Biochemical and functional analysis of TBP-containing complexes

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Biochemical and functional analysis of TBP-containing complexes

Biochemische en functionele analyse van TBP-bevattende complexen

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A mio padre

...L'industria operativa della natura e' cosi prolifica che verranno trovate un giorno macchine non solo a noi sconosciute ma neppure immaginabili pe la nostra mente..

..the operative industry of Nature is so prolific that machines will be eventually found not only unknown to us but also unimaginable by our mind..

Marcello Malpighi
De Viscerum Structura
1666

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Chapter 1

General Introduction

The regulation of transcription initiation is a key feature of gene expression control. RNA polymerase II is recruited to the DNA upon assembly of the Pre-Initiation Complex (PIC), which is in turn nucleated by the binding of TATA-binding protein (TBP) to the core promoter. Therefore the delivery and the removal of TBP to and from the promoter play a crucial role in the initiation of gene transcription. In this Chapter we discuss the first events in the regulation of transcription initiation focusing on the protein-complexes that regulate TBP activity on the promoter.

Gene expression

The diversity of eukaryotic cell types is reflected in different enzymatic and structural compositions of a variety of molecules. However, cellular diversity within the same organism does not correlate with genetic information. How to explain the cellular diversity within the same organism? How can the same genetic information be expressed to obtain different phenotypes? The answers reside in the differential regulation of the gene expression. The same genetic set within an organism can be differentially regulated and expressed such that the ultimate protein balance as well as metabolites, lipids, organelles and non-coding RNAs differ from cell to cell in composition.

Gene expression starts with transcription. In this step assembly of the transcriptional machinery is tightly controlled by a plethora of protein complexes. These occupy the convergence of a network of enzymatic pathways that integrate external and internal signals. The productive assembly of the transcriptional machinery culminates with the recruitment of the RNA polymerase II that will transcribe the genetic information into messenger RNA (mRNA). The mRNA is then assembled in a ribonucleic particle and exported to the cytoplasm where the translation takes place. The activity of the newly synthesized protein is then regulated by posttranslational modification and degradation. The possibility to alter the genetic program by transcription regulation guarantees the cell to respond to different stimuli as stress, damage, cell-cell interaction, development, growth and differentiation.

Transcription regulation

The regulation of gene expression requires interaction of transcriptional regulators with DNA. Such interactions occur via specific DNA elements with DNA-binding domains of the regulators. A rearrangement of the chromatin structure is required in order to expose the DNA elements. Furthermore, protein-protein cross talk allows to fine-tune the process and to integrate it with external signals. In the latter step of the process, RNA polymerase II is finally recruited and transcription of the gene can take place.

DNA elements and chromatin structure

In the yeast *Saccharomyces cerevisiae* three classes of genes (I, II, III) are transcribed by three different RNA polymerases (pol I, pol II and pol III). Each gene consists of a coding sequence (CDS) surrounded by untranslated upstream and downstream regulatory regions. Proteins or protein complexes binds the regulatory elements in the DNA. Generally, in class II genes the promoter region spans from -800 to the transcriptional start site (TSS) and is divided in the proximal and distal promoter. The proximal promoter contains general binding sites for core factors and co-activators. The distal promoter contains binding elements for sequence-specific activators or repressors (Upstream Activating Sequence (UAS) and Upstream Repressing Sequence (URS)) that can activate or repress transcription of the downstream gene respectively (reviewed in (86)). The binding sequences at the core (or proximal) promoter can be of several types and differ among the three classes of genes. In *S. cerevisiae* the TATA element is present in approximately 20% of all class II promoters (18). The TATA consensus sequence has been defined as TATA(A/T)A(A/T)(A/G) and can be recognized by TBP (18, 85). Another element is the initiator (Inr), which is defined as a DNA sequence distinct from TATA that can nucleate the PIC. Inr elements can also co-occur with TATA elements at the same gene promoter (177). In higher eukaryotes several other DNA elements can be found in the promoter region: DPE (Downstream Promoter Element), BREu (upstream

TFIIB Recognition Element), MTE (Motif Ten Element), DCE (Downstream Core Element) and XCPE1 (X Core Promoter Element 1) (43, 72).
The DNA is packed in a complex tertiary/quaternary structure called chromatin. Chromatin consists of DNA, histone proteins and a myriad of associated proteins. About 147 basepairs (bp) DNA is wrapped around a globular protein particle called the histone octamer. The DNA and the histone octamer form a defined element called the nucleosome. The histone octamer consists of two copies each of the canonical histone proteins H2A, H2B, H3 and H4 that assemble in a globular structure from which N-terminal unstructured tails are protruding (133). The tails can be posttranslationally methylated, acetylated, phosphorylated or ubiquitinated. Several modifications on the N-terminal tail define patterns that support functional DNA-protein or protein-protein interactions. This additional level of information is called the histone code (201). The chromatin structure is not static. Different classes of proteins modify the structure and function of chromatin using several mechanisms. The nucleosome remodeler complexes ISWIs and the SWI/SNFs, for instance, can slide or evict nucleosomes using the energy of ATP hydrolysis. The histones within the nucleosome can also be exchanged with histone variants by specific remodelers. The SWR1 complex for instance is responsible for the replacement of H2A with H2A.Z at the +1 and -1 nucleosome at promoters (147). Furthermore, posttranslational modifications can be deposited and erased by different classes of enzymes such as acetyltransferases/deacetylases, methylases/demethylases, kinases/phosphatases. The chromatin status influences the transcriptional activity. Regions with lower nucleosome occupancy are generally transcriptionally active and are termed euchromatin, whereas regions with higher nucleosome occupancy are transcriptionally inactive and are named heterochromatin (19, 188).

Polymerases and transcriptional factors

The three RNA polymerases are mutually exclusive and transcribe specific classes of genes. Pol I is required for transcription of the ribosomal RNAs (rRNAs), and pol III for transfer RNAs (tRNAs) and 5S rRNA. Pol II transcribes the bigger class of genes that include mRNAs, small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) (174, 175). A fourth polymerase (pol IV) has been recently discovered in plants which seems implicated in transcription of small interfering RNAs (siRNAs) (89, 153). The three polymerases share a structural conserved core formed by ten subunits. Additional specific subunits are located at the periphery of each polymerase (46).
The recruitment of the polymerases to the promoter sequence occurs upon assembly of the PIC (83). TBP is a universal factor required for the assembly of the PIC for all three classes of genes (44). In class I genes the PIC is formed by the CF (Core Factor) complex, the UAF (Upstream Activating Factor) complex and TBP. The assembly of the PIC starts with the binding of the UAF complex (6 subunits) to the upstream promoter element, and this triggers the binding of TBP to the DNA. The CF complex (3 subunits) is then recruited followed by the tethering of pol I. It has been shown that after promoter clearance the PIC is disassembled. However the UAF complex remains on the DNA as a scaffold facilitating transcription re-initiation (8). The loci transcribed by pol III are nearly 280 in the *S. cerevisiae* genome. The TFIIIC and TFIIIB complexes regulate pol III recruitment and transcription initiation at these loci, while the additional factor TFIIIA is required for transcription of 5S rRNA (183). The composition of promoter elements is variable at pol III genes. Together with the TATA consensus sequence, two other DNA elements can be found in these promoters: the A and B blocks. Although these consensus sequences are combined to form a variety of different pro-

moters, however, the PIC assembly follows a similar scheme in all of them. First, the TFIIIC complex is recruited to the A and B boxes. TFIIIC then recruits TFIIIB and TBP, followed by pol III tethering. Thus, the TFIIIC complex is important for promoter recognition, whereas TFIIIB-TBP and pol III are important for the transcript initiation and synthesis (171). The regulation of the class II gene expression requires the participation of a larger range of transcriptional factors. The molecular mechanisms involved in pol II transcription regulation will be summarized in the following section.

Pol II transcriptional cycle

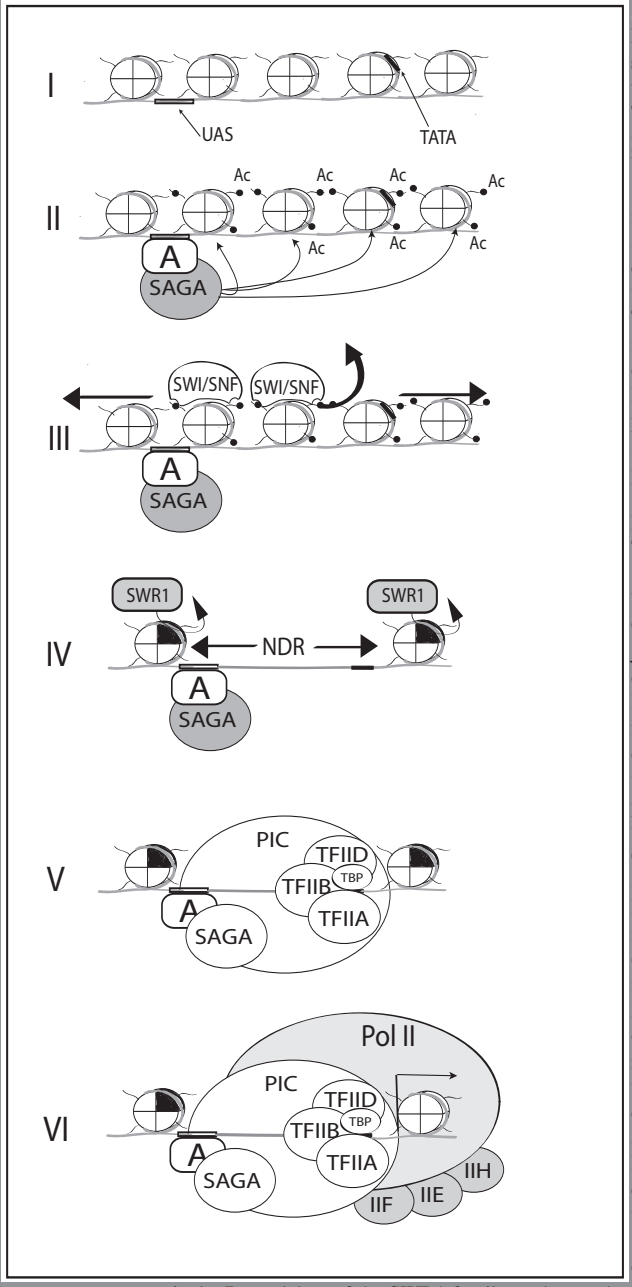
Transcription by RNA pol II follows several stages in a cyclical fashion. In general, three main processes can be defined: initiation, elongation and termination. All the three processes are well regulated by myriad of protein interactions between the transcriptional machinery, the chromatin and the DNA. Furthermore the transcriptional cycle co-occurs with several other transcription-related events. Below, a summary of the pol II transcriptional cycle and transcription-related events is given, focusing on the major steps of PIC assembly and initiation.

Initiation

In order to start the transcriptional cycle, the promoter structure has to be arranged in an open conformation [Box 1]. In the simplest form, this occurs by recruitment of an activator to an UAS, followed by the tethering of nucleosome remodeling and chromatin modifying complexes. The first complex uses ATP energy to mechanically remove or slide nucleosomes, whereas the second complex posttranslationally modifies the histone tails protruding from the nucleosome core particle by phosphorylation, acetylation, methylation, sumoylation, or ubiquitination. At the promoter, nucleosomes are modified, slided away or evicted by the aforementioned complexes leading to the formation of a Nucleosome Depleted Region (NDR) (228).

Once the promoter becomes accessible, the PIC machinery can be assembled (6). The binding of TBP to the DNA sequence triggers PIC nucleation. The regulation of TBP activity by positive [SAGA and TFIID] and negative [Mot1p/NC2] complexes is the main focus of this thesis and will be discussed in detail in the following section. PIC assembly proceeds with the binding to TBP of TFIIB and TFIIA, followed by the recruitment of TFIIE, TFIIF and TFIIH (reviewed in (83)). The tethering of the mediator complex assures the right positioning of RNA pol II that is recruited the last (192).

The numerous interactions occurring within the PIC have structural and functional roles. Among them, TFIIE and TFIIH interact with the DNA upstream the TSS stabilizing PIC-DNA interaction; the Rpb1p and Rpb2p subunits of the RNA pol II interact extensively with the DNA of the promoter region. The unstructured C-terminal domain (CTD) of the Rbp1 subunit of the RNA pol II contains heptapetide repeats (YSPTSPS) that are subject to serine phosphorylation (at Ser2, Ser5, Ser7). The modification pattern of the CTD offers a scaffold for protein-protein interactions and drives the transcriptional process. Interestingly, the modification pattern changes during the different stages of the transcriptional cycle allowing the recruitment of different sets of enzymatic activities. Once the PIC has been assembled, the helicase domain of TFIIH melts the TSS region in an ATP-dependent manner, allowing the open complex formation. The TFIIH-associated cyclin-dependent kinase, Kin28, phosphorylates the CTD at Ser5, which triggers pol II clearance and the transition to the next step of



BOX 1

Modulation of the chromatin landscape at promoters during transcription initiation.

The assembly of the PIC and RNA polymerase recruitment is preceded by a rearrangement of the promoter structure. The histone modification and nucleosome remodeling machineries are tethered to the promoter in order to expose the DNA elements necessary for the PIC nucleation.

The cartoon shows one of the possible mechanisms for promoter activation during transcription initiation.

I) The chromatin arrangement at the promoter of inactive genes is kept in a 'closed' conformation. Although the promoter region is occupied by nucleosomes, it has been shown that UAS is hypersensitive to DNAase digestion. Therefore the sequence is exposed possibly being localized in the linker DNA between consecutive nucleosomes. Conversely, the TATA box sequence is juxtapose to the nucleosome core particle, thus preventing the binding by TBP

II) During gene activation an activator factor (A) binds the exposed UAS as a downstream effect of a signaling pathway. The activator recruits SAGA to the promoter region via an interaction with the Tra1p subunit of the complex. The Gcn5p subunit of the complex will then acetylate the promoter nucleosomes at H3 tails (black circles). The acetylation mediates the electrostatic loosening up of the chromatin and the recruitment of nucleosome remodeling complexes.

III) Bromodomain-containing subunits of the SWI/SNF complex mediate the recruitment of the remodeling complex to the promoter. The RSC remodeling complex is recruited by the same mechanism.

IV) The ATPase-dependent nucleosome eviction or sliding allows the formation of a Nucleosome Depleted Region (NDR) delimited by the -1 and +1 nucleosome upstream and downstream of the promoter, respectively. Remodelers of the SWR1 family exchange the H2A with the histone variant H2A.Z at the periphery of the NDR. The presence of H2A.Z marks active promoters.

V) The exposition of the TATA element and the subsequent binding of TBP triggers PIC nucleation.

VI) Recruitment of RNA polymerase II and loss or acetylation of H2A.Z accompanies transcriptional activation of the downstream gene.

References:(6, 14, 18, 31, 37, 45, 64, 87, 95, 144, 147)

the cycle (reviewed in (60)). Kin28p phosphorylates Ser7 as well; however the functional role of this modification has still to be determined (101). It has been proposed that after promoter clearance by pol II, the PIC remains at the promoter as a scaffold complex (229). However, this hypothesis has being discarded from the current model of the pol II transcriptional cycle.

mRNA synthesis and related events

Elongation and termination. The synthesis of mRNA occurs during the second step of the pol II cycle termed elongation. The CTD phosporylation pattern orchestrates this process via interaction with specific enzymatic activities. The CTD code recruits the elongation factors TFIIS, Spt4/5p, Spt6p, FACT, the Bur1/2 and the PAF complex, which facilitate mRNA synthesis by interacting with chromatin modifiers and by offering a platform for protein interactions important for mRNA maturation, termination and quality control (97). The CTD phosphorylation pattern forms a linear gradient from the 5'-end to the 3'-end of the gene. The gradient decreases for Ser5-P and increases for Ser2-P. The kinase Kin28 phosphorylates Ser5 of the CTD residues to a greater extent at the 5'-end of the gene (111). This phosphorylated residue helps to recruit the mRNA capping machinery (184). In contrast, Ser2 phosphorylation increases towards the 3'-end of the gene by the activity of the Ctk1p kinase (160). The Ser2 modification orchestrates recruitment and activity of the termination complex at the 3'-end (4).

Ser5 and Ser2 are dephosphorylated by Ssu72p and Fcp1p phosphatases, respectively (142). Capping and polyadenylation occur at the 5'-end and 3'-end of the newly synthesized mRNA, respectively, and protect the molecule from degradation (27). Transcription termination co-occurs with mRNA polyadenylation. The termination complex is recruited to the termination site by interaction with the CTD Ser2-P that is highly enriched at the 3'-end of the gene. The polyadenylation machinery cleaves and polyadenylates the mRNA at the recognition site and the Rtt103p/Rat1p/Railp complex degrades the released RNA by its exonuclease activity. The ability to couple the two events has been linked to the recruitment to the 3'-end of Pcf11p and Ssu72p termination factors (103).

Histone modification. The histone tails are subjected to differential posttranslational modifications during the pol II cycle including acetylation, methylation and ubiquitination. Histone acetylation is mediated by acetyltransferases. In yeast, the SAGA and NuA4 complexes contain acetyltransferase enzymes and are recruited to promoters. Histone acetylation facilitates PIC recruitment and assembly. Histone deacetylases (HDACs) remove the acetyl mark leading to chromatin condensation. Histone acetylation is also required during the elongation stage. The acetyl marks on histones recruit nucleosome modifiers that slide or evict nucleosomes in order to allow pol II passage. The H3K4me3 mark occurs at the promoter region and marks transcriptionally active promoters. The Set1 methyltransferase in yeast is responsible for this mark (187). The Set1 complex is recruited via the Paf1 complex that binds to Ser5-P of the polymerase CTD (112). The H3K4me3 mark is deposited soon after the transient ubiquitination of H2BK123. In the current model, K123 of histone H2B is first ubiquitinated by the Bre1p/Rad6p ubiquitin ligase, and subsequently removed by the Ubp8p ubiquitin protease (187). Ubp8p is part of the DUB (de-ubiquitination) module of the SAGA complex discussed in detail later. The PAF complex also recruits the Set2p methyltransferase in the body of the gene, which writes the H3K36me3 mark (129). It has been suggested that the H3K4me3 and H3K36me3 marks lead to the recruitment of additional histone modifiers at different regions of the gene. In *S. cerevisiae* H3K4me3 recruits scYgn1p via its PHD domain to the promoter region. Ign1p is part of the NuA3 complex, which contains the Ssa3p

HAT enzyme. Therefore, it has been suggested that the H3K4me3 mediated recruitment of NuA3 would allow promoter hyperacetylation (93, 204). In contrast, H3K36me3 has been shown to recruit the Rpd3S HDAC complex at the gene body. This histone deacetylation is important to prevent cryptic transcription initiation during the pol II passage (35, 128).

mRNA processing, export and transcriptional memory. mRNA transcription is coupled to mRNA processing. Processing includes mRNA capping, splicing, cleavage and polyadenylation, and has an important role in mRNA stability, export and translation. The nascent mRNA is bound by RNA-binding proteins to form mRNPs (messenger ribonucleoproteins), which prepare mRNAs for export to the cytosol and also serve to protect it from degradation (2). Integration of transcription with mRNA export occurs via interaction of the SAGA co-activator complex with the TREX2 export complex. The Sus1p subunit mediates the interaction between the two complexes (47). Interaction of the TREX2 complex with the Mex67p export receptor allows targeting of the mRNA to the nuclear pore complex (NPC) (82). A model of gene re-localization upon activation has been proposed. The interactions between SAGA, TREX2, Mex67p and the NPC components would be responsible for the localization of the gene at the nuclear periphery. This interesting event has been shown for the GAL1 and INO1 genes upon activation (26, 36). Furthermore, the peri-nuclear localization of the genes would remain for several generations following repression, suggesting a model for transcriptional memory (3, 134). The initiation Scaffold complex has also been suggested as mechanism of transcriptional memory. In this model the formation of a stable PIC complex after the first transcription event would allow the re-initiation preventing the *de novo* recruitment of the entire PIC thus facilitating the gene expression (7).

Cryptic transcription. The Cryptic Unstable Transcripts (CUTs) were discovered as a very abundant class of non coding RNAs (ncRNAs) in yeast mutants defective for the RNA degradation machinery. In *S. cerevisiae* the exosome is a large complex with 3'- 5' nuclease activity implicated in RNA processing, which includes degradation, maturation, splicing and export (20). Linked to this is the Trf4p poly(A) polymerase containing complex TRAMP which activity has been shown to control exosome-dependent RNA degradation (226). Mutations in the exosome or in the TRAMP complex leads to accumulation of short ncRNAs of about 250 nt in length. The majority of these transcripts map to intergenic regions: 60% are generated in intergenic regions in between tandem arranged genes, 30% between divergent genes (sharing the same promoter region and transcribed in opposite directions) and 7% between convergent genes (sharing the same terminator region and transcribed in opposite directions) (150, 226). A more detailed analysis of ncRNAs in exosome mutants showed the enrichment of long non coding RNAs called SUTs (Stable Untranslated Transcripts). SUTs are about 800 nt in length, and 30% of them originate from divergent promoters (227). The study of the molecular mechanism involved unveiled a complicated model for the termination of cryptic transcription generated during the pol II transcription cycle. In this process, the participation of the Nrd1p-Nab3p proteins with the Sen1 helicase is fundamental. The proteins are recruited via the CTD Ser5-P to the promoter region. The Nrd1 complex recruits the exosome-TRAMP complexes and thereby mediates the fast degradation of cryptic transcripts. The Nrd1 recruitment is antagonized by Ser2-P of the CTD therefore the termination complex is excluded from the 3'-end of the CUTs (9, 81, 213). The discovery of cryptic transcripts unveiled a genome-wide pervasive transcription coupled with the pol II transcription cycle. Despite the initial idea of cryptic transcripts as 'transcription noise' products, the current picture of pervasive transcripts suggests an interesting mechanism of chromatin regulation that will challenge research in the transcription field for the next years.

PIC nucleation: delivery and removal of TBP at the promoter

The binding of TBP at promoters regulates the specificity, amplitude and timing of gene expression. The core promoter, TFIID, SAGA, Mot1p and NC2 regulate TBP turnover at the promoter, and hence control PIC assembly and transcription initiation. Hereafter a detailed description of structures and functions of TBP and TBP-binding complexes is given.

TBP

The TATA-binding protein (TBP) was identified using the adenovirus major late promoter system and mammalian cell extracts to be part of TFIID, one of the five intermediate complexes essential for transcription *in vitro* (28). Soon, it was discovered that the mammalian gene was homologous to the yeast *SPT15* gene isolated as a suppressor of the *Ty* insertion at the *HIS4* and *LYS2* promoters (62, 84). TBP is a universal transcription factor essential for all three eukaryotic polymerases (42, 44). The factor binds the TATA consensus sequence as well as non-canonical core promoter sequences with nanomolar affinities (15, 16). The binding to DNA drives the sequential recruitment of essential transcription factors (44). The isolation of TBP mutants unable to selectively initiate pol I, pol II or pol III transcription suggested that the TBP function is modulated by the binding of interactors that confer selectivity for different classes of genes (29, 185).

The crystal structure elucidated many functional aspects of the protein. TBP is a symmetric dyad, saddle-shaped protein. Co-crystals with the TATA consensus of the *CYC1* promoter unveiled its mechanism of action. TBP binds and bends the minor groove at the TATA element with the concave surface. Van der Waals forces and phenylalanine stacking between the protein and the DNA stabilize the interaction (102, 106). The TATA-TBP complex is more stable on TATA consensus sequences. It has been shown that this can be attributed to TFIIA. Indeed, binding of TFIIA stabilizes the TBP-DNA complex *in vitro* at TATA sequences but not at non-canonical sequences (200). The C-terminus of TBP is highly conserved and encodes for the core particle, whereas the N-terminal is divergent in different organisms (124). In yeast the N-terminal domain of the protein has an auto-inhibitory role and participates in protein-protein interactions (124). Interestingly, higher eukaryotes contain TBP-related factors (TRF) which differ from canonical TBP mostly in their N-terminal regions. At least three TRFs have been isolated in higher eukaryotes (TRF1, TRF2, TRF3). These have tissue-specific or stage-specific roles during embryonic development. In these variants the N-terminal domain would be involved in specific protein-protein interactions responsible for promoter selection or transcriptional factor recruitment (54, 159).

In yeast, TBP binding to the core promoter is highly regulated. TBP-binding complexes regulate transcription initiation by modulating the activity of TBP on the promoter. TFIID and SAGA regulate TBP positively, whereas Mot1p and NC2 are negative regulators of TBP (207). It has been shown that TBP turnover at the core promoter correlates with expression of the downstream gene. TBP turnover is high at pol II, low at pol I and intermediate at pol III promoters. The differential state of TBP dynamics correlates with different patterns of TBP-binding complexes occupancy and DNA elements at promoter. For instance low TBP turnover promoters have higher TBP occupancy and lack the canonical TATA, whereas high TBP turnover promoters are enriched for SAGA and contain the TATA sequence (212).

TFIID

The evolutionary conserved TFIID complex positively regulates TBP activity at promoters. TBP is assembled with 13-14 different TBP-Associated Factors (*TAF1-14*) to form TFIID.

The requirement of TAFs for gene expression defines the difference between TAF-dependent and TAF-independent gene classes (114). For TAF-dependent genes, TAFs are co-recruited with TBP during gene activation, whereas they are not at TAF-independent genes (131). However none of the individual TAF mutant alleles affects the full range of TAF-dependent genes. For instance, it has been shown that the inhibition of *TAF10* function affects the expression of a subset of TAF-dependent genes. This suggests that TFIID might exist in different subcomplexes or that the subunits form different rate-limiting steps at different promoters thus regulating specific genes (see later) (107).

