHD) fingers [170,220,222]. Recently, it has been described that the TAF3-H3K4me3 interaction in mammals is required for PIC assembly on a selective group of genes which are mainly involved in the response to DNA damage [85]. In addition to H3K4me3, promoter-associated modifications include hyperacetylation on histone H3 and the presence of a specific histone variant H2A.Z, which replaces the canonical H2A [77,283].

Here we show that the binding of TFIID to recombinant nucleosomes is synergistically enhanced by the presence of a TATA box in nucleosomes carrying H3K$_C$4me3 and H3K14ac. However, this binding is not affected by incorporation of histone variant H2A.Z or the H3K27me3 repressive mark. To further dissect the biochemistry of TFIID and to investigate the requirements for TFIID binding to nucleosomes *in vitro,* we determined the stoichiometry of endogenous human TFIID. These experiments revealed that TFIID consists of a stable symmetric core and a number of peripheral sub-stoichiometric TAFs. Finally, we show that binding of TAF3 is enriched on 'poised' stress gene promoters containing H3K4me3 in a PHD-finger dependent manner *in vivo*.

Results

TFIID binding to H3K4$_C$me3 nucleosomes.

TFIID is a large protein complex containing various subunits that can interact with specific DNA-elements and distinct histone modifications. Thus far, such interactions have mainly been studied using gel-shift and peptide pull-down assays [170,271,279,284]. Recently, several approaches have been developed to generate *in vitro* reconstituted nucleosomes containing specific histone modifications and DNA sequences [100,285]. In combination with quantitative mass spectrometry, affinity purifications using such immobilized nucleosomes can reveal proteins and protein complexes that can specifically interact with these *in vitro* assembled modified nucleosomes species [156,286,287]. We applied a methyl lysine analog (MLA) approach to produce recombinant nucleosomes carrying an H3K4me3 mimic (H3K$_C$4me3) with the aim to use these as bait for affinity purifications in crude nuclear extracts. To validate our approach we first tested the interaction between the TAF3 PHD-finger and different MLA peptides. As shown in Fig. 1A, the TAF3 PHD-finger specifically binds to the histone H3 N-terminus containing the H3K$_C$4me2 and H3K$_C$4me3 modification analogs. This binding is specific and comparable to H3 peptides containing natural methylated lysines (H3K4me2 and H3K4me3). This indicates that the MLA approach can be used as a tool to study TFIID-nucleosome interactions.

Next, we reconstituted MLA containing histone octamers with the 'Widom' 601 sequence labeled with a biotin on the 5'-end. The 'Widom' 601 sequence was used to prevent unintentional sliding of the nucleosome and transcription factor binding. Furthermore, the 'Widom' 601 sequence allows for efficient reconstitution of nucleosomes. Reconstituted nucleosomes were immobilized on streptavidin-conjugated magnetic beads and incubated with HeLa nuclear extract. To validate our assay we used western blotting to show the specific binding of the TFIID core subunit TAF5 to H3K$_C$4me3 containing nucleosomes. In contrast, TAF5 does not interact with unmodified or H3K36$_C$me3 marked nucleosomes, which validates the specificity of our approach (Fig. 1B).

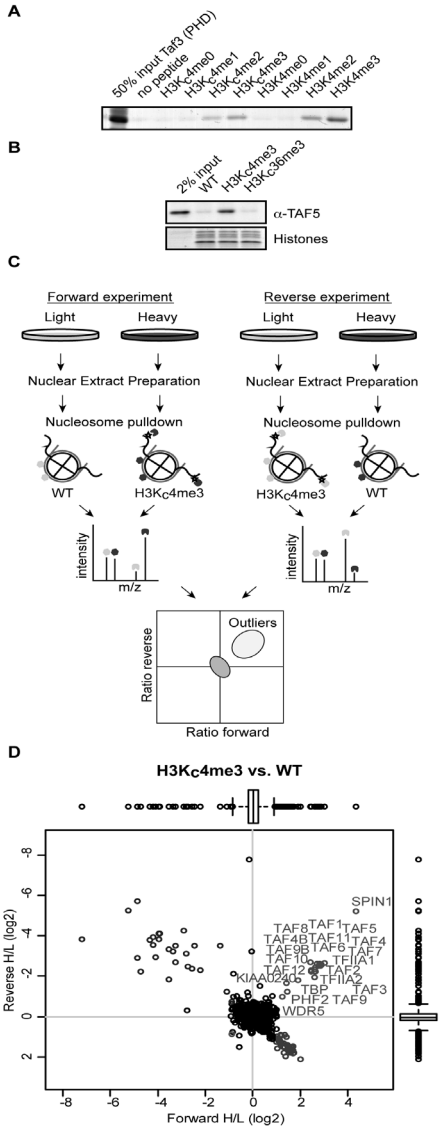


Figure 1: H3K4$_c$me3 nucleosomes bind endogenous TFIID and recombinant TAF3. (A) Pulldown with the indicated biotinylated peptides using streptavidin coated beads incubated with GST-TAF3 PHD protein lysates. Proteins are visualized using Coomassie blue staining. (B) Immunoblot analysis of endogenous TAF5 binding to immobilized recombinant nucleosomes with the indicated MLA modification. Histones are visualized using Coomassie blue staining. (C) Workflow as applied for quantitative analysis of nucleosome interactors. In brief, heavy and light labeled extracts are used for pulldowns with immobilized, differentially modified nucleosomes. Experiments are also performed with a label swap. Eluted proteins are measured using LC-MS/MS. Enriched proteins in both experiments are selected based on box plot statistics. (D) Scatter plot of SILAC ratios for H3K4$_c$me3 versus non-modified nucleosome interacting proteins. Upper right corner significant outliers are depicted and labeled based on the box plot analysis.

The H3K$_C$4me3 and unmodified control nucleosomes were then used for affinity purification in combination with SILAC-labeled HeLa nuclear extracts. Quantitative mass spectrometry was applied to identify specific interactors in an unbiased manner [205] (Fig. 1C). Nucleosomes with H3K4$_C$me3 showed enriched binding of all TFIID subunits and TBP (Fig. 1D). The SILAC ratio plots also reveal specific binding of TFIIA, which is known to functionally cooperate with TFIID during the early stages of PIC assembly. Several known H3K4me3 interactors were also identified, including PHF2 and SPIN1 [205,286]. In contrast, a number of known H3K4me3 interactors were not enriched in our experiments. This may be related to the use of the MLA instead of natural tri-methylated lysine, which can affect binding affinity. Indeed, although recombinant SGF29 specifically interacts with H3K4me3 [204,205], this protein does not bind to H3K$_C$4me3 peptides (data not shown). Interestingly, an uncharacterized protein (KIAA0240) was found to interact specifically with the H3K$_C$4me3 nucleosomes. This protein does not carry an annotated putative H3K4me3 interaction domain, indicating that it may interact with one of the H3K$_C$4me3 readers. In summary, these experiments reveal that a single histone modification (H3K4me3) contributes significantly to the overall affinity of TFIID for nucleosomes, despite the high basal affinity of the TBP subunit for DNA [288].

TFIID binding to nucleosomes is enhanced by acetylation of K9/K14 and a TATA box and not disrupted by the presence of H3K27me3.

The MLA approach can be used to study crosstalk between different chromatin modifications. One such cross-talk phenomenon has been described for embryonic stem cells, where H3K4me3 and H3K27me3 co-occur on silent but 'poised' developmentally regulated, bivalent genes [95,289]. We used both western blotting and quantitative mass spectrometry to study the interaction between TFIID and bivalent nucleosomes. As shown in Fig. 2A, the TAF3 PHD-finger, which directly binds to H3K4me3, binds equally well to H3K$_C$4me3- and H3K$_C$4me3/H3K$_C$27me3 containing nucleosomes. In agreement with this, the TFIID complex was identified as a specific reader for H3K$_C$4me3/H3K$_C$27me3 marked nucleosomes, as revealed by quantitative mass spectrometry (Fig. 2B). Together, these results demonstrate that TFIID binding to H3K$_C$4me3 is not disrupted by the presence of H3K$_C$27me3.

SPIN1 and KIAA0240 were again identified as specific interactors, as was TFIIA. Interestingly, PHF2 does not interact with bivalent nucleosomes but another PHD containing protein, PHF12, does. This protein is part of a complex containing the JARID1A H3K4me demethylase enzyme [205], which was not observed as a specific interactor in our experiments. Notably, experiments using nucleosomes containing only H3K$_C$27me3 did not yield significant interactors (data not shown).

The chromatin landscape around active gene promoters is characterized by the presence of several distinct features including the histone variant H2A.Z and acetylated histones H3 and H4 [290]. Additionally, distinct DNA-elements in the promoter region can contribute to PIC assembly. We investigated the contribution of these features to TFIID binding *in vitro* using recombinant nucleosomes (Fig. 2C).

Incorporation of the H2A.Z variant marks promoters and enhancers [291]. We first tested the effect of H2A.Z incorporation on TFIID binding to recombinant nucle-

osomes carrying H3K$_C$4me3 (Fig. 2D). This experiment revealed that the presence of unmodified H2A.Z in nucleosomes does not significantly influence TFIID binding *in vitro*. Next, we used an amber codon suppression approach to express recombinant histone H3 containing either acetylated K9 or K14 in bacteria [292] and combined this with the aforementioned MLA approach. Using these doubly modified nucleosomes, we observed enhanced binding (1.2-1.4 fold) of TFIID to nucleosomes decorated with both H3K$_C$4me3 and H3K9ac or H3K14ac as compared to H3K$_C$4me3 alone (Fig. 2E,G). We find a comparable level of enhancement using either H3K9ac or H3K14ac combined with H3K$_C$4me3. These agonistic binding effects can be explained by the tandem bromodomain of TAF1, which was previously shown to interact with double acetylated histone H4 peptides [151]. Furthermore, TFIID binding to histone H3K4me3 peptides has previously been shown to be enhanced by additional H3K9 and H3K14 acetylation [170]. Unfortunately, efforts to express recombinant H3 bearing both H3K9 and H3K14 acetylation proved to be unsuccessful (data not shown).

We then set out to study the potential interplay between histone modifications and specific DNA-elements in the regulation of TFIID binding to nucleosomes. To this end, recombinant nucleosomes were generated containing the classic 'Widom' 601 sequence, which carries a weak TATA sequence (GATATATAC) or a 601 variant carrying a consensus TATA sequence (TATATAAAAT) at super helical loop +6 (SHL +6) (Fig. 2F). As shown in Fig. 2G, H3K$_C$me3-dependent TFIID binding is not potentiated in the presence of a consensus TATA sequence (Fig. 2G). However, when the consensus TATA DNA was used in combination with nucleosomes carrying both H3KC4me3 and H3K14ac, binding was significantly enhanced as compared to nucleosomes carrying the weak TATA sequence or the methyl/acetyl combination (Fig. 2G). Together, these data reveal that diverse features including histone modifications and specific DNA-elements affect TFIID binding to nucleosomes. Furthermore, the importance of a functional DNA-element with regard to TFIID binding can be dependent on the modification state of the nucleosomes in cis, suggesting context-dependent synergy. TBP binding itself seems not affected in these experiments, which may be explained by the nonspecific binding of TBP to unmodified nucleosomes. In these cases, TBP binding is TFIID independent (Fig. 2D, E and G). In these experiments, no competitor DNA was used, which provides a possible explanation for the observed TBP binding.

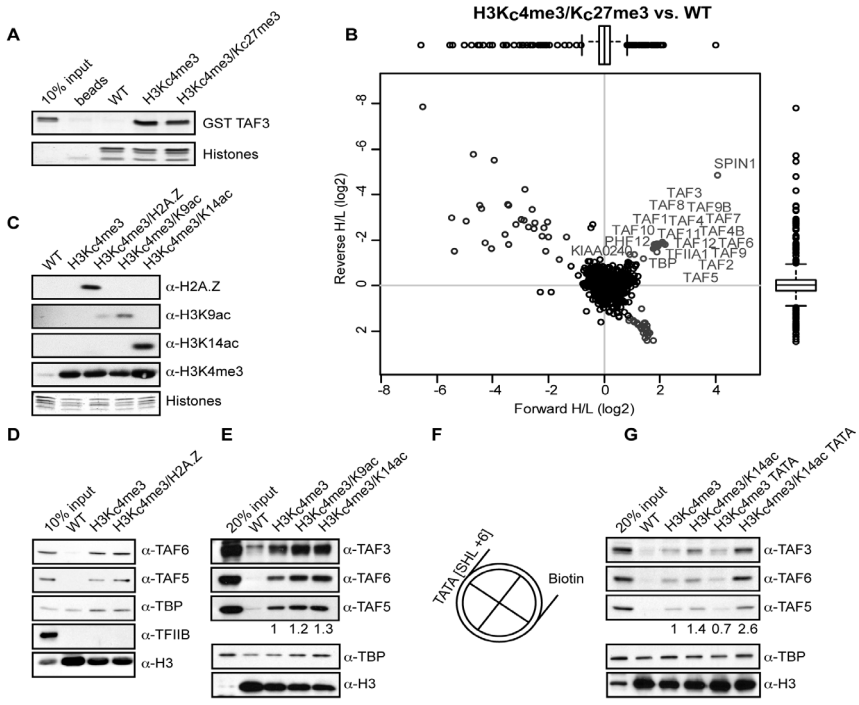


Figure 2: Presence of H2AZ, H3K9/K14ac and a TATA sequence enhances binding of endogenous TFIID to recombinant nucleosomes. (A) Analysis of pulldowns with recombinant nucleosomes immobilized on magnetic streptavidin coated beads and GST-TAF3 (PHD). Proteins are visualized by silver stain. (B) Scatter plot of SILAC ratios for H3K4$_c$me3/K27$_c$me3 versus non-modified nucleosome interacting proteins. In the upper right corner significant outliers are depicted and labeled in grey based on box plots analysis. (C) Immunoblot analysis of recombinant nucleosomes with the indicated antibodies showing the presence of modifications or variants. (D) TFIID binds to H3K4$_c$me3 nucleosomes independently of H2A.Z. Immunoblot analysis of eluted proteins using indicated antibodies. (E) TFIID binding is enhanced by histone H3 acetylation. Immunoblot analysis of eluted proteins using indicated antibodies. TAF5 antibody signal is quantified relative to the H3K$_c$4me3 pulldown. (F) Schematic representation of the nucleosome with the introduced TATA sequence and biotin group indicated. (G) Combination of TATA DNA and H3K14 acetylation on H3K4$_c$me3 nucleosomes increases the interaction with TFIID. Immunoblot analysis of eluted proteins using indicated antibodies. TAF5 antibody signal is quantified relative to the H3K$_c$4me3 pulldown.

Stoichiometry determination of human endogenous TFIID

So far, we have shown that TFIID is recruited to immobilized nucleosomes containing histone modifications and a consensus TATA box in a synergistic manner. This implies that TAF1 and TAF3, which are the subunits binding acetylated and methylated lysines, as well as TBP, which binds TATA containing DNA, have to be present together in substantial amounts in the same complex. To determine the composition of endogenous human TFIID, we analyzed the stoichiometry using label-free interaction proteomics combined with the iBAQ algorithm, which can be used to estimate relative protein abundance in a sample of interest [293,294]. We generated a cell line containing a doxycycline-inducible

GFP-fusion of TAF5. As a control, wildtype HeLa FRT cells were used (Fig. 3A). Nuclear extracts were made and these were subjected to single-step GFP-affinity purification in triplicate which was followed by on-bead trypsin digestion and LC-MS/MS analysis [295].

Purification of GFP-TAF5 resulted in the identification of all known TFIID subunits (Fig. 3B). iBAQ-based stoichiometry determination revealed the presence of a core complex containing a dimeric TAF6,4,9,10 and -12 module (Fig. 3C). TAF5 appears to be trimeric, which may be due to the moderate ~5 fold over-expression of the protein (Fig. 3A). Two TAFs, TAF8 and TAF7, are monomeric. The remaining TFIID subunits are substoichiometric, including TAF1, TAF3 and TBP (stoichiometry relative to TAF6 ~0.5). TAF11 and TAF13 are highly substoichiometric (~0.2 and 0.05, respectively). Thus, as expected, TAF1, TAF3 and TBP co-purify with core TFIID, although their stoichiometry is slightly lower compared to the dimeric core. This may hint towards the existence of distinct TFIID subcomplexes, each containing a specific set of peripheral TAFs. Alternatively, peripheral subunits may be partially dissociated from the core complex during affinity purification.

To further investigate this, we tagged and purified a peripheral TFIID subunit, TAF3. We also generated a stable cell line expressing a GFP-tagged inducible TAF3 mutant, M288A, which cannot bind to H3K4me3. As was shown for TAF5, purification of GFP-TAF3 and GFP-TAF3 M882A resulted in the co-purification of all TFIID subunits (Fig. 3D,E and F). The stoichiometry determination for wildtype and mutant TAF3 look almost identical, which illustrates the quality of the affinity purifications. Moreover, these data demonstrates that a mutated PHD-finger is not affecting the integrity of the TFIID complex. In both purifications, the stoichiometry of TAF3 is significantly higher compared to the other TAFs, indicating that a proportion of TAF3 is not incorporated into TFIID. Nevertheless, we again identify a stable dimeric core. In addition, the stoichiometry of TAF10 exceeds the dimeric core, which implies the existence of a ‘free’ TAF3/TAF10 dimer. This is in agreement with the fact that TAF10 can associate with TAF3 as well as TAF8 through their respective histone fold domains [184,296]. Notably, relatively high amounts of TAF1 and TBP co-purified with GFP-TAF3 and GFP-TAF3 M882A (stoichiometry ~0.5 relative to the dimeric core) (Fig. 3E,F). This indicates that the lysine methyl-, acetyl- and TATA-binding moieties co-exist within a single TFIID complex. These observations therefore substantiate our earlier results in which these three activities were found to act agonistically to anchor TFIID on ‘active’ promoter nucleosomes.