Electron microscopy structural studies on TFIID show that the complex is multi-lobed (lobes A-B-C1-C2-D) and organized in a clamp shape structure with A and B lobes at the extremities and the C and D lobes occupying the central part (127, 154) [BOX2]. The Taf1p and Taf5p proteins have a major structural role. The Taf1p subunit spans from the A to the C lobes and has a scaffold function. The C-terminal domain binds the DNA at the downstream core element (DCE) and the truncation of it abolishes TFIID recruitment to the promoter (143). Importantly, Taf1p regulates TBP binding to the TFIID complex via three TANDs domains. The binding of Taf1p to TBP on DNA leads to structural changes in the DNA conformation essential for transcription initiation (16, 203). However, binding of TBP to the TFIID complex occurs also in solution in a dynamic fashion (180). It has been proposed that the N-terminal region 4 (200-300) of Taf1p binds the Taf4p and Taf6p subunits and serves as a scaffold for TFIID nucleation (190). Interestingly, human Taf1p (hTaf1) contains a bromodomain at the N-terminus whereas the yeast Taf1p does not. It has been proposed that the lack of the bromodomain in yeast Taf1p is complemented by Bdf1p recruitment to the complex via interaction with Taf7p upon gene activation (141). Indeed Bdf1p contains a bromodomain that might allow the recruitment of TFIID to acetylated histones (181). Another structural important subunit of TFIID is Taf5p. Taf5p is present in two copies within the complex and has been hypothesized to mediate the pseudosymmetrical structure of the complex (127). The N-terminal domain mediates the dimerization of the two Taf5p subunits. The C-terminus contains WD-40 repeats organized in a tertiary propeller structure that orchestrates the interaction with the other TAF subunits (191). Nine out of 14 subunits of the complex contain the Histone Fold Domain (HFD). These domains mediate pair-wise interactions between TAFs: Taf4p-Taf12p, Taf6p-Taf9p, Taf3p-Taf10p, Taf10p-Taf8p, Taf11p-Taf13p (66). As mentioned before, the functional variety of TFIID might reflect the existence of multiple complex conformations (152). For instance, the observation of Taf2p-containing and Taf2p-lacking TFIID complexes opens the possibility for distinct complexes with specific roles or specific gene targeting activities (155). In higher eukaryotes a different subunit distribution between the SAGA/STAGA and TFIID complexes has been observed. TAF homologs are involved in SAGA and TFIID formation in *D. melanogaster* and *H. sapiens* (139, 169). A new complex called SMAT contains TAF8-TAF10 together with SPT7L, and might have a regulatory function on the subunit composition of TFIID and SAGA (57).

The peripheral TFIID TAFs might mediate specific functions of the complex. In human TAF3p contains a PHD domain that binds trimethylated K4 of histone H3, thereby recruiting TFIID to the promoter (215). In yeast the Taf4p and Taf5p subunits contain a Rap1 binding domain (RBD) that mediate the interaction with the Rap1p activator. The interaction TFIID-Rap1p is essential for the expression of ribosomal proteins (121). Rap1p regulates 40% of the pol II genes via interaction with TFIID (132). It has been shown that the Rap1p activator binds the UAS and interacting with TFIID leads to the formation of a DNA loop between the distal and the core promoter. This structure is further stabilized by the binding of TFIIA to the TFIID-TBP-DNA-Rap1p complex (154).

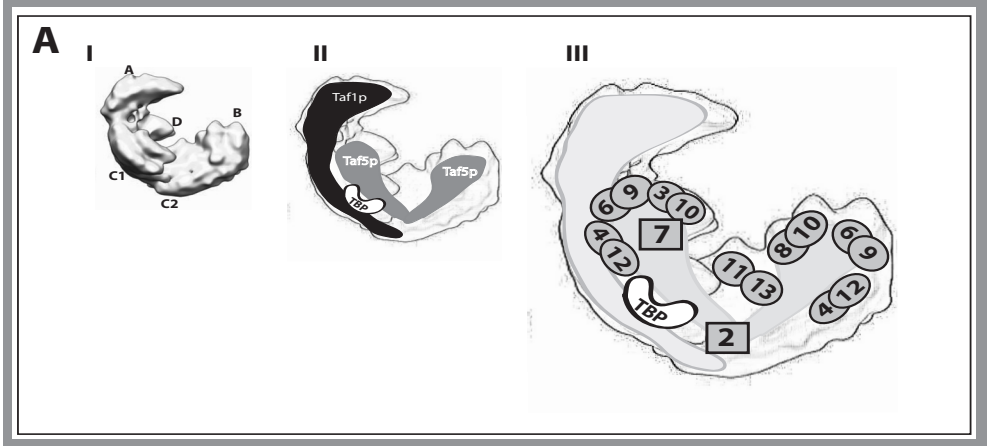
SAGA/SLIK

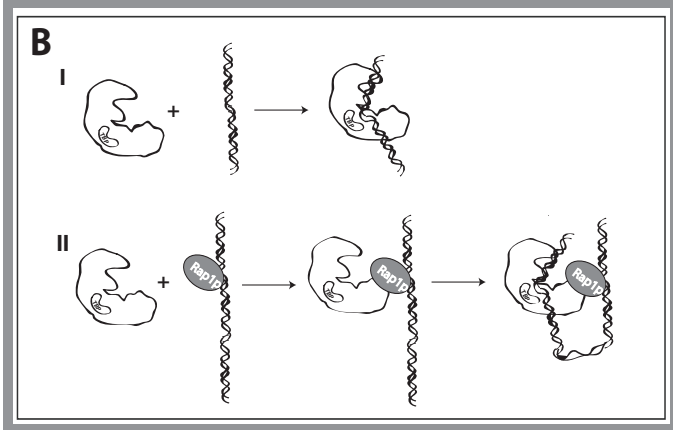
TBP delivery at promoters can also be mediated by the SAGA complex (Spt-Ada-Gcn5-acetyltransferase). The 20 subunits that form the complex are arranged in a modular structure. Electron microscopy analysis revealed that the Spt7p and Ada1p subunits form a core scaffold that interacts with the other subunits. The structure is organized in five domains (206) with the central part resembling the core of the TFIID complex (224). Early characterization of the subunits of the complex identified three main classes of gene products: the SPT (Suppressor of Ty), the ADA (from ADAptor) and the TAF (TBP-Associated Factor) genes (199). The SPT genes were isolated in a genetic test for trans-activation of the Ty transposon. The SPT8, SPT3, SPT7, SPT20 genes encoding subunits of the SAGA complex were isolated together with genes encoding histones proteins (SPT11, SPT12), elongation factors (SPT4/5) and TBP (SPT15). The acidic protein products Spt3p and Spt8p were found to interact directly with TBP (61, 68, 220). The adaptor proteins (ADA) were discovered in a genetic screen for proteins bridging the transactivator VP16 to the pol II holoenzyme. Ada2p and Ada3p were found to interact physically with the bromodomain-containing protein Gcn5p (91, 137). The adaptor proteins and Gcn5p can form a discrete complex *in vivo* required for gene transcription (32). Soon this complex was found to interact functionally with the Spt proteins via Spt20p/Ada5p. Interestingly, the SPT20 null mutant exerted both *ada-* and *spt-* phenotypes, therefore phenotypically merging the SPT and ADA classes of genes (136, 172). A similar phenotype was obtained for the ADA1 deletion strain (92).

The SAGA complex was identified when biochemical analysis revealed Gcn5p-mediated histone acetyltransferase activity on nucleosomes while Spt and Ada subunits co-fractionated (78). However the first complete panel of subunits of the complex was revealed by mass spectrometry. Together with the Spt and Ada proteins also a core of TAF proteins shared with the TFIID complex (Taf5p, Taf6p, Taf9p, Taf10p, Taf12p) was identified (79). More recently two other functional modules have been characterized: the Tra1p and the DUB (De-Ubiquitination) modules.

TAFs: The structural core of the SAGA subunit is constituted of TAF proteins and is shared with the TFIID complex. It has been hypothesized that two Taf5p subunits dimerize and organize the pseudosymmetry of the complex (156). It is interesting to note that also in the SAGA complex the TAF subunits are integrated via HFD protein-protein interactions. However, besides the dimer Taf6p-Taf9p that can be found in TFIID as well, the other TAF proteins have different partners in the SAGA complex. Indeed, Taf10p dimerizes with Spt7p and Taf12p with Ada1p (66).

ADA-GCN5: With the identification of Gcn5p as a subunit, the transcriptional role of SAGA could be extended to chromatin regulation. GCN5 was identified in a screen for mutants unable to grow under conditions of amino acid limitation (71). Gcn5p can acetylate H3 and H4 tails *in vitro* (230), and is a shared subunit of two distinct complexes: ADA (Ada2p, Ada3p and Gcn5p) and SAGA (59, 77). The two ADA subunits (Ada2p, Ada3p) have a regulatory function on the enzymatic activity of Gcn5p. In particular, it has been shown that the SANT domain of the Ada2p subunit modulates Gcn5p activity and affects the rate of chromatin remodeling *in vitro* (17). Also in higher eukaryotes Gcn5p is present in multiple complexes. In flies, the Gcn5p-containing complexes (SAGA, ATAC) can be distinguished by two different Ada2p variants (dADA2a and dADA2b). The dADA2b is incorporated in SAGA whereas dADA2a is part of the ATAC complex (115). In human, Gcn5p has two homologues (GCN5L and PCAF) which can both be present in different SAGA-like complexes: STAGA/PCAF/TFTC (recently renamed hSAGA (207)) and ATAC (139).





BOX 2

The TFIID complex: structure and mechanism of action.

A) TFIID structure. I) Electron microscopy analysis of the TFIID complex unveils a pseudosymmetric, clamp-shaped structure divided in five subdomains (A, B, C1, C2 and D). II) The dimerization of two Taf5p subunits confer the pseudosymmetry to the complex and form together with the Taf1p subunit the complex backbone. Taf1p spans from the A to the C domain and binds TBP via TAND domains located in the N-terminal part. III) The Taf5p WD-40 domain organized in a propeller-like structure mediates the interaction of the backbone with TAF heterodimers (6-9, 3-10, 4-12, 8-10, 11-13). Taf2p and Taf7p peripheral subunit mediate specific interactions and activities of the complex. B) Mechanism of TBP-delivering by TFIID complex. Rap1p-mediated TFIID recruitment occurs in two steps. First, Rap1p binds TFIID by interaction with Taf4p and Taf5p Rap1-binding domains. Second, TBP promoter binding and DNA bending occur upon DNA looping.

References: (40, 121, 152, 154, 155, 176)

SPTs: The Spt7p and Spt20p subunits together with Ada1p and Taf5p constitute the backbone of the SAGA complex [BOX3]. Null mutations of these genes disrupt the SAGA structure (21). In contrast, the other Spt subunits, Spt3p and Spt8p, mediate TBP delivery at SAGA-regulated promoters (22). For instance, it has been shown that the recruitment of TBP to the PHO5 promoter is depending on Spt3p, which exerts a positive role on transcription (17). On the other hand, Spt8p has both positive and negative roles. Spt8p competes with DNA for TBP binding (186). Structural studies have shown that Spt8p can bind the N-terminal domain of TFIIA, which is essential for PIC nucleation. Therefore, Spt8p recruitment to a promoter via SAGA might exert a negative function by blocking TFIIA function. However, Spt8p is also required for TBP recruitment at specific promoters (218). Genetic analyses have shown that *SPT3* interacts with *TBP* and modulates the *TBP-SPT8* interaction (117). Accordingly, biochemical cross-linking experiments showed that Spt3p interacts directly with TBP on the

same surface that is important for Mot1p and TFIIA interaction, suggesting competition between these proteins (148). The ySpt3 is highly homologous to hSpt3, which is a subunit of the human SAGA complex. However the functional link between the protein and TBP recruitment at a promoter has not been explored so far (138).

Tra1p: Tra1p was identified as the yeast homolog of the human TRAPP and ATM DNA kinases. Later it was isolated as a subunit of the SAGA complex in yeast (178). The Tra1p protein is a shared subunit with the NuA4 acetylase complex. NuA4 acetylates nucleosome at H2A and H4 tails *in vivo* by the enzymatic activity of the essential subunit Esa1p (5). In the SAGA and NuA4 complexes, Tra1p mediates the recruitment to the promoter via interaction with an acidic activator at the UAS (5). The interaction of Tra1p with SAGA or NuA4 occurs with the same surface of the protein, whereas a different surface is used for the interaction with the activator (109). FRET (Forster Resonance Energy Transfer) analyses have shown that during galactose induction Gal4p directly binds Tra1p. This binding is essential to recruit SAGA to the DNA, which is in turn important in order to assemble the PIC (24). Similar results have been shown for the interaction of Tra1p with Rap1p and Gcn4p activators (5).

DUB: The De-ubiquitination module of the SAGA complex has recently been discovered and characterized. The module contains the Ubp8p ubiquitin protease that forms a discrete structure together with the Sgf11p, Sus1p and Sgf73p subunits (110, 179). The catalytic and the zinc finger domains are required for the *in vivo* function of Ubp8p (96). The Sus1p and Sgf73p subunits exert a regulatory role on its enzymatic activity (96, 122, 110, 179). Furthermore, Sgf73p anchors the DUB module to the SAGA complex. It has recently been shown that the zinc finger domain of Sgf73p is implicated in nucleosome binding, suggesting a mechanism in which the nucleosome is recruited nearby the DUB module (25). The Ubp8p enzyme de-ubiquitinate H2B at K123. Histone ubiquitination is necessary for optimal gene expression by regulating H3K4 and H3K36 trimethylation (48, 88). It has been suggested that H2BK123 de-ubiquitination is required for Set1p and Ctk1p recruitment (225), which are involved in H3K4 trimethylation and CTD-Ser2 phosphorylation, respectively. However the deletion of the *UBP8* gene does not affect genome wide gene expression and H2BK123 is not strictly required for PIC assembly (189).

The DUB module of hSAGA is highly conserved. In higher eukaryotes USP22 (Ubp8p) ATXN7L3 (Sgf11p), ENY2 (Sus1p) and ATXN7 (Sgf73p) are assembled in the DUB module within the SAGA complex (173). The Sus1p subunit of the DUB module is also implicated in other processes. Sus1p is a shared subunit of the SAGA and the TREX complexes suggesting a functional link between transcription and mRNA export, respectively (173). However structural studies have shown that a single Sus1p subunit does not physically connect SAGA and TREX (63). Interestingly, Sus1p interacts with the Mlp1p nuclear pore subunit mediating SAGA-nuclear pore interactions. Microscopy and ChIP analyses have shown that the *GAL* genes re-localize to the nuclear pore upon activation, which is mediated by Sus1p and the SAGA complex (30).

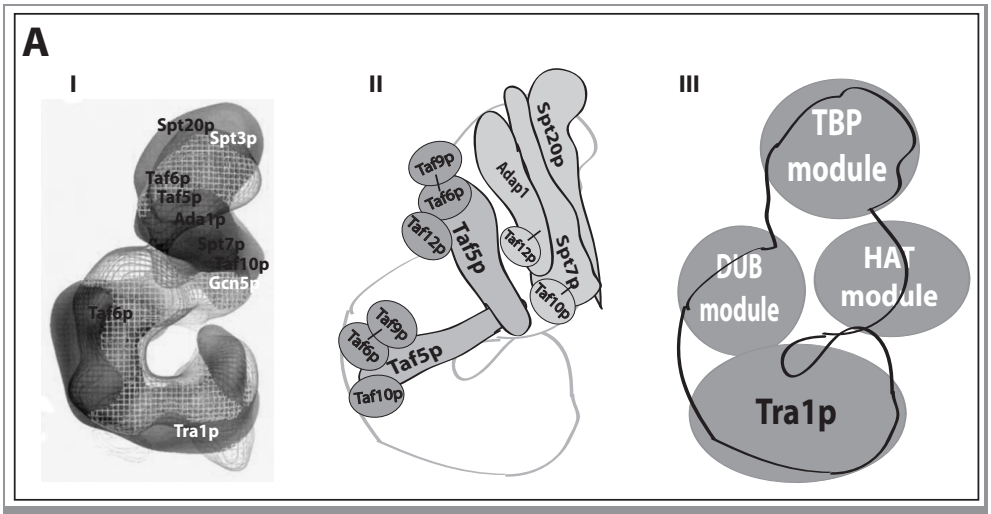
A poorly studied subunit of the SAGA complex is Sgf29p. The subunit has been found to be stably associated with the complex in several proteomic studies, however its function is not understood yet (123, 163). Human SGF29 contains a double tudor domain that mediates the recruitment of SAGA to H3K4me3 enriched regions (214).

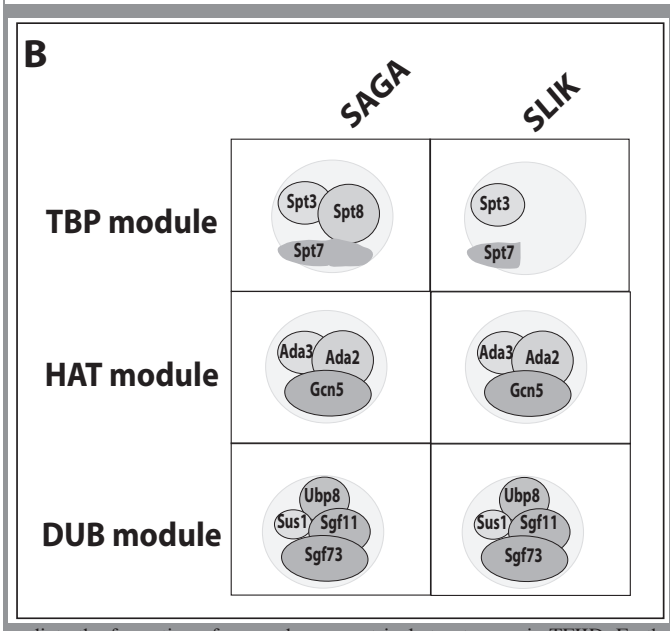
Promoter recruitment of SAGA is essential for transcription initiation of certain genes. The multi-modular composition of the complex allows a large variety of physical and functional interactions. Numerous modes of promoter tethering and protein-protein interactions have been described so far. Extensive studies on the *GAL* genes have shown that upon galactose

exposure the Gal4p activator recruits SAGA to the UAS via the Tra1p subunit (23, 118). SAGA binding is followed by the recruitment of the mediator complex, which stabilizes PIC assembly (119). The recruitment of SAGA to osmotic stress related genes occurs upon Hog1p kinase mediated conversion of the Tup1p-Cyc8p repressor to an activator (167, 157). Also in human it has been shown that SAGA is recruited to retinoic acid responsive elements by the interaction of ADA3 with nuclear receptors (NR). hSAGA is also recruited to adenovirus E1A induced activation promoters (113, 130).
It has been shown that cross-talk between SAGA and nucleosome remodeling complexes is also required for optimal gene expression. For instance, the recruitment of SAGA to *HXT2* and *HXT4* promoters depends on the Snf1p kinase (210). Interaction with the SWI/SNF complex has been show to be required for TBP delivery at the promoter (146). Using *PHO8* as model gene, it has been shown that SAGA and the SWI/SNF remodeling complex are required to propagate the transcriptional signal from the UAS to the core promoter (80). According to the mechanism proposed, histone acetylation mediated by Gcn5p creates a platform for the interaction of the SWI/SNF complex with the chromatin. Indeed, the Swi2p/Snf2p bromodomain can bind acetylated nucleosomes thereby tethering the SWI/SNF complex to the chromatin. Acetylation-mediated nucleosome displacement has been shown to be exerted by both the SWI/SNF and the RSC remodeling complexes (37). A similar mechanism could take place during transcription elongation (76). Gcn5p and Elp3p HAT activities are overlapping during elongation. The double mutant is synthetic lethal and can be rescued by null mutations of the *HDA1* and *HOS2* histone deacetylases (HDACs) (221). Nucleosome acetylation at the gene body has been shown to be dependent on the overlapping function of Gcn5p an Esa1p (74).

Slik/Salsa
A complex related to SAGA is SLIK (SAGA-like) or SALSA (SAGA-altered Spt8 absent). SLIK contains a truncated Spt7p subunit and lacks Spt8p. The truncation releases the Spt8p-binding-domain from Spt7p and, thereby, the Spt8p subunit from the complex (223). Although a human SLIK complex has not being identified yet, the conservation of the Spt8p-binding domain in hSpt7 may suggest the existence of this complex in human as well (198). The transcriptional function of SLIK has not being elucidated with certainty so far. Some authors found a subtle functional role of SLIK overlapping with the SAGA complex (223). Others define SLIK as the activated state of the SAGA complex. According to this model, Spt8p inhibits TBP binding at the *HIS3* and *TRP1* promoters under normal conditions. The activation of these genes occurs parallel to the loss of Spt8p from the SAGA complex and the translocation of newly formed SLIK into the ORF of the gene. Furthermore the isolation of the specific SLIK subunit Rtg2p has suggested a role of the complex in the retrograde response (164). Although previous data report that Spt7p truncation also occurs in the absence of an integral SAGA complex (90), detailed analyses on the mechanism of the SLIK complex formation are lacking. The description of the protease responsible for Spt7p cleavage and SLIK formation is the subject of Chapter 3 in this thesis.

Mot1
In *S. cerevisiae* the Modifier Of Transcription (*MOT1*) gene was isolated as a temperature sensitive mutant capables to increase the basal expression of pheromone responsive genes in the absence of the *STE12* activator (55). The functional link with activator factors was unveiled later in a similar genetic test, in which Mot1 was isolated as a mutant that Bypass-





BOX 3

Functional organization of the SAGA and SLIK complexes.

A) Functional organization of the SAGA complex. I) Electron microscopy structure of SAGA (222). Location of the examined subunit is indicated: structural subunits are depicted in black whereas subunits of functional modules are in white. II) The cartoon shows the subunits important for the structural integrity of the SAGA complex, of which most of them have a current unclear localization. SAGA contains a TAF core structure shared with the TFIID complex (light grey). The depicted position of the TAFs is derived by EM localization studies (see I), suggested by biochemical and genetic studies or indicated by overlay of the SAGA structure with the TFIID core structure (206, 222). The dimerization of the Taf5p subunits might mediate the formation of a pseudo-symmetrical structure as in TFIID. Furthermore histone fold domain mediated dimerization occurs between some subunits of the complex: Spt7p-Taf10p, Ada1p-Taf12p, and Taf6p-TAf9p. III) Cartoon of functional modules of SAGA. It is hypothesized that the SAGA complex is organized in functional submodules. The TBP-module is important for TBP delivery at the promoter. The nucleosome modification modules HAT (Histone acetyltransferase module) and DUB (De-Ubiquitination module) are important for H3K9/K14 acetylation and H2BK123 de-ubiquitination at the promoter region, respectively. The Tra1p protein mediates the recruitment of the SAGA complex via interaction with Activator Factors.

B) Modular composition of the SAGA and SLIK complexes. The HAT module consists of the Histone acetyltransferase enzyme Gcn5p and the Ada2p and Ada3p regulatory subunits. The DUB module is formed by the ubiquitin protease enzyme Ubp8p and Sgf11p, Sus1p and Sgf73p subunits. Sgf73p anchors the DUB module to the SAGA complex. The TBP module composition differs between the SAGA and SLIK comlexes. In SAGA, Spt3p and Spt8p interact with TBP. A full length Spt7p subunit contains an Spt8p binding domain at the C-terminus, which recruits Spt8p to the complex. SLIK contains a C-terminal truncated form of Spt7p lacking the Spt8p-binding domain therefore leading to a different composition of the TBP module of the complex.

References:(109, 116, 123, 148, 179, 186, 206, 222, 223)

es the UAS Requirement (BUR) of the *SUC2* gene (166). Biochemical characterization in mammalian cells identified the Mot1p homolog as a TAF distinct from TFIID TAFs termed BTAF1, which forms the B-TFIID complex. (38, 161, 205). As for TFIID, B-TFIID was able to activate transcription *in vitro* of pol II genes (205) .

The interaction with TBP was characterized in an *in vitro* system using ATPase defective (D1408N) Mot1p mutants and truncations. It has been shown that the N-terminal domain of Mot1p interacts with the C-terminus of TBP and requires DNA for optimal binding. This binding disrupts the TBP-TFIIA complex suggesting a competition mechanism for TBP binding with transcriptional co-activators (1, 10).

However *in vitro* transcription experiments have led to controversial results on the B-TFIID/Mot1p activity. In fact, both positive and negative effects of Mot1p have been observed. The authors have hypothesized that these difference are linked to the relative level of TFIIA in the system used (38, 205). It has been shown that TFIIB overexpression does not affect the repressive function of Mot1p suggesting a differential interplay with these TBP-binding factors (38, 11).

Pioneering studies of Auble and co-workers showed that both *in vivo* and *in vitro* systems the repressive function of Mot1p was dependent on its C-terminal ATPase domain (11). Although the mutation in the catalytic site of the ATPase domain does not affect the interaction with TBP (1), it prevents its specific disassembly from the DNA (13). The ATPase domain of yeast Mot1p is highly conserved in human and Drosophila (hBTAF1 and dBTAF1) (38, 208). Moreover the homology of the ATPase domain with those of the helicases Snf2p and Rad54p (55) places Mot1p in the SNF2/SWI2 ATPase family (13).

Structural studies on BTAF1 unveiled a double domain structure. The protein is arranged in a C-terminal ATPase domain and a N-terminal TBP-binding domain with a protruding thumb-like structure. It has been hypothesized that the curved shape of the N-terminal thumb structure, due to the presence of HEAT repeats, forms a groove that might allocate TBP (158). Moreover it has been shown that Mot1p binds directly to the DNA via its N-terminal domain. This domain is highly conserved within the SNF2/SWI2 ATPases family and its deletion prevents Mot1p-TBP-DNA complex formation and inhibits the ATPase activity (194).

Although several mechanisms of action have been proposed, the exact mechanism of Mot1p-TBP interaction and TBP-DNA complex disruption is not clear yet. It has been proposed that the interaction of Mot1p with TBP occurs in two steps. Mot1p would first bind TBP via the N-terminal domain, followed by ATPase driven TBP ejection. This 'powerstroke' leads to DNA-TBP complex disruption (12, 13). Accordingly it has been found that Mot1p can bind a handle upstream of the TATA box suggesting that a possible tracking of Mot1p along the handle would disrupt the DNA-TBP binding (50). In another possible mechanism of action ATPase driven structural changes of the N-terminal thumb of Mot1p might occur. This would allow sandwiching the TBP-DNA complex between the N-terminal and C-terminal domains of Mot1p therefore moving TBP away from a transcriptionally favorable position (158).

The latest hypothesized molecular mechanism of TBP removal from the core promoter is the 'spring-loading 'model (207). The authors suggest that the bending of the DNA by the TBP binding acts as a 'spring' for the TBP-promoter dissociation. Moreover the TATA and TATA-less promoter contain a different intrinsic 'spring' power due to the differential affinity for TBP binding and the bendability of the DNA sequence. The action of Mot1p and NC2 would eventually release TBP rapidly from TATA containing promoters and slowly from TATAless promoters (207).

The repressive function of Mot1p has been linked to the *in vivo* ratio of Mot1p to TBP. In-

deed, protein levels of Mot1p are in excess compared to TBP. *In vitro* assay using approximately similar stoichiometric amount of purified Mot1p and TBP activates transcription.
The authors suggest that the positive effect on transcription occurs via an indirect mechanism in which Mot1p might liberate TBP from non-promoter regions, thus increasing the TBP availability for core promoter regions (149). According to this the Mot1p protein levels might play a role in the regulation of transcriptional activity *in vivo*. Recent studies shows that Mot1p undergoes a quality control process mediated by sumoylation via the Slx5p-Slx8p and proteosome pathway. The modulation of Mot1p degradation could therefore affect its transcriptional role (216, 217). The dual transcriptional function of Mot1p has been addressed *in vivo* by genome wide mRNA expression profiling. It has been found that functional inhibition of Mot1p results in differential gene activation and repression, with most of the genes being upregulated and related to stress-response, diauxic shift and sporulation. Interestingly, these genes were shown to be upregulated upon functional loss of NC2 as well (52). However, the pattern of regulated genes was different in another study that showed that Mot1p activates transcription more than repressing it (15% and 5% respectively) (69). The inconsistency among different studies might be related to the genetic system and the different temperature sensitive mutants of mot1 used. In Chapter 4 of this thesis we describe a new Mot1p genome wide expression profile using a new unbiased genetic system.
Using gene models, it has been observed that Mot1p activating or repressing function does not correlate with TBP recruitment at the promoter. For example, the *INO1* and *HSP26* genes are repressed by Mot1p via inhibition of TBP recruitment to the promoter, whereas the URA1 and BNA1 genes are activated by an identical effect on TBP recruitment (53). The mechanism of activation by Mot1p has been explored in more detail using the *URA1* gene model. The authors show that TBP binds the URA1 promoter in the inverted orientation in the absence of Mot1p. In the Mot1p dependent activation, TBP is removed from the promoter allowing the binding of a new molecule in the right orientation, which will promote the expression of the downstream gene. Accordingly, the inhibition of Mot1p function results in repression of *URA1* transcription (196). This result suggests that the nature and sequence of the promoter might intrinsically control the DNA-TBP-Mot1p interplay and therefore the transcriptional output. Interesting results have been obtained on transcription from the two-element containing promoter of the *HIS3* gene using a ts (temperature sensitive) mutant of Mot1p. The promoter region contains two different promoters that mediate the initiation from two transcriptional start site (TSS): a canonical TATA-containing promoter (+13 TSS) and a non-canonical TATA-less promoter (+1 TSS). It has been shown that Mot1p is required for transcription from the TATA-less promoter, whereas it represses transcription from the TATA-containing promoter of the same gene (41, 69). Although the primary sequence of the DNA might have a direct effect on Mot1p, the modulation of protein activity might be related to the interaction with transcription factors in the PIC at the promoter. It has been shown that Mot1p co-occupies promoters with TBP under normal conditions. Upon heat shock or osmotic stress Mot1p co-occupies the promoter with TBP and TFIIB but not with TFIIA. Therefore a differential composition on PIC could determine the transcriptional output (70).
Recent findings on Mot1p have expanded its transcriptional role from initiation to elongation. The control of TBP targeting to a promoter influences RNA synthesis and thus the elongation process. Tiling array profiles of Mot1 ts mutants showed that loss of Mot1p function correlates with an increase in spurious transcription initiation and termination, comparable to a *SET2* deletion mutant. Thus the authors suggest that the PIC disassembly function of Mot1p ensures a dynamic turnover at the promoter, which is essential to maintain the precision and productivity of mRNA synthesis (162).

NC2

The NC2 complex has been shown to negatively regulate TBP activity. NC2 can repress transcription *in vitro* (65) and consists of the NC2α (Bur6p) and NC2β (Ydr1p) proteins. The two subunits dimerize via an N-terminal HFD similar to the H2A/H2B heterodimer (182). Yeast *NC2α* and *NC2β* are homologous to the human DRAP1 and DR1, respectively, with high sequence conservation of the HFDs (165). Human Dr1 can rescue an *NC2β* null mutation whereas complementation does not occur for DRAP1 and *NC2α* (104).

The repressive function via modulation of TBP activity links the transcriptional function of NC2 to Mot1p. Interestingly, in *in vitro* assays it was found that human B-TAF1 interacts with human NC2 via direct binding of NC2α C-terminal region and that the binding of NC2 to the promoter increases the B-TAF1 recruitment to the DNA-TBP complex (108). As hypothesized for Mot1p, a possible mechanism of action of NC2 is the disassembly of the PIC. Accordingly it has been shown that the inhibition of transcription occurs by NC2 competition for TBP binding with TFIIA/TFIIB. However NC2 does not compete with TFIID (105, 182). FRET analyses show that NC binds TBP on the underside of the pre-bent DNA via electrostatic interaction. The new formed TBP-DNA-NC2 complex is dynamic and favors the TBP sliding along the core promoter region therefore inhibiting the PIC nucleation property of TBP and thus transcription (182). The repressive activity exerted by NC2 might depend on its protein level. It has been shown *in vitro* that increasing concentrations of TBP can revert NC2 function (104). It is interesting to note that, as mentioned for Mot1p, also for NC2 the DNA primary sequence has an effect on its transcriptional function. In a Droshophila system, purified dNC2 is able to activate transcription at DPE-containing promoters, and represses transcription at TATA-containing promoters (94). Accordingly, it has been shown that selective mutations in NC2 can abolish the repressive function on TATA promoters, suggesting a role of the primary DNA sequence in the modulation of NC2 transcriptional activity (219).

The dual function of NC2 on TATA and TATA-less transcription has been tested on the TATA-containing and TATA-less promoter of the HIS3 gene. Using *NC2β* mutants, it was observed that NC2 was required for transcription from the TATA less promoter of *HIS3*, showing a similar transcriptional function to Mot1p *in vivo* (126). However also for NC2, PIC composition and interaction with specific TAFs modulate transcriptional output. It has been shown for instance that generally NC2 positively affects TFIIB target genes. However NC2 represses the TFIIB association at certain promoters via the C-terminal region of NC2β, hence inhibiting PIC assembly (140). In another study it was shown that recruitment of TFIIA and NC2 is mutually exclusive at a TFIIB- recognition element (BRE) containing promoter. Moreover TFIIA has a negative effect whereas NC2 has a positive transcriptional effect at BRE-lacking promoters suggesting a different interplay between TFIIB-TFIIA and NC2 at BRE-containing versus BRE-lacking promoters.

Functional interplay among TBP-binding complexes

Modulation of TBP activity at promoters involves a functional interplay among TBP binding complexes. TFIID and SAGA complexes positively affect TBP recruitment to the DNA. The functional similarity between the two complexes is reflected in their structural organization. TFIID and SAGA share a similar TAF composition. In both complexes, HFD-mediated interaction of TAF pairs mediates the structural organization (67). Functional studies show that TFIID regulates the expression of housekeeping genes whereas SAGA is important for stress related genes. The TFIID dominated genes (90%) are TAF-dependent genes whereas the SAGA dominated genes (10%) are TAF-independent (95). However, target gene regulation

by the two complexes is not mutually exclusive. Indeed, the complexes co-occupy a large part of genes in yeast (125). Interplay between the TBP-binding complexes TFIID and SAGA has been documented at certain promoters. During DNA damage or heat shock a sequential recruitment of SAGA followed by TFIID occurs. Therefore, it has been suggested that the SAGA pathway would be used to mount a fast response to the stress stimulus and later this would be settled by recruitment and stabilization of the TFIID complex (73).

The functional interaction between Mot1p and NC2 is well documented. As mentioned earlier Mot1p and NC2 have a negative role on TBP activity. It has been shown that Mot1p forms a discrete complex on DNA with TBP and NC2 that is disassembled in the presence of ATP (211). *In vitro* experiments show that Mot1p and NC2 can co-occupy the same promoters and NC2 does not affect Mot1p dependent TBP displacement (49). It is interesting to note that both *MOT1* and *NC2α* (*BUR3* and *BUR6* respectively) have been isolated in a screen for mutants that Bypass UAS Requirement (165). This suggests that both proteins are repressors of basal transcription and are functionally linked to activator factors. Also TBP mutants were selected to be able to bypass the *SUC2* UAS. Interestingly these mutations span in a region important for NC2 interaction (33). Although Mot1p and NC2 repress basal transcription *in vivo*, their function was found to be also important during gene induction. Temperature sensitive mutants of Mot1p and NC2α are unable to fully activate *GAL1* and *GAL10* in the presence of galactose (34, 165), and mutation on NC2β leads to a suboptimal gene activation of *HSP12* during heat shock (140).

Interplay between the complexes that have a positive (TFIID, SAGA) or negative (Mot1p, NC2) effect on TBP activity has been documented genetically and biochemically. A genome-wide study showed that TFIID, SAGA, Mot1p, NC2 and TBP co-occupy the same gene promoters (211). The authors suggest that the co-occurrence of complexes with opposite effects on TBP activity on the same promoter in a cell population can be explained by the dynamic nature of TBP on DNA (211). TBP is highly mobile *in vivo* (56, 195). It is widely assumed that cycles of PIC assembly/disassembly can take place during gene transcription. The dynamic nature of TBP is linked to the Mot1p activity *in vivo* in yeast (195). Studies *in vivo* using mammalian cells have shown that BTAF1 (Mot1p) as well as NC2 regulate TBP dynamics by affecting the DNA dissociation rate (56). TBP turnover modulates the transcriptional output of the downstream gene. Furthermore, TBP-binding complexes rather than DNA elements at the promoter affect TBP turnover. Low TBP turnover correlates with higher TFIID binding, TATA-less promoters, and high transcription. Higher TBP turnover correlates with higher SAGA binding, TATA-containing promoters, Mot1p-repression, and low transcription (212). The modulation of transcription of this group of genes must be well-controlled in order to obtain a fast and productive response. Hence, the interplay between TBP-binding complexes coordinates TBP turnover and, thus, gene transcription. For instance, Mot1p genetically interacts with the SAGA complex. Genetic interactions between *SPT3* and *MOT1* alleles produce synthetic lethal phenotypes (135). A functional connection between SAGA and Mot1p at the *HXT2* and *HXT4* promoter has also been described. *SPT3* and *SPT8* deletion reduce Mot1p and TBP binding whereas Gcn5p activity controls both Mot1p and Taf1p (TFIID) recruitment to promoters (209). The positive transcriptional role of Mot1p has been suggested to operate indirectly by a redistribution of TBP from high to low affinity sites. The low affinity interactions are governed by Spt3p-TBP binding, and mutations affecting Spt3p-TBP interaction can suppress the mot1-*1* mutant (41). Interestingly, mutations of TBP that bypass Mot1p function were also found on the DNA binding domain

and on the TFIIA, TFIIB, and NC2 binding residues suggesting that these interactors are competing for TBP binding (197).

Transcription dynamics

In the past few years a new picture of the transcription process has emerged. The use of *in vivo* imaging, FRET and FRAP (Fluorescence Recovery After Photobleaching) technologies have allowed the analysis of transcription in real time (51, 145, 193). In particular, the use of single-molecule methods has unveiled that transcription is more stochastic and dynamic than expected. Indeed, transcription in individual cells does not follow the average measured in a population but rather fluctuates. The transcriptional fluctuations arise from oscillations within the transcription machinery that stochastically meet the need of genes to be expressed in 'burst-like' transcriptional events (39, 75, 170). Dynamic oscillations have been found during subcellular sequestration of transcription factors (151), activator-DNA binding (99), for the stimulating effectors of transcription factors (58) and for chromatin architecture (98). In a recent issue of Science, Larson *et al.,* (120) and Suter *et al.,* (202) presented interesting findings on transcription dynamics. These authors showed that in yeast and in mammalian cells transcription occurs in burst-like rather than a continuous fashion, and that transcription initiation is stochastic whereas transcription elongation is deterministic. Furthermore, initiation bursts correlate with the 'searching time' of transcriptional factors, which is the diffusion time that a factor needs to meet its DNA element. This is in line with the idea that transcription activation is determined by the recruitment of a rate-limiting factor, which nucleates the PIC (100, 168). Moreover, once transcription initiates, the RNA polymerization proceeds at a steady state, making elongation a deterministic process. In addition, the authors show that gene inactivation is controlled by a single rate-limiting step whereas reactivation requires sequential or parallel processes, suggesting the absence of transcriptional memory between separated transcriptional events.

It is interesting to note how these new findings on transcription using real-time methods agree with previous mechanistic models suggested by biochemical data. This is the case for the sequential recruitment of factors during PIC nucleation, during which the rate-limiting step is binding of TBP to the promoter. Similarly, transcription inactivation is dependent on the removal of TBP from the promoter. However, findings on transcription dynamics do not correlate with processes that are thought to facilitate re-initiation of transcription, including formation of a PIC scaffold or promoter-terminator looping (7, 8). The use of the new microscopy and real-time methodologies will certainly shed light on the understanding of the complex mechanisms of transcriptional control in the future.

Summary and outline of this thesis

The key feature of transcription initiation is the nucleation of PIC assembly determined by TBP binding to the promoter. Therefore, this event is subjected to tight control by many proteins and protein complexes. Here we describe the state of the art on the regulation of TBP activity.
The interplay among TBP-binding complexes and DNA elements modulates the transcriptional output. Furthermore, modulation of TBP activity occurs in a dynamic fashion by controlling the TBP delivering and removal rates. TFIID and SAGA have a positive role on TBP activity whereas Mot1p and NC2 have a negative role. Although a myriad of data exists on this issue, the current working model is far from being complete.

In this thesis the biochemical and functional characterization of TBP-binding complexes is reported. In Chapter 2 we review the diversification of SAGA complexes during evolution from yeast to human, focusing on the composition and conservation of domains and subunits and on the interaction of the complexes with signaling pathways. Chapter 3 describes the profiling of posttranslational modifications on TFIID and SAGA TBP-binding complexes. The pattern of phosphorylation and acetylation on shared and specific subunits of the two complexes offers an interesting framework for transcription regulation and its molecular mechanisms. The mapping of the cleavage site on the C-terminus of the Spt7p subunit of the SAGA complex is also reported. In Chapter 4 we identify the Pep4p protease as being responsible for Spt7p cleavage. The proteolytic processing of the Spt7p subunit affects the composition of the TBP-module of SAGA leading to formation of the SLIK protein complex. With Chapter 5 we move the focus from the TBP-activating complexes to the TBP-repressing proteins. An assessment of genome-wide regulation of gene expression by Mot1p and NC2 is presented. The Mot1p-NC2 activity for removal of TBP from promoters appears to be crucial to maintain basal expression and to shut-down *HSP26* transcription following heat shock activation. In Chapter 6 we discuss how TBP binding complexes can regulate TBP activity, and what the implications are for the control of gene expression.

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Chapter 2

Diversification of the SAGA transcriptional coactivator in higher eukaryotes: interplay with signaling pathways

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The multisubunit SAGA complex function as transcriptional co-activator and is highly conserved. In yeast, in flies and human SAGA regulates transcription and chromatin by acetylating and de-ubiquitinating histones and by delivering TBP to promoters. Recently, the identification of a SAGA-related complex termed ATAC in higher eukaryotes, has been reported. Here, we review the current knowledge on SAGA and ATAC, focusing on their structural and functional similarities from an evolutionary prospective. SAGA and ATAC exert complementary functions in higher eukaryotes, which suggest a possible evolutionary diversification from a common ancestor. We provide a detailed analysis of the domain and subunit composition of the complexes in S. cerevisiae, D. melanogaster and H. sapiens. Moreover, we focus on the interaction of SAGA and ATAC and signaling pathways, unveiling conservation at the functional level.

Introduction

Over 15 years have passed since Allis and coworkers discovered that a protein of 55 kDa purified from *Tetrahymena termophyla* macronuclei was homologous to Gcn5p in *Saccharomyces cerevisiae*, and that both exhibited acetyltransferase activity towards histones (12). Yeast Gcn5p was a well-studied coactivator of gene transcription (13). This finding initiated a new field of research that has expanded enormously and has revolutionized molecular models of genetic structure, inheritance, and gene expression. Since then, the concept of DNA as a linear molecule has progressed into several different novel directions. Within chromatin DNA is wrapped around histone octamers forming discrete units called nucleosomes. Histone N-termini protrude from the nucleosome core particle and are subjected to posttranslational modifications. These can modify the physical-chemical property of the nucleosome but they also offer an interaction platform for a myriad of so-called writers/readers/erasers. Histone acetylation has been one of the most studied histone modifications, and the acetyltransferase complexes involved have become major research topics.

Few years after its first characterization, the acetyltransferase (AT) Gcn5p was found to be part of a multiprotein complex named SAGA (Spt-Ada-Gcn5-Acetyltransferase) (32). The unveiling of the modular architecture of the SAGA complex has permitted the deciphering of its multifunctional role. Characterization of the ySAGA complex revealed several layers of transcription-related functions, including transcription initiation, transcription elongation, gene activation, histone de-ubiquitination, TBP binding, and mRNA export. SAGA also has been isolated from flies and human and it has been shown to preserve an extraordinary grade of conservation of subunit composition and function. yGCN5 is paralogous to two human genes: GNC5 and PCAF (p300/CBP-associated factor). Both genes code for acetyltransferases that share 73% identity and were found to be assembled in at least 3 similar protein complexes: STAGA, TFTC and PCAF. Today, it is suggested that GNC5 and PCAF are mutually exclusive in these complexes, now collectively termed hSAGA (80).

More recently, the complexity of GCN5/PCAF-containing complexes has been expanded by the discovery of the related ATAC complex (5, 51, 65, 76). The multiprotein ATAC complex contains a novel HAT subunits (ATAC2) and both SAGA and ATAC can be assembled either with GCN5 or PCAF acetyltransferases.

Interestingly, the existence of SAGA and ATAC in higher eukaryotes correlates with the duplication of the yeast *ADA2* gene in ADA2a and ADA2b. The products of the two variants code for two related proteins that are exclusively present either in ATAC or SAGA respectively. Together with the ADA2 variants, the panel of specific subunits for each complex highlights differential protein-protein interactions and binding to histone marks, suggesting specific and unique transcriptional roles for each complex.

Whereas the composition of SAGA is conserved from yeast to human, ATAC is specific for higher eukaryotes and shows conservation from flies to human. The two complexes share a common set of subunits besides a large number of complex-specific subunits (figure 1). Domain analysis of subunits of the two complexes reveals, with a few exceptions, a high level of evolutionary conservation between the species investigated (Figure 2, Table S1).

This review focuses on the GCN5/PCAF-containing complexes in *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Homo sapiens*. The assortment of subunits, domains and their evolutionary conservation is analyzed. We also discuss current knowledge on the signaling pathways that control activation, gene-specific recruitment, and protein-protein interactions of SAGA and ATAC. Although far from being complete, our analysis is based on the strong conservation among different species, and allows us to assemble 15 years of research in a cumulative manner to delineate a functional profile of GCN5/PCAF-containing complexes.

SAGA and ATAC complexes: domains, modules and functional conservation

HATs

Based on similarities in their active domains, histone acetyltransferases (HATs) can be grouped in different classes. The GNAT class of acetyltransferases includes GCN5, PCAF, ELP3, HAT1, HPA2, NUT1 and ATAC2, whereas the MYST class includes MORF, YBF2 (SAS3), SAS2 and TIP60. Another important protein that has HAT activity is p300/CBP in humans.

In yeast and flies, a single mRNA codes for the GCN5 HAT protein. In humans, the GCN5 mRNA is differentially spliced in long (GCN5L) and short (GCN5S) transcripts: only GCN5L is involved in the formation of acetyltransferase-containing complexes. Human GCN5L shares 73% of similarity with hPCAF and 50% with dGNC5, whereas the homology with yGNC5 is only 48% (reviewed in (68)). yGcn5p lacks an N-terminal PCAF-homology domain that is shared between hGCN5, dGCN5 and PCAF. However, the C-terminal acetyltransferase domain and the bromodomain are highly conserved in all species mentioned. The ATAC2 HAT that has recently been identified in the dATAC and hATAC complexes is not homologous to GNC5. However its AT domain belongs to the GCN5 class of acetyltransferases.

GCN5, PCAF and ATAC2 acetylate a specific pattern of histone lysines. It was shown first in yeast that yGCN5 is specific for histone H3 and H4 acetylation at residues located in the N-termini. Soon after the isolation from yeast, it was discovered that the C-terminal bromodomain of yGcn5p is essential for interaction with histones H3 and H4 (74). It was shown later that the Gcn5p bromodomain binds residues between position 16 and 19 at the N-terminus of H4, and that lysine acetylation increases the binding affinity. Therefore, a preceding histone acetylation can increase the specificity and recruitment of Gcn5p to chromatin (75). Interestingly, despite the high sequence conservation, the bromodomain present in Gcn5p has a higher affinity for H4K16Ac peptides, whereas the PCAF bromodomain preferentially binds acetylated H4K8 and H3K14 residues (23, 43).

In yeast, Grant and co-workers showed that recombinant Gcn5p (rGcn5p) is not able to acetylate nucleosomes. However, when assembled in the SAGA complex, the authors could show *in vitro* acetylation of nucleosomes (32, 33). Interestingly, rGcn5p efficiently acetylates H3K14 and H4K8/K16 histone proteins outside a nucleosomal context; however, H3K9 is the most preferred residue *in vivo* (50). Also for human PCAF, as for the yGcn5p, it has been observed that it harbors acetyltransferase activity on naked histones but not on nucleosomes (117). However, both hGCN5 and PCAF, when assembled in their complexes (STAGA/TFTC/PCAF) were able to acetylate nucleosomes (11, 57).

The specificity of Gnc5p acetylation has been linked to its biological function, such as transcription and cell cycle progression (120). The pattern of lysine acetylation by Gcn5p has been expanded lately by the discovery of new residues and their functional role in transcription. In particular, in yeast H3K36 acetylation occurs at pol II promoters and is Gcn5p-dependent. It is shown that the pattern of H3K36 acetylation resembles the H3K9/K14Ac pattern and is highly conserved in mammals. Interestingly, H3K36 can also be trimethylated, which occurs in the gene body, suggesting that H3K36 acetylation/methylation might act as a chromatin switch between the promoter and ORF to modulate two different transcriptional events: initiation and elongation of transcription (63). On the same line, it has been shown recently that in yeast, Gcn5p is also responsible, together with Rtt109p, for H3K4 acetylation. The location of the H3K4Ac mark has been found to be upstream of the promoter region. The authors therefore suggest a boundary role for the newly discovered histone mark (38).

In flies, dGCN5 contains the PCAF-homology domain suggesting a common ancestor gene for dGCN5, hGCN5 and PCAF (94). dGCN5 is the major HAT for H3K9/K14 acetylation but not for H4 acetylation (16). Interestingly, the recently discovered GCN5-related ATAC2 protein has been shown to be responsible for H4 acetylation at the K16 residue (99). rATAC2 has a weak acetyltransferase activity towards histone H4, however, the assembly in the ATAC complex enhances its

acetyltransferase activity. Knockdown of mammalian ATAC2 expression leads to decrease of H3K9 and H4K5/12/16 acetylation. However, since ATAC2 also has a structural role in ATAC, it is likely that its knockdown also affects GCN5 activity within the ATAC complex, therefore making it difficult to discern the roles of these two acetyltransferase activities *in vivo* (35).

HAT ADAptors

A major role in regulating the HAT activity towards histone tails is played by the adaptor proteins (ADA). In yeast, Gcn5p binds Ada2p and Ada3p, and this forms the ADA/HAT module (4). Assembly with Ada2p and Ada3p allows acetylation of nucleosomes by Gcn5p. The binding of the Ada3p adaptor to Gcn5p specifies the pattern of lysine acetylation on histone tails, similar to the pattern of the SAGA complex [H3K18>K14>K9] (4). In flies dADA3 is the product of the gene dik (diskette) (35, 83) The gene is essential for growth and development, and mutational analysis shows effects on histone acetylation and phosphorylation. Null mutations lead to loss of H3K9/K14ac and H4K12ac, whereas H3K18ac and H4K5/K8/K16ac remain unchanged (34).

In yeast, it has been shown that Ada2p is important to potentiate Gcn5p activity on histone tails (4). The interaction between Gcn5p and Ada2p is conserved from yeast to human (15). In flies both ADA2a and ADA2b interact with GCN5 and occupy different but overlapping loci at the X chromosome (51, 65). Characterization of the two variants has shown that the SAGA and ATAC complexes have different roles in chromatin acetylation: disruption of dADA2b affects H3K9 and K14 acetylation, whereas disruption of dADA2a does not. In addition, ADA2a disruption affects H4K12Ac and H4K5Ac levels, while neither ADA2a nor ADA2b disruption affect H3K18 and H4K8/K16 acetylation (18). Although the ATAC complex does not exist in yeast, the appearance of the complex might have happened early in evolution. In *Arabidopsis thaliana* two variants of the ADA2 gene exist. By genetic characterization it has been found that loss of ADA2a or ADA2b leads to different phenotypes. Furthermore, GCN5 and ADA2b null mutants have a similar phenotype in Arabidopsis suggesting that they are part of the SAGA complex (40, 104). Taken together, the ADA2 gene has been duplicated during evolution from lower to higher eukaryotes, which presumably has been the starting point for the generation of two similar HAT-containing complexes with specific transcriptional roles.

SAGA-specific modules

The SAGA complex is composed of 21 subunits organized in a modular structure, and has a molecular mass of 2 MDa. The following subunits are present in SAGA but not in ATAC: TAFs (TAF5, TAF6, TAF9, TAF 10, TAF12) SPTs (SPT3, SPT7, SPT8, SPT20) together with DUB-module subunits (SGF11, SGF73, UBP8, SUS1), TRA1 and ADA2/2b. Human SAGA was discovered by Roeder and coworkers (56). They unveiled the association of human SPT3 to TAFs and to the newly discovered GCN5L protein. The complex was called STAGA (for Spt3-Taf31II-GCN5L-acetyltransferase) (56). A few years later, the same authors identified a large number of additional complex subunits (57). TFTC (TBP-free TAF Complex) was discovered by Tora and coworkers as a TAF-containing complex lacking TBP (112). Purification of TFTC revealed that the complex has a composition similar to STAGA (11) and to the PCAF complex, which contains the PCAF acetyltransferase instead of GCN5L (71). It is generally accepted that these complexes are very similar and they are referred to as human SAGA (80).