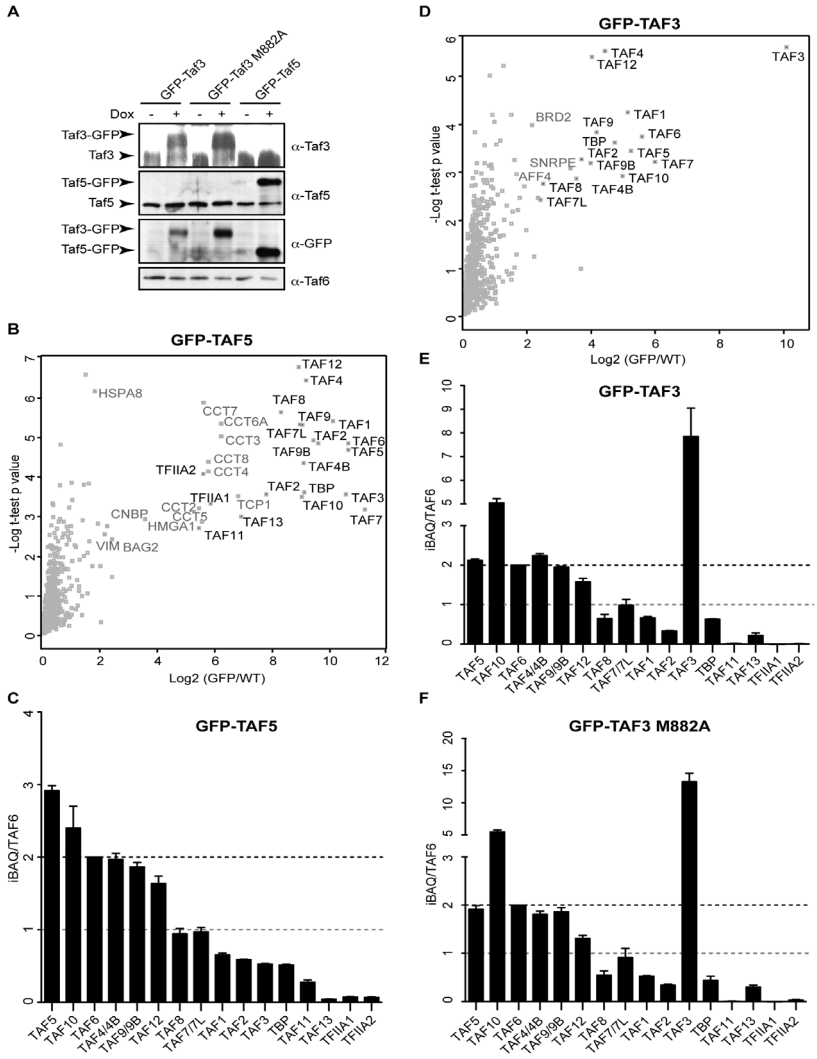


Figure 3: Stoichiometry analysis of endogenous TFIID. (A) Immunoblot analysis of GFP-TAF3, GFP-TAF3 M882A and GFP-TAF5 expression after 24 hours of doxycycline induction with the indicated antibodies (right). Endogenous proteins and GFP-fusions are indicated on the left. (B) Identification of interacting proteins for GFP-TAF5 by volcano plot. The ratio of identified proteins in all fusion lines over wildtype in label-free quantification are plotted against the -log10 of the false discovery rate (FDR) calculated by a permutation-based FDR adapted t-test. In all experiments FDR was set to <0.05 and S_0 = 1.5. Significant outliers are labeled. (C) Bar graphs indicate the stoichiometry of TFIID subunits (indicated at bottom) relative to TAF6. Black dashed line indicates a ratio to the total TAF6 protein. Error bars indicate the standard deviations of the technical triplicate. (D) Identification of interacting proteins for GFP-TAF3 by volcano plot. (E) Bar graphs indicate the stoichiometry of TFIID subunits (indicated at bottom) relative to TAF6 in GFP-TAF3 (E) and GFP-TAF3 M882A (F) purifications.

TAF3 requires its PHD-finger for binding to H3K4me3 in vivo.

We have demonstrated that incorporation of TAF3 lacking a functional PHD domain does not change the composition and stoichiometry of TFIID. Next, we wanted to determine how TFIID recruitment to target sites is affected by the absence of a functional PHD-finger. Recent experiments in our lab have shown that H3K4me3 is present on ER stress responsive genes prior to stress, presumably to maintain these genes in a 'poised' chromatin state. Furthermore, in the absence of TAF3, activation of ER stress genes such as *GRP78* and *CHOP* is impaired [245]. Therefore, we used the GFP-TAF3 and GFP-TAF3 M882A cell lines to determine if the PHD-H3K4me3 interaction is important for the binding to and expression of these ER stress responsive genes *in vivo*. Data mining published ENCODE ChIP sequencing data for H3K4me3 in different human cell lines revealed that H3K4me3 is found at the TSS of *GRP78* and *CHOP* in the absence of ER stress (Fig. 4A and B, upper panels). Scanning GFP ChIPs of the *GRP78* and *CHOP* loci revealed that TAF3 binding correlates well with the presence of H3K4me3 (Fig. 4A and B). As was observed for H3K4me3, TAF3 is present at these promoters prior to ER stress, which is indicative of a 'poised' state. Strikingly, the TAF3 mutant M882A, which can no longer bind to H3K4me3, shows impaired binding to the ER stress gene promoters (Fig. 4A and B). These results mirror recent findings showing that TAF3 is recruited to a specific subset of promoters enriched for DNA damage response related genes [85].

Next, the inducible GFP-TAF3 and GFP-TAF3 M882A cell lines were used to investigate the mRNA expression of ER stress target genes using the ER stress inducing agent tunicamycin. Strikingly, the induction of both GRP78 and CHOP is impaired in the TAF3 mutant cell line compared to the TAF3 wildtype (Fig. 4C and D). These results indicate that the interaction with H3K4me3 is required for the recruitment to and/or stabilization of TAF3 on ER stress gene promoters *in vivo*. Furthermore, this interaction is required for the rapid induction of ER stress responsive genes. Taken together, these *in vivo* observations strengthen our biochemical data and reveal that interactions with active histone modifications are relevant for TFIID binding to nucleosomes.

Discussion

The TFIID complex is important for the transcription initiation process and plays a major role in setting up the PIC at pol II promoters. Here, we have shown that synergistic effects of functional DNA-elements and histone modifications mediate high affinity binding of TFIID to promoters.

Stoichiometry analyses of affinity purified TFIID complexes through a core (TAF5) and a peripheral (TAF3) subunit revealed the presence of a stable core complex consisting of two copies of TAF4, TAF5, TAF6, TAF9, TAF10 and TAF12. These results are in agreement with recent work by Berger and colleagues who used recombinantly expressed TFIID (sub)complexes for structural (cryo-EM) studies [157].

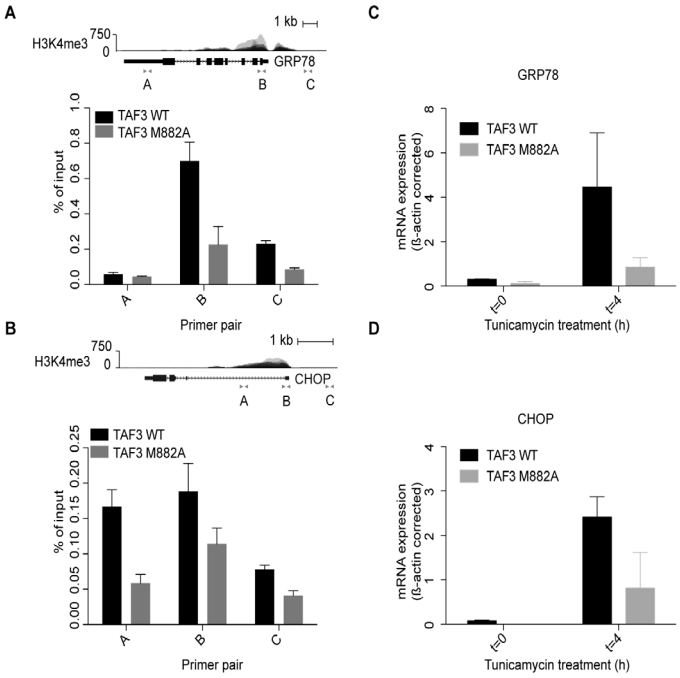


Figure 4: TAF3 binding to ER stress gene promoters is dependent on the PHD-finger. (A) Overlay of ENCODE H3K4me3 profiles from seven human cell lines at the GRP78 locus (upper panel). ChIP analysis of GFP-TAF3 and GFP-TAF3 M882A at *GRP78* with the indicated primer sets (lower panel). Standard deviations represent technical triplicates and similar results were observed in at least three independent experiments. (B) Similar labeling as in (A) but for the *CHOP* locus. (C and D) Analysis of mRNA expression levels of *GRP78* and *CHOP* by quantitative RT-PCR. Levels were normalized to β-ACTIN and are presented as change compared to a control DMSO-treated sample. Samples were analyzed 4 hours after tunicamycin treatment. Standard deviations represent two biological duplicates.

Outside of this stable symmetric core, peripheral TAFs appear to be present in sub-stoichiometric amounts. These observations could be indicative of heterogeneity within holo-TFIID complexes, as was suggested previously by Tora and colleagues [160]. Each of these distinct holo-TFIID complexes, all bearing a subset of peripheral TAF proteins, might serve its own specific target genes in a particular tissue or cell state. Indeed, individual TAFs and TAF variants have been shown to specify certain cell fates during development. Deletion of TAF7l in mice, for example, results in defective spermatogenesis [297]. It was also suggested that TFIID adopts different structural configurations dependent on the subunit composition as incorporation of TAF4b induces a more open configuration compared to TAF4 containing complexes [298].

We observed a functional interplay between DNA-elements and histone modifications on TFIID binding to promoter nucleosomes. Interestingly, the positive effect of

a canonical TATA box was only apparent in the context of $H3K_C4me3$ and H3K14 acetylation. This result could indicate that acetylation of histone tails affects accessibility of the TATA sequence for TBP binding. However, recent structural studies on TFIID binding to a TATA-containing template showed that a structural rearrangement of TAF1/2 in TFIID can be induced by TATA DNA binding [15]. These observations combined with our data suggest that structural changes in TFIID induced by interactions with DNA-elements or histone modifications could result in the exposure of additional chromatin binding surfaces, which would potentiate the complex for multivalent engagement. Additionally, the spacing between the +1 nucleosome and the TATA element could affect TFIID binding to different promoters in a specific manner [63].

We used ChIP experiments to show that TAF3 binds to the promoters of 'poised' stress genes. This binding is severely compromised when expressing a TAF3 mutant containing a point mutation (M882A), which cannot bind to H3K4me3. Roeder and colleagues recently showed that knock-down of TAF3 only results in a minor change in global pol II-dependent transcription. However, for the induction of early p53 response genes as well as ER stress genes, the interaction between TAF3 and H3K4me3 appears to be critical [85]. Together these data illustrate that H3K4me3 binding by TFIID is only required when rapid induction of transcription is demanded. When unchallenged, acetyl and TATA binding can be sufficient for TFIID loading. These experiments from Roeder and colleagues, together with our data, further suggest that H3K4me3 can act either independently or cooperatively with a TATA box to regulate PIC formation and transcription. This, together with the fact that only ~10% of human pol II promoters contain a canonical TATA box, implies a certain degree of structural plasticity regarding TFIID engagement on different target genes. Interestingly, yeast TFIID lacks an H3K4me3 binding domain. Nevertheless, recent high resolution ChIP in yeast revealed that TFIID binding partially overlaps with the position of the +1 nucleosome [63], indicating that TFIID can bind simultaneous to the nucleosome depleted region and to the first nucleosome. This observation is more pronounced on Taf1 depleted genes, indicating that SAGA and TFIID regulated genes are different in promoter architecture, at least in yeast [299]. A systematic analysis of human promoter architecture and TFIID association however remains to be done.

Future experiments can be directed towards determining the exact position of TFIID subunits in promoter bound complexes. Furthermore, functional domain mapping experiments could be pursued to dissect the molecular mechanisms underlying the multivalent engagement of TFIID at various promoter nucleosomes. Additional stoichiometric analysis on nucleosome bound TFIID using different promoter and/or enhancer nucleosomes for affinity purification could reveal the exact composition of the TFIID complex binding to these nucleosomes. In these experiments, we only made use of '601'-based DNA sequences to avoid nucleosome sliding and transcription factor binding. Future experiments using promoter DNA sequences should reveal the contribution of additional DNA-elements to TFIID binding. Finally, deciphering the genome-wide profile of individual core and peripheral TFIID subunits in different cellular systems and stress conditions will increase our understanding regarding the assembly and composition of TFIID (sub)complexes and their role in the regulation of transcription initiation.

MATERIALS AND METHODS

Plasmids and cell culture

The ORF of the bait protein was amplified by PCR using the relevant human cDNA constructs and introduced into pDONR2.1. The DNA sequence of the amplified ORF was verified and introduced into a GATEWAY-compatible version of pCDNA5/FRT/TO essentially as described before [112]. Mouse TAF3 and mutant M882A were tagged by GFP at the N-terminus. Stable doxycycline-inducible cell lines were created by transfecting pCDNA5/FRT/TO and pOG44 into HeLa FRT cells carrying the TET repressor using polyethyleneimine followed by antibiotic selection. Cells were grown in plates (Greiner Bio-One, Frickenhausen, Germany) in DMEM with high glucose supplemented with pen/strep and L-Glutamine (all LONZA) under blasticidin and hygromycin B selection. pRPN-mTAF3 (PHD) was described previously [170]. pDUET-H3K4$_C$,K14X was derived by introducing K4C, C110A mutations and an amber codon at position 14, into Drosophila melanogaster histone H3. All histone H3 plasmids carried a C110A mutation. Amber codon histones were inserted into the pDUET plasmid using Nco1/Xho1 and transformed in bacteria that already carried pAC-KRS (kind gift of Robert Schneider). Point mutations in H3 were introduced using the Quickchange protocol (Stratagene) and verified by DNA sequencing. Other histone proteins were expressed from pET21b (gift from Y. Bai).

GFP affinity purification and sample preparation

Extract preparation [257] and affinity purifications using GFP-beads [295] were performed essentially as described before. Briefly, nuclei were isolated using hypotonic lysis and nuclear extracts were prepared by using 420 mM NaCl Purifications for GFP lines and WT HeLa cells were performed in triplicate using 1 mg of nuclear extract per purification and GFP binder beads (CHROMOTEK) in 20 mM HEPES-KOH pH 7.9, 20% glycerol, 300 mM NaCl, 2 mM MgCl2, 0.2 mM EDTA, 0.1% NP-40, 0.5 mM DTT and complete protease inhibitors (Roche). All purifications included 50 µg/ml ethidium bromide to suppress DNA mediated interactions. After 2.5 hours incubation at 4°C the beads were extensively washed and on-bead digestion was performed using 0.1 µg trypsin (Promega).

Protein expression and nucleosome reconstitution

GST-mTAF3 PHD was expressed in E. coli strain BL21DE3 at 37°C in LB medium. Drosophila histones were expressed in E. coli strain BL21DE3 codon+ or Rosetta and prepared essentially as previously described [300]. For expression of acetylated histones 20 mM Nicotinamide and 10 mM N-acetyl-L-Lysine (Sigma) was added to the cultures at OD600=0.6 and protein expression was induced after 30 minutes using 0.5 mM IPTG as described before [292]. Histone H3K4$_C$ and derivatives were alkylated essentially as described before [285,301]. 167-bp DNA (‘601 Widom’ positioning sequence or TATA mutants) was produced by PCR amplification using one biotinylated primer, purification using DEAE anion exchange and ethanol precipitation. After octamer refolding, nucleosomes were reconstituted with the DNA using salt displacement.

Extract preparation and nucleosome pulldowns

Hela S3 cells were cultured in large quantities using a bioreactor setup in MEM deplet-

ed from Lysine and Arginine supplemented with dialyzed FBS, Pen/Strep, L-Glutamine (all LONZA) and normal or $^{13}C^{15}N$- labeled arginine and lysine (Isotec). Nuclear extracts were prepared by isolating the nuclei and hypertonic lysis as described before [170,257]. For nucleosome pulldown assays magnetic Streptavidin beads (Sigma, MyOne) were coated with 130 pmol nucleosome and incubated in pulldown buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 20% glycerol, 0.1% NP-40 and 1mM DTT) for one hour at 4 °C. After washing twice, 500 µg nuclear extract was added and beads were incubated for 2-3 hours rotating at 4°C. For mass-spec experiments, heavy-labeled modified nucleosome pulldowns and light controls were mixed at this point. Proteins were eluted from the beads in sample buffer after extensive washing and the bound proteins were analyzed by immunoblotting or processed for LC-MS/MS. Peptide pulldown experiments were performed essentially as described [170]. Briefly, biotinylated peptides were alkylated as described before [285] and incubated with magnetic Streptavidin beads (Sigma, MyOne). After incubation and extensive washing beads were incubated with GST-TAF3 (PHD) lysate. Bound protein was visualized using Coomassie blue staining.

Mass-spectroscopy

Eluted proteins were separated on a SDS-PAGE gel and stained using Colloidal blue staining (Invitrogen). Lanes were sliced into eight pieces and samples were subjected to in-gel digestion with 0.1 µg trypsin (Promega) as described before [170]. Tryptic peptides were extracted from the individual gel slices and concentrated using stage-tips with C18 material. The peptides were applied to online nanoLC-MS/MS, using a 120 minutes acetonitrile gradient. Mass spectra were recorded on a LTQ-Orbitrap-Velos mass spectrometer (Thermo) selecting the 15 most intense precursor ions of every full scan for fragmentation. The data was analyzed using the Max-Quant software package [302].

Chromatin immunoprecipitation

Cells were cross-linked at 80-90% confluency using 1% paraformaldehyde in PBS for 10 minutes at room temperature. Reactions were quenched by addition of 125 mM glycine for 5 minutes on ice. After a cold PBS wash cells were scraped and collected by centrifugation (5 min, 400 g, 4°C). Pelleted cells were resuspended in ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 7.9, 1 mM DTT, 5 µM sodium butyrate (Merck) and complete protease inhibitors (Roche)) and disrupted by sonication (Bioruptor, Diagenode: seven cycles, 30 seconds on/off, high setting) to produce an average DNA fragment size of ~400-bp. Samples were centrifuged (5 minutes, 200 g, 4°C) and supernatant collected. For immunoprecipitation, chromatin was diluted in IP buffer (0.5% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 7.9, 150 mM NaCl, 1 mM DTT, 5 µM sodium butyrate and complete protease inhibitors (Roche)), 1-5 µg antibody was added and rotated overnight at 4°C. Immunocomplexes were collected for 4 hrs at 4°C on protein A/G PLUS-agarose beads (Santa-Cruz), after o/n blocking in 1.5% fish gelatin and washing. Subsequently beads were washed four times at 4°C with wash buffer (0.25% NP-40, 0.05% SDS, 2 mM EDTA, 20 mM Tris-HCl pH 7.9, 250 mM NaCl, 5 µM sodium butyrate and complete protease inhibitors) and once with TE (10 mM Tris-HCl pH 6.8, 1 mM EDTA). Cross-links of protein-DNA were reversed by overnight incubation at 65°C in 100 µl elution buffer (100 mM NaHCO3, 1% SDS). Samples were treated with 1 mg/ml proteinase K (Roche) and 1 mg/ml

RNase A for 2 hours at 37°C. DNA was purified using PCR purification kit (Qiagen) and amplified in a 25 µl reaction mixture (iQ SYBR green supermix (Biorad)) in a real-time PCR machine (CFX96, Biorad). Primer sequences are available upon request

mRNA expression analysis

Total RNA was isolated using RNeasy kit (Qiagen) and cDNA was synthesized using the First-strand cDNA synthesis kit (Qiagen) both according to the manufacturers manual. Subsequently the cDNA was amplified in a 25 µl reaction mixture (iQ SYBR green supermix (Biorad)) in a real-time PCR machine (CFX96, Biorad). Primer sequences are available upon request.