The first extensive characterization of yeast SAGA was performed in the Berger lab (96). The authors showed that the complex contains the protein products of three main classes of genes: TAFs, SPTs and ADAs (96) The TAF subunits form a structural core which is shared between the SAGA and TFIID complexes. Structural studies suggest that yTaf5p is present in two copies within the SAGA complex and orchestrates the pseudo-symmetric structure of the complex. Taf5p dimerization occurs via the N-terminus, whereas the C-terminus is organized in a propeller structure medi-

ated by the WD-40 repeats, which are important for protein-protein interactions.
Recent data from Workman and coworkers report a new gene isolated from flies, termed WDA, with a highly similar domain organization compared to yeast TAF5. Although yeast Taf5p is highly similar to WDA inWD flies, WDA deletion does not affect dSAGA complex stability as does yTAF5, suggesting a different mechanism for SAGA complex assembly in flies (37). Interactions between the TAFs occur via their histone fold domains (HFD). Figure 2 shows that all TAF homologs in the three species contain a HFD, suggesting conservation of the TAF-interactome within SAGA. Recently, the SAF6 protein has been isolated in flies as the TAF6 ortholog (109).
Together with the TAF module, Spt20p, Spt7p and Ada1p have important structural roles in yeast. In flies and human, the SPT20 homolog is involved in both complex stability and in interaction with the MAP kinase pathway (67, 109). Indeed, the protein, originally named p38IP, binds the p38 MAP kinase. The implications of this interaction are discussed later in this review. Although orthologs of Spt7p have been found in flies and human, these proteins haven not been well characterized. While the human and the yeast SPT othologs contain a C-terminal bromodomain, the fly counterpart does not (36). In yeast, Spt3p and Spt8p form the TBP-interaction module. Although *SPT3* is highly conserved, *SPT8* does not have orthologs in flies and human. Interestingly, in contrast to ySAGA, the dSAGA and hSAGA complexes do not bind TBP. It is therefore possible that the lack of *SPT8* in these complexes leads to loss of the TBP binding activity of SAGA.
An important contribution to our understanding of SAGA function comes from the Tra1p subunit. Tra1p in yeast (TRRAP in fly and human) is important for the tethering of the complex to the chromatin. TRA1 in the three species belongs to the phosphatidylinositol 3-kinase (PI3K)-related protein kinase (PIKK) family, but the protein is catalytically inactive. It has been shown that the N-termini of yTra1p and hTRA1 are highly similar and that they contain HEAT and TRP repeats, which are important for the tethering of the complex to the DNA and for structural integrity of the complex. Tra1p in yeast has been shown to bind acidic activators at the UAS, whereas the human counterpart has been shown to bind c-Myc at myc-responsive genes. The dTRA1 has not extensively been characterized biochemically, but the high similarity to the yeast and human orthologs suggest similar functions.
More recently, another module of the SAGA complex has been characterized: the De-ubiquitination (DUB) module. In yeast, the module is formed by the catalytic subunit Ubp8p and by the adaptors Sgf11p, Sgf73p, Sus1p. H2AK123 deubiquitination in yeast is required for H3K4 trimethylation, which occurs at gene promoters. The components of the DUB module have been well characterized from yeast to human (90). Recently, Workman and collaborators have isolated the SGF73 ortholog in flies, indicating that the panel of DUB subunits is well conserved in all three species (109).

Shared subunits, shared functions

GCN5/PCAF, ADA3 and SGF29 are subunits shared between SAGA and ATAC. The HAT module of the yeast SAGA complex is formed by Gcn5p, Ada3p and Ada2p. Ada2p contains a SANT domain involved in modulation of the HAT activity (9, 95) and a zinc finger that might be involved in protein-protein or protein-DNA interaction. Domain analysis (Figure 2) reveals that the ADA2a and ADA2b variants differ in the SWIRM domain, which is present in yeast ADA2 and in the ADA2a variant, but not in ADA2b. The SWIRM domain has been shown to bind nucleosomes, preferentially at acetylated H3 tails (87). Therefore, it is possible that the presence of the SWIRM domain in ATAC might distinguish the function of this complex from SAGA.
It has been recently shown that the double tudor domain at the C-terminus of SGF29 is highly conserved from yeast to human, and mediates the recruitment of SAGA to H3K4me3 (8, 100). Since SGF29 is a shared subunit of both ATAC and SAGA, it is possible that the two complexes might compete for binding to the same promoters.

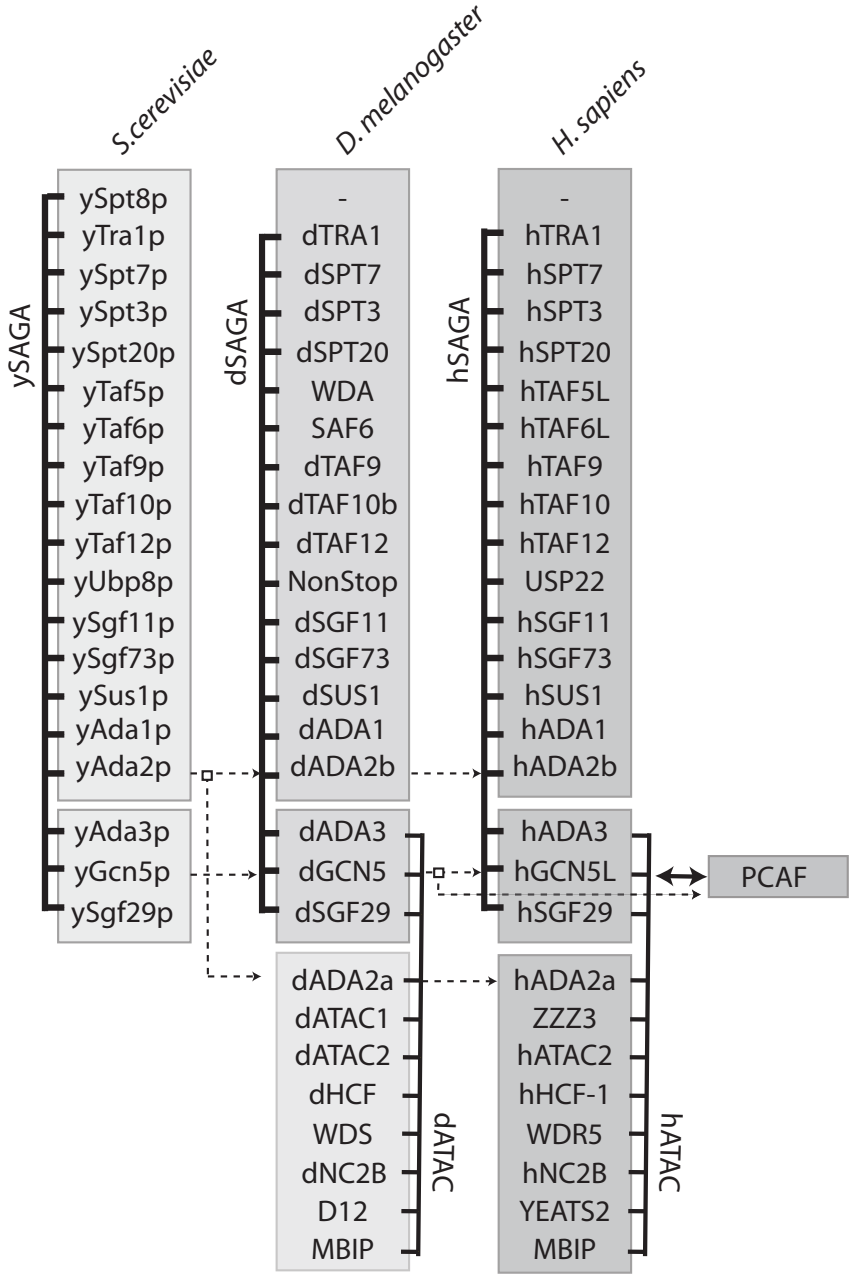
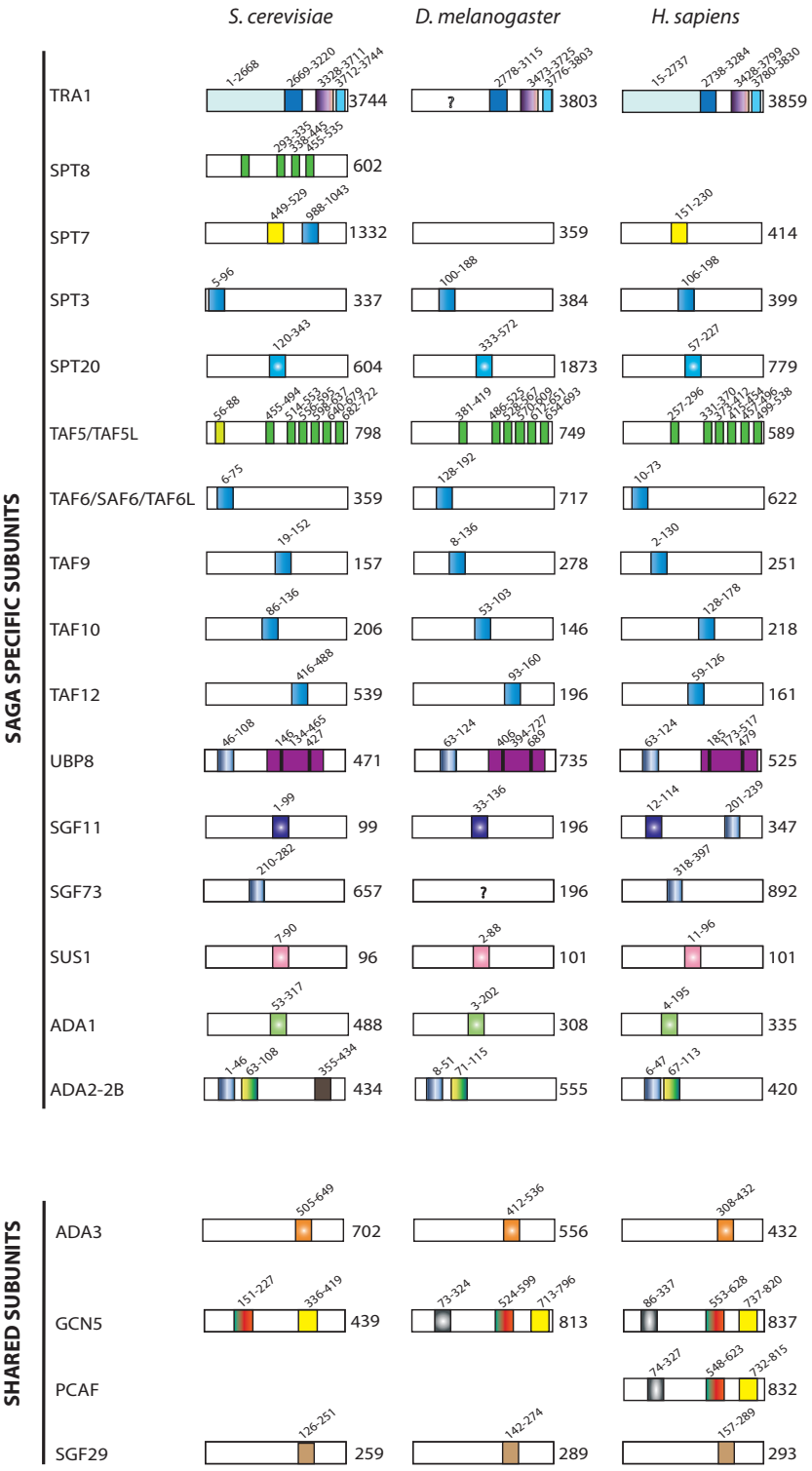
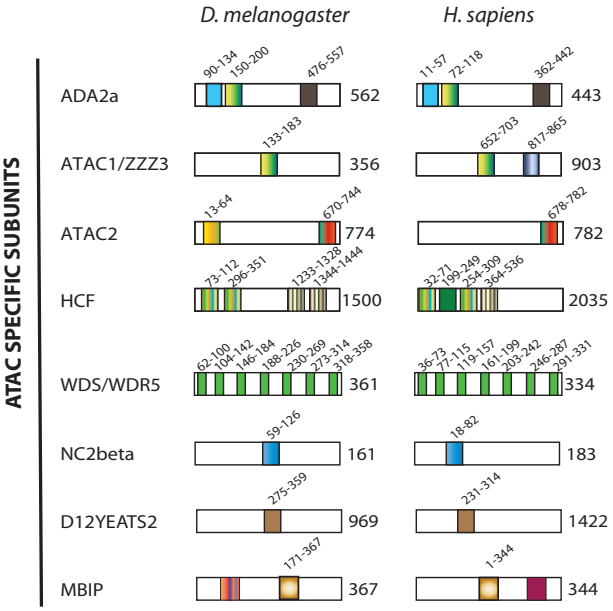


Figure 1. Subunit compositions of SAGA and ATAC complexes in *S. cerevisiae*, *D. melanogaster* and *H. sapiens*. Disputed or transient subunits have been omitted. SAGA-specific (upper), ATAC-specific (lower) and shared (middle) subunits are shown. The orthologous subunits in the three species are shown in the same row. PCAF and GCN5L are mutually exclusive in both hATAC and hSAGA. The evolutionary link (dashed lines) between GCN5L and PCAF in human and ADA2a and ADA2b in fly and human has likely originated from a gene duplication (squares) of a common ancestor.

Figure 2.
Assortment of domains in SAGA and ATAC complexes in *S. cerevisiae*, *D. melanogaster* and *H. sapiens*. The orthologous subunits are grouped in SAGA-specific, ATAC-specific and shared subunits. The protein structure shown is not to scale. Polypeptide length and domain coordinates are indicated for each subunit.




D. melanogaster
H. sapiens
ATAC SPECIFIC SUBUNITS
ADA2a
90-134
150-200
476-557
562
11-57
72-118
362-442
443
ATAC1/ZZZ3
133-183
356
652-703
817-865
903
ATAC2
13-64
670-744
774
678-782
782
HCF
73-112
296-351
1233-1328
1344-1444
1500
32-71
199-249
254-309
364-536
2035
WDS/WDR5
62-100
104-142
146-184
188-226
230-269
273-314
318-358
361
36-73
77-115
119-157
161-199
203-242
246-287
291-331
334
NC2beta
59-126
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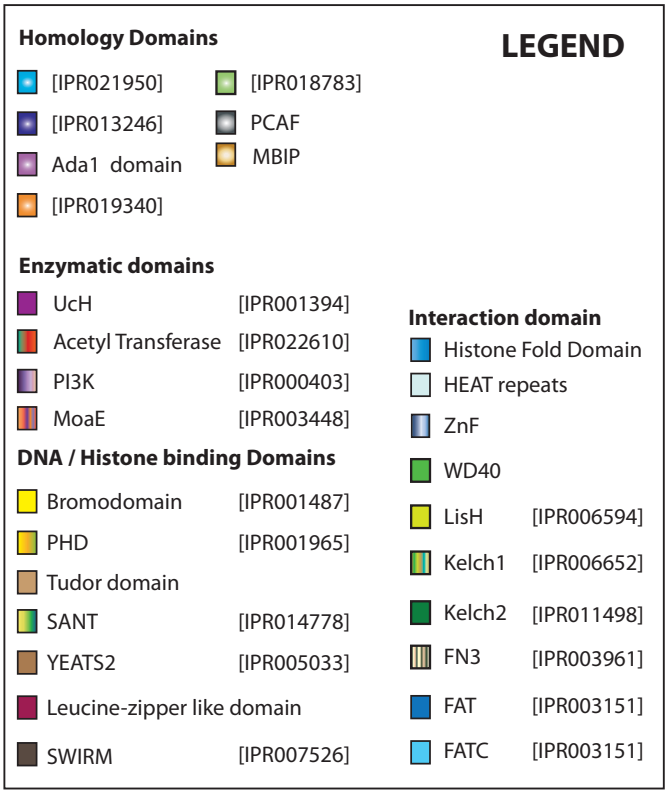
18-82
183
D12YEATS2
275-359
969
231-314
1422
MBIP
171-367
367
1-344
344


LEGEND
Homology Domains
[IPR021950]
[IPR013246]
Ada1 domain
[IPR019340]
[IPR018783]
PCAF
MBIP
Enzymatic domains
UcH [IPR001394]
Acetyl Transferase [IPR022610]
PI3K [IPR000403]
MoaE [IPR003448]
DNA / Histone binding Domains
Bromodomain [IPR001487]
PHD [IPR001965]
Tudor domain
SANT [IPR014778]
YEATS2 [IPR005033]
Leucine-zipper like domain
SWIRM [IPR007526]
Interaction domain
Histone Fold Domain
HEAT repeats
ZnF
WD40
LisH [IPR006594]
Kelch1 [IPR006652]
Kelch2 [IPR011498]
FN3 [IPR003961]
FAT [IPR003151]
FATC [IPR003151]

ATAC-specific subunits

ATAC and SAGA share roles in transcription, the cell cycle, and development (see later). The composition of the ATAC complex is well conserved in flies and human and includes: ADA2a, ATAC1/ZZZ3, ATAC2, HCF, WDS/WDR5, NC2β, D12/YEATS2 and MBIP (Figure 2). The complex contains two acetyltransferases: GCN5/PCAF and ATAC2. The ATAC2 HAT does not have a strong catalytic activity *in vitro*, however its function is essential during development (35).

It is interesting to note that in flies ATAC2 contains a PHD domain, which might mediate the specific recruitment of the complex to H3K4me3-enriched regions, thereby functioning in concert with SGF29. This feature is specific for dATAC2 and is not present in hATAC, suggesting different mechanisms for chromatin recruitment of the complex in different species.

It has been shown that the YEATS2 subunit of hATAC has a scaffold function and can interact with NC2β via the histone fold domain (108). The homologous MBIP (MAP kinase binding inhibitory protein) genes in flies and human (dMBIP and hMBIP) are conserved, however the N-terminus of dMBIP contains a MoaE domain (named after a subunit of the molybdopterin (MPT) synthase), which is responsible for JNK inhibition and is not present in hMBIP. Interestingly, the N-terminal sequence of hMBIP has presumably evolved from the MoaE domain (99). Under cellular stress conditions, including UV irradiation, heat shock, and osmotic shock, the kinase MUK (MAPKKK) induces JNK/SAPK activity by direct phosphorylation (41, 61). MBIP has been shown to bind MUK via the leucine-zipper domain, and inhibits its kinase activity and thereby inhibits JNK activation (26). In agreement, overexpression of MBIP inhibits activation of JNK responsive genes during osmotic stress. The functional implication of the MBIP-MUK interaction in the MAP kinase signaling will be discussed later in this review.

In addition to the subunits listed in Figure 1, other subunits have been identified in hATAC preparations: the MAP3K7/TAK1 subunit involved in MAP kinase signaling; the histone fold containing POLE3 (CHRACK14) and POLE4 subunits which interact with GCN5. Although Chrack14 also has been isolated in dATAC preparations (108), more experimental data are needed in order to confirm the integration of these proteins in the ATAC complex.

Molecular models for the regulation of transcription and chromatin by SAGA and ATAC

The molecular mechanism by which SAGA interacts with chromatin has been extensively investigated in yeast. Although a similar wealth of data is not available for dSAGA and hSAGA, several studies have highlighted that the mechanism of recruitment and activity of the complexes follows mechanisms similar to ySAGA. In yeast, the Tra1p subunit mediates the recruitment of the SAGA complex to DNA by binding to an acidic activator via N-terminal HEAT repeats (2, 7, 45). In flies, genetic data suggest that dTRA1 and dGCN5 activities are required for Notch signaling-induced gene expression (29), whereas hTRA1 interacts with the potent c-MYC and E2F activators (58). Recruitment of SAGA to chromatin facilitates histone acetylation and deubiquitination in all three species (reviewed in (46, 90)). It has been shown that SAGA-dependent acetylation facilitates recruitment of the SWI/SNF complex, which in turn remodels chromatin allowing promoter opening and assembly of the transcription initiation machinery (5, 17, 52).

Also the SAGA de-ubiquitination module has been shown to play an essential role in gene activation in all three species (reviewed in (25)). In flies and human, the ATAC complex has both overlapping and specific functions with respect to SAGA (see later in this review). The new, recently proposed mechanism derived from studies on ATAC in flies and human have revealed binding and regulation of enhancer sequences. In flies, ATAC binds to the enhancers of the JNK-responsive genes jra and chickadee (Figure 3), and modulates the functions of downstream gene products by regulating kinases of the MAP pathway (99). In human cells, ADA3 and ADA2a regulate β-catenin activity

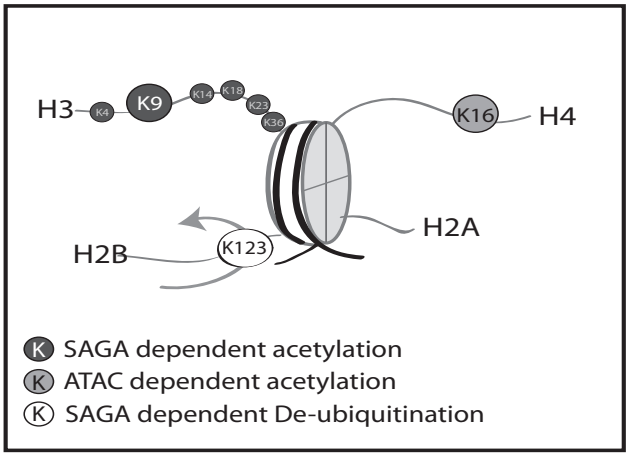
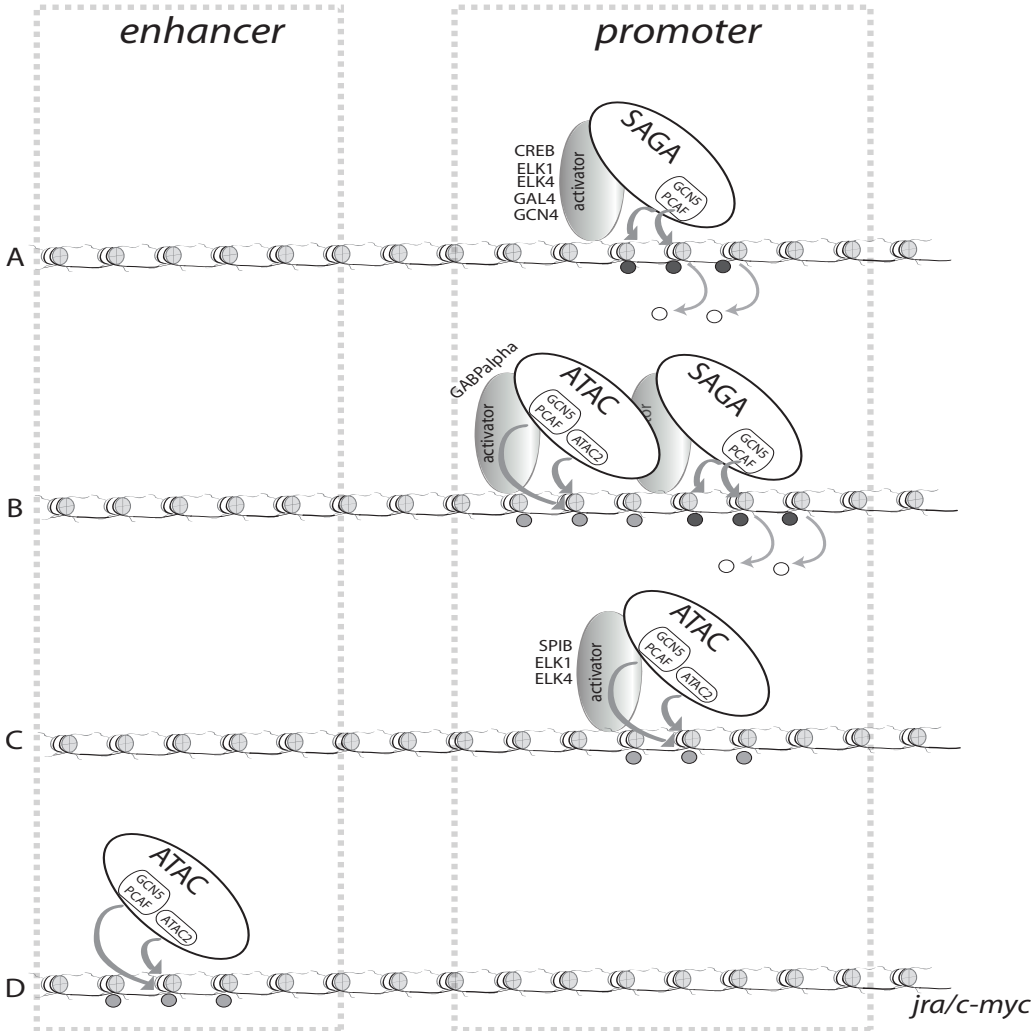


Figure 3. *Upper panel:* Histone acetylation and de-ubiquitination SAGA or ATAC dependent. *Lower Panel:* Possible molecular mechanisms of transcriptional control by SAGA and ATAC. (A) SAGA regulates expression of specific genes upon recruitment mediated by CREB, ELK1 and ELK4 in mammalian cells or by Gcn4p and Gal4p in yeast. (B) SAGA and ATAC can also co-regulate genes at certain promoters including CREB-responsive genes. (C) ATAC can be recruited to promoters by SPIB, ELK1 and ELK4 or to enhancers by jra (in fly) or c-myc (in human).



at the enhancer of the c-MYC gene (116). More recently, Tora and coworkers have shown that in mammalian cells, ATAC binds a subset of p300-independent enhancers [Tora personal communication], suggesting the presence of a new class of enhancers. Interestingly, ATAC and SAGA regulate distinct sets of genes but can also co-occur on the same promoter [Tora personal communication] (Figure 3). In addition, *in vitro* experiments indicate that ATAC mediates nucleosome remodeling by ISWI, SWI/SNF and RSC complexes, suggesting similar mechanism compared to SAGA (98).

Interplay between signaling pathways and GCN5/PCAF-containing protein complexes

Transcriptional responses can be evoked by signaling pathways that are induced either within or outside the cell membrane. The overlapping and specific functions of SAGA and ATAC are likely to be controlled by protein-protein interactions between complex subunits and signaling molecules, or by posttranslational modifications. Below, we have analyzed this intricate network by dividing it in five different signaling pathways in which SAGA and ATAC function as transcriptional effectors. The conservation of interactions between SAGA/ATAC and signaling pathways is investigated in three different species, and shows how molecular networks are adjusted and modeled during evolution by keeping some aspects while modifying others.

ER Stress signaling

The participation of SAGA in the transcriptional program of the endoplasmic reticulum (ER)-stress response has been described both in human and yeast. Recently, Nagy and co-workers have focused on the hSPT20 (p38IP) subunit. They find that hSAGA is recruited to the promoters of ER-stress genes in response to the ATPase pump-inhibitor thapsigargin, and is essential for their transcription (67). The pattern of stress genes regulated by SAGA is specific, since Na-arsenite treatment, which induces the p38 MAPK pathway, does not result in SAGA recruitment on the canonical MAPK-responsive gene EGR-1.

It has been reported that hSAGA interacts with the NF-Y transcriptional factor. In mammals, NF-Y is involved in the ER response (reviewed in (91)). It interacts with GCN5 or PCAF *in vitro* and *in vivo*, and GCN5 potentiates NF-Y-dependent activation of the α2(I) collagen promoter (21).

In yeast, the interplay between SAGA and the ER-stress pathway occurs at several levels. GCN5 was isolated in a two-hybrid screen for Ire1p interactors. In yeast, the endoribonuclease Ire1p is an ER transmembrane protein containing a sensor domain in the ER lumen. The stimulation of this sensor activates the cytosolic ribonuclease moiety of Ire1p that can then splice an intron present in the HAC1 transcript, which allows its translation (reviewed in (91)). GCN5 and Ire1p interact both *in vivo* and *in vitro* (110). Moreover, it was found that also Ada2p, Ada3p and Spt20p interact with Ire1p, suggesting that Ire1p may bind the SAGA complex during the ER-stress response (110). This interaction affects the efficiency of processing of the *HAC1* transcript, but the mechanism involved is unknown. SAGA also interacts with Hac1p, possibly to regulate Hac1p-dependent gene expression during the transcriptional reprogramming following ER-stress (111). In yeast, Hac1p and Gcn4p co-regulate transcriptional activation of target genes (6). Interestingly, the essential transcription factor GCN4 activates target genes in response to amino acid starvation and other stresses by recruiting SAGA to promoters (49, 69). Therefore, both in yeast and in human, SAGA interacts with the ER-stress pathway, and its transcriptional activity is essential to modulate the transcriptional response to stress.

MAP kinase signaling

MAPK signaling pathways are activated by a variety of stress signals such as osmotic stress, heat shock, UV, growth factors and ligands (reviewed in (24)). In yeast, the MAP kinase Hog1p (p38 in higher eukaryotes) promotes the recruitment of SAGA, mediator and pol II to osmostress promoters.

The SAGA complex (but not TFIID) is essential for gene activation under high osmolarity conditions but is dispensable at low osmolarity conditions. It has been shown that SAGA is recruited to the promoter and coding regions of osmostress-related genes by direct interaction with Hog1p (118). Hog1p activates several transcriptional activators, including Hot1p, Smp1p, Msn2/4p and Sko1p (1). In yeast, Sko1p (bZIP) represses osmostress genes by interaction with the co-repressor Tup1p-Ssn6p (84). Upon phosphorylation, the Sko1p-Tup1-Ssn6 complex switches from a repressor to an activator and recruits SAGA and SWI/SNF to the promoter, leading to the initiation of transcription of osmostress genes (85).
In human, the hSPT20 subunit was first identified as a p38 interacting protein (p38IP) in a two-hybrid screening using p38α as bait (Y.L and J.H unpublished data (126)). However, besides SPT20, other subunits also interact with the kinase. PCAF, for instance, interacts and acetylates p38 at K53 and K152, which leads to an increase in its kinase activity and downstream transcriptional effects (62, 81, 107). A relationship between hATAC, dATAC, and MAPK signaling during osmotic stress has been reported. Both hATAC and dATAC inhibit the activity of the MAPK c-Jun N-terminal kinase (JNK) during osmotic stress (99). dATAC co-immunoprecipitates with proteins involved in MAPK signaling such as jra and MSN. The authors show that ATAC stimulates expression of JNK target genes in the absence of osmotic stress (via the ATAC2 HAT), but negatively regulates gene expression during osmotic stress (via MBIP subunit). In the absence of osmotic stress, ATAC occupies both the promoter and enhancer of a JNK responsive gene (jra), which is accompanied by H4K16 acetylation. Upon osmotic stress, ATAC binding and H4K16 acetylation are reduced at the enhancer but are unchanged at the promoter. Interestingly, it has been observed that upon activation, the H4K16 acetylation spreads to the gene body and is ATAC-dependent. H4K16Ac has been shown to be important for transcription elongation at certain genes (122). Taken together, the proposed model is that ATAC is required for basal expression of JNK responsive genes and is dependent on ATAC2 activity. Upon osmotic stress, ATAC suppresses the induced activation of the target gene by localizing to the promoter and via the ATAC subunit MBIP-mediated inhibition of the JNK kinase; in a second step, ATAC relocalizes to a position upstream to the enhancer. Therefore the complex orchestrates chromatin acetylation and kinase inhibition during the osmotic response in order to modulate transcription of the target gene (99).

Nuclear receptor signaling
In higher eukaryotes, SAGA and ATAC subunits have been shown to interact with nuclear receptors (NR) and to regulate transcription of their responsive genes. NRs can interact with SAGA via the hTRA1 subunit. Three LXXLL motifs present in the hTRA1 protein have been shown to bind directly to ERα and other NRs (115). However, the interaction of the NR with the complex might also occur via other subunits. Yeast two hybrid screens have shown that the mammalian AF-2 (activation domain) of NRs interacts with ADA3 and activates transcription. Retinoid X receptor (RXRα) and estrogen receptor (ERα) require ADA3, ADA2 and GCN5 for maximal activity (105). In particular, hADA3 directly binds RXR and RAR via an LXXLL motif in a ligand-dependent manner both *in vivo* and *in vitro* (53, 119). The interaction with hADA3 enhances ERα and ERβ-mediated transactivation of ER responsive genes such as c-Myc and progesterone receptor (PR), which are involved in the oncogenic pathway (59). Following estrogen treatment of MCF-7 breast cancer cells, hADA3, GCN5 and PCAF co-immunoprecipitate and co-occupy the ER-responsive pS2 promoter suggesting a positive role for SAGA/ATAC in the transcriptional regulation of NRs-responsive genes (31). H3K14 acetylation by PCAF has been shown to play a major role in progesterone receptor (PR)-dependent gene activation by recruiting the chromatin remodeling complex BAF and by promoting PIC assembly (60, 89, 102, 103).
Acetylation by GCN5/PCAF can also occur on non-nucleosome substrates and can result in repression of gene expression. The mouse orphan receptor ERRα interacts with and is acetylated by PCAF

on the K129/K138/K160/K162 residues. The acetylation results in reduced DNA affinity, thereby affecting the activating function of the receptor. The ERRα is dynamically de-acetylated by SIRT1 (113).
The transcriptional role of SAGA in the nuclear receptor-signaling pathway is also mediated by the DUB module of the complex. In flies and in human cells, the subunits of the DUB module of SAGA have been shown to play a major role in androgen receptor (AR)-mediated transactivation and chromatin rearrangement (121). In particular, nonstop and dSGF11 subunits have been shown to have a role in chromatin silencing as shown by position effect variegation (PEV) analysis. The authors tested whether DUB subunits could mediate chromatin de-silencing via an AR reporter, and found that mutation of SAGA-specific DUB subunits indeed abrogates de-silencing. Similar results were obtained using a human cell system (121).

Cell cycle signaling
GCN5/PCAF-containing SAGA and ATAC complexes have been found to play differential roles in cell cycle regulation (28). First, ChIP analysis of GCN5, and PCAF in human cells has shown that occupancy of the two acetyltransferases is differentially regulated: GCN5 positively regulates G1/S-specific genes including PCNA, whereas PCAF is important for activating expression of G2/M-specific genes including Cyclin B. Second, the distribution of the SAGA and ATAC complexes varies on different genes. On the PCNA promoter, for instance, SAGA and GCN5 are enriched on the +1 and +2 nucleosomes at G1/S, and at the -1 nucleosome at G2/M, whereas ATAC is only present at the +1 nucleosome during G1/S. Furthermore, the Cyclin D1 and Cyclin A2 genes are enriched for SAGA and GCN5, whereas the promoter of the SON gene is enriched only for ATAC and GCN5 (28). ATAC and SAGA (containing either GCN5 or PCAF) therefore bind to specific target genes and to different locations within these genes. Remarkably, in yeast deletion of *GCN5* leads to accumulation of cells in the G2/M phase. Moreover mutation of H3K14 and H4K8-K16 in a GCN5 null mutant results in synthetic lethality, suggesting a major role for acetylation of these residues in survival (120).
Mammalian ATAC regulates cellular viability and cell cycle progression via several mechanisms (35). In human cells, it has been shown the ATAC localizes to the mitotic spindle during mitosis. The deletion of ADA2a leads to polychromosomes and appearance of midbodies in human cells, and to chromosome fragility in flies (76). Disruption of ATAC, but not SAGA, in mammalian cells leads to hyperacetylation of α-tubulin via an indirect mechanism involving the SIRT2 deacetylase, and of cyclin A, further strengthening the link between ATAC and cell cycle regulation.
SAGA and ATAC are involved in the Wnt signaling pathway via interaction with β-catenin. Mutation in any component of this pathway results in various forms of human cancer (19, 82, 88). It has been shown that PCAF acetylates β-catenin at K19 and K49 and that this significantly increases protein stability. The subcellular localization of β-catenin acetylation is not well understood yet; it is likely that it occurs on promoter DNA such as the TCF4 promoter. However, PCAF-dependent regulation of β-catenin is shared with other p300/CBP ((30) and references therein). Results from another study also suggest that the ATAC complex has a role in β-catenin regulation. Indeed, in mammalian cells ADA2a/ADA3 interact with β-catenin via Armadillo repeats and mediate acetylation of β-catenin. The loss of ada2a/ada3 negatively impacts the transcriptional activity of β-catenin on target genes such as c-myc. Furthermore, it has been shown that ADA2a/ADA3 localize at the enhancer of the c-myc gene. (116). In addition, it has been shown that the N-terminal domain of c-MYC binds hSAGA via interaction with multiple subunits (TRA1/SPT3/GCN5/TAF5-9). The interaction occurs when MYC is present as dimer with MAX ((55) and references therein). Therefore SAGA and ATAC regulate Wnt pathway members both at the transcriptional level and by direct protein-protein interactions.
The regulation of protein stability by acetylation has also been suggested for another important

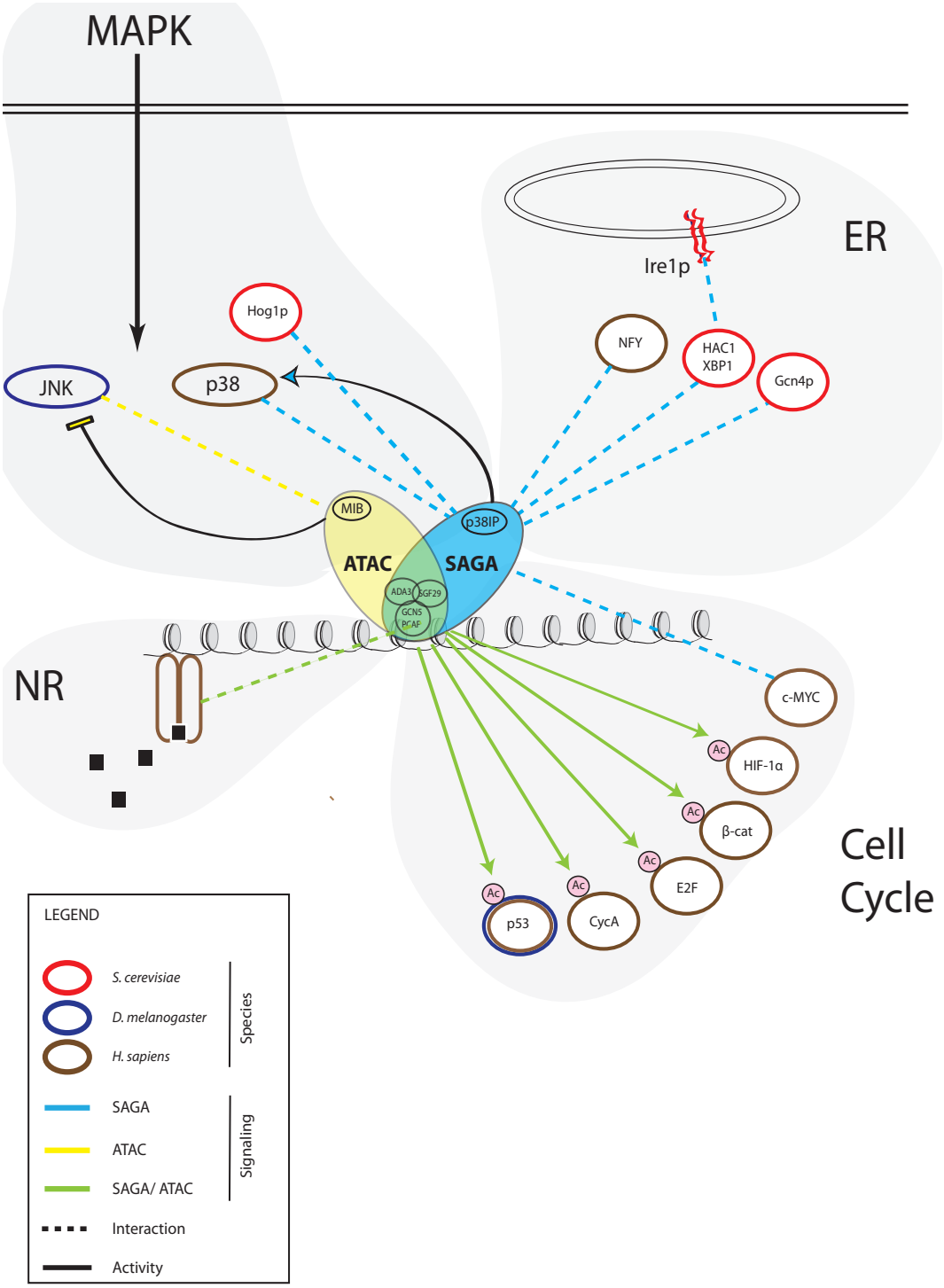


Figure 4. SAGA and ATAC function in different signaling pathways. Cumulative analysis of the interplay between SAGA/ ATAC and different signaling pathways in yeast, fly and human. ER-stress signaling, MAP kinase, cell cycle and nuclear receptor signaling pathways are shown.

factor involved in the cell cycle and apoptosis: the hypoxia-inducible factor-1 (HIF-1). It has been shown that PCAF is responsible for HIF-1α acetylation in mammalian cells. The acetylation activates HIF-1α, which in turn activates transcription under conditions of redox imbalance. The SIRT proteins are responsible for deacetylation of HIF-1, suggesting a regulatory mechanism of acetylation/deacetylation to regulate the hypoxia-induced transcriptional program (54).

The available data are not sufficient to discriminate between SAGA and ATAC for controlling the cell cycle via p53. It has been shown that hADA3 directly binds p53 *in vitro* and *in vivo*. Binding is enhanced by γ-irradiation-induced DNA damage, is dependent on the N-terminal phosphorylation of p53, and is important for p53-target gene activation (27, 48, 66, 70, 73, 93, 106). The interaction of hADA3 with the E6 protein of HPV blocks p53 function, thereby positively activating the oncogenic pathway (48). However, it cannot be excluded that p53 is acetylated by different acetyltransferases than GCN5 or PCAF. For example, it has been shown that hADA3 regulates p300-mediated p53 acetylation as part of the p14ARF-induced senescence pathway (92). Also in flies, the p53 homologue Dmp53 directly binds dADA2b (51,72).

In human cells, p53 regulates the E2F transcriptional factor, which controls apoptosis and the cell cycle via the targets pRB, p107, p103 and p73 (22, 44, 64). It has been shown that upon DNA damage E2F is phosphorylated and acetylated by CHK2 and PCAF, respectively (79, 97). E2F forms a complex with PCAF on the p73 promoter and is regulated by the deacetylase hSirT1 (79). More recently it has been shown that upon UV-dependent DNA damage, E2F recruits GCN5 to the site of damage and favors H3K9 acetylation, which, in turn, is required for the tethering of the nucleotide excision repair (NER) machinery (39). However, the recruitment of GCN5 might also occur via SAP130, a subunit of the SF3b-SAP spliceosome complex, which has previously been proposed to be a component of the hSAGA complex (10).

In yeast, the acetyltransferase activity of Gcn5p for H3K14 is shared with other two HATs: Hpa2p and Ssa3p. Loss of both *GCN5* and *SSA3* results in a synthetic lethal phenotype. Interestingly, conditional ablation of the two acetyltransferases results in a cell cycle block at the G2/M phase, suggesting an essential role for H3 acetylation in cell cycle progression (42).

The role of Gcn5p in cell cycle progression is linked to the activity of the SWI/SNF complex. In yeast, it has been shown that during mitosis the Gcn5p-independent recruitment of SWI/SNF, followed by the recruitment of Gcn5p to mitotic loci, are important in order to acetylate and open the chromatin surrounding the gene that need to be expressed (47). Moreover, yeast Gcn5p occupies centromeric DNA and GCN5 genetically interacts with kinetocore components. The importance of GNC5 in G2/M progression and the effect of its deletion on spindle dynamics and minichromosome loss have suggested an important role of this acetyltransferase in cell cycle control (101). Also in higher eukaryotes GCN5 plays a role in chromosome stability. In human cells, GCN5 depletion causes chromosomal fusion. It has been shown that the molecular mechanism involves telomere integrity by the action of the USP22 de-ubiquitinase activity. The authors propose that hSAGA including GCN5 and the DUB module are responsible for stability of the shelterins and thereby of telomere maintanance (3). Finally, the effect of SAGA/ATAC activity on the cell cycle also involves the regulation of DNA replication via modulation of CDC6 protein activity (78).

Developmental signaling

Extensive experimental evidence suggests that the SAGA/ATAC acetyltransferase complexes have an important role during development. In mouse, it has been shown that PCAF is dispensable whereas GCN5 is essential during embryonic development. In particular, pcaf-/- mice are viable and fertile whereas gcn5-/- embryos grow slowly and die at 10.5 days post coitum (d.p.c). This suggests that PCAF is redundant to GCN5 during development. However the double mutant GCN5-/-/PCAF-/- results in a more severe phenotype. The GCN5 KO results in defects in specific lineages. In particular, the dorsal mesoderm, including chordamesoderm and paraxial mesoderm have severe growth

defects, whereas extraembryonic and cardiac mesoderm follows a normal development (114). This phenotype is likely caused by an increase in cellular death in specific tissues, as suggested by the fact that p53-/- GCN5-/- embryos survive longer than GCN5 null mutants. Furthermore, GCN5-/- mice exhibit severe cranial neural tube closure and exencephaly (14, 20).

In the mouse, p38IP/SPT20 has been found to be required for regulating the activity of p38α kinase and mesoderm lineage development during embryogenesis. A p38IP dominant negative allele results in multiple phenotypic defects at E9.5-E10.5. In more detail, at this developmental stage p38IP is important for downregulation of E-cadherin expression. Indeed a p38IP single mutation (drey) shows severe defects in cell migration and neural tube closure, similarly as reported for GCN5-/- mice (123). These results suggest that the p38 pathway is linked to GCN5 during development.

The ATAC complex is also involved in embryonic development. In the mouse, it has been shown that a null mutation of the ATAC-specific subunit ATAC2 results in embryonic lethality at 11 d.p.c. Embryos exhibit global defects in growth in which all tissues are affected, suggesting, therefore, a role different from that of SAGA (35).

ATAC and SAGA also control developmental programs in flies. It has been shown that the dATAC complex specifically regulates genes involved in the biosynthetic pathway of the steroid hormone ecdysone, which results in arrest of the larval-prepupal transition (77). A comparative study of developmental effects of mutations in ADA2a and ADA2b in flies suggests differential roles for these two complex-specific subunits. Indeed, a mutation in ADA2b results in reduction of global H3K9/K14 Ac levels and lethality at the late pupal stage. In contrast, ADA2a mutation does not affect these histone acetylations, but results in a more severe phenotype (76). Furthermore, mutation of ADA2b, but not ADA2a, affects the localization of TAF10 on polytene chromosomes, in agreement with the fact that TAF10 is a subunit of the SAGA complex (ADA2b) but not of the ATAC complex (ADA2a) (76). Moreover, as mentioned before for SAGA/ATAC in mouse, also in fly the p53 transcription factor (DMp53) is linked to SAGA. Although p53-dependent genes were not affected in ADA2b mutants under normal conditions, upon UV DNA damage, the mutants exhibit extensive p53-dependent apoptosis. This suggests a specific role for SAGA in p53-dependent gene regulation (86).

Concluding remarks

Here, we have discussed the biochemical and functional aspects of two related GCN5/PCAF-containing complexes: SAGA and ATAC. It appears that the complexity of the functional networks around these complexes increases during evolution. *S. cerevisiae* only contains the SAGA complex, whereas *D. melanogaster* and *H. sapiens* in addition contain the ATAC complex. In mammals, the existence of the GCN5 homolog PCAF allows the formation of two different SAGA and two different ATAC complexes, each containing either the GCN5 or the PCAF acetyltransferases. Therefore, one, two or four GNAT-containing complexes exist in yeast, fly and human, respectively. This increase in complexity correlates with the increase in cellular and transcriptional pathways in the three different species. The molecular model for the transcriptional roles of SAGA and ATAC supports this picture. Indeed, whereas SAGA is recruited to the promoter region from yeast to human, ATAC seems to preferentially bind to enhancer sequences in both fly and human cells. Therefore, it is possible that the ATAC complex has evolved in need for regulation of the transcriptional functions of enhancers in higher eukaryotes.

We have also described the signaling pathways involved in SAGA and ATAC regulation, and found a high degree of consistency and conservation of these interactions in the three different species investigated. However, we have observed that specific mechanisms evolve within the same pathway in different species to interconnect the complex with upstream signaling molecules. For instance, in yeast, Hog1p kinase activity on activators mediates SAGA recruitment to the promoter during cellular stress. However in human cells, the SPT20 subunit (p38IP) of SAGA directly interacts with

p38 kinase (the Hog1p ortholog) in order to modulate transcription at MAP related target genes. In addition to their activities towards histone proteins, GCN5 and PCAF can also acetylate non-nucleosomal substrates involved in cell cycle and developmental pathways. It is still unclear whether the SAGA/ATAC complexes, the HAT modules, or the acetyltransferases alone are involved in this function. It would therefore be interesting to investigate the loss of acetylation on nucleosomal or non-nucleosomal substrates using depletion of shared or specific subunits of ATAC and SAGA.

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Supplemental Table 1 **(***next page)***.** Proteins are grouped in SAGA-specific, ATAC-specific and shared subunits. For each subunit the following information is presented: the Uniprot Accession number, Gene aliases, Protein aliases, Isoform accession number, primary reference for identification of the subunit. SMART [http://smart.embl-heidelberg.de/], PFAM [http://pfam.sanger.ac.uk/], UniProt [http://www.uniprot.org/] and reported references were used for domain analysis and annotation.