Antibodies

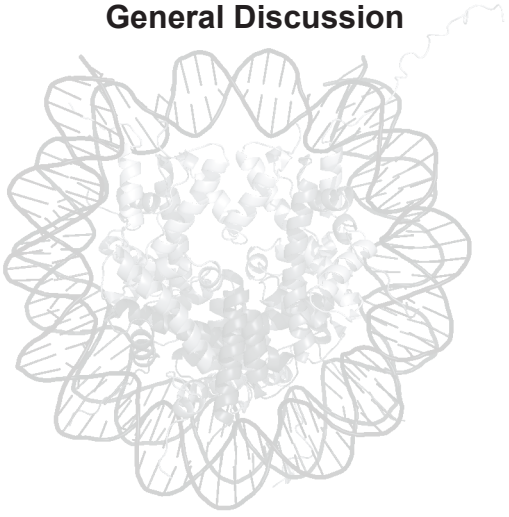
α-TBP (SL30) (gift from Henk Stunnenberg), α-TAF3 and α-TAF5 (obtained from Robert Roeder), α-TAF6 (25TA-2G7, Euromedex), α-TFIIB (Santa Cruz), α-H3 (Abcam Ab1791), α-H3K4me3 (home made), α-H3K9ac (Cell Signaling 9671), α-H3K14ac [101] and α-H2A.Z (Abcam Ab18263) were used for immunoblotting. For ChIP an immunoblotting α-GFP (gift from Geert Kops) was used. Quantification of antibody signals was performed using Adobe Photoshop.

Acknowledgements

We thank members of the Timmers and Vermeulen lab for useful suggestions and discussions. We are very grateful to Susan Kloet for critical reading of the manuscript. We acknowledge Dr. Robert Schneider for reagents and discussions. Dr. Geert Kops and Dr. Robert G. Roeder kindly provided antibodies. Furthermore, we thank Arne Smits for advice on the iBAQ quantifications.

Chapter 5

General Discussion



In 1995, David Allis and co-workers discovered that the transcription co-factor p55 from Tetrahymena is a histone acetyltransferase. This groundbreaking paper clearly linked enzymatic acetylation of histones to activation of transcription. Since then, studying the involvement of histone PTMs in regulating processes such as transcription, DNA repair and replication has remained a major theme in the transcription and chromatin field. Researchers are continuously identifying novel histone modifications and their enzymes and are determining their genomewide localization, thereby trying to shed light on their function. Other studies include characterization of 'reader' proteins that specifically get recruited to certain histone modifications. The binding of these histone readers is thought to be important for the biological function of a certain modification. Recently, the term 'chromatin signaling', which refers to processes in which multiple histone modifications serve as a layer of information to coordinate cellular functioning, is gaining popularity. The studies described in this thesis focus on various aspects of chromatin signaling, including the 'writing' and 'reading' of histone marks in the context of transcription. In chapter 2 we investigated the function of SAGA subunit SGF29, which is known to bind to H3K4me3. In the addendum a mass spectrometric-based method to determine histone modification levels is explained. In chapter 3 the identification of a writer for H3K4ac is described. In chapter 4 we studied the multivalent engagement of TFIID to promoter nucleosomes. Novel aspects of important chromatin readers, SAGA and TFIID were characterized. These complexes are also chromatin modifiers. Below these results and their implications will be further discussed.

Stress genes require a 'poised' chromatin state

In chapter 3 we studied the function of the chromatin reader SGF29. We discovered an evolutionary conserved role for this protein in the crosstalk between H3K4me3 and acetylation of H3, both of which are histone modifications associated with active transcription. To our surprise, however, we also discovered that SGF29 is necessary for the presence of H3K4me3 on the transcription start site of ER stress target genes. Furthermore, H3K4me3 and SGF29 itself are already present prior to ER stress, when these genes are not highly expressed. The generality of the presence of H3K4me3 prior to ER stress is suggested by a similar observation in HepG2 cells [240]. Finally, a writer for H3K4me3, ASH2, also binds to ER stress genes prior to ER stress and this binding is dependent on the presence of SGF29. These observations lead us to speculate that ER stress response genes are maintained in a 'poised' state, which facilitates rapid transcriptional induction upon exposure to ER stress. In this scenario (Figure 1), acetylation of histone H3 serves as a final trigger to switch ER stress genes from a transcriptionally repressed state into an active one.

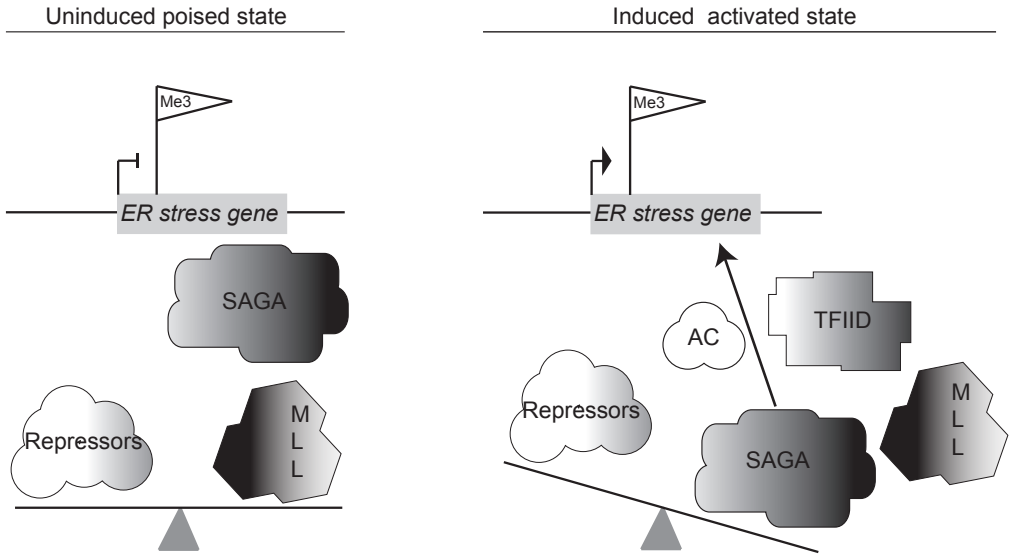


Figure 1: Model for the poised and activated stress genes.

The role of SAGA in these two states is clearly different. In the uninduced state, SGF29 is responsible for the maintenance of H3K4me3 (Figure 1). The underlying mechanism is not quite clear yet, based on our ChIP experiments however, SGF29 may be directly involved in the recruitment of writers of H3K4me3. Alternatively, SGF29 bound to H3K4me3 may prevent H3K4 demethylases from gaining access to the stress gene promoters. ChIP experiments for H3K4me3 demethylases, such as JARID1A in wildtype and SGF29 knockdown cells, could be pursued to investigate this possibility further.

Regulation of histone deacetylase activity could also be involved in the maintenance of the 'poised state'. The continuous recruitment or presence of HDACS in the absence of stress could be required to maintain a deacetylated state which may be necessary to prevent leaky expression in a non-stress situation. Our ChIP experiments show that the promoter is not acetylated prior to ER stress. This indicates that presence of SGF29 probably does not repel HDACs in the poised state. Experiments using HDAC inhibitors alongside monitoring the activation of ER stress genes could shed more light on their role in the maintenance of the poised state. Furthermore, ChIP experiments for HDACs should be performed prior to ER stress to unravel the situation at the promoter. In summary, we hypothesize that the presence of SGF29 on stress genes in the absence of stress, balances the chromatin state such that the gene is silenced, but ready for action, much like the bivalency described for pluripotent genes.

The second or induced state occurs after ER stress (Figure 1). We hypothesize that the role of SGF29 and SAGA now shifts from maintenance of H3K4me3 to acetylation of the promoter, which ultimately results in recruitment of TFIID and transcriptional activation of stress genes. TFIID and SAGA share a common binding platform, the

H3K4me3 mark. TFIID and SAGA have highly redundant roles in transcription. Studies in yeast have revealed the concurrent co-localization of TFIID and SAGA on ribosomal gene promoters [207,303]. Thus what determines which complex binds to the H3K4me3 modification at the ER stress genes? Since each nucleosome contains two histone H3 tails, in theory two complexes can bind. However, figure 1 depicts that –at least- on stress genes, TFIID succeeds the binding of SAGA on the promoter. Further time course experiments are required to determine the exact order of events at these stress promoters. Interestingly, the binding affinity of TAF3 for H3K4me3 is much higher than of SGF29 [170,204,304]. Recently it has also been shown that the interaction of TAF3 with H3K4me3 is particularly important for a subset of genes, mainly stress related [85]. Furthermore, in yeast it was observed that sustained and transient gene expression implies different assembly of SAGA and TFIID [209]. Which are the mechanisms that assure assembly of SAGA instead of TFIID at least in the uninduced state on ER stress genes (Figure 1)? Primarily the H3K14ac created by SAGA could have a role in the binding of TFIID, which is also suggested by the binding data from chapter 4. Perhaps the two-chromatin modifying modules of SAGA have another role, by either deubiquitination or acetylation of subunits of TFIID. It has previously been shown that posttranslational modifications on subunits can determine the composition of both SAGA and TFIID [215]. Therefore these modifications might also direct binding affinity of the complexes. After binding to and acetylation of the promoter, SAGA itself could be posttranslationally modified, causing dissociation from the promoter [172]. Furthermore, SAGA recruitment is often facilitated by gene-specific transcription factors, such as ATF6, to recruit SAGA to ER stress genes [227]. These transcription factors might also have a role in preferential SAGA binding to the promoter in an uninduced state. Studying the binding of SAGA and TFIID to an H3K4me3 recombinant nucleosome reconstituted with *GRP78* promoter DNA, in the presence and absence of ATF6 could be pursued to further elucidate this. Furthermore, an *in vivo* ChIP analysis of SAGA and TFIID binding to the stress gene promoters can be tested in an ATF6 knockdown background. The chromatin itself might also have an important role in determining which complex can bind, through crosstalk with other modifications. An obvious candidate is mono-ubiquitinated H2B. It is possible that upon stress induction, H2B de-ubiquitination by SAGA clears the path for TFIID to bind H3K4me3. The dynamics of this modification during ER stress could be determined by ChIP analysis.

The data presented in chapter 4 provide some clues regarding the issues raised here. First of all, these data indicate that TFIID binding to a general promoter is multivalent and determined both by histone modifications and DNA elements. Similar studies in SAGA could reveal whether there is overlap or differences in synergistic binding to promoters. These studies can possibly help elucidate the answer to when SAGA will bind to H3K4me3 and when TFIID will. This could be explored in gel shift assays, using recombinant TFIID and SAGA, also including subunits with mutations in their binding domains.

The stoichiometric analyses in chapter 4 together with previous studies suggest the existence of TFIID subcomplexes [160]. These subcomplexes might have specialized functions such as stress gene induction. It is conceivable that in order to rapidly activate a stress gene, the cell does not require complete assembly of TFIID, but rather only a small essential complex. This smaller complex allows for more rapid assembly than the recruitment of the entire holo-complex. The existence of TFIID subcom-

plexes has also been found in differentiating myotubes, where the TFIID holo-complex is replaced by the TRF3/TAF3 containing subcomplex [165,166]. It is likely that for the ER stress genes at least TAF3 will be involved in the TFIID complex, since the subset of genes for which it was found that the TAF3-H3K4me3 interaction is important, is stress related [85]. Monitoring ER stress gene induction after systematic knockdown of TFIID subunits can reveal the existence of specific TFIID subcomplexes on ER stress target genes. Such an analysis in yeast revealed three classes of promoters, those that depend on all Tafs, those that depend on a subset of Tafs and those that do not require any Tafs [305]. We hypothesize that the stress genes would fall in the second category. Stoichiometric analysis of SAGA could reveal whether subcomplexes also exist for this complex. Composition of the different subcomplexes of TFIID and SAGA might also determine their binding capacity to H3K4me3.

In conclusion more research is required to completely understand what happens at a promoter in case of transcription activation. Specifically this investigation is needed for promoters of stress genes that form a special case and for which rapid activation only in a stress situation is essential.

SAGA versus ATAC

It is important to note that SGF29 is not only part of SAGA, but also of the ATAC complex. Interestingly, only higher order species have acquired the ATAC complex. ATAC contains the same HAT module compared to SAGA, but both complexes have discrete functions [172]. This is also reflected in their genome-wide localization. ATAC is found on a different set of promoters compared to SAGA. Furthermore, ATAC binds to enhancer elements and it is required for the transcription of those genes that it directly binds [210]. In contrast to yeast, human SAGA is not only found on stress related genes [208], but also on promoters of housekeeping and tissue specific genes [210]. The transcription of these genes has been shown to be dependent on the presence of SAGA [210]. Our work described in chapter 2 suggests that not ATAC, but SAGA is responsible for the chromatin landscape at the ER stress target genes. However, ChIP experiments for ATAC subunits should be performed to confirm this. Again, the question arises here what determines the recruitment of SAGA and not ATAC, since they contain the same chromatin reader (SGF29). The specific transcription factors, which recruit each of the complexes, most likely play an important role in this. Stoichiometric analysis of both complexes could reveal which transcription factors bind to ATAC and SAGA. This will contribute to understand the functional differences between the two complexes.

HBO1 complex is responsible for acetylation of H3K4

Chapter 3 aimed at identifying the writer for H3K4ac. Our results suggest a role for the HBO1 complex in acetylating H3K4. Nevertheless, it is not entirely clear how the two splice forms of JADE1 play a role in the acetylation of H3K4. Further experiments are required to fully understand the role of HBO1 and the link between H3K4ac and activation of transcription. In our HAT assays only the small JADE1S/HBO1 complex

was used to acetylate histone peptides. Purification of the larger JADE1L/HBO1 complex in combination with HAT assays on recombinant nucleosomes with and without H3K4me3 could reveal whether this complex can specifically target H3K4 for acetylation. Luciferase assays could be used to directly determine the link between HBO1, its catalytic activity and transcription. Finally, nucleosome pull downs could determine readers for H3K4ac, which could provide clues regarding its role in transcription.

The *in vivo* effects of HBO1 knockdown could also appear due to loss of H4 acetylation, a known substrate for HBO1 [260]. Systematic knockdown of all HBO1 complex subunits is therefore required to dissect the exact role of HBO1 in H3K4ac and gene expression. Comparison and correlation of ChIP-seq data for H4ac and H3K4ac, with microarray data in individual subunit knockdown cell lines, could reveal which genes are specifically affected by loss or redistribution of H3K4ac and which aspects of HBO1 complex play a role herein.

The incidence of indirect effects may occur in studies related to identification and characterization of writers and erasers for histone modifications. Large-scale genetic interaction studies based on systematic knockdown of chromatin modifiers allow for revealing protein networks. The characterization of (novel) networks of chromatin modifiers can improve the understanding of their indirect effects. These genetic interaction studies require a robust phenotype as read-out. Therefore, when histone modifications or combinations thereof are used as a phenotype the mass spectrometric-based method described in the addendum could be useful.

Chromatin in the context of evolution

Interestingly, whereas core histones are extremely well conserved from yeast to humans, their respective writer and eraser proteins display a large degree of diversification throughout evolution. One such an example is the H3K4me3 mark. In yeast this modification has one writer, Set1, which has a function in the repression of anti-sense transcription at the 3' end of genes [306]. Mammals, in contrast, contain at least 10 methyltransferases for H3K4. Their activity is not redundant, as illustrated by the embryonic lethality of both MLL1 and MLL2 knockout mice [307,308].

Although TFIID is mostly conserved between yeast and humans, yeast TAF3 lacks a PHD finger. Therefore yeast TFIID appears to lack H3K4me3 binding affinity. In contrast the reader function of SAGA and SGF29 for H3K4me3 is conserved between yeast and humans (chapter 2 and [204]). These observations indicate that higher order species acquired extra writers, erasers and readers for the H3K4me3 modification. These writers, erasers and readers probably allow for more balanced and fine-tuned regulation of histone modifications in a spatio-temporal manner. The proposed ´poised´ state of the ER stress genes forms an example of such balanced regulation of chromatin.

Another exciting phenomenon of chromatin in light of evolution is the existence of histone variants. It is thought that histone variants, next to the posttranslational modifications and chromatin remodelers, evolved to provide the cell an opportunity to alter the rigid chromatin structure [309]. The process of transcription

requires a non-static chromatin structure; incorporation of histone variants provides a means to generate dynamic chromatin signatures. Histone variant H2A.Z is one such a histone variant that has been identified and that appears to play a role in transcription. Future experiments could be directed at determining whether histone variants are particularly important in the regulation of stress related genes and contribute to their poised chromatin state. We hypothesize H2A.Z, supplemented maybe by other histone variants, to be present prior to ER stress to ensure rapid transcriptional activation. However, further experiments are required to elucidate this.

Role of non-histone proteins in chromatin and transcription regulation

The biological outcome of a histone modification can be context dependent. One example of this is H3K4me3, which around the TSS is responsible for recruitment of TFIID and activation of transcription. In contrast, H3K4me3 marking sites of DNA damage has a role in transcriptional repression [153,170]. This context-dependent function might be brought about by the specific regulated recruitment of different readers and gene-specific transcription factors to a promoter. The important role of gene-specific transcription factors is also suggested by the absence of correlation between binding events of chromatin factors and the effect on expression in case of deletion [310-312]. Thus gene-specific transcription factors are probably involved in recruitment and function of chromatin readers and thereby also in determining the biological outcome of a modification.

Furthermore, the extensive chromatin modifying machinery might be involved by decorating the gene-specific transcription factors and other non-histone substrates. An average of 75 proteins occupy the promoter at the time of transcription initiation [311] and it is conceivable that also these proteins will have PTMs to determine their recruitment and function. To date a few examples exist of non-histone proteins getting post-translationally modified also suggesting a role for this type of modifications in regulation of transcription [139,140]. To identify novel non-histone targets of a certain chromatin modifier, an approach as essentially described in [313] can be followed. Briefly, whole cell lysates are first treated with inhibitors, followed by the addition of recombinant enzyme (chromatin modifier) to one half of the sample and no or inactive enzyme to the other half. Subsequently, the treated samples can be tagged using dimethyl label [314] and novel substrates can be identified by mass spectrometric analysis. In our case, cell lysates could be treated with HDAC inhibitors and novel non-histone substrates for recombinant SAGA could be identified. The identified targets and their mutants can then be used in live cell imaging to monitor recruitment events.