PROTEIN

Species	Accession Number	Gene aliases	Protein aliases	Isoforms	Ident. - Ref	Domains Ref

SAGA SPECIFIC SUBUNITS

SPT8

H.s.	-	-	-	-	-	-
D.m.	-	-	-	-	-	-
S.c	P38915	-	-	-	(1, 28)	SMART

TRA1

H.s.	Q9Y4A5	TRRAP/PAF400	PAF400/STAF40	Q9Y4A5-2	(20, 21)	(12)
D.m.	Q8I8U7	Nipped-a/Tra1	dTRA1	-	(16)	(16) pFAM
S.c	P38811	-	-	-	(5)	(11, 12)

SPT7

H.s.	O94864	SUPT7L	Staf65γ	O94864-2	(20)	SMART, (3, 20)
D.m.	Q9VX12	CG6506	CG6506	-	(8)	-
S.c	P35177	*GIT2*	-	-	(28)	(3)

SPT3

H.s.	O75486	SUPT3H	SPT3-like protein	O75486-2/O75486-3	(20)	pFAM
D.m.	Q9VGE2	Dmel_CG3169	FI01013p	-	(16)	pFAM
S.c	P06844	-	-	-	(28)	pFAM

SPT20

H.s.	Q8NEM7	FAM48A/ FP757	p38IP	Q8NEM7-2	(25)	pFAM
D.m.	Q9VU86	CG17689	LD34837p/ CG17689	Q2PDX8	(34)	pFAM
S.c	P50875	ADA5	-	-	(28)	pFAM

TAF5

H.s.	O75529	TAF5L/ PAF65B	PAF65-beta	O75529-2	(20)	SMART
D.m.	Q9VCN9	Wda/ CG4448	WDA/LD16387p	-	(8)	SMART(8)
S.c	P38129	TAF90	TAFII-90	-	(4, 28)	SMART

TAF6

H.s.	Q9Y6J9	TAF6L/PAF65A	PAF65-alpha	-	(20)	SMART
D.m.	Q9VPT4	Saf6/ CG3883	CG3883/ LD32410p	-	(34)	(34)
S.c	P53040	TAF60	TAFII60	-	(4, 28)	SMART

TAF9						
H.s.	Q9HBM6	TAF2G/TAFII31	TAFII31/32/STAF31/32	Q16594-1/ Q9Y3D8-1	(20)	pFAM
D.m.	Q27272	e(y)1/ TAF40	TAFII40/ p42/ TAFII42	-	(8)	pFAM
S.c	Q05027	TAF17	TAFII-17/ TAFII20	-	(4)	pFAM
TAF10						
H.s.	Q12962	TAF2A/TAF2H/TAFII30	STAF28/ TAFII30	-	(20)	pFAM
D.m.	Q9XZT7	TAf10b/TAF16/CG3069	TAFII16/dTAF(II)16	-	(8)	pFAM
S.c	Q12030	TAF23/TAF25	TAFII23/ TAFII25/p25	-	(4)	pFAM
TAF12						
H.s.	Q16514	TAF15/TAF2J/TAFII20	TAFII20/TAFII15	Q16514-2	(20)	pFAM
D.m.	P49905	TAF30α	TAFII30α/ p28-α	P49905-2	(8)	pFAM
S.c	Q03761	TAF61/TAF68	TAFII61/ TAFII68	-	(4)	pFAM
UBP8						
H.s.	Q9UPT9	USP22/USP3L/KIAA1063	USP22	Q9UPT9-2	(35, 36)	pFAM
D.m.	Q9VVR1	NOT/CG4166	Non-stop	-	(33)	pFAM
S.c	P50102	-		-	(10)	pFAM
SGF11						
H.s.	Q14CW9	ATXN7L3	hSGF11	Q14CW9-2	(36)	pFAM
D.m.	Q9VVR6	CG13379	-		(33)	pFAM
S.c	Q03067	-	-		(17)	pFAM
SGF73						
H.s.	O15265	ATXN7/SCA7	Ataxin-7	O15265-2	(9)	pFAM
D.m.	Q9VQB6	CG9866 isoform A	CG9866/LD40170	CG9866 isoform B	(34)	-
S.c	P53165	-	-	-	(22)	pFAM
SUS1						
H.s.	Q9NPA8	ENY2/DC6	ENY2 TF homolog	-	(36)	pFAM
D.m.	Q9VYX1	E(y)2/CG15191	ENY2 TF	-	(14)	pFAM
S.c	Q6WNK7	-	-	-	(26)	pFAM
ADA1						
H.s.	Q96BN2	TADA1/TADA1L	STAF42	-	(20)	pFAM
D.m.	Q8IPA1	Ada1-2/ CG31866	Ada1-2/ RE72529p	-	(8)	pFAM
S.c	Q12060	HFI1/SUP110	HFI1	-	(28)	pFAM
ADA2/2B						
H.s.	Q86TJ2	TADA2B	ADA2-beta	Q86TJ2-2/3	(20)	pFAM
D.m.	Q8I8V0	Ada2S/CG9638		Q8I8V0-2	(15)	pFAM
S.c	Q02336	-	-	-	(18, 28)	pFAM

SAGA AND ATAC SHARED SUBUNITS

ADA3

H.s.	O75528	TADA3/TADA3L	STAF54/ADA3-like	O75528-2	(20)	pFAM
D.m.	Q9VWZ1	Dik/CG7098	Diskette/TADA3		(16)	pFAM
S.c	P32494	NGG1/SWI7	NGG1		(28)	pFAM

GCN5

H.s.	Q92830	KAT2A/GCN5L2/HGCN5	KAT2A/HsGCN5/STAF97	Q92830-2	(20)	pFAM
D.m.	O76216	Pcaf/CG4107	-	-	(23)	pFAM
S.c	Q03330	ADA4/SWI9	-	-	(28)	pFAM

PCAF

H.s.	Q92831	KAT2B	KAT2B		(19, 20)	pFAM
D.m.			-	-		
S.c			-	-		

SGF29

H.s.	Q96ES7	CCDC101	CCDC101	-	(13)	pFAM
D.m.	Q9W2I4	CG30390	-	-	(8)	PFAM
S.c	P25554	-	-	-	(27)	PFAM

ATAC SPECIFIC SUBUNITS

ADA2A

H.s.	O75478	TADA2A/TADA2L	ADA2-like	O75478-2	(6, 31)	pFAM
D.m.	Q7KSD8	Rpb4/CG33520		Q7KSD8-2/3/4 Q9VEA5-1	(16)	pFAM/SMART
S.c						

ATAC1/ZZZ3

H.s.	Q8IYH5	ZZZ3	ZZZ3	Q8IYH5-2/3/4		pFAM
D.m.	Q9VM59	CG9200	ATAC1/LD22161p	-	(7)	pFAM
S.c	-	-	-	-	-	-

ATAC2

H.s.	Q9H8E8	CSRP2BP	CSRP2BP/ ATAC2	Q9H8E8-2		pFAM
D.m.	Q9VJ73	CG10414	CG10414/LD17982p		(29)	pFAM/SMART
S.c	-	-	-	-	-	-

HCF

H.s.	P51610	HCFC1/HCF1	HCF/HCF-1/C1 factor/CFF/VCAF	P51610-2/3		pFAM/SMART
D.m.	Q9V4C8	CG1710	dHCF	Q9V4C8-2/3/4	(7)	pFAM/SMART
S.c	-	-	-	-	-	-

WDS

H.s.	P61964	WDR5/BIG3	WDR5/BIG3	-	(6)	pFAM
D.m.	Q9V3J8	Wds/CG17437/I(1)3Ad	WDS	-	(29)	pFAM
S.c	-	-	-	-	-	-

NC2beta

H.s.	**Q01658**	DR1	NC2-beta/Dr1	-	(6)	pFAM
D.m.	**Q9VJQ5**	Dr1/CG4185	Dr1/dNC2	-	(29)	pFAM
S.c						

D12

H.s.	Q9ULM3	YEATS2/KIAA1197	YEATS2	-	(6) (32)	pFAM
D.m.	Q9VLL1	DmeI_CG13400	D12	-	(29)	pFAM
S.c						

MBIP

H.s.	Q9NS73	BM-015	MBIP	Q9NS73-2/3/4	(6, 30)	Uniprot/(2)
D.m.	Q9VBX2	CG10238	MOCS2B		(24, 29, 30)	pFAM
S.c						

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Chapter 3

In-Depth Profiling of Post-Translational Modifications on the Related Transcription Factor Complexes TFIID and SAGA

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The basal transcription factor TFIID and the chromatin-modifying complex SAGA, which have several subunits in common, are crucial for transcription regulation. Here, we describe an in-depth profiling of post-translational modifications (PTMs) on both TFIID and SAGA from yeast. We took a multipronged approach using high-resolution mass spectrometry (LC-MS) in combination with the proteases Trypsin, Chymotrypsin and Glu-C. The cumulative peptide identification data, at a false discovery rate <1%, allowed us to cover most TFIID and SAGA subunit sequences to near completion. Additionally, for TFIID/SAGA subunits, we identified 118/102 unique phosphorylated and 54/61 unique lysine acetylated sites. Especially, several lysine residues on the SAGA subunits Spt7p and Sgf73p were found to be acetylated. Using a spectral counting approach, we found that the shared subunit Taf5p is phosphorylated to a significant greater extent in SAGA than in TFIID. Finally, we were able to map for the first time the cleavage site in Spt7p that is related to formation of the SAGA-like complex SLIK/SALSA. In general, our combination of tandem affinity enrichment, digestion with different proteases, extensive prefractionation and high-resolution LC-MS identifies a large number of PTMs of TFIID and SAGA/ SLIK that might aid in future functional studies on these transcription factors.

Introduction

Proteome-wide studies of protein-protein interactions in *Saccharomyces cerevisiae* have substantiated the view that most proteins are organized into multimeric protein complexes (15, 16) These studies also show that a large set of proteins can be assembled into several different protein complexes. For instance, the TATA-box binding protein (TBP) associated factors (TAFs) Taf5p, Taf6p, Taf9p, Taf10p and Taf12p are known to be present in both the basal transcription factor TFIID and the histone acetyl transferase complex SAGA (3, 20, 31, 58, 59). However, it is still poorly understood how shared subunits incorporate into one or the other protein complex. Both TFIID and SAGA are large and heterogeneous complexes, containing 15 and 19 different subunits, respectively. Although most of the subunits of the TFIID and SAGA complexes have been known for a few years, several more targeted proteomics approaches still identified new SAGA subunits including Ubp8p, Sgf73p and Sgf29p8 as well as Sgf11p (30, 47). At present the view is that, besides these four integral SAGA subunits and the five shared TAFs, SAGA canonically contains SPT class gene products (Spt3p, Spt7p, Spt8p, Spt20p), ADA class gene products (Ada1p, Ada2p, Ada3p, Gcn5p), as well as Tra1p and Sus1p (18, 19, 25). Putatively, also Chd1p and Rtg2p are subunits of SAGA (48, 49). TFIID is composed of TBP as well as the 14 TAFs Taf1p-TAF14p (51). Electron microscopy combined with immunolabeling suggests that certain subunits of both TFIID and SAGA are likely to be present in more than one copy (32, 65). Functionally, both TFIID and SAGA are involved in regulated transcription of RNA polymerase II dependent genes. (21, 32). While TFIID functions as transcriptional coactivator by mediating interactions between activators, core promoter elements and preinitiation complex (PIC) components, SAGA not only activates transcription of highly regulated genes (12, 24, 57) but also has a role as transcriptional repressor on some genes (4, 61). In addition to its interactions with activators (7, 29), it exerts its coactivator function either through TBP recruitment (42, 53), through Gcn5p-dependent acetylation of histones H3, H4 and H2B or through Ubp8p-mediated histone deubiquitination, thereby generating transcriptionally active or removing repressive chromatin marks (5, 45). The negative function of SAGA is closely linked to the SAGA-like complex SLIK or SALSA (49, 56). SLIK contains all SAGA subunits except Spt8p which is involved in TBP binding (53) and is involved in the retrograde response (49). Instead of full-length Spt7p, SLIK contains a shorter form of Spt7p (Spt7pΔC) that lacks a C-terminal portion of the gene product required for Spt8p binding (66). Moreover, the transition from full-length Spt7p to Spt7pΔC does not require functional SAGA complex (22). The site of truncation has been mapped in a deletion study down to somewhere between amino acids 1125 and 1151 (66). In general, little is known about post-translational modifications (PTMs) of SAGA and TFIID. The publicly available data are not comprehensive as they are derived from proteome-wide studies. To increase confidence about the PTM state of SAGA and TFIID, we decided to analyze both complexes by an in-depth LC-MS based peptide sequencing approach. A viable approach for this is to use various proteases in parallel to generate different sets of peptides from the same sample (14, 38, 41, 52, 64, 67). The accompanying variation of physicochemical properties of the generated peptides usually increases protein sequence coverage, as the fraction of peptides that are successfully sequenced is constrained for each set by solubility after proteolytic cleavage, ionizability during transition to vacuum and good fragmentation behavior during peptide sequencing. The approach also beneficially affects sequence analysis on the amino acid level, especially with respect to PTMs, as confidence of identification of single (modified) residues increases if established from fragment ion spectra originating from different peptides covering the same sequence stretch. Another major requirement in approaching comprehensive sequence coverage at the protein level is the reduction of the complexity of the peptide mixture provided to the mass spectrometer, such that low-abundance peptides have an increased chance of being selected for sequencing. This is generally achieved by using multiple protein and peptide prefractionation techniques such as strong cation exchange (SCX)(62), isoelectric focusing (IEF)(8, 27), hydrophilic interaction chromatography (HILIC)(6) or SDS PAGE (44) in the first

dimension with the commonly used reversed phase (RP) LC-MS system as the second dimension (62). As a side effect of extensive prefractionation, many peptides are sequenced multiple times during their elution from the RP column, rendering an ideal situation for spectral-counting based relative quantification (13, 37, 43). In this study, we describe an in-depth analysis of the SAGA and TFIID complexes that were tandem-affinity purified from yeast and digested using multiple proteases, namely, Trypsin, Chymotrypsin and Glu-C. We combined this approach with extensive prefractionation by SDS-PAGE or SCX to achieve good separation orthogonality in the first dimension. With this strategy, we were able to map the nearly complete sequence of most subunits. Our data also revealed a high number of nonredundant phosphorylated (118/102) and acetylated (54/ 61) amino acid residues of subunits of TFIID/SAGA, including several present on common subunits. Spectral counting hints at differential abundance of PTMs on some of these common subunits. We also identified a C-terminal portion of Spt7p and used it to map in detail the cleavage site within Spt7p that is linked to SLIK/SALSA formation from SAGA.

Materials and methods

Cell Extraction

Cell extract was prepared from *S. cerevisiae* strains FY2031 (genotype MATa HA-*SPT7-TAP::TRP1 ura3Δ0 leu2Δ1 trp1Δ63 his4-917δ lys2-173R2*)(65) and SC1064 (genotype MATa *TAF1-TAP::URA3 ade2 arg4 leu2-3,112 trp1-289 ura3-52*) (15) as described previously(35) with minor modifications. In brief, cells were harvested from suspension culture in rich medium at an optical density at 600 nm of 2.0, disrupted by glass bead homogenization in extraction buffer (20 mM HEPES-NaOH, pH 8.0, 150 mM sodium chloride, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 1 μg/mL pepstatin, 0.5 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM PMSF, 1% (v/v) phosphatase inhibitor cocktails #1 and #2 (Sigma-Aldrich), and 10 mM sodium butyrate) and cleared by ultracentrifugation.

Tandem Affinity Purifications

Tandem-affinity purifications of the endogenously expressed protein complexes were carried out as previously published (50) with the following specifications. A total of 200 μL of 50% (v/v) immunoglobulin G sepharose (Sigma-Aldrich) was incubated for 2 h with 10 mL of cell extract and subsequently washed with 30 mL of extraction buffer and 10 mL of cleavage buffer (10 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 0.5 mM EDTA, pH 8.0, 0.1% (v/v) Tween 20, and 1 mM DTT). The protein A moiety of the tag was cleaved off with 10 μL of TEV protease (Invitrogen) in 1 mL of cleavage buffer for 2 h. Binding to calmodulin agarose (Stratagene) for 1 h in binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM calcium chloride, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, and 10 mM mercaptoethanol) was followed by washing with 30 mL of binding buffer and elution in 300 μL of elution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, pH 8.0, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, and 10 mM mercaptoethanol).

Protein Prefractionation, Digestion, and Desalting

Proteins were precipitated from the eluate according to Wessel *et al*. (63). The total protein content measured after precipitation was around 3-5 μg as determined by a micro-BCA assay (Pierce). Proteins were either reduced, alkylated and digested with Trypsin in the gel matrix as described by Shevchenko *et al*. (54) after visualization by SDS-PAGE (NuPAGE Novex Bis-Tris 4-12% gradient gel, Invitrogen) or digested in solution using Lys-C under denaturing conditions followed by digestion with Chymotrypsin or Glu-C (all proteases from Roche Applied Science). In detail for the in-solution digestion, the precipitate was suspended in 20 μL of 50 mM Tris-HCl, pH 8.0, 8 M urea

and 2.5 mM DTT, incubated at 55 °C for 10 min, and alkylated by addition of 5 μL of 50 mM Tris-HCl, pH 8.0, 8 M urea, and 20 mM iodoacetamide. Subsequently, 20 μL of diluent (50 mM Tris-HCl, pH 8.0) was added followed by addition of 5 μL (0.5 μg) of Lys-C and incubation at 37 °C for 4 h. For the second digestion, 145 μL of diluent and 5 μL (0.5 μg) of Chymotrypsin or Glu-C were added and the mixture was incubated at 25 °C for 18 h. The digest was dried in a vacuum centrifuge, resuspended in 20 μL of 10% (v/v) formic acid and desalted using C18 membrane (Empore C18 extraction disk, 3M) packed into a pipet tip.

Peptide Prefractionation

Insolution digests were prefractionated by SCX, which was performed using a Shimadzu LC- 9A binary pump with two ZORBAX BioSCX-Series II columns (50 mm × 0.8 mm, 3.5 μm particle size; Agilent Technologies) in series, connected to a SPD-6A UV-detector (Shimadzu) and a FAMOS autosampler (Dionex). The desalted digest was redissolved in 10 μL of 10% (v/v) formic acid and loaded onto the columns at 50 μL/min solvent A (0.05% (v/v) formic acid in 1:4 acetonitrile/water) for 10 min, followed by linear gradient elution of 1.3% (v/v) min-1 solvent B (500 mM NaCl in solvent A) at 50 μL/min. A total of 50 SCX fractions (1 min each) were collected and dried in a vacuum centrifuge.

Nano-LC-MS

Vacuum-dried in-gel digests (for Trypsin experiments) and SCX fractions (for Lys-C combined with Chymotrypsin and Lys-C combined with Glu-C experiments) were dissolved in 20 μL of 10% (v/v) formic acid and 10 μL of each sample was analyzed on a nano-LC-coupled LTQ-Orbitrap (Thermo Fisher Scientific). An Agilent 1200 series HPLC system was equipped with a 20 mm Aqua C18 (Phenomenex) trapping column (packed in-house, 100 μm i.d., 5 μm particle size) and a 200 mm ReproSil-Pur C18-AQ (Dr. Maisch, GmbH) analytical column (packed in-house, 50 μm i.d., 3 μm particle size). Trapping was performed at 5 μL/min solvent C (0.1 M acetic acid in water) for 10 min, and elution was achieved with 10-40% (v/v) solvent D (0.1 M acetic acid in 1:4 acetonitrile/water in either 22 or 35 min, followed by 38-100% (v/v) solvent D in 3 min and 100% solvent D for 2 min. The flow rate was passively split from 0.45 mL/min to 100 nL/min as previously described (46). Nanoelectrospray was achieved using a distally coated fused silica emitter (360 μm o.d., 20 μm i.d., 10 μm tip i.d.; New Objective) biased to 1.7 kV. The LTQ-Orbitrap was operated in the data dependent mode to automatically switch between MS and MS/MS. Survey full scan MS spectra were acquired from m/z 150 to m/z 1500 in the Orbitrap with a resolution of 60 000 at m/z 400 after accumulation to a target value of 500 000 in the linear ion trap. The two most intense ions at a threshold of above 500 were fragmented in the linear ion trap using collisionally induced dissociation at a target value of 30 000.

Data Processing and Analysis

Spectra were processed with Bioworks 3.3 (Thermo Fisher Scientific) and the subsequent data analysis was carried out using Mascot 2.2.1 (Matrix Science) licensed in-house. Mascot was set up to search the Saccharomyces genome database (10) with carbamidomethyl cysteine as fixed modification and the following variable modifica- tions: oxidation of methionine, acetylation of lysine, phosphorylation of serine and threonine, methyl esterification of aspartic and glutamic acid (only for samples digested in gel) and carbamamylation of the peptide N-terminal amino group (only for samples digested in solution). Up to two missed cleavages were allowed. The mass tolerance of the precursor ion was set to 5 ppm and that of fragment ions to 0.6 Da. In all six data sets, protein identifications were established at a significance threshold <0.01 which corresponds to individual Mascot ion scores >31 and false discovery rates <1% as determined by decoy database searching as implemented in Mascot. Calculation of sequence coverage was performed using the in-house build

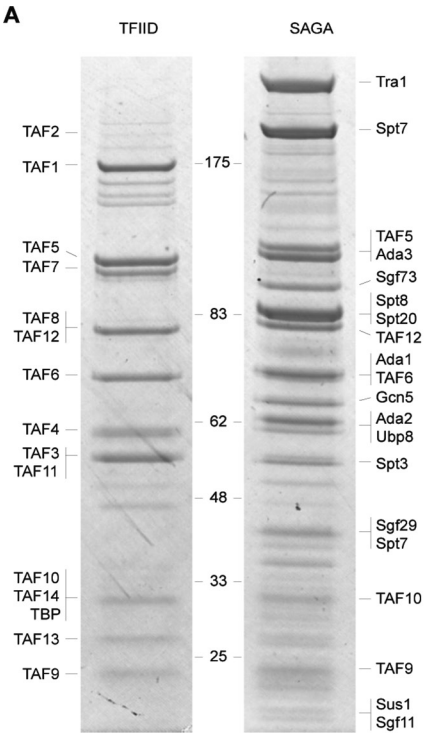
program SpectraMapper (41). Fragment ion spectra were visualized using Scaffold 2.1.03 (Proteome Soft- ware). Fragment ion spectra and tables of fragment ion matches of peptides mapping modified amino acids which are explicitly discussed in the text can be found in Supporting Information 1 and 2. RAW data files are available on the public repository Tranche (https: //proteomecommons. org/) using the following hash code: VYyYWAkbdUaOOrcKoz1eQXHQSxxUHv1oJL6nFxmMB-1J0M9NgyFStOC9Fqp8BYx87Vx8XF6I7POD9/oZDFid5ZMXACM8AAAAAAACk1Q)Spectral abundance factors were calculated similar to a method previously published by the Washburn group (13, 43). In detail, for each of the six digests, peaklists from all MS runs were combined and searched with Mascot. In the search results, the number of spectra assigned to a protein was divided by the molecular weight of that protein and the total number of queries that were submitted. Extracted ion chromatograms (EIC) were generated using Xcalibur 2.0.5 (Thermo Fisher Scientific). For every peptide precursor EIC, the first two isotopes were considered using a m/z window of 0.02 or 0.03 Th. Gaussian smoothing was applied over nine m/z data points. Protein sequences of fungal Spt7p homologues found in the UniProt database (http://www.uniprot.org) were aligned with MUSCLE as implemented on the European Bioinformatics Institute's Web site (http://www.ebi.ac.uk/tools/muscle). The full alignments of Spt7p and Sgf73p can be found in FASTA format as Supporting Information 4 and 5. Aligned residues were colored in JalView 2.4 (http://www.jalview.org) by conservation at a visibility threshold of 30 using BLOSUM62 homology definitions.

Analysis of Taf5p Mutants and of Truncated Spt7p

Yeast strains used for the analysis of *TAF5* phosphosite mutants are listed in Supplemental Table 1. *TAF5* mutants were created in pRS415-*TAF5* (11) by PCR site-directed mutagenesis using oligo- nucleotides listed in Supplemental Table 2. pRS415-*TAF5* mutant plasmids were transformed in YJR501 (Mata *taf5::TRP1* [pRS316-*TAF5*] *his4-917δ lys2-17R2 leu2 ura3-52*) and plated onto leucine dropout plates. Two independent clones were grown overnight in YPD, plated onto 5-fluoroorotic acid, and grown for 3 days at 30 °C. Two independent clones were verified by sequence analysis and used in the spot assay. For this, cells were grown to saturation overnight in YPD medium and spotted in a dilution series from 108 to 105 cells/mL. The plates were incubated at 30 °C for the following times: YPD, 2 days; YPGal, 3 days; SC-Lys, 3 days; SC-His, 4 days; SC-Ino, 3 days. For the immunoblot analysis of Taf5p mutants, yeast extracts were made as described previously (28). Samples were blotted onto PVDF membrane and probed with a Taf5p antibody kindly provided by Jerry Workman. Yeast strains used for the analysis of truncated *SPT7* were W303-1B derivatives and are listed in Supplemental Table 1. N-terminal TAP tagging and C-terminal truncation of the genomic copy of *SPT7* were performed using standard methods (36, 50). Strains were grown to midlog phase, whole extracts were prepared as above, and TAP- Spt7p was immunoprecipitated using IgG affinity resin. Elution was performed in Laemmli buffer for 5 min at 100 °C. Eluted proteins were separated by SDS-PAGE, transferred to PVDF membrane and analyzed for SAGA/SLIK subunits. PAP antibody (Sigma-Aldrich) was used to detect the protein A moiety of the TAP tag. Antibodies recognizing Spt8p, Taf10p, Taf12p, and Taf5p were kindly provided by Jerry Workman.

Results and Discussion

To comprehensively map PTMs of SAGA and TFIID, especially with respect to the TAFs that occur in both complexes, both SAGA and TFIID were tandem-affinity purified using Spt7p and TAF1p as tagged subunits, respectively, and initially separated by SDS-PAGE, digested with Trypsin, and analyzed by high-resolution LC-MS. As concluded from the gel pattern, both purifications were highly enriched for their respective canonical subunits (Figure 1A). The high degree of enrichment of both complexes was also reflected by the high numbers of peptide queries detected for the SAGA/TFIID subunits, ranging in the hundreds for the larger components, as well as the high sequence



B

TFIID

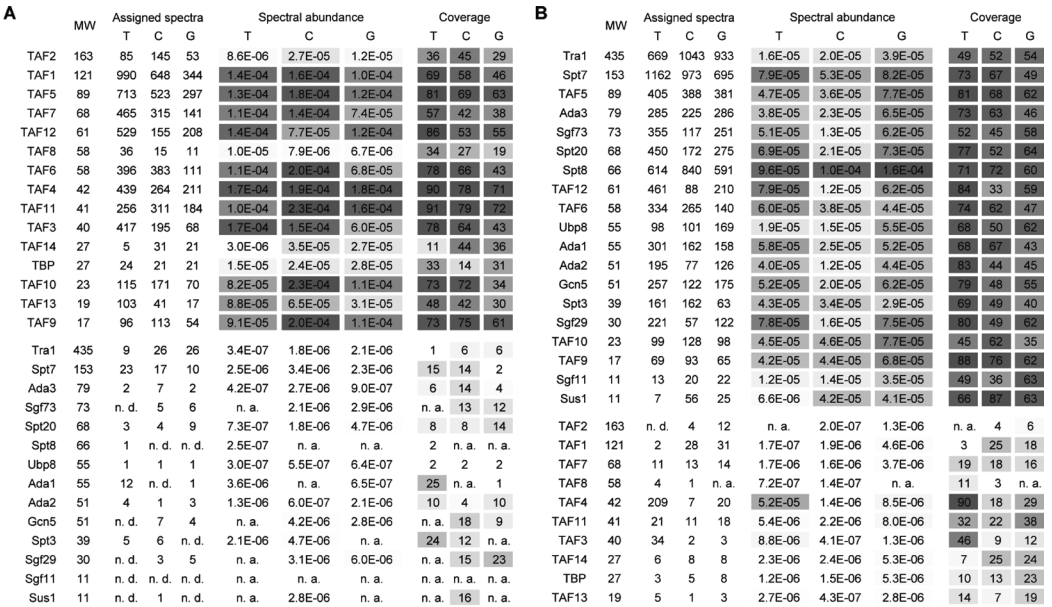
Subunit	MW	Coverage
TAF2	163	65
TAF1	121	86
TAF5	89	94
TAF7	68	66
TAF12	61	96
TAF8	58	55
TAF6	58	92
TAF4	42	92
TAF11	41	96
TAF3	40	91
TAF14	27	59
TBP	27	49
TAF10	23	93
TAF13	19	74
TAF9	17	96

SAGA

Subunit	MW	Coverage
Tra1	435	84
Spt7	153	91
TAF5	89	93
Ada3	79	90
Sgf73	73	79
Spt20	68	80
Spt8	66	89
TAF12	61	93
TAF6	58	89
Ubp8	55	92
Ada1	55	87
Ada2	51	89
Gcn5	51	92
Spt3	39	90
Sgf29	30	88
TAF10	23	88
TAF9	11	100
Sgf11	17	84
Sus1	11	96

Figure 1. TFIID and SAGA purifications and cumulative sequence coverage. (A) Coomassie Blue stained 4-12% gradient gel of the tandem-affinity purified complexes TFIID (left lane) and SAGA (right lane) with the main protein subunits indicated. (B) Overview of all known TFIID/SAGA subunits, their molecular weight (MW, in kDa) and the cumulative sequence coverage (in %) when adding up the individual sequence coverage from the three different digests. The color bars (linear gradient between white, corresponding to the lowest value, 49%, and black, corresponding to the highest value, 100%) illustrate the numerical values in a heat-map like representation.

coverage (Figures 1B and 2). More importantly, the amount of TFIID copurified with SAGA, and vice versa, the amount of SAGA copurified with TFIID, was around 5% as estimated from normalized spectral counts (Figure 2). This is particularly important for a differential comparison of the PTM state of the subunits present in both complexes. The fact that we still identify a few of these copurifying subunits suggests that TFIID and SAGA may transiently interact, in agreement with the shared role of these complexes in transcription initiation (23, 32). Although the median of sequence coverage of all canonical SAGA/TFIID subunits was already above 70% from the in-gel tryptic digests, we also digested both SAGA and TFIID preparations in solution with Lys-C in combination with Chymotrypsin and Lys-C in combination with Glu-C, in particular to further enhance sequence coverage and to independently verify PTMs. Lys-C was chosen because of its ability to cleave under strong denaturing conditions (34). Indeed, when projecting the identified peptides from all three analyses onto their respective subunits, around 90% (median) of primary sequence could be covered (Figures 1B and 2). In some cases, this increase can be directly related to the bias in amino acid composition toward hydrophobic or acidic residues in some stretches; for this particular reason, the proteases Glu-C and Chymotrypsin were chosen. Another gain of the multiprotease approach is an improved confidence in PTM identification. In some cases, PTMs could only be identified from Chymotrypsin- or Glu-C-derived peptides, showing the limitations of a Trypsin-only approach. To illustrate the potency of this approach, we concentrate on Taf1p, which we found to be phosphorylated at multiple residues. Phosphorylated Taf1p S34 and Taf1p S189 were exclusively identified on peptides with a chymotryptic N- terminus because the corresponding tryptic peptides are likely



A

	MW	Assigned spectra			Spectral abundance			Coverage		
		T	C	G	T	C	G	T	C	G
TAF2	163	85	145	53	8.6E-06	2.7E-05	1.2E-05	36	45	29
TAF1	121	990	648	344	1.4E-04	1.6E-04	1.0E-04	69	58	46
TAF5	89	713	523	297	1.3E-04	1.8E-04	1.2E-04	81	69	63
TAF7	68	465	315	141	1.1E-04	1.4E-04	7.4E-05	57	42	38
TAF12	61	529	155	208	1.4E-04	7.7E-05	1.2E-04	86	53	55
TAF8	58	36	15	11	1.0E-05	7.9E-06	6.7E-06	34	27	19
TAF6	58	396	383	111	1.1E-04	2.0E-04	6.8E-05	78	66	43
TAF4	42	439	264	211	1.7E-04	1.9E-04	1.8E-04	90	78	71
TAF11	41	256	311	184	1.0E-04	2.3E-04	1.6E-04	91	79	72
TAF3	40	417	195	68	1.7E-04	1.5E-04	6.0E-05	78	64	43
TAF14	27	5	31	21	3.0E-06	3.5E-05	2.7E-05	11	44	36
TBP	27	24	21	21	1.5E-05	2.4E-05	2.8E-05	33	14	31
TAF10	23	115	171	70	8.2E-05	2.3E-04	1.1E-04	73	72	34
TAF13	19	103	41	17	8.8E-05	6.5E-05	3.1E-05	48	42	30
TAF9	17	96	113	54	9.1E-05	2.0E-04	1.1E-04	73	75	61
Tra1	435	9	26	26	3.4E-07	1.8E-06	2.1E-06	1	6	6
Spt7	153	23	17	10	2.5E-06	3.4E-06	2.3E-06	15	14	2
Ada3	79	2	7	2	4.2E-07	2.7E-06	9.0E-07	6	14	4
Sgf73	73	n. d.	5	6	n. a.	2.1E-06	2.9E-06	n. a.	13	12
Spt20	68	3	4	9	7.3E-07	1.8E-06	4.7E-06	8	8	14
Spt8	66	1	n. d.	n. d.	2.5E-07	n. a.	n. a.	2	n. a.	n. a.
Ubp8	55	1	1	1	3.0E-07	5.5E-07	6.4E-07	2	2	2
Ada1	55	12	n. d.	1	3.6E-06	n. a.	6.5E-07	25	n. a.	1
Ada2	51	4	1	3	1.3E-06	6.0E-07	2.1E-06	10	4	10
Gcn5	51	n. d.	7	4	n. a.	4.2E-06	2.8E-06	n. a.	18	9
Spt3	39	5	6	n. d.	2.1E-06	4.7E-06	n. a.	24	12	n. a.
Sgf29	30	n. d.	3	5	n. a.	3.1E-06	6.0E-06	n. a.	15	23
Sgf11	11	n. d.	n. d.	n. d.	n. a.	n. a.	n. a.	n. a.	n. a.	n. a.
Sus1	11	n. d.	1	n. d.	n. a.	2.8E-06	n. a.	n. a.	16	n. a.

B

	MW	Assigned spectra			Spectral abundance			Coverage		
		T	C	G	T	C	G	T	C	G
Tra1	435	669	1043	933	1.6E-05	2.0E-05	3.9E-05	49	52	54
Spt7	153	1162	973	695	7.9E-05	5.3E-05	8.2E-05	73	67	49
TAF5	89	405	388	381	4.7E-05	3.6E-05	7.7E-05	81	68	62
Ada3	79	285	225	286	3.8E-05	2.3E-05	6.5E-05	73	63	46
Sgf73	73	355	117	251	5.1E-05	1.3E-05	6.2E-05	52	45	58
Spt20	68	450	172	275	6.9E-05	2.1E-05	7.3E-05	77	52	64
Spt8	66	614	840	591	9.6E-05	1.0E-04	1.6E-04	71	72	60
TAF12	61	461	88	210	7.9E-05	1.2E-05	6.2E-05	84	33	59
TAF6	58	334	265	140	6.0E-05	3.8E-05	4.4E-05	74	62	47
Ubp8	55	98	101	169	1.9E-05	1.5E-05	5.5E-05	68	50	62
Ada1	55	301	162	158	5.8E-05	2.5E-05	5.2E-05	68	67	43
Ada2	51	195	77	126	4.0E-05	1.2E-05	4.4E-05	83	44	45
Gcn5	51	257	122	175	5.2E-05	2.0E-05	6.2E-05	79	48	55
Spt3	39	161	162	63	4.3E-05	3.4E-05	2.9E-05	69	49	40
Sgf29	30	221	57	122	7.8E-05	1.6E-05	7.5E-05	80	49	62
TAF10	23	99	128	98	4.5E-05	4.6E-05	7.7E-05	45	62	35
TAF9	17	69	93	65	4.2E-05	4.4E-05	6.8E-05	88	76	62
Sgf11	11	13	20	22	1.2E-05	1.4E-05	3.5E-05	49	36	63
Sus1	11	7	56	25	6.6E-06	4.2E-05	4.1E-05	66	87	63
TAF2	163	n. d.	4	12	n. a.	2.0E-07	1.3E-06	n. a.	4	6
TAF1	121	2	28	31	1.7E-07	1.9E-06	4.6E-06	3	25	18
TAF7	68	11	13	14	1.7E-06	1.6E-06	3.7E-06	19	18	16
TAF8	58	4	1	n. a.	7.2E-07	1.4E-07	n. a.	11	3	n. a.
TAF4	42	209	7	20	5.2E-05	1.4E-06	8.5E-06	90	18	29
TAF11	41	21	11	18	5.4E-06	2.2E-06	8.0E-06	32	22	38
TAF3	40	34	2	3	8.8E-06	4.1E-07	1.3E-06	46	9	12
TAF14	27	6	8	8	2.3E-06	2.4E-06	5.3E-06	7	25	24
TBP	27	3	5	8	1.2E-06	1.5E-06	5.3E-06	10	13	23
TAF13	19	5	1	3	2.7E-06	4.3E-07	2.8E-06	14	7	19

Figure 2. Mapping the sequence of TFIID and SAGA subunits using a three-enzyme approach. Number of assigned spectra, the spectral abundance (dimensionless) and sequence coverage (in %) for all TFIID and SAGA subunits, whereby T, C, and G abbreviate the data obtained from the three different digests with Trypsin, Chymotrypsin, and Glu-C. (A) TFIID analysis. The table shows a breakdown for the Taf1p purification with the canonical TFIID subunits in the upper half and the SAGA subunits in the lower half. As concluded from the spectral abundance, the amount of SAGA copurifying with TFIID is low (around 5%). (B) SAGA analysis. As in panel A but now with a breakdown for the Spt7p purification with the canonical SAGA subunits in the upper half and the low-abundance copurifying TFIID subunits in the lower half. The color bars in panels A and B illustrate the numerical values as linear gradient between white, corresponding to the lowest value (1% for sequence coverage, 2.3 × 10 -4 for spectral abundance) and black, corresponding to the highest value (91% for sequence coverage, 1.4 × 10 -7 for spectral abundance).

too large for detection or fragmentation. In other cases, a PTM could be identified from different proteolytic peptides with different precursor m/z and different fragment ion spectra patterns, which increases confidence about the identity of the observed PTMs. For example, phosphorylated Taf1p T355 was detected in all three digests and could be inferred from a fully Lys-C derived peptide of 22 residues. In addition, phosphorylated Taf1p T355 was also detected on a smaller peptide of 10 amino acids with a chymotryptic N-terminus. In this case, where the large peptide contains 4 serine and 4 threonine residues, the small peptide significantly increased the confidence in the site-assignment of the phosphorylation to Taf1p T355 by its specific fragment ion spectrum. Other examples of novel phosphosites of SAGA/TFIID subunits, not previously detected in large-scale phosphoproteomic data sets (1, 9, 33, 55), were phosphorylated residues of Spt7p (S100, S341, T367, S636, S637), Spt8p (S25, S372, S385, S409, S506, S545), Ada3p (S134) and Taf4p (S301, S321, S338), nicely illustrating how our targeted affinity-enrichment and multiprotease approach enabled an in-depth analysis of the PTM status of these transcription factor complexes.

In total, for TFIID/SAGA subunits, we detected in our combined data set 481/624 peptide queries related to serine or threonine phosphorylated peptides, and 69/232 peptide queries related to lysine acetylated peptides (Supplemental Table 3). These numbers reduce to 183/200 unique phosphorylated peptides and 56/108 unique peptides with lysine acetylation (Supplemental Table 4), mapping 118/102 serine or threonine phosphorylated sites and 54/61 lysine acetylated sites for TFIID/SAGA.

Post-Translational Modifications of TAFs Common to Both SAGA and TFIID

Some of the TAFs present in both SAGA and TFIID were also found to be highly phosphorylated in

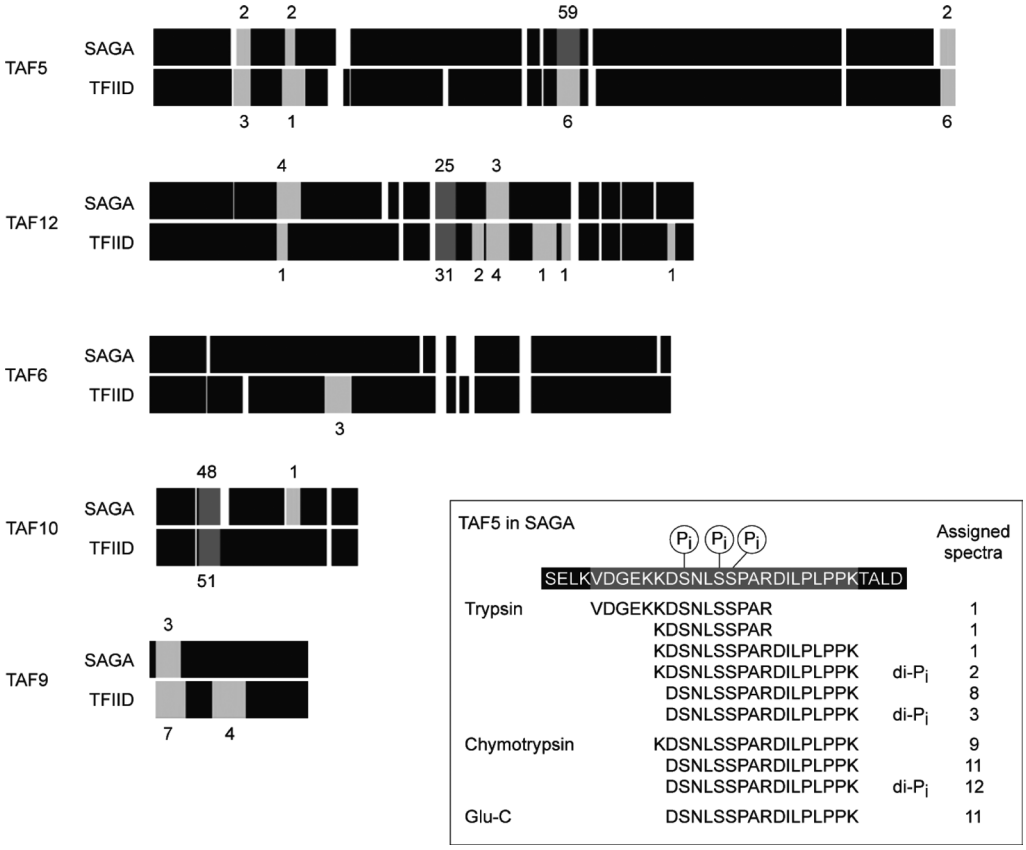


Figure 3. Differential phosphorylation of TFIID and SAGA. Color-coded sequence coverage maps of the TAFs common to SAGA and TFIID produced from data cumulatively obtained from all three digests. Black sequences stretches indicate protein sequence coverage by nonmodified peptides, red stretches indicate coverage by a large number (>25, black) or smaller number (<10, grey) of phosphorylated peptides, and white indicates no coverage. Above or below the red colored sequence stretches, the cumulative numbers of phosphorylated peptides assigned to that stretch are given. The inset illustrates using TAF5p in SAGA as example that the length of a black stretch does not necessarily correlate with the number of phosphorylated residues, but rather with the length of the phosphorylated peptides that form this stretch.

specifically defined sequence stretches (Figure 3). Three out of these stretches, which in two cases contain multiple adjacent serine/threonine residues, namely, S411, S414 and S415 in Taf5p; S58 in TAF10p; and S286, S287, S288, S290, and T291 in Taf12p, were identified by a large number of spectra, which allowed a semiquantitative estimation of the phosphorylation levels based on spectral counts. While the number of phos- phorylated peptide queries assigned to S58 in Taf10p (25 in SAGA versus 31 in TFIID) and to the phosphorylated stretch in Taf12p (48 in SAGA versus 51 in TFIID) was found to be similar in both complexes, the phosphorylated stretch in TAF5p has a roughly 10-fold higher number of assigned phosphorylated spectra in SAGA (59 spectra) than in TFIID (6 spectra). In addition to a difference in phosphorylation, we found Taf5p to be differentially acetylated as well. In the cumulative data from the SAGA preparation, TAF5p K103 was identified as acetylated in only a single query, while in TFIID, 23 spectra mapped to this residue.

Both the differential phosphorylation and acetylation of TAF5p could be confirmed by extracted ion chromatograms (EICs, Figure 4). These were generated from the analyses of the two TAF5p-containing gel bands of the TFIID and SAGA preparation. In case of the phosphorylated stretch of TAF5p, the most prominent (as judged by spectral counts, see inset of Figure 3) monophosphorylated peptide DSNLSSPARDILPLPPK was found to be more abundant in the context of SAGA then

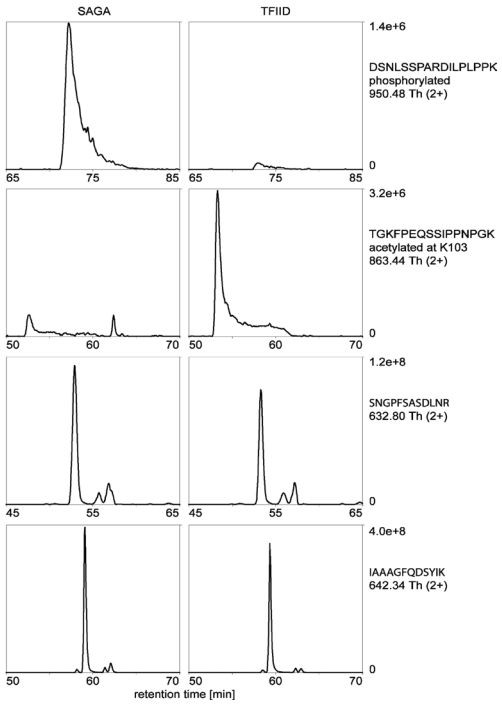


Figure 4. Peptide ion intensities correlate with spectral counts and confirm differential PTM status. Extracted ion chromatograms (EICs) of Taf5p-derived peptides generated from the MS analyses of the two Taf5p-containing gel bands of the SAGA and TFIID purifications (left and right column, respectively), illustrating the differences in Taf5p S411/S414/S415 phosphorylation (covered by the phosphorylated peptide DSNLSSPARDILPLPPK, first row) and in Taf5p K103 acetylation levels (acetylated TGKFPEQSSIPPNPGK, second row) in the two complexes. The third and fourth row present EICs of unmodified spectra used for normalization of the TFIID and SAGA analyses.

in TFIID. For TAF5p K103, which is exclusively identified on the acetylated peptide TGKFPEQSSIPPNPGK, this relation was opposite. For normalization between the two runs, several TAF5p-derived peptides were selected from the pool of non- modified, fully cleaved peptides, of which two are shown in Figure 4.

Concerning the phosphorylated residues on Taf5p, Taf12p and Taf10p, all residues (or residues within sequence stretches) have been reported previously (1, 9, 33, 55), but to our knowledge, this is the first time that the phosphorylation state can be linked to the presence of the TAFs in either SAGA or TFIID, which would be impossible in high-throughput analysis at the lysate level without prior selective enrichment of the two different transcription complexes.

To test whether Taf5p phosphorylation is essential for the integrity and/or function of SAGA, we changed the Taf5p integrity and/or function of SAGA, we changed the Taf5p serine residues 411, 414, 415, alone or in combination, to alanine, and tested the strains for known SAGA functions. Mutants in SAGA subunits are able to suppress Ty insertions, known as the SPT phenotype (11, 57, 66). SPT phenotypes were tested using the *his4-917Δ* and *lys2-17R2* alleles and correspond to growth on medium lacking histidine and inhibition of growth on medium lacking lysine. In addition, SAGA mutants lack the ability to grow on medium containing alternative carbon sources like galactose and to grow on medium lacking inositol (11, 57, 66). None of these phenotypes were observed in single, double, or triple mutant Taf5p strains, whereas the *Δspt3* control strain displayed all of these phenotypes (Supplemental Figure 1). This suggests that Taf5p phosphorylation at S411, S414 and S415 in the context of SAGA is not essential for these SAGA functions and for SAGA integrity. It remains possible that phosphorylation on these residues has alternative (e.g., gene specific) functions not tested here, or its functions may be redundant with other PTMs present on Taf5p or on other SAGA subunits.

Lysine Acetylation of Spt7p and Sgf73p

The SAGA subunits Spt7p and Sgf73p were found to be significantly lysine acetylated. Although other SAGA subunits were found to be acetylated as well, Spt7p and Sgf73p stand out by the high

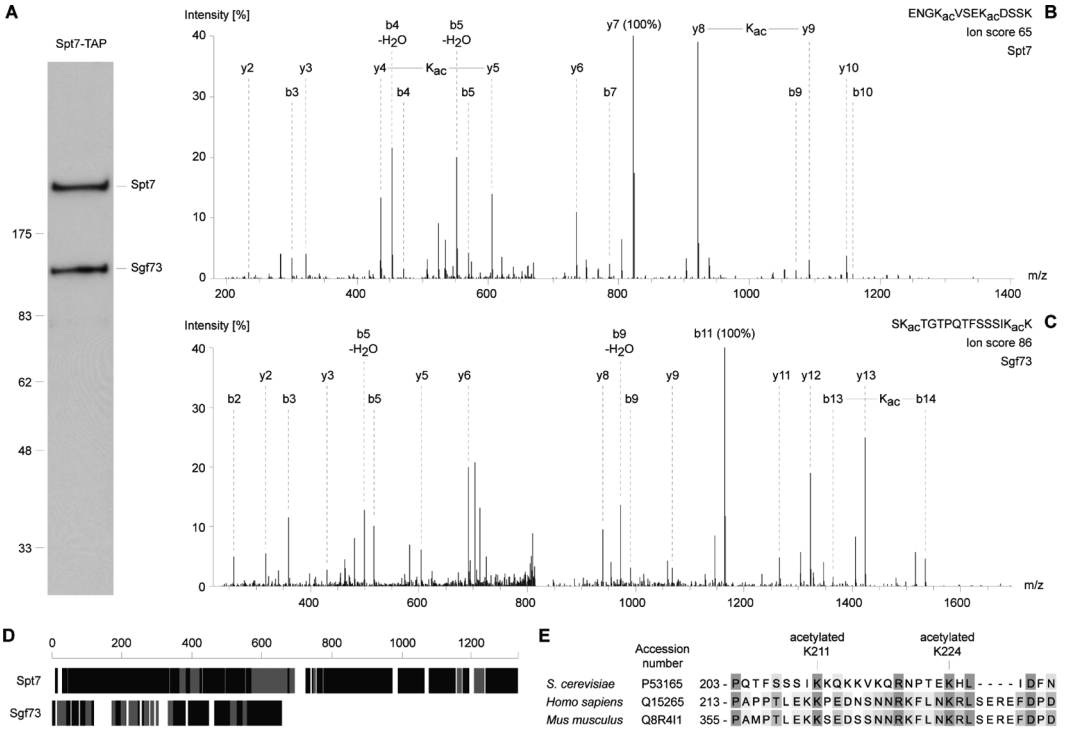


Figure 5. Detection of SAGA acetylation. (A) Immunoblot of purified SAGA complex (Spt7p-TAP) probed with an antiacetyl-lysine antibody and visualized by immunoperoxidase staining. The antigen identities denoted on the right (Spt7p, Sgf73p) are based on consistency with their molecular weight and LC-MS results in Figure 1. (B and C) Typical fragment ion spectra of representative doubly lysine acetylated peptides originating from Spt7p and Sgf73p, enabling unambiguous site localization. Only main fragment ion series are annotated with many of the nonannotated peaks originating from additional neutral losses. (D) Sequence coverage maps of Spt7p and Sgf73p produced from data cumulatively obtained from all three digests. Red stretches indicate sequence coverage by acetylated peptides, black stretches indicate sequence coverage by nonmodified peptides, and white stretches indicate no coverage. (E) Sequence alignment of Sgf73p to its mouse and human homologues ataxin-7.

number of unique spectra, the number of assigned spectra, as well as by the quality of the fragmentation spectra, enabling confident assignments (Figure 5B,C). Moreover, the high degree of lysine acetylation of Spt7p and Sgf73p could be confirmed by Western blotting using an antiacetyllysine antibody (Figure 5A). We found that Spt7p is acetylated at multiple lysine residues that group to mainly two sequence stretches adjacent to the bromodomain (Figure 5D). In the stretch that precedes the bromodomain, between K379 and K410, three out of five lysine residues present were detected in the acetylated form (K379, K400, K410), and in the sequence stretch that follows the bromodomain, between K584 and K680, notably 10 out of 17 lysine residues (K584, K599, K603, K607, K610, K629, K633, K647, K667, K680) were detected in their acetylated state. In the case of K599, K603, K607 and K610, lysine residues are so close together that two acetylated residues could be detected on a single peptide, indicating that a single Spt7p molecule can carry at least two acetylated lysine residues at once. Sgf73p was found to be acetylated at multiple lysine residues (K171, K199, K211, K224, K288, K300) in a region adjacent to the second Zn finger motif. As with Spt7p, two lysine residues (K199 and K211) were found to be acetylated on a single peptide, showing that also a single Sgf73p molecule can be multiply acetylated. Moreover, phosphorylated T212 could be detected together with acetylated K224 on the same peptide.

From our data alone, the possible implication of this significant lysine acetylation for Spt7p or

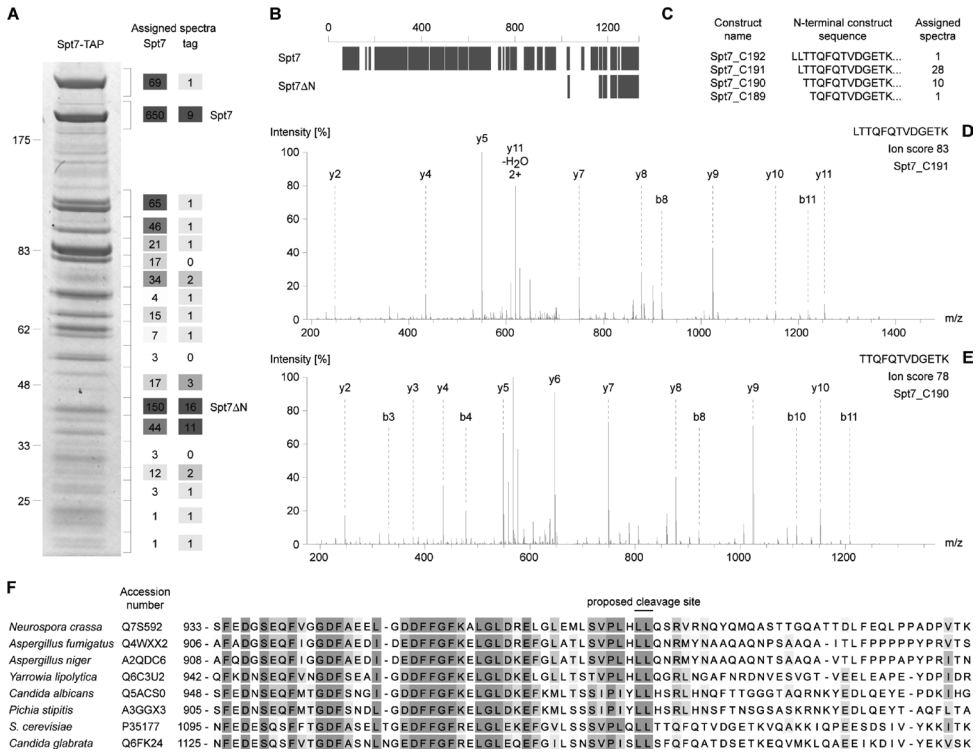


Figure 6. Mapping of the Spt7p truncation site. (A) Coomassie Blue stained gel of the tandem-affinity purified SAGA complex, purified via TAP-tagged Spt7p. Next to the gel on the right, the cutting scheme applied to the gel-separated SAGA complex is indicated as well as the number of identified spectra assigned to either Spt7p (first column) or the TAP-tag (second column) in every gel piece. The color bars (linear gradient between white, corresponding to the 10th percentile, and black, corresponding to the 90th percentile) illustrate the numerical values and highlight the maximum of Spt7p-derived spectra between 33 and 48 kDa, the gel band which is annotated as Spt7pΔN. (B) Peptide identifications made in the Spt7p and Spt7pΔN band as indicated in panel A. Nearly all Spt7p-derived peptides in Spt7pΔN map exclusively to approximately the last 175 amino acids. (C) Putative truncation site of Spt7pΔN as revealed by the search using a database containing consecutive N-terminally truncated forms of Spt7p. The number of N-terminal peptides unique to each truncated form clearly peaks at Spt7p_C191 (amino acids 1142-1332) and Spt7p_C190 (1143-1332) with only a few N-terminal peptides spectra occasionally assigned to shorter or longer truncated forms. (D and E) Representative fragment ion spectra of N-terminal peptides originating from Spt7p_C191 and Spt7p_C190, with only the main ion series annotated. (F) Sequence alignment of Spt7p to its fungal homologues.