Interestingly, reader proteins are being identified that contain a RKS motif, which is repeatedly found in histone H3. This motif is similarly modified, the lysine is methylated and this is required to bind chromatin [315]. This makes them excellent non-histone targets for the chromatin modifying machinery. The RKS motif probably exists in more reader proteins, a search amongst the important players in transcription might reveal whether it also has a role in this process. Thus experiments in these directions could reveal whether an additional layer of information by PTMs on non-histone sub-

strates, such as gene-specific transcription factors, exists. Furthermore, this could reveal a possible role in the determination of biological outcome of a histone modification.

Importance of chromatin related research

The studies described in this thesis have investigated a few aspects of chromatin biology and from the discussion it is clear that many unsolved issues remain. These issues are important to solve not only from a basic scientific interest, but also from a clinical perspective. It is becoming increasingly clear that writers, readers and erasers of histone modifications are implicated in a number of disorders, including cancer (reviewed in [316,317]). The reversible nature of histone modifications makes them excellent targets for therapy. However, in order to efficiently use histone writers, erasers and readers as targets for anticancer drugs, a deeper understanding of their biology is required. To achieve this, researchers should both make use of global, systems-wide approaches as well as dedicated mechanistic experiments at the single gene level. Integration of these diverse approaches eventually may result in the development of 'epi'-drugs that can be used to treat cancer and other disorders in which histone modifications and their writers, readers and erasers are implicated.

References

1. Crick F (1970) Central dogma of molecular biology. Nature 227: 561-563.

2. Roeder RG, Rutter WJ (1969) Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. Nature 224: 234-237.

3. Gniazdowski M, Mandel JL, Jr., Gissinger F, Kedinger C, Chambon P (1970) Calf thymus RNA polymerases exhibit template specificity. Biochem Biophys Res Commun 38: 1033-1040.

4. Kedinger C, Gniazdowski M, Mandel JL, Jr., Gissinger F, Chambon P (1970) Alpha-amanitin: a specific inhibitor of one of two DNA-pendent RNA polymerase activities from calf thymus. Biochem Biophys Res Commun 38: 165-171.

5. Weinmann R, Raskas HJ, Roeder RG (1974) Role of DNA-dependent RNA polymerases II and III in transcription of the adenovirus genome late in productive infection. Proc Natl Acad Sci U S A 71: 3426-3439.

6. Weinmann R, Roeder RG (1974) Role of DNA-dependent RNA polymerase 3 in the transcription of the tRNA and 5S RNA genes. Proc Natl Acad Sci U S A 71: 1790-1794.

7. Haag JR, Pikaard CS (2011) Multisubunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing. Nat Rev Mol Cell Biol 12: 483-492.

8. Matsui T, Segall J, Weil PA, Roeder RG (1980) Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. J Biol Chem 255: 11992-11996.

9. Samuels M, Fire A, Sharp PA (1982) Separation and characterization of factors mediating accurate transcription by RNA polymerase II. J Biol Chem 257: 14419-14427.

10. Hahn S (2004) Structure and mechanism of the RNA polymerase II transcription machinery. Nat Struct Mol Biol 11: 394-403.

11. He Y, Fang J, Taatjes DJ, Nogales E (2013) Structural visualization of key steps in human transcription initiation. Nature 495: 481-486.

12. Goodrich JA, Cutler G, Tjian R (1996) Contacts in context: promoter specificity and macromolecular interactions in transcription. Cell 84: 825-830.

13. Holstege FC, Tantin D, Carey M, van der Vliet PC, Timmers HT (1995) The requirement for the basal transcription factor IIE is determined by the helical stability of promoter DNA. EMBO J 14: 810-819.

14. Holstege FC, van der Vliet PC, Timmers HT (1996) Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIH. EMBO J 15: 1666-1677.

15. Cianfrocco MA, Kassavetis GA, Grob P, Fang J, Juven-Gershon T, et al. (2013) Human TFIID binds to core promoter DNA in a reorganized structural state. Cell 152: 120-131.

16. Gershenzon NI, Ioshikhes IP (2005) Synergy of human Pol II core promoter elements revealed by statistical sequence analysis. Bioinformatics 21: 1295-1300.

17. Chalkley GE, Verrijzer CP (1999) DNA binding site selection by RNA polymerase II TAFs: a TAF(II)250-TAF(II)150 complex recognizes the initiator. EMBO J 18: 4835-4845.

18. Burke TW, Kadonaga JT (1997) The downstream core promoter element, DPE, is conserved from Drosophila to humans and is recognized by TAFII60 of Drosophila. Genes Dev 11: 3020-3031.

19. Shao H, Revach M, Moshonov S, Tzuman Y, Gazit K, et al. (2005) Core promoter binding by histone-like TAF complexes. Mol Cell Biol 25: 206-219.

20. Lagrange T, Kim TK, Orphanides G, Ebright YW, Ebright RH, et al. (1996) High-resolution mapping of nucleoprotein complexes by site-specific protein-DNA photocrosslinking: organization of the human TBP-TFIIA-TFIIB-DNA quaternary complex. Proc Natl Acad Sci U S A 93: 10620-10625.

21. Benoist C, Chambon P (1981) In vivo sequence requirements of the SV40 early promotor region. Nature 290: 304-310.

22. Smale ST, Baltimore D (1989) The “initiator” as a transcription control element. Cell 57: 103-113.

23. Bird A, Taggart M, Frommer M, Miller OJ, Macleod D (1985) A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. Cell 40: 91-99.

24. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, et al. (2001) Initial sequencing and analysis of the human genome. Nature 409: 860-921.

25. Larsen F, Gundersen G, Lopez R, Prydz H (1992) CpG islands as gene markers in the human genome. Genomics 13: 1095-1107.

26. Saxonov S, Berg P, Brutlag DL (2006) A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc Natl Acad Sci U S A 103: 1412-1417.

27. Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, et al. (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39: 457-466.

28. Jones PA (2012) Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 13: 484-492.

29. Vinson C, Chatterjee R (2012) CG methylation. Epigenomics 4: 655-663.

30. Kinney SR, Pradhan S (2013) Ten eleven translocation enzymes and 5-hydroxymethylation in mammalian development and cancer. Adv Exp Med Biol 754: 57-79.

31. Carninci P, Sandelin A, Lenhard B, Katayama S, Shimokawa K, et al. (2006) Genome-wide analysis of mammalian promoter architecture and evolution. Nat Genet 38: 626-635.

32. Juven-Gershon T, Hsu JY, Kadonaga JT (2006) Perspectives on the RNA polymerase II core promoter. Biochem Soc Trans 34: 1047-1050.

33. Juven-Gershon T, Hsu JY, Theisen JW, Kadonaga JT (2008) The RNA polymerase II core promoter - the gateway to transcription. Curr Opin Cell Biol 20: 253-259.

34. Kim TH, Barrera LO, Zheng M, Qu C, Singer MA, et al. (2005) A high-resolution map of active promoters in the human genome. Nature 436: 876-880.

35. Sandelin A, Carninci P, Lenhard B, Ponjavic J, Hayashizaki Y, et al. (2007) Mammalian RNA polymerase II core promoters: insights from genome-wide studies. Nat Rev Genet 8: 424-436.

36. Smale ST, Kadonaga JT (2003) The RNA polymerase II core promoter. Annu Rev Biochem 72: 449-479.

37. Serizawa H, Conaway JW, Conaway RC (1993) Phosphorylation of C-terminal domain of RNA polymerase II is not required in basal transcription. Nature 363: 371-374.

38. Heidemann M, Hintermair C, Voss K, Eick D (2013) Dynamic phosphorylation patterns of RNA polymerase II CTD during transcription. Biochim Biophys Acta 1829: 55-62.

39. Buratowski S (2009) Progression through the RNA polymerase II CTD cycle. Mol Cell 36: 541-546.

40. Mischo HE, Proudfoot NJ (2013) Disengaging polymerase: terminating RNA polymerase II transcription in budding yeast. Biochim Biophys Acta 1829: 174-185.

41. Cho H, Kim TK, Mancebo H, Lane WS, Flores O, et al. (1999) A protein phosphatase functions to recycle RNA polymerase II. Genes Dev 13: 1540-1552.

42. Luger K, Mader AW, Richmond RK, Sargent DF, Richmond TJ (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature 389: 251-260.

43. Luger K, Rechsteiner TJ, Flaus AJ, Waye MM, Richmond TJ (1997) Characterization of nucleosome core particles containing histone proteins made in bacteria. J Mol Biol 272: 301-311.

44. Han M, Kim UJ, Kayne P, Grunstein M (1988) Depletion of histone H4 and nucleosomes activates the PHO5 gene in Saccharomyces cerevisiae. EMBO J 7: 2221-2228.

45. Kayne PS, Kim UJ, Han M, Mullen JR, Yoshizaki F, et al. (1988) Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell 55: 27-39.

46. Kim UJ, Han M, Kayne P, Grunstein M (1988) Effects of histone H4 depletion on the cell cycle and transcription of Saccharomyces cerevisiae. EMBO J 7: 2211-2219.

47. Hewish DR, Burgoyne LA (1973) Chromatin sub-structure. The digestion of chromatin DNA at regularly spaced sites by a nuclear deoxyribonuclease. Biochem Biophys Res Commun 52: 504-510.

48. Olins AL, Olins DE (1974) Spheroid chromatin units (v bodies). Science 183: 330-332.

49. McCall M, Brown T, Kennard O (1985) The crystal structure of d(G-G-G-G-C-C-C-C). A model for poly(dG).poly(dC). J Mol Biol 183: 385-396.

50. Nelson HC, Finch JT, Luisi BF, Klug A (1987) The structure of an oligo(dA). oligo(dT) tract and its biological implications. Nature 330: 221-226.

51. Segal E, Widom J (2009) Poly(dA:dT) tracts: major determinants of nucleosome organization. Curr Opin Struct Biol 19: 65-71.

52. Struhl K, Segal E (2013) Determinants of nucleosome positioning. Nat Struct Mol Biol 20: 267-273.

53. Suter B, Schnappauf G, Thoma F (2000) Poly(dA.dT) sequences exist as rigid DNA structures in nucleosome-free yeast promoters in vivo. Nucleic Acids Res 28: 4083-4089.

54. Iyer V, Struhl K (1995) Poly(dA:dT), a ubiquitous promoter element that stimulates transcription via its intrinsic DNA structure. EMBO J 14: 2570-2579.

55. Raveh-Sadka T, Levo M, Shabi U, Shany B, Keren L, et al. (2012) Manipulating nucleosome disfavoring sequences allows fine-tune regulation of gene expression in yeast. Nat Genet 44: 743-750.

56. Jiang C, Pugh BF (2009) A compiled and systematic reference map of nucleosome positions across the Saccharomyces cerevisiae genome. Genome Biol 10: R109.

57. Jiang C, Pugh BF (2009) Nucleosome positioning and gene regulation: advances through genomics. Nat Rev Genet 10: 161-172.

58. Mavrich TN, Ioshikhes IP, Venters BJ, Jiang C, Tomsho LP, et al. (2008) A barrier nucleosome model for statistical positioning of nucleosomes throughout the yeast genome. Genome Res 18: 1073-1083.

59. Mavrich TN, Jiang C, Ioshikhes IP, Li X, Venters BJ, et al. (2008) Nucleosome

organization in the Drosophila genome. Nature 453: 358-362.

60. Shivaswamy S, Bhinge A, Zhao Y, Jones S, Hirst M, et al. (2008) Dynamic remodeling of individual nucleosomes across a eukaryotic genome in response to transcriptional perturbation. PLoS Biol 6: e65.

61. Yen K, Vinayachandran V, Batta K, Koerber RT, Pugh BF (2012) Genome-wide nucleosome specificity and directionality of chromatin remodelers. Cell 149: 1461-1473.

62. Zhang Y, Moqtaderi Z, Rattner BP, Euskirchen G, Snyder M, et al. (2009) Intrinsic histone-DNA interactions are not the major determinant of nucleosome positions in vivo. Nat Struct Mol Biol 16: 847-852.

63. Rhee HS, Pugh BF (2012) Genome-wide structure and organization of eukaryotic pre-initiation complexes. Nature 483: 295-301.

64. Zhang Z, Wippo CJ, Wal M, Ward E, Korber P, et al. (2011) A packing mechanism for nucleosome organization reconstituted across a eukaryotic genome. Science 332: 977-980.

65. Badis G, Chan ET, van Bakel H, Pena-Castillo L, Tillo D, et al. (2008) A library of yeast transcription factor motifs reveals a widespread function for Rsc3 in targeting nucleosome exclusion at promoters. Mol Cell 32: 878-887.

66. Floer M, Wang X, Prabhu V, Berrozpe G, Narayan S, et al. (2010) A RSC/nucleosome complex determines chromatin architecture and facilitates activator binding. Cell 141: 407-418.

67. Henikoff S (2008) Nucleosome destabilization in the epigenetic regulation of gene expression. Nat Rev Genet 9: 15-26.

68. Hake SB, Allis CD (2006) Histone H3 variants and their potential role in indexing mammalian genomes: the “H3 barcode hypothesis”. Proc Natl Acad Sci U S A 103: 6428-6435.

69. Loyola A, Bonaldi T, Roche D, Imhof A, Almouzni G (2006) PTMs on H3 variants before chromatin assembly potentiate their final epigenetic state. Mol Cell 24: 309-316.

70. Talbert PB, Henikoff S (2010) Histone variants--ancient wrap artists of the epigenome. Nat Rev Mol Cell Biol 11: 264-275.

71. Adam M, Robert F, Larochelle M, Gaudreau L (2001) H2A.Z is required for global chromatin integrity and for recruitment of RNA polymerase II under specific conditions. Mol Cell Biol 21: 6270-6279.

72. Hardy S, Jacques PE, Gevry N, Forest A, Fortin ME, et al. (2009) The euchromatic and heterochromatic landscapes are shaped by antagonizing effects of transcription on H2A.Z deposition. PLoS Genet 5: e1000687.

73. Zlatanova J, Thakar A (2008) H2A.Z: view from the top. Structure 16: 166-179.

74. Fan JY, Rangasamy D, Luger K, Tremethick DJ (2004) H2A.Z alters the nucleosome surface to promote HP1alpha-mediated chromatin fiber folding. Mol Cell 16: 655-661.

75. Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature 403: 41-45.

76. Hathaway NA, Bell O, Hodges C, Miller EL, Neel DS, et al. (2012) Dynamics and memory of heterochromatin in living cells. Cell 149: 1447-1460.

77. Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, et al. (2007) High-resolution profiling of histone methylations in the human genome. Cell 129: 823-837.

78. Chen S, Ma J, Wu F, Xiong LJ, Ma H, et al. (2012) The histone H3 Lys 27 de-

methylase JMJD3 regulates gene expression by impacting transcriptional elongation. Genes Dev 26: 1364-1375.

79. Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, et al. (2007) Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature 448: 553-560.

80. Lee JS, Shilatifard A (2007) A site to remember: H3K36 methylation a mark for histone deacetylation. Mutat Res 618: 130-134.

81. Minsky N, Shema E, Field Y, Schuster M, Segal E, et al. (2008) Monoubiquitinated H2B is associated with the transcribed region of highly expressed genes in human cells. Nat Cell Biol 10: 483-488.

82. Batta K, Zhang Z, Yen K, Goffman DB, Pugh BF (2011) Genome-wide function of H2B ubiquitylation in promoter and genic regions. Genes Dev 25: 2254-2265.

83. Lang G, Bonnet J, Umlauf D, Karmodiya K, Koffler J, et al. (2011) The tightly controlled deubiquitination activity of the human SAGA complex differentially modifies distinct gene regulatory elements. Mol Cell Biol 31: 3734-3744.

84. Weake VM, Workman JL (2008) Histone ubiquitination: triggering gene activity. Mol Cell 29: 653-663.

85. Lauberth SM, Nakayama T, Wu X, Ferris AL, Tang Z, et al. (2013) H3K4me3 Interactions with TAF3 Regulate Preinitiation Complex Assembly and Selective Gene Activation. Cell 152: 1021-1036.

86. Bernstein BE, Kamal M, Lindblad-Toh K, Bekiranov S, Bailey DK, et al. (2005) Genomic maps and comparative analysis of histone modifications in human and mouse. Cell 120: 169-181.

87. Schneider R, Bannister AJ, Myers FA, Thorne AW, Crane-Robinson C, et al. (2004) Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. Nat Cell Biol 6: 73-77.

88. Hon G, Wang W, Ren B (2009) Discovery and annotation of functional chromatin signatures in the human genome. PLoS Comput Biol 5: e1000566.

89. Rada-Iglesias A, Bajpai R, Prescott S, Brugmann SA, Swigut T, et al. (2012) Epigenomic annotation of enhancers predicts transcriptional regulators of human neural crest. Cell Stem Cell 11: 633-648.

90. Shyh-Chang N, Locasale JW, Lyssiotis CA, Zheng Y, Teo RY, et al. (2013) Influence of threonine metabolism on S-adenosylmethionine and histone methylation. Science 339: 222-226.

91. Kim J, Hake SB, Roeder RG (2005) The human homolog of yeast BRE1 functions as a transcriptional coactivator through direct activator interactions. Mol Cell 20: 759-770.

92. Zhu B, Zheng Y, Pham AD, Mandal SS, Erdjument-Bromage H, et al. (2005) Monoubiquitination of human histone H2B: the factors involved and their roles in HOX gene regulation. Mol Cell 20: 601-611.

93. Henry KW, Wyce A, Lo WS, Duggan LJ, Emre NC, et al. (2003) Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. Genes Dev 17: 2648-2663.

94. Vastenhouw NL, Schier AF (2012) Bivalent histone modifications in early embryogenesis. Curr Opin Cell Biol 24: 374-386.

95. Voigt P, LeRoy G, Drury WJ, 3rd, Zee BM, Son J, et al. (2012) Asymmetrically modified nucleosomes. Cell 151: 181-193.

96. Smolle M, Workman JL (2013) Transcription-associated histone modifications

and cryptic transcription. Biochim Biophys Acta 1829: 84-97.

97. Creyghton MP, Cheng AW, Welstead GG, Kooistra T, Carey BW, et al. (2010) Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc Natl Acad Sci U S A 107: 21931-21936.

98. Heintzman ND, Hon GC, Hawkins RD, Kheradpour P, Stark A, et al. (2009) Histone modifications at human enhancers reflect global cell-type-specific gene expression. Nature 459: 108-112.

99. Rada-Iglesias A, Bajpai R, Swigut T, Brugmann SA, Flynn RA, et al. (2011) A unique chromatin signature uncovers early developmental enhancers in humans. Nature 470: 279-283.

100. Shogren-Knaak M, Ishii H, Sun JM, Pazin MJ, Davie JR, et al. (2006) Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science 311: 844-847.

101. Karmodiya K, Krebs AR, Oulad-Abdelghani M, Kimura H, Tora L (2012) H3K9 and H3K14 acetylation co-occur at many gene regulatory elements, while H3K14ac marks a subset of inactive inducible promoters in mouse embryonic stem cells. BMC Genomics 13: 424.

102. Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, et al. (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473: 43-49.

103. Grant PA, Duggan L, Cote J, Roberts SM, Brownell JE, et al. (1997) Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes Dev 11: 1640-1650.

104. Millar CB, Grunstein M (2006) Genome-wide patterns of histone modifications in yeast. Nat Rev Mol Cell Biol 7: 657-666.

105. Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, et al. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature 406: 593-599.

106. Black JC, Van Rechem C, Whetstine JR (2012) Histone lysine methylation dynamics: establishment, regulation, and biological impact. Mol Cell 48: 491-507.

107. Dillon SC, Zhang X, Trievel RC, Cheng X (2005) The SET-domain protein superfamily: protein lysine methyltransferases. Genome Biol 6: 227.

108. Dou Y, Milne TA, Ruthenburg AJ, Lee S, Lee JW, et al. (2006) Regulation of MLL1 H3K4 methyltransferase activity by its core components. Nat Struct Mol Biol 13: 713-719.

109. Milne TA, Briggs SD, Brock HW, Martin ME, Gibbs D, et al. (2002) MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol Cell 10: 1107-1117.

110. Nakamura T, Mori T, Tada S, Krajewski W, Rozovskaia T, et al. (2002) ALL-1 is a histone methyltransferase that assembles a supercomplex of proteins involved in transcriptional regulation. Mol Cell 10: 1119-1128.

111. Schneider J, Wood A, Lee JS, Schuster R, Dueker J, et al. (2005) Molecular regulation of histone H3 trimethylation by COMPASS and the regulation of gene expression. Mol Cell 19: 849-856.

112. van Nuland R, Smits AH, Pallaki P, Jansen PW, Vermeulen M, et al. (2013) Quantitative Dissection and Stoichiometry Determination of the Human SET1/MLL Histone Methyltransferase Complexes. Mol Cell Biol 33: 2067-2077.

113. Eissenberg JC, Shilatifard A (2010) Histone H3 lysine 4 (H3K4) methylation in development and differentiation. Dev Biol 339: 240-249.

114. Shilatifard A (2008) Molecular implementation and physiological roles for histone H3 lysine 4 (H3K4) methylation. Curr Opin Cell Biol 20: 341-348.

115. Shilatifard A (2012) The COMPASS family of histone H3K4 methylases: mechanisms of regulation in development and disease pathogenesis. Annu Rev Biochem 81: 65-95.

116. Lee JH, Skalnik DG (2005) CpG-binding protein (CXXC finger protein 1) is a component of the mammalian Set1 histone H3-Lys4 methyltransferase complex, the analogue of the yeast Set1/COMPASS complex. J Biol Chem 280: 41725-41731.

117. Miller T, Krogan NJ, Dover J, Erdjument-Bromage H, Tempst P, et al. (2001) COMPASS: a complex of proteins associated with a trithorax-related SET domain protein. Proc Natl Acad Sci U S A 98: 12902-12907.

118. Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, et al. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell 119: 941-953.

119. Shi Y, Sawada J, Sui G, Affar el B, Whetstine JR, et al. (2003) Coordinated histone modifications mediated by a CtBP co-repressor complex. Nature 422: 735-738.

120. Cloos PA, Christensen J, Agger K, Maiolica A, Rappsilber J, et al. (2006) The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. Nature 442: 307-311.

121. Fodor BD, Kubicek S, Yonezawa M, O'Sullivan RJ, Sengupta R, et al. (2006) Jmjd2b antagonizes H3K9 trimethylation at pericentric heterochromatin in mammalian cells. Genes Dev 20: 1557-1562.

122. Klose RJ, Yamane K, Bae Y, Zhang D, Erdjument-Bromage H, et al. (2006) The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36. Nature 442: 312-316.

123. Trojer P, Zhang J, Yonezawa M, Schmidt A, Zheng H, et al. (2009) Dynamic Histone H1 Isotype 4 Methylation and Demethylation by Histone Lysine Methyltransferase G9a/KMT1C and the Jumonji Domain-containing JMJD2/KDM4 Proteins. J Biol Chem 284: 8395-8405.

124. Whetstine JR, Nottke A, Lan F, Huarte M, Smolikov S, et al. (2006) Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. Cell 125: 467-481.

125. Loidl P (1994) Histone acetylation: facts and questions. Chromosoma 103: 441-449.

126. Glozak MA, Sengupta N, Zhang X, Seto E (2005) Acetylation and deacetylation of non-histone proteins. Gene 363: 15-23.

127. Zhang K, Dent SY (2005) Histone modifying enzymes and cancer: going beyond histones. J Cell Biochem 96: 1137-1148.

128. Dekker FJ, Haisma HJ (2009) Histone acetyl transferases as emerging drug targets. Drug Discov Today 14: 942-948.

129. Hodawadekar SC, Marmorstein R (2007) Chemistry of acetyl transfer by histone modifying enzymes: structure, mechanism and implications for effector design. Oncogene 26: 5528-5540.

130. Wang L, Tang Y, Cole PA, Marmorstein R (2008) Structure and chemistry of the p300/CBP and Rtt109 histone acetyltransferases: implications for histone acetyltransferase evolution and function. Curr Opin Struct Biol 18: 741-747.

131. Kasper LH, Lerach S, Wang J, Wu S, Jeevan T, et al. (2010) CBP/p300 double null cells reveal effect of coactivator level and diversity on CREB transactivation. EMBO J 29: 3660-3672.

132. Vo N, Goodman RH (2001) CREB-binding protein and p300 in transcriptional regulation. J Biol Chem 276: 13505-13508.

133. Brownell JE, Zhou J, Ranalli T, Kobayashi R, Edmondson DG, et al. (1996) Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. Cell 84: 843-851.

134. Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87: 953-959.

135. de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J 370: 737-749.

136. Feng Q, Zhang Y (2001) The MeCP1 complex represses transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes. Genes Dev 15: 827-832.

137. Spruijt CG, Bartels SJ, Brinkman AB, Tjeertes JV, Poser I, et al. (2010) CDK2AP1/DOC-1 is a bona fide subunit of the Mi-2/NuRD complex. Mol Biosyst 6: 1700-1706.

138. Tong JK, Hassig CA, Schnitzler GR, Kingston RE, Schreiber SL (1998) Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. Nature 395: 917-921.

139. Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, et al. (2002) HDAC6 is a microtubule-associated deacetylase. Nature 417: 455-458.

140. Juan LJ, Shia WJ, Chen MH, Yang WM, Seto E, et al. (2000) Histone deacetylases specifically down-regulate p53-dependent gene activation. J Biol Chem 275: 20436-20443.

141. Ropero S, Fraga MF, Ballestar E, Hamelin R, Yamamoto H, et al. (2006) A truncating mutation of HDAC2 in human cancers confers resistance to histone deacetylase inhibition. Nat Genet 38: 566-569.

142. Zhu P, Martin E, Mengwasser J, Schlag P, Janssen KP, et al. (2004) Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis. Cancer Cell 5: 455-463.

143. Wagner JM, Hackanson B, Lubbert M, Jung M (2010) Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy. Clin Epigenetics 1: 117-136.

144. Kristensen LS, Nielsen HM, Hansen LL (2009) Epigenetics and cancer treatment. Eur J Pharmacol 625: 131-142.

145. Prebet T, Vey N (2011) Vorinostat in acute myeloid leukemia and myelodysplastic syndromes. Expert Opin Investig Drugs 20: 287-295.

146. Vigna E, Recchia AG, Madeo A, Gentile M, Bossio S, et al. (2011) Epigenetic regulation in myelodysplastic syndromes: implications for therapy. Expert Opin Investig Drugs 20: 465-493.

147. Yoshida M, Kijima M, Akita M, Beppu T (1990) Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. J Biol Chem 265: 17174-17179.

148. Song SH, Han SW, Bang YJ (2011) Epigenetic-based therapies in cancer:

progress to date. Drugs 71: 2391-2403.

149. Musselman CA, Lalonde ME, Cote J, Kutateladze TG (2012) Perceiving the epigenetic landscape through histone readers. Nat Struct Mol Biol 19: 1218-1227.

150. Varier RA, Outchkourov NS, de Graaf P, van Schaik FM, Ensing HJ, et al. (2010) A phospho/methyl switch at histone H3 regulates TFIID association with mitotic chromosomes. EMBO J 29: 3967-3978.

151. Jacobson RH, Ladurner AG, King DS, Tjian R (2000) Structure and function of a human TAFII250 double bromodomain module. Science 288: 1422-1425.

152. Saksouk N, Avvakumov N, Champagne KS, Hung T, Doyon Y, et al. (2009) HBO1 HAT complexes target chromatin throughout gene coding regions via multiple PHD finger interactions with histone H3 tail. Mol Cell 33: 257-265.

153. Shi X, Hong T, Walter KL, Ewalt M, Michishita E, et al. (2006) ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature 442: 96-99.

154. Binda O (2013) On your histone mark, SET, methylate! Epigenetics 8.

155. Cler E, Papai G, Schultz P, Davidson I (2009) Recent advances in understanding the structure and function of general transcription factor TFIID. Cell Mol Life Sci 66: 2123-2134.

156. Sanders SL, Garbett KA, Weil PA (2002) Molecular characterization of Saccharomyces cerevisiae TFIID. Mol Cell Biol 22: 6000-6013.

157. Bieniossek C, Papai G, Schaffitzel C, Garzoni F, Chaillet M, et al. (2013) The architecture of human general transcription factor TFIID core complex. Nature 493: 699-702.

158. Papai G, Tripathi MK, Ruhlmann C, Layer JH, Weil PA, et al. (2010) TFIIA and the transactivator Rap1 cooperate to commit TFIID for transcription initiation. Nature 465: 956-960.

159. Brand M, Leurent C, Mallouh V, Tora L, Schultz P (1999) Three-dimensional structures of the TAFII-containing complexes TFIID and TFTC. Science 286: 2151-2153.

160. Muller F, Demeny MA, Tora L (2007) New problems in RNA polymerase II transcription initiation: matching the diversity of core promoters with a variety of promoter recognition factors. J Biol Chem 282: 14685-14689.

161. Falender AE, Freiman RN, Geles KG, Lo KC, Hwang K, et al. (2005) Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. Genes Dev 19: 794-803.

162. Geles KG, Freiman RN, Liu WL, Zheng S, Voronina E, et al. (2006) Cell-type-selective induction of c-jun by TAF4b directs ovarian-specific transcription networks. Proc Natl Acad Sci U S A 103: 2594-2599.

163. Kolthur-Seetharam U, Martianov I, Davidson I (2008) Specialization of the general transcriptional machinery in male germ cells. Cell Cycle 7: 3493-3498.

164. Frontini M, Soutoglou E, Argentini M, Bole-Feysot C, Jost B, et al. (2005) TAF9b (formerly TAF9L) is a bona fide TAF that has unique and overlapping roles with TAF9. Mol Cell Biol 25: 4638-4649.

165. Deato MD, Marr MT, Sottero T, Inouye C, Hu P, et al. (2008) MyoD targets TAF3/TRF3 to activate myogenin transcription. Mol Cell 32: 96-105.

166. Deato MD, Tjian R (2008) An unexpected role of TAFs and TRFs in skeletal muscle differentiation: switching core promoter complexes. Cold Spring Harb Symp Quant Biol 73: 217-225.

167. Indra AK, Mohan WS, 2nd, Frontini M, Scheer E, Messaddeq N, et al. (2005) TAF10 is required for the establishment of skin barrier function in foetal, but not in adult mouse epidermis. Dev Biol 285: 28-37.

168. Liu Z, Scannell DR, Eisen MB, Tjian R (2011) Control of embryonic stem cell lineage commitment by core promoter factor, TAF3. Cell 146: 720-731.

169. Pijnappel WW, Esch D, Baltissen MP, Wu G, Mischerikow N, et al. (2013) A central role for TFIID in the pluripotent transcription circuitry. Nature 495: 516-519.

170. Vermeulen M, Mulder KW, Denissov S, Pijnappel WW, van Schaik FM, et al. (2007) Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4. Cell 131: 58-69.

171. Moriniere J, Rousseaux S, Steuerwald U, Soler-Lopez M, Curtet S, et al. (2009) Cooperative binding of two acetylation marks on a histone tail by a single bromodomain. Nature 461: 664-668.

172. Spedale G, Timmers HT, Pijnappel WW (2012) ATAC-king the complexity of SAGA during evolution. Genes Dev 26: 527-541.

173. Samara NL, Datta AB, Berndsen CE, Zhang X, Yao T, et al. (2010) Structural insights into the assembly and function of the SAGA deubiquitinating module. Science 328: 1025-1029.

174. Grant PA, Schieltz D, Pray-Grant MG, Steger DJ, Reese JC, et al. (1998) A subset of TAF(II)s are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation. Cell 94: 45-53.

175. Wu PY, Ruhlmann C, Winston F, Schultz P (2004) Molecular architecture of the S. cerevisiae SAGA complex. Mol Cell 15: 199-208.

176. Ogryzko VV, Kotani T, Zhang X, Schiltz RL, Howard T, et al. (1998) Histone-like TAFs within the PCAF histone acetylase complex. Cell 94: 35-44.

177. Gangloff YG, Romier C, Thuault S, Werten S, Davidson I (2001) The histone fold is a key structural motif of transcription factor TFIID. Trends Biochem Sci 26: 250-257.

178. Gangloff YG, Werten S, Romier C, Carre L, Poch O, et al. (2000) The human TFIID components TAF(II)135 and TAF(II)20 and the yeast SAGA components ADA1 and TAF(II)68 heterodimerize to form histone-like pairs. Mol Cell Biol 20: 340-351.

179. Selleck W, Howley R, Fang Q, Podolny V, Fried MG, et al. (2001) A histone fold TAF octamer within the yeast TFIID transcriptional coactivator. Nat Struct Biol 8: 695-700.

180. Wu PY, Winston F (2002) Analysis of Spt7 function in the Saccharomyces cerevisiae SAGA coactivator complex. Mol Cell Biol 22: 5367-5379.

181. Lang D, Thoma R, Henn-Sax M, Sterner R, Wilmanns M (2000) Structural evidence for evolution of the beta/alpha barrel scaffold by gene duplication and fusion. Science 289: 1546-1550.

182. Sterner DE, Grant PA, Roberts SM, Duggan LJ, Belotserkovskaya R, et al. (1999) Functional organization of the yeast SAGA complex: distinct components involved in structural integrity, nucleosome acetylation, and TATA-binding protein interaction. Mol Cell Biol 19: 86-98.

183. Brown CE, Howe L, Sousa K, Alley SC, Carrozza MJ, et al. (2001) Recruitment of HAT complexes by direct activator interactions with the ATM-related Tra1 subunit. Science 292: 2333-2337.

184. Demeny MA, Soutoglou E, Nagy Z, Scheer E, Janoshazi A, et al. (2007) Identification of a small TAF complex and its role in the assembly of TAF-containing

complexes. PLoS One 2: e316.

185. Martinez E, Palhan VB, Tjernberg A, Lymar ES, Gamper AM, et al. (2001) Human STAGA complex is a chromatin-acetylating transcription coactivator that interacts with pre-mRNA splicing and DNA damage-binding factors in vivo. Mol Cell Biol 21: 6782-6795.

186. Nagy Z, Riss A, Romier C, le Guezennec X, Dongre AR, et al. (2009) The human SPT20-containing SAGA complex plays a direct role in the regulation of endoplasmic reticulum stress-induced genes. Mol Cell Biol 29: 1649-1660.

187. Wang YL, Faiola F, Xu M, Pan S, Martinez E (2008) Human ATAC Is a GCN5/PCAF-containing acetylase complex with a novel NC2-like histone fold module that interacts with the TATA-binding protein. J Biol Chem 283: 33808-33815.

188. Nagy Z, Tora L (2007) Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. Oncogene 26: 5341-5357.

189. Barbaric S, Reinke H, Horz W (2003) Multiple mechanistically distinct functions of SAGA at the PHO5 promoter. Mol Cell Biol 23: 3468-3476.

190. Bhaumik SR, Green MR (2001) SAGA is an essential in vivo target of the yeast acidic activator Gal4p. Genes Dev 15: 1935-1945.

191. Larschan E, Winston F (2001) The S. cerevisiae SAGA complex functions in vivo as a coactivator for transcriptional activation by Gal4. Genes Dev 15: 1946-1956.

192. Yu J, Madison JM, Mundlos S, Winston F, Olsen BR (1998) Characterization of a human homologue of the Saccharomyces cerevisiae transcription factor spt3 (SUPT3H). Genomics 53: 90-96.

193. Lee KK, Swanson SK, Florens L, Washburn MP, Workman JL (2009) Yeast Sgf73/Ataxin-7 serves to anchor the deubiquitination module into both SAGA and Slik(SALSA) HAT complexes. Epigenetics Chromatin 2: 2.

194. Rodriguez-Navarro S, Fischer T, Luo MJ, Antunez O, Brettschneider S, et al. (2004) Sus1, a functional component of the SAGA histone acetylase complex and the nuclear pore-associated mRNA export machinery. Cell 116: 75-86.

195. Galan A, Rodriguez-Navarro S (2012) Sus1/ENY2: a multitasking protein in eukaryotic gene expression. Crit Rev Biochem Mol Biol 47: 556-568.

196. Bonnet J, Wang YH, Spedale G, Atkinson RA, Romier C, et al. (2010) The structural plasticity of SCA7 domains defines their differential nucleosome-binding properties. EMBO Rep 11: 612-618.

197. Koutelou E, Hirsch CL, Dent SY (2010) Multiple faces of the SAGA complex. Curr Opin Cell Biol 22: 374-382.

198. Krebs AR, Demmers J, Karmodiya K, Chang NC, Chang AC, et al. (2010) ATAC and Mediator coactivators form a stable complex and regulate a set of non-coding RNA genes. EMBO Rep 11: 541-547.

199. Balasubramanian R, Pray-Grant MG, Selleck W, Grant PA, Tan S (2002) Role of the Ada2 and Ada3 transcriptional coactivators in histone acetylation. J Biol Chem 277: 7989-7995.

200. Muratoglu S, Georgieva S, Papai G, Scheer E, Enunlu I, et al. (2003) Two different Drosophila ADA2 homologues are present in distinct GCN5 histone acetyltransferase-containing complexes. Mol Cell Biol 23: 306-321.

201. Nagy Z, Riss A, Fujiyama S, Krebs A, Orpinell M, et al. (2010) The metazoan ATAC and SAGA coactivator HAT complexes regulate different sets of inducible target genes. Cell Mol Life Sci 67: 611-628.

202. Orpinell M, Fournier M, Riss A, Nagy Z, Krebs AR, et al. (2010) The ATAC acetyl transferase complex controls mitotic progression by targeting non-histone substrates. EMBO J 29: 2381-2394.

203. Gamper AM, Kim J, Roeder RG (2009) The STAGA subunit ADA2b is an important regulator of human GCN5 catalysis. Mol Cell Biol 29: 266-280.

204. Bian C, Xu C, Ruan J, Lee KK, Burke TL, et al. (2011) Sgf29 binds histone H3K4me2/3 and is required for SAGA complex recruitment and histone H3 acetylation. EMBO J 30: 2829-2842.

205. Vermeulen M, Eberl HC, Matarese F, Marks H, Denissov S, et al. (2010) Quantitative interaction proteomics and genome-wide profiling of epigenetic histone marks and their readers. Cell 142: 967-980.

206. Sanders SL, Jennings J, Canutescu A, Link AJ, Weil PA (2002) Proteomics of the eukaryotic transcription machinery: identification of proteins associated with components of yeast TFIID by multidimensional mass spectrometry. Mol Cell Biol 22: 4723-4738.

207. Lee TI, Causton HC, Holstege FC, Shen WC, Hannett N, et al. (2000) Redundant roles for the TFIID and SAGA complexes in global transcription. Nature 405: 701-704.

208. Huisinga KL, Pugh BF (2004) A genome-wide housekeeping role for TFIID and a highly regulated stress-related role for SAGA in Saccharomyces cerevisiae. Mol Cell 13: 573-585.

209. Ghosh S, Pugh BF (2011) Sequential recruitment of SAGA and TFIID in a genomic response to DNA damage in Saccharomyces cerevisiae. Mol Cell Biol 31: 190-202.

210. Krebs AR, Karmodiya K, Lindahl-Allen M, Struhl K, Tora L (2011) SAGA and ATAC histone acetyl transferase complexes regulate distinct sets of genes and ATAC defines a class of p300-independent enhancers. Mol Cell 44: 410-423.

211. Daniel JA, Torok MS, Sun ZW, Schieltz D, Allis CD, et al. (2004) Deubiquitination of histone H2B by a yeast acetyltransferase complex regulates transcription. J Biol Chem 279: 1867-1871.

212. Zhang XY, Pfeiffer HK, Thorne AW, McMahon SB (2008) USP22, an hSAGA subunit and potential cancer stem cell marker, reverses the polycomb-catalyzed ubiquitylation of histone H2A. Cell Cycle 7: 1522-1524.

213. Zhang XY, Varthi M, Sykes SM, Phillips C, Warzecha C, et al. (2008) The putative cancer stem cell marker USP22 is a subunit of the human SAGA complex required for activated transcription and cell-cycle progression. Mol Cell 29: 102-111.

214. Zhao Y, Lang G, Ito S, Bonnet J, Metzger E, et al. (2008) A TFTC/STAGA module mediates histone H2A and H2B deubiquitination, coactivates nuclear receptors, and counteracts heterochromatin silencing. Mol Cell 29: 92-101.

215. Mischerikow N, Spedale G, Altelaar AF, Timmers HT, Pijnappel WW, et al. (2009) In-depth profiling of post-translational modifications on the related transcription factor complexes TFIID and SAGA. J Proteome Res 8: 5020-5030.

216. Ruthenburg AJ, Allis CD, Wysocka J (2007) Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. Mol Cell 25: 15-30.

217. Martin C, Zhang Y (2005) The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol 6: 838-849.

218. Vermeulen M, Timmers HT (2010) Grasping trimethylation of histone H3 at lysine 4. Epigenomics 2: 395-406.

219. Eissenberg JC (2012) Structural biology of the chromodomain: form and function. Gene 496: 69-78.

220. Li H, Ilin S, Wang W, Duncan EM, Wysocka J, et al. (2006) Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. Nature 442: 91-95.

221. Yap KL, Zhou MM (2010) Keeping it in the family: diverse histone recognition by conserved structural folds. Crit Rev Biochem Mol Biol 45: 488-505.

222. Wysocka J, Swigut T, Xiao H, Milne TA, Kwon SY, et al. (2006) A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. Nature 442: 86-90.

223. Tora L (2002) A unified nomenclature for TATA box binding protein (TBP)-associated factors (TAFs) involved in RNA polymerase II transcription. Genes Dev 16: 673-675.

224. Samara NL, Wolberger C (2011) A new chapter in the transcription SAGA. Curr Opin Struct Biol 21: 767-774.

225. Shukla A, Lahudkar S, Durairaj G, Bhaumik SR (2012) Sgf29p facilitates the recruitment of TATA box binding protein but does not alter SAGA's global structural integrity in vivo. Biochemistry 51: 706-714.

226. Lee KK, Sardiu ME, Swanson SK, Gilmore JM, Torok M, et al. (2011) Combinatorial depletion analysis to assemble the network architecture of the SAGA and ADA chromatin remodeling complexes. Mol Syst Biol 7: 503.

227. Sela D, Chen L, Martin-Brown S, Washburn MP, Florens L, et al. (2012) Endoplasmic reticulum stress-responsive transcription factor ATF6alpha directs recruitment of the Mediator of RNA polymerase II transcription and multiple histone acetyltransferase complexes. J Biol Chem 287: 23035-23045.

228. Gorman AM, Healy SJ, Jager R, Samali A (2012) Stress management at the ER: regulators of ER stress-induced apoptosis. Pharmacol Ther 134: 306-316.

229. Tighe A, Staples O, Taylor S (2008) Mps1 kinase activity restrains anaphase during an unperturbed mitosis and targets Mad2 to kinetochores. J Cell Biol 181: 893-901.

230. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, et al. (2011) Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res 10: 1794-1805.

231. Samali A, Fitzgerald U, Deegan S, Gupta S (2010) Methods for monitoring endoplasmic reticulum stress and the unfolded protein response. Int J Cell Biol 2010: 830307.

232. Raciti M, Lotti LV, Valia S, Pulcinelli FM, Di Renzo L (2012) JNK2 is activated during ER stress and promotes cell survival. Cell Death Dis 3: e429.

233. Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol 8: 519-529.

234. Dorner AJ, Wasley LC, Raney P, Haugejorden S, Green M, et al. (1990) The stress response in Chinese hamster ovary cells. Regulation of ERp72 and protein disulfide isomerase expression and secretion. J Biol Chem 265: 22029-22034.

235. Takahashi YH, Schulze JM, Jackson J, Hentrich T, Seidel C, et al. (2011) Dot1 and histone H3K79 methylation in natural telomeric and HM silencing. Mol Cell 42: 118-126.

236. Lee JH, Tate CM, You JS, Skalnik DG (2007) Identification and characterization of the human Set1B histone H3-Lys4 methyltransferase complex. J Biol Chem

282: 13419-13428.

237. Grant PA, Eberharter A, John S, Cook RG, Turner BM, et al. (1999) Expanded lysine acetylation specificity of Gcn5 in native complexes. J Biol Chem 274: 5895-5900.

238. Eberl HC, Spruijt CG, Kelstrup CD, Vermeulen M, Mann M (2013) A Map of General and Specialized Chromatin Readers in Mouse Tissues Generated by Label-free Interaction Proteomics. Mol Cell 49: 368-378.

239. Hung T, Binda O, Champagne KS, Kuo AJ, Johnson K, et al. (2009) ING4 mediates crosstalk between histone H3 K4 trimethylation and H3 acetylation to attenuate cellular transformation. Mol Cell 33: 248-256.

240. Donati G, Imbriano C, Mantovani R (2006) Dynamic recruitment of transcription factors and epigenetic changes on the ER stress response gene promoters. Nucleic Acids Res 34: 3116-3127.

241. McMahon SB, Van Buskirk HA, Dugan KA, Copeland TD, Cole MD (1998) The novel ATM-related protein TRRAP is an essential cofactor for the c-Myc and E2F oncoproteins. Cell 94: 363-374.

242. Jenuwein T, Allis CD (2001) Translating the histone code. Science 293: 1074-1080.

243. Dignam JD, Martin PL, Shastry BS, Roeder RG (1983) Eukaryotic gene transcription with purified components. Methods Enzymol 101: 582-598.

244. Wisniewski JR, Zougman A, Nagaraj N, Mann M (2009) Universal sample preparation method for proteome analysis. Nat Methods 6: 359-362.

245. Schram AW, Baas R, Jansen PW, Riss A, Tora L, et al. (2013) A Dual Role for SAGA-Associated Factor 29 (SGF29) in ER Stress Survival by Coordination of Both Histone H3 Acetylation and Histone H3 Lysine-4 Trimethylation. PLoS One 8: e70035.

246. Allfrey VG, Faulkner R, Mirsky AE (1964) Acetylation and Methylation of Histones and Their Possible Role in the Regulation of Rna Synthesis. Proc Natl Acad Sci U S A 51: 786-794.

247. Struhl K (1998) Histone acetylation and transcriptional regulatory mechanisms. Genes Dev 12: 599-606.

248. Tropberger P, Pott S, Keller C, Kamieniarz-Gdula K, Caron M, et al. (2013) Regulation of transcription through acetylation of H3K122 on the lateral surface of the histone octamer. Cell 152: 859-872.

249. Sterner DE, Berger SL (2000) Acetylation of histones and transcription-related factors. Microbiol Mol Biol Rev 64: 435-459.

250. Prinjha RK, Witherington J, Lee K (2012) Place your BETs: the therapeutic potential of bromodomains. Trends Pharmacol Sci 33: 146-153.

251. Sanchez R, Zhou MM (2009) The role of human bromodomains in chromatin biology and gene transcription. Curr Opin Drug Discov Devel 12: 659-665.

252. Xhemalce B, Kouzarides T (2010) A chromodomain switch mediated by histone H3 Lys 4 acetylation regulates heterochromatin assembly. Genes Dev 24: 647-652.

253. Guillemette B, Drogaris P, Lin HH, Armstrong H, Hiragami-Hamada K, et al. (2011) H3 lysine 4 is acetylated at active gene promoters and is regulated by H3 lysine 4 methylation. PLoS Genet 7: e1001354.

254. Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, et al. (2008) Combinatorial patterns of histone acetylations and methylations in the human genome. Nat Genet 40: 897-903.

255. Marmorstein R (2001) Structure of histone acetyltransferases. J Mol Biol 311: 433-444.

256. Iizuka M, Sarmento OF, Sekiya T, Scrable H, Allis CD, et al. (2008) Hbo1 Links p53-dependent stress signaling to DNA replication licensing. Mol Cell Biol 28: 140-153.

257. Dignam JD, Lebovitz RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11: 1475-1489.

258. Barneda-Zahonero B, Parra M (2012) Histone deacetylases and cancer. Mol Oncol 6: 579-589.

259. Avvakumov N, Cote J (2007) The MYST family of histone acetyltransferases and their intimate links to cancer. Oncogene 26: 5395-5407.

260. Foy RL, Song IY, Chitalia VC, Cohen HT, Saksouk N, et al. (2008) Role of Jade-1 in the histone acetyltransferase (HAT) HBO1 complex. J Biol Chem 283: 28817-28826.

261. Thomas T, Voss AK (2007) The diverse biological roles of MYST histone acetyltransferase family proteins. Cell Cycle 6: 696-704.

262. Jeong KW, Kim K, Situ AJ, Ulmer TS, An W, et al. (2011) Recognition of enhancer element-specific histone methylation by TIP60 in transcriptional activation. Nat Struct Mol Biol 18: 1358-1365.

263. Utley RT, Cote J (2003) The MYST family of histone acetyltransferases. Curr Top Microbiol Immunol 274: 203-236.

264. Miotto B, Struhl K (2008) HBO1 histone acetylase is a coactivator of the replication licensing factor Cdt1. Genes Dev 22: 2633-2638.

265. Doyon Y, Cayrou C, Ullah M, Landry AJ, Cote V, et al. (2006) ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. Mol Cell 21: 51-64.

266. Panchenko MV, Zhou MI, Cohen HT (2004) von Hippel-Lindau partner Jade-1 is a transcriptional co-activator associated with histone acetyltransferase activity. J Biol Chem 279: 56032-56041.

267. Georgiakaki M, Chabbert-Buffet N, Dasen B, Meduri G, Wenk S, et al. (2006) Ligand-controlled interaction of histone acetyltransferase binding to ORC-1 (HBO1) with the N-terminal transactivating domain of progesterone receptor induces steroid receptor coactivator 1-dependent coactivation of transcription. Mol Endocrinol 20: 2122-2140.

268. Garcia BA, Hake SB, Diaz RL, Kauer M, Morris SA, et al. (2007) Organismal differences in post-translational modifications in histones H3 and H4. J Biol Chem 282: 7641-7655.

269. Rando OJ (2007) Global patterns of histone modifications. Curr Opin Genet Dev 17: 94-99.

270. Kueh AJ, Dixon MP, Voss AK, Thomas T (2011) HBO1 is required for H3K14 acetylation and normal transcriptional activity during embryonic development. Mol Cell Biol 31: 845-860.

271. Buratowski S, Hahn S, Guarente L, Sharp PA (1989) Five intermediate complexes in transcription initiation by RNA polymerase II. Cell 56: 549-561.

272. Thomas MC, Chiang CM (2006) The general transcription machinery and general cofactors. Crit Rev Biochem Mol Biol 41: 105-178.

273. Wright KJ, Marr MT, 2nd, Tjian R (2006) TAF4 nucleates a core subcomplex of

TFIID and mediates activated transcription from a TATA-less promoter. Proc Natl Acad Sci U S A 103: 12347-12352.

274. Kaufmann J, Smale ST (1994) Direct recognition of initiator elements by a component of the transcription factor IID complex. Genes Dev 8: 821-829.

275. Bajic VB, Tan SL, Christoffels A, Schonbach C, Lipovich L, et al. (2006) Mice and men: their promoter properties. PLoS Genet 2: e54.

276. FitzGerald PC, Shlyakhtenko A, Mir AA, Vinson C (2004) Clustering of DNA sequences in human promoters. Genome Res 14: 1562-1574.

277. Geiger JH, Hahn S, Lee S, Sigler PB (1996) Crystal structure of the yeast TFIIA/TBP/DNA complex. Science 272: 830-836.

278. Tan S, Hunziker Y, Sargent DF, Richmond TJ (1996) Crystal structure of a yeast TFIIA/TBP/DNA complex. Nature 381: 127-151.

279. Burke TW, Kadonaga JT (1996) Drosophila TFIID binds to a conserved downstream basal promoter element that is present in many TATA-box-deficient promoters. Genes Dev 10: 711-724.

280. Berger SL (2007) The complex language of chromatin regulation during transcription. Nature 447: 407-412.

281. Kouzarides T (2007) Chromatin modifications and their function. Cell 128: 693-705.

282. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, et al. (2007) Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet 39: 311-318.

283. Li B, Pattenden SG, Lee D, Gutierrez J, Chen J, et al. (2005) Preferential occupancy of histone variant H2AZ at inactive promoters influences local histone modifications and chromatin remodeling. Proc Natl Acad Sci U S A 102: 18385-18390.

284. Workman JL, Roeder RG (1987) Binding of transcription factor TFIID to the major late promoter during in vitro nucleosome assembly potentiates subsequent initiation by RNA polymerase II. Cell 51: 613-622.

285. Simon MD, Chu F, Racki LR, de la Cruz CC, Burlingame AL, et al. (2007) The site-specific installation of methyl-lysine analogs into recombinant histones. Cell 128: 1003-1012.

286. Bartke T, Vermeulen M, Xhemalce B, Robson SC, Mann M, et al. (2010) Nucleosome-interacting proteins regulated by DNA and histone methylation. Cell 143: 470-484.

287. Nikolov M, Stutzer A, Mosch K, Krasauskas A, Soeroes S, et al. (2011) Chromatin affinity purification and quantitative mass spectrometry defining the interactome of histone modification patterns. Mol Cell Proteomics 10: M110 005371.

288. Hahn S, Buratowski S, Sharp PA, Guarente L (1989) Yeast TATA-binding protein TFIID binds to TATA elements with both consensus and nonconsensus DNA sequences. Proc Natl Acad Sci U S A 86: 5718-5722.

289. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, et al. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125: 315-326.

290. Juven-Gershon T, Kadonaga JT (2010) Regulation of gene expression via the core promoter and the basal transcriptional machinery. Dev Biol 339: 225-229.

291. Calo E, Wysocka J (2013) Modification of enhancer chromatin: what, how, and why? Mol Cell 49: 825-837.

292. Neumann H, Hancock SM, Buning R, Routh A, Chapman L, et al. (2009) A

method for genetically installing site-specific acetylation in recombinant histones defines the effects of H3 K56 acetylation. Mol Cell 36: 153-163.

293. Hubner NC, Mann M (2011) Extracting gene function from protein-protein interactions using Quantitative BAC InteraCtomics (QUBIC). Methods 53: 453-459.

294. Smits AH, Jansen PW, Poser I, Hyman AA, Vermeulen M (2013) Stoichiometry of chromatin-associated protein complexes revealed by label-free quantitative mass spectrometry-based proteomics. Nucleic Acids Res 41: e28.

295. Hubner NC, Bird AW, Cox J, Splettstoesser B, Bandilla P, et al. (2010) Quantitative proteomics combined with BAC TransgeneOmics reveals in vivo protein interactions. J Cell Biol 189: 739-754.

296. Leurent C, Sanders S, Ruhlmann C, Mallouh V, Weil PA, et al. (2002) Mapping histone fold TAFs within yeast TFIID. EMBO J 21: 3424-3433.

297. Cheng Y, Buffone MG, Kouadio M, Goodheart M, Page DC, et al. (2007) Abnormal sperm in mice lacking the Taf7l gene. Mol Cell Biol 27: 2582-2589.

298. Liu WL, Coleman RA, Grob P, King DS, Florens L, et al. (2008) Structural changes in TAF4b-TFIID correlate with promoter selectivity. Mol Cell 29: 81-91.

299. Basehoar AD, Zanton SJ, Pugh BF (2004) Identification and distinct regulation of yeast TATA box-containing genes. Cell 116: 699-709.

300. Dyer PN, Edayathumangalam RS, White CL, Bao Y, Chakravarthy S, et al. (2004) Reconstitution of nucleosome core particles from recombinant histones and DNA. Methods Enzymol 375: 23-44.

301. van Nuland R, van Schaik FM, Simonis M, van Heesch S, Cuppen E, et al. (2013) Nucleosomal DNA binding drives the recognition of H3K36-methylated nucleosomes by the PSIP1-PWWP domain. Epigenetics Chromatin 6: 12.

302. Cox J, Mann M (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol 26: 1367-1372.

303. Ohtsuki K, Kasahara K, Shirahige K, Kokubo T (2010) Genome-wide localization analysis of a complete set of Tafs reveals a specific effect of the taf1 mutation on Taf2 occupancy and provides indirect evidence for different TFIID conformations at different promoters. Nucleic Acids Res 38: 1805-1820.

304. van Ingen H, van Schaik FM, Wienk H, Ballering J, Rehmann H, et al. (2008) Structural insight into the recognition of the H3K4me3 mark by the TFIID subunit TAF3. Structure 16: 1245-1256.

305. Shen WC, Bhaumik SR, Causton HC, Simon I, Zhu X, et al. (2003) Systematic analysis of essential yeast TAFs in genome-wide transcription and preinitiation complex assembly. EMBO J 22: 3395-3402.

306. Margaritis T, Oreal V, Brabers N, Maestroni L, Vitaliano-Prunier A, et al. (2012) Two distinct repressive mechanisms for histone 3 lysine 4 methylation through promoting 3'-end antisense transcription. PLoS Genet 8: e1002952.

307. Lubitz S, Glaser S, Schaft J, Stewart AF, Anastassiadis K (2007) Increased apoptosis and skewed differentiation in mouse embryonic stem cells lacking the histone methyltransferase Mll2. Mol Biol Cell 18: 2356-2366.

308. Yu BD, Hess JL, Horning SE, Brown GA, Korsmeyer SJ (1995) Altered Hox expression and segmental identity in Mll-mutant mice. Nature 378: 505-508.

309. Pusarla RH, Bhargava P (2005) Histones in functional diversification. Core histone variants. FEBS J 272: 5149-5168.

310. Lenstra TL, Holstege FC (2012) The discrepancy between chromatin factor

location and effect. Nucleus 3: 213-219.

311. Venters BJ, Wachi S, Mavrich TN, Andersen BE, Jena P, et al. (2011) A comprehensive genomic binding map of gene and chromatin regulatory proteins in Saccharomyces. Mol Cell 41: 480-492.

312. Lenstra TL, Benschop JJ, Kim T, Schulze JM, Brabers NA, et al. (2011) The specificity and topology of chromatin interaction pathways in yeast. Mol Cell 42: 536-549.

313. Knight JD, Tian R, Lee RE, Wang F, Beauvais A, et al. (2012) A novel whole-cell lysate kinase assay identifies substrates of the p38 MAPK in differentiating myoblasts. Skelet Muscle 2: 5.

314. Boersema PJ, Raijmakers R, Lemeer S, Mohammed S, Heck AJ (2009) Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat Protoc 4: 484-494.

315. Fischle W, Franz H, Jacobs SA, Allis CD, Khorasanizadeh S (2008) Specificity of the chromodomain Y chromosome family of chromodomains for lysine-methylated ARK(S/T) motifs. J Biol Chem 283: 19626-19635.

316. Lee TI, Young RA (2013) Transcriptional regulation and its misregulation in disease. Cell 152: 1237-1251.

317. Suva ML, Riggi N, Bernstein BE (2013) Epigenetic reprogramming in cancer. Science 339: 1567-1570.

Summary/Samenvatting

Curriculum vitae

List of Publications

Acknowledgements

SUMMARY

In eukaryotic cells, the process of copying a DNA template into RNA is called gene transcription or expression. This is highly regulated at many different levels by a wide plethora of proteins. Chromatin is one of the important regulators of gene expression. Nucleosomes are the basic building blocks of chromatin and are formed by DNA wrapped around a histone octamer (two copies of histones H2A, H2B, H3 and H4) and the linker histone H1. For a long time the histones were regarded as merely being packaging material to fit the DNA into the cell's nucleus. However, later it became apparent that the protruding (N-terminal) tails of the histones can be post-translationally modified. They are subjected to chemical modifications like acetyl, methyl, phosphor and ubiquitin. These modifications can act alone or context-dependent to activate or repress transcription. Furthermore, one modification can lead to the addition or removal of the next. Genome-wide analyses of different modifications have correlated certain modification states, their genome-wide loci and level of gene expression. For example, nucleosomes surrounding the transcription start site (TSS) of active genes are often hyperacetylated on histones H3 and H4. Also trimethylation of histone H3 of lysine residue 4 (H3K4me3) is associated with the promoter of active genes. Promoters are the minimal DNA sequence at the start of a gene required to start transcription. The three categories of proteins involved in chromatin biology are writers; proteins that add the modification, erasers; proteins that remove modifications and readers; proteins that are recruited to modifications. Two important readers in the process of transcription are TFIID and SAGA, which both contain a subunit that binds to H3K4me3. SAGA also contains a writer subunit to acetylate histones. This thesis describes the role of SAGA and TFIID in the regulation of transcription through chromatin. Furthermore, a writer was identified for a novel acetylation of histone H3 on lysine 4 (H3K4ac).

The role of SAGA in transcription was investigated using genes involved in the cell's reaction to stress in the endoplasmic reticulum (ER), which is a compartment of the cell specialized in folding of proteins. It is known that SAGA is recruited to these ER stress genes upon ER stress. These genes were used to dissect the exact role of SAGA and its H3K4me3 binding subunit SGF29. Experiments showed that not only acetylation of the promoter of ER stress genes but also presence of H3K4me3 is dependent on SGF29. This is surprising because by itself SAGA cannot establish this modification. Thus most likely SAGA and SGF29 binding to H3K4me3 recruits other protein complexes to maintain presence of H3K4me3 to ensure transcription in case of a stress situation. Lastly, both SAGA and SGF29 are required for survival of the cells after ER stress.

H3K4ac is a relatively new identified modification and is also associated with active transcription. With the help of a screen, the HBO1 protein was identified to be a writer for this modification. This was confirmed by an experiment were HBO1 was over-expressed in cells leading to an increase of H3K4ac.

TFIID is a very important player in the process of transcription initiation. Previously, its subunit TAF3 was identified to bind to H3K4me3. In this thesis we further characterized the binding of TFIID to promoter nucleosomes. We discovered that this binding is multivalent and depends on several different features found in the

promoter region. In addition to H3K4me3, acetylation of histone H3 and the presence of the TATA sequence in the DNA increase the binding of TFIID. We have also determined the composition of the TFIID complex in human cells, which turned out to be similar to yeast TFIID. These analyses also suggested that TFIID exists in different compositions, probably dependent on the situation at the specific genes.

Taken together the results described in this thesis have added to our knowledge of SAGA, TFIID and chromatin in the regulation of transcription.

SAMENVATTING

Het kopiëren van het DNA in RNA in eukaryote cellen is een proces genaamd transcriptie of gen-expressie. Dit is een belangrijk proces, omdat de beslissing wanneer welk gen wel of niet afgeschreven wordt van belang is voor veel verschillende zaken, zoals de functie van een cel en haar groei. In het geval van kanker bijvoorbeeld, kan verkeerde gen-expressie leiden tot wildgroei van cellen, een tumor. Transcriptie wordt daarom op veel verschillende niveaus gereguleerd door een enorme verzameling eiwitten.

Het chromatine, de context waarin het DNA verpakt is in de celkern, speelt een belangrijke rol in de regulatie van gen-expressie. Nucleosomen zijn de meest basale bouwstenen van het chromatine en worden gevormd door DNA, omwonden om een octameer van histonen (2 kopieën van elk histon H2A, H2B, H3 en H4) en het linker histon H1. De histonen hebben (N-terminale) staarten die uit het nucleosoom steken en onderhevig zijn aan chemische modificaties, zoals acetyl, methyl, phosphor en ubiquitine. Deze modificaties kunnen alleen of in context bijdragen aan de activering of de repressie van transcriptie. De aanwezigheid van één modificatie kan ook leiden tot het ontstaan of verwijderen van de volgende modificatie. Studies die in het hele genoom hebben gekeken vinden een correlatie tussen de staat van een modificatie, zijn locatie in het genoom en de mate van gen-expressie. Een voorbeeld is de acetylatie van histon H3, welke altijd wordt gevonden rondom de plaats op een gen waar transcriptie start. Ook de aanwezigheid van ge-tri-methyleerd lysine 4 op histon H3 (H3K4me3) op de promotor van een gen wordt geassocieerd met actieve expressie van het desbetreffende gen. De promoter is het minimale stukje DNA dat je nodig hebt om transcriptie te kunnen starten en bevindt zich aan het begin van een gen. De eiwitten die betrokken zijn bij histonmodificaties worden in drie categorieën onderverdeeld: de schrijvers, de verwijderaars en de lezers. De eerste categorie zijn eiwitten die de modificaties plaatsen, de tweede eiwitten die de modificaties verwijderen en de laatste categorie, lezers, zijn eiwitten gespecialiseerd in het herkennen en binden van histonmodificaties.

SAGA en TFIID zijn voor transcriptie belangrijke eiwitcomplexen (die uit meerdere eiwitten bestaan). Allebei bevatten ze een eiwit dat specifiek H3K4me3 herkent en bindt. SAGA huist daarnaast een schrijvend eiwit, dat histonen kan acetyleren. Dit proefschrift beschrijft onderzoek naar de rol van SAGA en TFIID in de regulatie van transcriptie en hoe zij daarbij het chromatine gebruiken. Daarnaast is de schrijver voor een nieuwe histonmodificatie, namelijk de acetylatie van lysine 4 op histon H3 (H3K4ac) geïdentificeerd.

Allereerst is de rol van SAGA onderzocht. Hiervoor is gebruik gemaakt van de

zogenaamde endoplasmatische reticulum (ER) stressgenen. Het ER is een compartiment van de cel dat gespecialiseerd is in de vouwing van eiwitten. De ER-stressgenen worden door de cel geactiveerd in een ER stress-situatie en het is bekend dat SAGA in die situatie naar de ER-stressgenen gebracht wordt. Onze experimenten laten zien dat SAGA's onderdeel SGF29, dat verantwoordelijk is voor de binding aan H3K4me3, noodzakelijk is voor zowel acetylatie van histon H3 als voor H3K4me3 op de promoter van deze ER-stressgenen. Dit is interessant omdat SAGA zelf niet de H3K4me3-modificatie kan maken, maar andere eiwitcomplexen moet aantrekken om te zorgen dat H3K4me3 aanwezig is, zodat transcriptie kan plaatsvinden in geval van een acute ER stress-situatie. Tenslotte is duidelijk geworden dat zowel SAGA als SGF29 absoluut noodzakelijk zijn voor het overleven van cellen van ERstress.

H3K4ac is een redelijk recent geïdentificeerde histonmodificatie en correleert ook met actieve gen-expressie. We hebben verschillende schrijvers voor acetylatie vergeleken en vonden dat het HBO1-eiwit verantwoordelijk is voor het schrijven van H3K4ac. Experimenten waarbij HBO1 tot over-expressie gebracht werd in cellen leidden tot een verhoging van H3K4ac en bevestigden dat HBO1 een schrijver zou kunnen zijn voor H3K4ac.

TFIID is een belangrijke speler in de initiatie van transcriptie. Van zijn onderdeel TAF3 is aangetoond dat het zeer sterk kan binden aan H3K4me3. In dit proefschrift hebben we de binding van TFIID aan promotor nucleosomen verder gekarakteriseerd. Hieruit is gebleken dat de binding multivalent en afhankelijk van meerdere kenmerken die gevonden worden op het promotor nucleosoom is. Niet alleen H3K4me3 is belangrijk voor de binding, maar ook de acetylatie van histon H3 en de aanwezigheid van de TATA-sequentie in het DNA. We hebben de samenstelling van humaan TFIID in cellen bepaald en vonden een gelijkenis met de samenstelling van TFIID uit gist. Uit deze analyse bleek ook dat het aannemelijk is dat TFIID voorkomt in verschillende composities, waarschijnlijk mede afhankelijk van de situatie op specifieke genen.

Onze resultaten dragen tezamen bij aan het begrip van de rol van SAGA, TFIID en chromatine in de regulatie van gen-expressie.

Curriculum Vitea

Andrea Wilhelmina Schram werd op 2 december 1984 in Tilburg geboren. In 2002 behaalde zij haar gymnasium diploma *cum laude* met het profiel Natuur & Gezondheid aan het Vossius Gymnasium te Amsterdam. Datzelfde jaar startte zij haar bachelor Biologie aan de Universiteit Utrecht te Utrecht. Deze bachelor rondde zij in 2005 af, om in 2006 te starten met de master Biomolecular Sciences opnieuw aan de Universiteit Utrecht. Gedurende deze master deed zij twee stages, de eerste onder begeleiding van Dr. Jan-Andries Post in de Electron Microscopy Group aan de Universiteit Utrecht. Na haar eerste stage besloot ze haar master te onderbreken voor het volgen van het minor programma Bussines and Economics for Science students van de Utrecht University School of Economics. Hierna besloot zij alsnog een tweede stage te doen, welke zij deed onder begeleiding van Dr. Bart Westerman en Dr. Koen Braat in de groep van professor Dr. Maarten van Lohuizen aan het Nederland Kanker Instituut te Amsterdam. Met het afronden van deze stage behaalde zij haar Master of Science diploma in 2008. In november van datzelfde jaar startte zij het promotieonderzoek dat staat beschreven in dit proefschrift. Dit werd uitgevoerd onder begeleiding en in het laboratorium van professor dr. Marc Timmers in het UMC Utrecht.

List of publications

Andrea W. Schram, Roy Baas, Pascal W.T.C. Jansen, Anne Riss, Laszlo Tora, Michiel Vermeulen and H. Th. Marc Timmers
A dual role for SAGA-associated factor 29 (SGF29) in ER stress survival by coordination of both histone H3 acetylation and histone H3 lysine-4 trimethylation
PLoS One. 2013 Jul 23;8(7):e70035

Rick van Nuland[#], **Andrea W. Schram[#]**, Frederik M.A van Schaik, Pascal W.T.C Jansen, Michiel Vermeulen and Marc H.T.M. Timmers
Multivalent engagement of TFIID to active promoter nucleosomes
# These authors contributed equally
PLoS One. 2013 Sep 11;8(9):e73495.

Altelaar AF, Frese CK, Preisinger C, Hennrich ML, **Schram AW**, Timmers HT, Heck AJ, Mohammed S.
Benchmarking stable isotope labeling based quantitative proteomics
Journal of Proteomics 2012 Oct. doi: 10.1016

Dankwoord

Het was een reis van twaalf uur. Reisgenoot G. viel enkele minuten na vertrek in slaap, maar ik was klaarwakker. Ik peinsde over mijn toekomst: in principe was ik klaar met studeren, maar ik had nog een stage nodig om eventueel te kunnen promoveren. Terwijl de bus door de Cubaanse sterrennacht reed, besloot ik de mogelijkheid om te promoveren open te laten, ik ging nog een stage te lopen.

De Cubaanse epifanie bracht mij naar het Nederlands Kanker Instituut (NKI). Ik deed daar onderzoek naar stamcellen en toen begon het echt te kriebelen. Halverwege mijn stage nam ik het definitieve besluit: ik zou gaan promoveren.

In maart 2008 reageerde ik op een vacature voor een promotieplaats. Hoewel de sluitingsdatum al op 16 januari was, leek het ze goed dat ik toch nog zou reageren. Drie dagen lang heb ik mijn hoofd gebogen op de beste brief en een paar dagen later hoorde ik dat ik op gesprek mocht komen. Er volgde zelfs een tweede gesprek en een presentatie, waarna ik bij de baas werd geroepen en hij zei: ‘ik wil jou als mijn AIO'.

Op 1 november 2008 begon dat waarvan je het resultaat in handen hebt: het onderzoektraject dat op 19 november 2013 leidde tot mijn promotie.

Als ik aan mijn promotie denk, denk ik aan moleculen en eiwitten. Ik zie ze voor me, visueel. Ik zie ze voor me als ik naar mijn eigen handen kijk, en naar die van jullie. Maar voor de meesten van jullie is dat abracadabra. De meesten van jullie zullen dan ook niets meer lezen dan dit dankwoord. Maar ha, zo makkelijk komen jullie niet van mij af. Met enige inspanning heb ik daarom dit dankwoord uit kunnen bouwen tot meer dan 3500 woorden, en in deze fysieke versie kun je natuurlijk niet met ctrl+F op zoek naar je eigen naam.

Maar nu dan echt, waarde lezers, Hora est! om wat mensen bij naam te noemen:

Geachte professor Timmers, beste **Marc**, de eerste alinea kan alleen maar over jou gaan. Ik wil je namelijk bedanken voor mijn plek in jouw lab. In de afgelopen (bijna) vijf jaar heb ik ontzettend veel geleerd: dat biochemie veel minder eng is dan het klinkt, van transcriptie en chromatine; de mooiste processen in een cel toch? Ik heb geleerd om niet te vaak in herhaling te vallen in een tekst. Je leerde me het belang van vragen stellen en opmerkingen maken bij andermans werk, maar bovenal leerde jij mij het belang van wetenschap en onderzoek. Dat deed je met je grenzenloze kennis, je enorme ervaring in het lab en in de wetenschap, en met je bereidwilligheid en geduld. Ik vind het heel jammer dat we het niet samen hebben kunnen afmaken, vooral die SGF29 rakker. Maar het is gelukt en ik ben blij dat we 19 november de echte afronding van dit traject samen gaan meemaken.

Geachte (nog net) Dr. Vermeulen, beste **Michiel**, toen ik begon, begon jij ook. Dit betekende onder meer dat je bij de zojuist gearriveerde Orbitrap wegtrokken moest worden, om deze AIO verder te helpen tijdens haar eerste evaluatie. En dat heb je met verve gedaan. Niet alleen toen, je hebt me de afgelopen bijna vijf jaar geholpen met al je tips en adviezen in en buiten werkbesprekingen en evalua-

ties. En in de laatste fase heb je me bovendien nog geholpen met een tweede publicatie. Ik vind het leuk om je promotie naar volwaardig groepsleider en straks zelfs professor te hebben meegemaakt; je onbeteugelde enthousiasme en razende intelligentie maken dat dit niet meer dan terecht is. Maar Kapitein Schuimbaard, ik wil wel wat in de melk te brokkelen hebben als er een filmpje voor je oratie komt.

Geachte professor Holstege, beste **Frank**, we hebben nooit veel met elkaar te maken gehad en toch wil ik je heel erg bedanken. Ik waardeer het enorm dat je in de laatste weken zoveel tijd en energie in mijn promotie hebt gestoken. Dankzij jou lees ik al mijn sollicitatiebrieven, eigenlijk al mijn post en geschreven teksten, hardop alvorens het te versturen.

De leden van mijn AIO- en/of leescommissie: **Sander** bedankt voor alle nuttige aio-evaluaties en **Sander, Susanne** en **Gert-Jan** bedankt voor het lezen en beoordelen.

Wim en team, **Marjoleine, Marcel, Betty, Marianne, Christina** en **Marian, Cheuk,** bedankt voor het regelen van allerlei rand-, maar zeer belangrijke, zaken. Jullie maken het afronden van een promotie echt makkelijker.

Lazlo, Anne and **Jacques**, thank you very much for my instructive and pleasant stay in your lab. Even though the HAT assays did not make it into the book, our collaboration was very fruitful to me.

Leden en oud-leden van de **Holstege, Bos, Burgering, Kops, Lens, Kalkhoven en van Mil** groepen, hartelijk dank voor de gezelligheid bij borrels op de tweede en derde, vooral ook **Marrit, Marten, Nanette, Wilco, Timo, Tobias, Marieke, David** en **Saskia.**

The Vermeulen-lab, thank you very much for your input scientific (work discussions) and non-scientific (probably more important such as during lunches and retreats). **Irem**, hang in there with this project, it will come to an end and you will do great. **Radhika,** thank you so much for your support through the years, including the check of my introduction and discussion. Specifically for your compliments for me as a person and as a scientist, it really helped me through at times. Good luck in your future inside and outside of work. **Anneloes**, jij ook het beste in de nieuwe omgeving straks. **Nelleke**, NuRD, succes het komende jaar, dat komt vast allemaal goed, dat weet je al, want je hebt het al zo top gedaan de afgelopen jaren. **Susan**, our overlap is not too long, but I wish you all the best with your projects and with moving again.

Het Timmers lab. Zonder jullie geen gezellig lab om in te promoveren, dus allereerst de oud-leden: **Pim, Nikolay, Sjoerd** en **Marijke,** bedankt en hopelijk gaat het jullie allen goed op jullie nieuwe plek en natuurlijk ook buiten het werk. De huidige leden: **Roy**, ik hoop dat je mindere start, met het ontbreken van een promoter, een slechte generale blijkt te zijn, waarna natuurlijk een hele goede première volgt. Aan je enthousiasme zal het niet liggen. **Richard**, de enige echte (bio)chemicus van het hele stratenum, wat zijn er veel projecten waar je me direct of indirect bij hebt geholpen. Realiseer je je dat ik al die tijd bij je in de minibespreking heb gezeten? Bedankt dus, en succes met je nieuwe projecten en het bridgen. **Hetty**, pietje precies en altijd in voor een praatje, ik wens je voor het komende jaar veel gezondheid, zodat je lekker kunt genieten van

nog een paar jaartjes pipetteren en vooral van al je leuke hobby's (fotografie!). **Petra**, de moeder van het lab, naast het relativeren van wetenschap, heb je ook mijn angst voor bevallingen en moederschap weg weten te nemen bij de lunch of in het lab. Dit deed je door met enorm veel liefde over je gezin te praten. Ik hoop oprecht dat je dat ook heel snel weer over je werk doet, dat verdien je. **Maria,** nu ben je echt de senior en weet je, dat kan jij. Jij hebt zo vaak goede opmerkingen bij mijn en andere presentaties en jij hebt je eigen project al zover gebracht, je komt er wel. Twijfel niet te veel het komende jaar, dat is niet nodig: je bent een tof wijf en een topwetenschapper.

De kwak-groep. In zijn geheel heeft deze groep een aardige poging gedaan om mijn lever om zeep te helpen in het stratenum en tot ver over de landsgrenzen, maar wat was het leuk om jullie de afgelopen jaren te hebben leren kennen. **Rick, Boki, Allez-den-Book**, wat ben jij sterk, fysiek en mentaal. Soms ben je hard, meestal ben je heel lief. Jij hebt me echt geholpen met je ideeën en doordat je naar mijn geklaag wilde luisteren – en ik naar dat van jou mocht luisteren. En je gaf me (gewilde en ongewilde) tips over wetenschap, auto's en het leven. Ik vind het heel leuk dat onze samenwerking bekroond is met een paper. Ik wens jou en **Tess** alle geluk in Zuid- en Noord-Amerika. **Maaike**, ik laat die stomme bijnaam weg, want dat ben je niet, tenzij het over je doorzettingsvermogen gaat. Wat ben ik blij voor je dat ook jij klaar bent. Ik wens je veel geluk in Engeland. **Arne**, wat een focus heb jij, daar heb ik erg veel bewondering voor, vooral omdat je het nog altijd goed weet te combineren met lekker koekjes eten of een goede borrel. **Pascal, Pasqualle**: die Michiel die werd misschien wel helemaal geen professor als hij niet zo een slimme en hardwerkende analist had gehad. Ik hoop dat je snel je draai vindt in Nijmegen, zolang je nog in Utrecht woont, kunnen we misschien eindelijk die mared-sous kroegentocht gaan doen? Sowieso goed tegen een kater weet ik toevallig. **Tale**, ik weet niet zo goed of ik je moet bedanken voor je inleiding tot de wondere wereld van de carrousel, ik weet wel dat ik je moet bedanken voor je optimisme en voor de talloze keren dat ik bij je kon uithuilen. Ik vind jou supergrappig en heel creatief, en veel slimmer dan je zelf af en toe denkt dat je bent. Ik wens dat je in de buurt blijft werken, maar niet in de keuken van het UMC, zodat we bakkies kunnen blijven doen en je nog vaak trots foto's van je mooie dochter kunt laten zien. **Astrid, Assie**: superslim en een *heul* aardig meisje. Wat ben je goed en wat ben je lief, met je bubbels voor mijn paper, het nakijken van datzelfde paper, je overgebleven QPCR slots en vooral al je gezellige praatjes tijdens en na het werk; een borrel met een hand voor je mond. Jij gaat shinen, succes popje.

Via kwak kom ik dan uit bij mijn paranimfen die een bijzonder plekje verdienen hier, allereerst **Mathijs**. Lieve Mattie, wat ben ik blij dat je naast me kwam zitten op de retraite. Het was goed dat het eten lekker op tijd was, zodat we meer dan voldoende energie hadden om onze projecten grondig en geïnspireerd te bespreken. Ik moet zo vaak zo hard met jou lachen en dat heeft me, samen met je vele strenge, lieve en troostrijke woorden, vaak geholpen op moeilijke momenten. Je bent echt een hele slimme jongen, ik hoef jou niet uit te leggen dat er een verschil is tussen een half glas huiswijn en helemaal starnakel, en dat je alleen jezelf er mee hebt als je een glas huiswijn neemt. Je intellect, je inzicht en je intuïtie maken je een uitstekende wetenschapper, een gepassioneerde muzikant en een levensgenieter, maar vooral een hele goede vriend. Zo en open nou die baco-bar maar.

Lieve **Annika**, dat jij mijn paranimf zou worden, wist ik al voordat ik überhaupt be-

gon met promoveren. Er zouden immers genoeg moeilijke momenten komen in de wetenschap en daarbuiten waar jij mij met je nuchtere en rationele analyses bij zou kunnen helpen. Ik wist, voordat ik wist dat ik zou gaan promoveren, dat je me veel nieuwe dingen en nieuwe muziek zou leren, die mij dan op mijn werk weer konden helpen. En ik wist dat jij niet alleen in mijn aio-periode belangrijk voor mij zou zijn, maar ook in de rest van mijn leven. Je intelligentie, je creativiteit, je laid-back-instelling zou ik, evenals je analyses en je ovenschoteltjes, niet willen missen. Net als onze gesprekken over festivals, onze mannen, mensen in het algemeen en muziek, maar kun je dan nu zorgen dat er geen Monique Smit wordt opgezet op mijn feestje?

Annie is niet alleen mijn beste vriendin en mijn paranimf, maar ook lid van mijn jaarclub: JC Obesitas. Een volstrekt losgeslagen zooitje vrouwen die, op een enkeling na misschien, nooit in aanmerking zouden komen voor een plekje bij een echte studentenvereniging. Daarom hebben we ons zelf maar verenigd. Obesitas staat in binnen- en buitenland bekend om haar vernietigende kracht. Maar Obesitas is meer: toen ik op de whatsapp vertelde dat ik een datum aan mocht vragen voor mijn promotie hadden ze allemaal binnen 20 minuten gereageerd. Negen vrouwen die van mij houden en trots op mij zijn. Zonder jullie had ik het ook heus wel gekund, deze promotie, maar wat was het saai geweest de afgelopen vijf jaar. Bijvoorbeeld zonder **Mellie**, die zomaar naar het diepen zuiden verhuisde, wat baal ik daarvan, maar wat vind ik het moedig. Je bent sterk, lief en grappig en wat is het ontzettend fijn om jou als gezelschap te hebben. **Nynke**, wat ben ik trots op je dat je de Tour for Life hebt gefietst. Natuurlijk vind ik het jammer dat ik je daardoor het afgelopen jaar minder heb gezien, ik heb je namelijk graag in mijn leven. En **Mim**, wat geniet ik van je grenzeloze gastvrijheid en gulheid. Dat is fijn als je, zoals ik, in de WW zit, maar natuurlijk vooral omdat het me zo'n geborgen gevoel geeft. Je geeft me bovendien een enorme portie zelfvertrouwen, bijvoorbeeld omdat jij zo hard lacht om mijn grappen. Ik kijk nu al uit naar de volgende avond op de KDS. **Meik**, wat ben je een stoer wijf en wat heb jij mooie keuzes in je carrière gemaakt waar ik veel van kan leren. Je opmerkingen om mij te steunen waren vaak heel erg raak, je hebt me daardoor erg geholpen. **Manonski**, de enige echte vrouw in de jaarclub, wat ben je lief en zachtaardig. En wat voelde ik me groeien toen je in Rockanje-sur-mer steeds zei: 'noem je toekomstige titel nog eens, ik ben zo trots als je dat zegt'. **Nora**, wat ben ik blij dat jij vier jaar geleden ook geen zin had om te liften naar Berlijn en we daardoor samen konden reizen en ik je leerde kennen. Als jij zegt dat ik je altijd mag bellen, dan mag ik je dus ook altijd bellen; jij hebt mij minstens 1000 keer horen huilen de afgelopen jaren. Wat ben jij grappig en lief en wat ben ik je dankbaar voor onze vriendschap. **Gep**, wij hebben een robuuste vriendschap, die heel waardevol voor mij is. Ik vind je supergrappig, super ontnuchterend. Spontane avonden en tripjes met jou hebben mij de afgelopen jaren veel plezier bezorgd. Wanneer gaan we naar Leuven? **Eve**, je luisterend oor en adequate adviezen hebben me geholpen in een moeilijke periode, daarvoor ben ik je erg dankbaar. En dan zijn er nog drie mannen en een fantastische dame die ik in dit gezelschap ook nog wil noemen: **Wouter**, we moeten zo snel mogelijk weer de trein doen op een goed techno nummer. Ik waardeer je nuchtere adviezen over muziek en ver daarbuiten heel erg. **Davski**: je grenzeloze optimisme is erg inspirerend en **Kees**, wat ben jij een toffe dude. **Fleurtje,** lieve dame, wat fijn dat je wilde helpen met dit stuk. Ik ben zo blij met het resultaat en met jou en je luisterend oor, niet alleen voor dit dankwoord, maar vooral in het algemeen.

Genoeg gewicht voor hen met overgewicht. Er zijn namelijk ook een aantal individuen die voor mij de afgelopen jaren heel belangrijk zijn geweest: **Miesje**, jij bent één van hen. Geduldig, lief en met een heel fijn luisterend oor. Ik wens dat je binnenkort een beroep kiest waar je gelukkig van wordt. Ik weet zeker dat het kleine babymeisje jou en **Sander** ontzettend veel geluk zal brengen, ik kijk er naar uit om met haar te knuffelen. **Twinkie**, je grenzeloze geloof in mij geeft mij ontzettend veel zelfvertrouwen. Maar ik ben vast niet de enige die door jou een beter gevoel krijgt, want Twink: de wereld is mooier met jou. **Natas**, het liefste meisje en het meisje dat het meest van roddelen houdt ineen. Wat een heerlijke combinatie. Ik hoop dat er nog vele kopjes thee zullen volgen, de afgelopen jaren waren ze namelijk een grote steun. **Gerrie**, valse nicht dat je er bent. Ik ben blij dat we de afgelopen twee jaar goede vrienden zijn geworden. Jouw humor en nuchtere kijk op het leven maken dat het heerlijk is om met je te eten, groene en roze cocktails te drinken en uiteindelijk altijd weer in een homokroeg te belanden. Ik heb zin in al onze dates, ook doubles met **Larsje**. En misschien kunnen we binnenkort met zijn vieren naar Neeeeeeeeew York. **GP**, the day I realized I would not see you every single day anymore, I cried. Now I realize that it might take another year to see you again, but it does not matter, it will be good once that day arrives. You are actually the whole package, you understand me as a person and as a scientist, and so your advices have been of tremendous value. I admire how you have built your own life, integrating you Sicilian identity into this amazing cosmopolitan and scientist that you are now. I wish you so much luck with **Gene** in London and maybe later in Amsterdam again?

En dan een woord van dank voor hen die zo vanzelfsprekend zijn en die juist daarom zo bijzonder: mijn familie. Allereerst een dank aan al mijn **tantes** en **ooms,** voor het warme gevoel dat jullie mij van kleins af aan hebben gegeven. En in het bijzonder een woord van dank aan **Kinny** voor de lekkere broodjes die zij gaat verzorgen.

Ria en **John:** ik ben jullie heel erg dankbaar dat jullie mij meteen een thuisgevoel hebben gegeven. Jullie hebben nooit een kans voorbij laten gaan om mij te laten merken dat jullie van mij houden. Jullie niet aflatende interesse in mijn werk en mijzelf hebben me de afgelopen jaren heel erg geholpen. De weekendjes in Hengelo, met de leesmap, maakten dat ik tot rust kon komen. Ook jullie **Jolanda** en **Bart**, gaven me direct een liefdevol gevoel. Jolanda, ik wens dat je gaat knallen in je nieuwe functie. En Bart, ik wens jou alle geluk samen met **Caitlin.**

Oma, jij hebt het ongekende talent om gewoon lekker te kletsen waardoor ik ontzettend veel rust bij je vind. Het is een genoegen om bij jou aan de keukentafel te zitten met een kopje thee en ik wens dat we nog heel veel gesprekjes samen zullen hebben. **Opa** en **oma**: niemand geeft me zo'n gevoel van trots dat ik dit bereikt heb als mijn opa. Ik wens dan ook opa, dat jij je aan meer waagt dat alleen de samenvatting en dit dankwoord, dat zou ik een echte eer vinden. En **oma**, laten we wel wezen wat voor man zou opa zijn zonder deze sterke, feministe avant-la-lettre aan zijn zijde, dat maakt dat jullie alle twee altijd grote voorbeelden zijn geweest. Ik houd veel van jullie drieën en ik wens dat jullie nog vele jaren in goede gezondheid in mijn leven zullen blijven.

Eva, Alexander en **Daniel**: ik zie jullie niet vaak, maar ik weet dat jullie er altijd zullen zijn en daarom zijn jullie misschien wel de meest belangrijke mensen in mijn leven. Ik besef me sinds een jaar dat mensen gelijk hebben: ik lijk heel erg op jou **Eva** en daar

ben ik heel erg trots op. **Alexander,** ook wij lijken op elkaar. Er is iets wat ik alleen nog graag van je zou leren: je vermogen om te relativeren en te nuanceren. Je bent net als Eva daarin en daarnaast zijn jullie heel erg slim en maken jullie door je zijn, jullie grapjes en je intelligentie de wereld een stukje mooier. **Lucie,** lief meiske, wat fijn dat je mijn broer zo gelukkig maakt, ik wens jullie een prachtige toekomst samen. Lieve **Daniel**: ik vind jou lief sinds het sms'jes dat aankondigde dat je er was. Ik zou meer spelletjes met je willen spelen, en zwemmen. Kom je logeren als je straks terug in Nederland bent?

Klara, vrouwen die weten wat goede rode wijn is, zijn mooie vrouwen. Ik heb veel bewondering voor de manier waarop jij invulling geeft aan je leven. Ik vind het fijn om met je naar balletuitvoeringen te gaan, met name vanwege de gesprekken die we voor en na de voorstelling voeren. Maar bovenal ben ik blij dat je papa zo gelukkig maakt en dat je een hele goede moeder voor Daniel bent.

Papa: Je koos er bewust voor om mij mijn ding te laten doen, je wilde mij geen druk opleggen. Je had immers in een eerder stadium al een grote rol gespeeld in mijn uiteindelijke promoveren, toen je me op mijn hart drukte om toch wiskunde B te doen. Je bent voor mij de personificatie van optimisme. Je kunt enorm toeleven naar een schaats- of voetbalwedstrijd, even is dat het allerbelangrijkste in je leven, maar als we dan verliezen, geef jij er direct een positieve draai aan. En zo doe je dat met alles, dat maakt je een uitstekende wetenschapper, maar een nog fijnere vader. Jouw keuze om wat afstand te bewaren tot mijn promotie begreep ik niet altijd even goed. Maar uiteindelijk kan ik uit die boodschap alleen maar je immense vertrouwen in mij opmaken.

Mam: je bent de allerliefste mens die ik ken. Ik heb zoveel bewondering voor de wijze waarop je ons hebt opgevoed. Hoewel je soms ten onrechte weinig vertrouwen hebt in je eigen capaciteiten (je kunt namelijk alles, behalve wiskunde B) heb je ons juist altijd het gevoel gegeven dat wij alles kunnen. Ik vind dat je een boek moet schrijven mam, of een tentoonstelling moet organiseren om je eigen schilderwerken te tonen. Ik vind dat de wereld mag zien wat je kunt en daarom wil ik je in dit meest gelezen stuk van mijn proefschrift danken voor wie je bent. En zeggen dat ik enorm trots op je ben.

Zonder jullie, papa en mama, had ik dit niet gekund, echt niet. Jullie opvoeding, begeleiding, trouw en liefde maken dat ik ben geworden wie ik ben en daarom draag ik dit boekje dan ook aan jullie op.

Maar niet voordat ik nog iets heb geschreven aan **Rik**. Jij bent de liefde van mijn leven Rik. Je bent slim en grappig en hoewel ik soms onbegrijpelijk ben, lijk jij mij toch altijd te begrijpen; je weet zo vaak de juiste dingen op het juiste moment te zeggen. En ik ben trots op je, je bent een prachtige man en je wordt een hele goede kno-arts. De weg daar naartoe is niet eenvoudig, maar hé, dat heeft ons nooit ergens van weerhouden. Jij komt er wel en wij zijn er al.
Laat je even weten wanneer we naar Moldavië gaan?

Andrée,

Utrecht, september 2013