Sgf73p function can just be speculated, although it is well-known that lysine acetylation of transcription factors generally affects protein function in a very diverse way (17). One intriguing possibility for Spt7p acetylation could be the regulation of the binding affinity of the Spt7p or the Gcn5p bromodomain, similar to the autoregulative effect of acetylated Rsc4p K24, as recently described for the Rsc4 bromodomain (60). Concerning Sgf73p, a regulatory effect on Gcn5 activity can be envisaged, as Sgf73p has been shown to regulate SAGA and SLIK acetyl transferase activity (40). Comparative genome analysis revealed that none of the lysine residues found to be acetylated on Spt7p are conserved in fungal *SPT7* homologues (data not shown). However, in Sgf73p, which is generally a much more conserved protein than Spt7p, K211 and K224 are homologous to K321 and K334 in human and K307 and K320 in mouse ataxin-7 (Figure 5E). Although conserved, this region of Sgf73p is not implicated in the binding of Sgf73p to SAGA (26). In summary, although no real function can yet be attributed to these lysine acetylations, their prominence implies that they are likely functionally or structurally important.

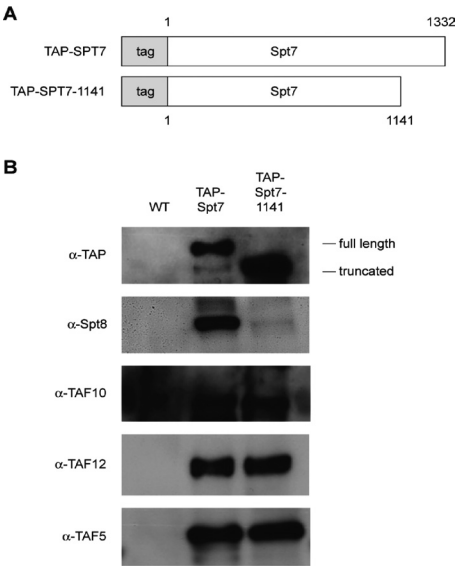


Figure 7. Truncation of Spt7p at the natural cleavage site results in loss of the Spt8p subunit from the SAGA complex mimicking SLIK formation. (A) Cartoons of the *SPT7* alleles. Strains were either wild-type (WT), or contained an N-terminal TAP-tag fused to the WT full-length *SPT7* gene *(TAP-SPT7)* or a C-terminal truncation at residue 1141 *(TAP-SPT7-1141)*. (B) Immunoblot analysis of anti-TAP-tag immunoprecipitates from the *WT, TAP-SPT7* and *TAP-SPT7-1141* strains. Purified material was analyzed by immunoblotting using the indicated antibodies.

Truncation Isoforms of Spt7p

Spt7p, besides being assembled into SAGA in its full-length form, can also be present in a truncated form that lacks a C-terminal part of approximately 200 amino acids as revealed by a deletion study (66). This remaining N-terminal part of Spt7p, which we name Spt7pΔC, is uniquely present in the SLIK (or SALSA) complex (49, 56). We copurified the putative complementary piece to Spt7pΔC, which we name Spt7pΔN, along with the SAGA complex. We conclude this in first instance from the shape of the distribution of the number of Spt7p-derived peptides over the gel lane (Figure 6A). This distribution has two peaks, one in the MW region of full-length Spt7p at around 180 kDa and another one, unexpectedly, in the low MW region at approximately 40 kDa. Second, in contrast to the 650 Spt7p-derived peptides identified in the 180 kDa MW region of the gel, which maps the whole Spt7p sequence, the 150 Spt7p-derived peptides from the 40 kDa region of the gel exclusively map the C-terminal 174 amino acids of the Spt7p sequence (Figure 6B). Third, if the hypothetical Spt7pΔN was copurified with the SAGA complex, it had to carry a TAP tag since the yeast cells express only Spt7p TAP- tagged at its C-terminus. In this case, peptides derived from the TAP-tag of Spt7pΔN can be detected equally well as peptides originating from full-length Spt7p. This hypothesis was tested by observing the shape of the distribution of the number of TAP-tag derived peptides over the gel lane (Figure 6A). The distribution has two peaks that coincide perfectly with the two peaks observed for the distribution of the number of Spt7p-derived peptides over the gel lane. Taking this evidence together, we conclude that we have indeed copurified Spt7pΔN.

As the length of Spt7pΔN is in very good agreement with the one proposed from the genetic deletion study mentioned above, we decided to try to map the N-terminus of Spt7pΔN to the exact residue. For this purpose, we generated a database that contains 313 potential C-terminal cleavage products of Spt7p ranging between 138 (Spt7p residues 1195-1332, named Spt7p_C138) and 450 (Spt7p residues 883-1332, named Spt7p_C450) amino acids long and all differing by a single N-terminal amino acid in length. We searched the in gel- digestion data set against this database for peptides with a nontryptic N-terminus and indeed found a significant number of semitryptic peptides that could be uniquely assigned to Spt7p_C191 and Spt7p_C190 (Figure 6C). These unambiguous peptide identifications (with 28 and 10 spectra, respectively) were found to be well above the normal, likely false-positive background of 1-2 semitryptic peptides that were sometimes assigned to other Spt7p variants in the database. Representative fragment ion spectra characterizing N-terminus of the truncated forms Spt7p_C191 and Spt7p_C190 are shown in Figure 6D, E, showing that the terminus of Spt7pΔN is likely either Spt7p L1142 or T1143. This result strongly hints to a protease that specifically processes Spt7p by cleaving it between L1141 and L1142 or L1142 and T1143.

To test whether cleavage of Spt7p at this position results in formation of SLIK rather than SAGA,

we generated N-terminally TAP tagged strains fused to full-length Spt7p (TAP-Spt7p) or to Spt7p truncated at the C-terminus at L1141 (TAP-Spt7p- 1141) as depicted in Figure 7A. Tandem-affinity purification followed by immunoblotting indicated that C-terminal truncation at L1141 results in specific loss of the Spt8p subunit from SAGA, indicative of formation of SLIK (Figure 7B). In addition, truncated Spt7p present in TAP-Spt7p-1141 migrated on SDS-PAGE at the same position as endogenously truncated Spt7p generated in TAP-Spt7p. As expected, shared SAGA/SLIK subunits including Spt7p, Taf10p, Taf12p, and Taf5p were present at similar levels, consistent with previous reports on the compositions of these complexes. We conclude that genetic truncation of the *SPT7* gene at L1141 results in SLIK formation and hence validate the identified cleavage site. Interestingly, this cleavage site, as well as a large sequence stretch preceding it, is highly conserved both in fungal *SPT7* homologues (Figure 6F) and also shows conservation in human STAF65γ (39). As the formation of Spt7pΔC or its incorporation into SAGA is an important step in the transition from SAGA to SLIK, a protease catalyzing this step would have an important regulatory function. Previous studies suggest that this protease might be an upstream element of the retrograde response pathway (2). Another possibility is that Spt7pΔN has a transcriptional role by itself, as it represents the portion of Spt7p that interacts with Spt8p and through this subunit with TBP. Our results should aid in the further elucidation of the protease responsible for this regulation.

Conclusions

In this study, we show that, by combining digestion of the highly enriched complexes with different proteases, extensive prefractionation, and high-resolution LC-MS, both SAGA and TFIID can be comprehensively analyzed. Using our multipronged approach, we identify a large number of previously unidentified PTMs on many SAGA and TFIID subunits with high confidence. Most notably, we find lysine acetylation to be a major PTM on the SAGA subunits Spt7p and Sgf73p. We also show by spectral counting that TAF5p is phosphorylated and acetylated to a different extend in SAGA and TFIID. Finally, we identify Spt7pΔN, a previously unreported portion of Spt7p that is probably linked to the transition from SAGA to SLIK, and pinpoint the potential cleavage site within Spt7p by mapping the N-terminus of Spt7pΔN.

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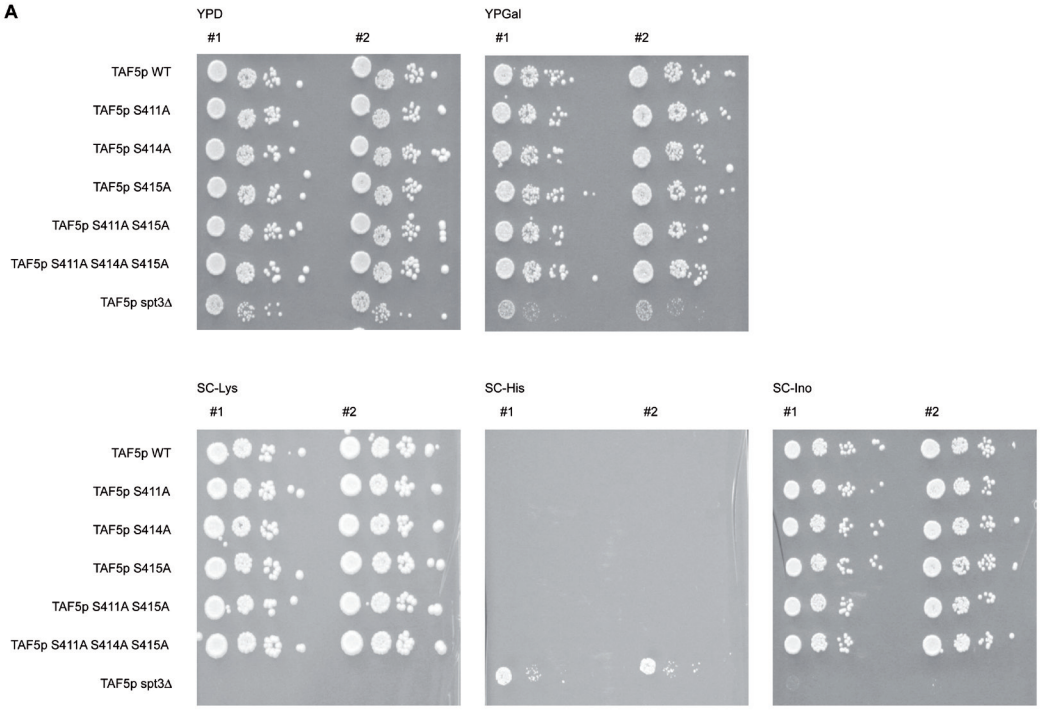
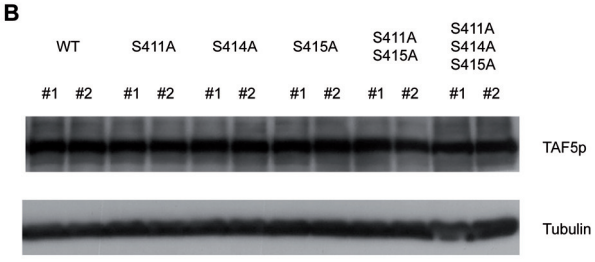
Supplemental Table 1. Yeast strains used in this study.

Strain	Genotype	Source
YJR501	*Mata taf5::TRP1 (pRS316-TAF5) his4-917d lys2-17R2 ura3-52 leu2*	Durso *et al.*, MCB 21: 7731-7344 (2001)
W303 1B	*Matα ura3-52 trp1D2 leu2-3_112 his3-11 ade2-1 can1-100*	
YGP000	*Mata taf5::TRP1 (pRS415-TAF5) his4-917d lys2-17R2 ura3-52 leu2*	this study
YGP001	*Mata taf5::TRP1 (pRS415-TAF5 S411A) his4-917d lys2-17R2 ura3-52 leu2*	this study
YGP002	*Mata taf5::TRP1 (pRS415-TAF5 S414A) his4-917d lys2-17R2 ura3-52 leu2*	this study
YGP003	*Mata taf5::TRP1 (pRS415-TAF5 S415A) his4-917d lys2-17R2 ura3-52 leu2*	this study
YGP004	*Mata taf5::TRP1 (pRS415-TAF5 S411/415A) his4-917d lys2-17R2 ura3-52 leu2*	this study
YGP005	*Mata taf5::TRP1 (pRS415-TAF5 S411/414/415A) his4-917d lys2-17R2 ura3-52 leu2*	this study
YGP006	*Mata taf5::TRP1 (pRS316-TAF5) his4-917d lys2-17R2 ura3-52 leu2 spt3::kan*	this study
YGP101	*Matα TAP-SPT7 ura3-52 trp1D2 leu2-3_112 his3-11 ade2-1 can1-100*	this study
YGP102	*matα TAP-SPT7-1141::HIS3 ura3-52 trp1D2 leu2-3_112 his3-11 ade2-1 can1-100*	this study

Supplemental Table 2. Plasmid used in this study.

Plasmid	Forward oligonucleotide sequence	Reverse oligonucleotide sequence
pRS415 -*TAF5 S411OA*	ggtgaaaaaaaagacGCcaatttatcttcgccagcacgtgatatt	aatatcacgtgctggcgaagataaattgGCgtctttttttttcacc
pRS415 -*TAF5 S414*	ggtgaaaaaaaagacagcaatttaGcttcgccagcacgtgatatt	aatatcacgtgctggcgaagCtaaattgctgtctttttttttcacc
pRS415 -*TAF5 S415A*	ggtgaaaaaaaagacagcaatttatctGcgccagcacgtgatatt	aatatcacgtgctggcgCagataaattgctgtctttttttttcacc
pRS415 -*TAF5 S411/415A*	ggtgaaaaaaaagacGCcaatttatctGcgccagcacgtgatatt	aatatcacgtgctggcgCagataaattgGCgtctttttttttcacc
pRS415 -*TAF5 S411/414/415A*	ggtgaaaaaaaagacGCcaatttaGctGcgccagcacgtgatatt	aatatcacgtgctggcgCagCtaaattgGCgtctttttttttcacc





Supplemental Figure 1. (A) Phenotypic analysis of *TAF5* mutants. Two independent clones (#1 and #2) expressing wild-type or mutant *TAF5* were grown overnight in YPD, spotted in a dilution series of 108 to 105 cells/ml (from left to right) and incubated at 30°C. All strains carry the *his4-917Δ* and *lys2-17R2* alleles. As a positive control, an isogenic Δspt3 strain was used. A SPT phenotype corresponds to growth in medium lacking histidine, and inhibition of growth in medium lacking lysine. In addittion, several SAGA mutants are auxotrophic for galactose and inositol. The data indicate that these TAF5p mutants are not impaired in the SAGA functions tested. (B) Expression levels of wild-type and mutant Taf5p in the strains used in A. Whole cell extracts were analyzed by immunoblottng using a Taf5p antibody. Tubulin was used as a loading control.

Supporting Information Available at http://pubs.acs.org.

Supplemental Table 3, list of all serine or threonine phosphorylated and lysine acetylated peptides identified in the three digests. The columns contain the following information. "Protein", the protein to which the modified peptide is assigned; "Peptide sequence", the sequence of the modified peptide; "MASCOT ion score", the ion score assigned to this peptide match; "Variable modi- fication(s)", descriptor of kind and position of the variable modification(s) detected on that peptide (see inset table description below); "Protease(ses)", the digest in which the peptide was identified; "Fraction number", the SCX fraction number or gel piece number in which the fragment ion spectrum identifying the peptide was recorded; "LTQ scan number", the scan number that links to the fragment ion spectrum identifying the peptide. The inset table shows the link between the numbers used in the main table's "Variable modification(s)" field and the variable modifications to which they correspond.

Supplemental Table 4, list of all unique serine or threonine phosphorylated and lysine acetylated peptides identified in the three digests. The columns contain the following information. "Protein", the protein to which the modified peptide is assigned; "Peptide sequence", the sequence of the modified peptide; "MASCOT ion score", the ion score assigned to this peptide match; "Modification(s)", kind of variable modification(s) of the peptide; "Site(s)", the amino acid residue(s) identified as modified by this peptide; "Proteas- e(ses)", the digest in which the peptide was identified; "Fraction number", the SCX fraction number or gel piece number in which the fragment ion spectrum identifying the peptide was recorded; "LTQ scan number", the scan number that links to the fragment ion spectrum identifying the peptide.

Supplemental Material 1, fragment ion spectra of peptide matches mapping modified amino acids which are explicitly discussed in the text. The visualization was generated automatically by using Scaffold 2.1.03 (Proteome Software).

Supplemental Material 2, fragment ion tables that belong to fragment ion spectra in Supplemental Material 1. The tables contain all fragment ions used for peptide identification. Supplemental Materials 3 and 4, FASTA files of alignments of Spt7p and Sgf73p. The files contain the alignments of Spt7p to its fungal homologues and Sgf73 to human and mouse ataxin-7 in FASTA format.

Chapter 4

Identification of Pep4p as the Protease Responsible for Formation of the SAGA-related SLIK Protein Complex

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The Saccharomyces cerevisiae SAGA protein complex is a co-activator for transcription by RNA polymerase II and has various activities including acetylation and deubuiqitination of histones and recruitment of TATA binding protein to promoters. The Spt7p subunit is subject to proteolytic cleavage at its C-terminus resulting in removal of the Spt8p binding domain and generation of the SAGA-related SLIK protein complex. Here, we report identification of the protease responsible for this cleavage. Screening of a protease knockout collection revealed PEP4 to be required for cleavage of Spt7p within SAGA in vitro. Endogenous formation of truncated Spt7p was abolished in cells lacking PEP4. Purified Pep4p but not catalytic dead mutant Pep4p or unrelated Prc1p protease specifically cleaved Spt7p within SAGA into SLIK-related Spt7p. Interestingly, SAGA lacking Spt8p was more sensitive to Pep4p-mediated truncation of Spt7p, suggesting that Spt8p counteracted its own release from SAGA. Strains mimicking constitutive SLIK formation showed increased resistance to rapamycin treatment, suggesting a role for SLIK in regulating cellular responses to nutrient stress.

Introduction

Transcription initiation by RNA polymerase II is a dynamic process that is regulated by the interplay of a large number of factors. The class of coactivators orchestrates pre-initiation complex (PIC) formation by recruiting nucleosome remodelers, basal transcription factors and histone modifiers. The evolutionary highly conserved SAGA (Spt-Ada-Gcn5 acetyltransferase) complex is a 1.8 MDa coactivator crucial for PIC assembly and transcriptional regulation of 10% of the genes in *S. cerevisiae* (2, 5, 16, 26, 27). SAGA contains ~21 different subunits organized in a multifunctional sub-modular architecture comprising a structural core, a TRA1 subunit, a HAT (histone acetyltransferase) module, a DUB (de-ubiquitination) module, and the Spt3p/Spt8p/TBP module (31, 40, 45). It is well established that the SAGA coactivator complex is recruited to upstream activating sequences by interaction of its Tra1p subunit with transcriptional activators (7, 23). On chromatin SAGA acetylates the tails of histone H3 and H2B via its Gcn5p subunit, and it deubiquitinates histone H2B at lysine 123 via its Ubp8p subunit (10, 17, 21). SAGA is also involved in recruitment of basal transcription factors like TBP to the PIC via the Spt3p/Spt8p module (4, 48). Furthermore, SAGA interacts with other protein complexes extending its functions to transcription elongation, mRNA export (22, 33, 34) and nucleosome remodeling (6, 41). SAGA in higher eukaryotes operates in telomere maintenance (1) and development (43). The Spt3p and Spt8p subunits of SAGA interact with TBP modulating both positive and negative effects on gene expression (42). It is not clear which molecular mechanism governs the Spt8p-Spt3p-TBP interaction within SAGA but several genetic and biochemical studies have described different aspects of this interplay. For instance it has been documented that Spt8p directly binds TBP (31) and can compete with TATA box DNA for TBP binding (38). Furthermore, genetic evidence suggests that both Spt3p and Spt8p interact with TBP (24). This is indicated by the identification of mutations in TBP that are able to suppress *SPT3* mutations in an allele-specific manner. The same TBP mutations also bypass *SPT8* null mutations.

The identification of the SALSA/SAGA-LIKE (SLIK) complex, here referred to as SLIK, has been previously reported in yeast (3, 35, 39). SLIK subunit composition is identical to that of SAGA except that in SLIK the Spt7p subunit is C-terminally truncated by proteolytic cleavage leading to release of the Spt8p subunit. Furthermore, Rtg2p has been reported to be uniquely present in SLIK but not in SAGA (35), linking SLIK to the retrograde response pathway. Rtg2p has been shown to be required for the integrity of SLIK formation, and yeast deleted for either *RTG2* or *SPT8* displayed differential sensitivity to growth on alterative carbon sources like acetate and glycerol (26). It has also been shown that SLIK is actively involved in transcription of the HIS3 gene, thereby counteracting the inhibiting action of SAGA (39). Other analyses indicate redundancy of SLIK with SAGA (46). For instance, it has been shown that the Sgf73p subunit serves as anchor for the DUB module in both SAGA and SLIK complexes (25). So far the molecular function of SLIK has not been established with certainty. A possible function can be inferred from its distinct complex composition. In SLIK the balance between the TBP binding proteins Spt3p and Spt8p is broken since Spt8p is lost. This might affect the TBP-delivery function of SAGA globally or specifically at promoters. We previously showed that cleavage of Spt7p occurs between positions 1141 and 1142 (30), which map in a cleavage domain described previously by Winston and coworkers (46). This cleavage is essential for formation of the SLIK protein complex but the protease responsible for this process has not been identified so far.

Site-specific proteolysis is involved in the regulation of gene expression by cleavage of transcription factors. Regulated intramembrane proteolysis (RIP), for instance, regulates the activation of SREBP, Notch and ATF6 transcriptional factors (8). The cleavage of HCF regulates cell proliferation (44) whereas protein truncation of TFIIA (49) or MLL (20) by Taspase1 protease serves to fine-tune transcription regulation of a subset of genes during differentiation and development. Interestingly, it has also been shown that proteolytic cleavage of histone tails acts in chromatin regulation. In yeast the histone H3 tail clipping after Ala21 regulates gene expression (37) and in mouse cathepsin L cleaves

H3 tails at position 21-22 and 27-28 during embryonic stem cell differentiation (12). The identification of proteases responsible for such functional protein truncation has been crucial for a better understanding of the biological processes listed above. Here we identify Pep4p as the protease responsible for the cleavage of Spt7p and for the formation of the SLIK protein complex. Furthermore, we found that the SAGA-specific subunit Spt8p negatively regulates the Pep4p-mediated cleavage of Spt7p. Pep4p-mediated SLIK formation caused increased resistance to rapamycin treatment, suggesting a role for SLIK in the response to nutrient starvation.

Matherials and methods

Yeast Strains, plasmids and growth conditions

Yeast strains used in this study were FY or W303-1B derivatives and are listed in Supplemental Table 1. Gene TAP-tagging and generation of null mutations were performed by standard methods as described previously (30). Protease expressing strains were constructed in W303-1B background. Plasmids for protease expression are listed in Supplemental Table 2 and were obtained from the Open Biosystem ORF collection. ORF constructs C-terminally tagged with 6xHis-HA-Prescission-Protein A were expressed under the inducible *GAL1* promoter. *PEP4* and *PRC1* ORFs were verified by sequence analysis and the pep4 D294A plasmid was obtained by PCR site-directed mutagenesis using the primers 5'-ATGGTGCCGCCATCGCTACTGGTACTTCTTT-3' and 5'- AAAGAAG-TACCAGTAGCGATGGCGGCACCAT-3'). For enzymatic activity assays using whole cell extracts (WCEs), *in vivo* Spt7p truncation analysis and TAP purifications, cells were cultured in standard YPD at 30°C until an OD600 of 2. For protease expression, cells where cultured in SC-URA supplemented with 2% raffinose until an OD600 of 2, followed by galactose addition (2% final concentration). Cells were grown for a subsequent period of 3 h at 30 °C prior to harvesting. Protease knockout strains were obtained from Open Biosystems and are listed in Supplemental Table 3. Strains were selected on G418 plates and grown until saturation in standard liquid YPD.

Spot assay and rapamycin treatment

Exponential phase growing strains were harvested and spotted in a dilution series from 10^6 to 10^4 cells/ml on synthetic complete medium (SC) supplemented with glucose (glu) and containing rapamycin at a final concentration of 10, 20, or 30 nM. The plates shown were incubated at 30° C for the following times: SC/glu (2 days), rapa 10 nM (3 days) rapa 20 nM and rapa 30 nM (4 days). Strains used were: WT (FY2031 (46)), SLIK-only (FY2032 (46)), *Δspt8* (YGP160), *Δpep4* (YGP161).

Yeast extracts and affinity purifications

Yeast extracts for *in vitro* cleavage assay were prepared as described previously (30). Briefly cell pellets were washed once with water and snap-frozen in dry ice. Pellets were thawed, resuspended in 2 ml of Buffer E (10 mM HEPES-NaOH pH 8.0, 150 mM NaCl, 0.1% Tween 20, 10% glycerol) without protease inhibitors and transferred to 2 ml screw cap eppendorf tubes with 1 ml of glass beads (BioSpec). Proteins were extracted by 8 cycles (30 sec power/ 2 min pause) in a minibeadbeater (BioSpec). Extracts were cleared by centrifugation at 14,000 rpm and supernatants were aliquoted and snap frozen for further analysis. Yeast extracts for *in vivo* Spt7p truncation analysis were prepared as above, but using buffer E with protease inhibitor cocktail for yeast extracts (Sigma).
SAGA and SLIK, SAGA-only and SAGA *Δspt8* were purified respectively from WCEs of Coy142, FY2031 or YGP160 by TAP purification as described previously (30) with the following modifications: for the first binding step, 200 µl of streptavidin M-280 Dynabeads (Invitrogen) were washed with 3 ml of Buffer E and resuspended in 180 µl of the same buffer. Beads were coated with 20

µl of biotinylated IgG (Bethyl) by incubation at 37 °C for 30 min with shaking at 1000 rpm. The beads were washed with 3 ml of buffer E and incubated with 10 ml of yeast cell extract for 2 hr. Beads were collected, washed with 3 ml of Buffer E, 1 ml of cleavage buffer (10mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 0.1% Tween-20 and 1 mM DTT) and resuspended in 1 ml of cleavage buffer. The protein A moiety of the TAP tag was cleaved off with 10 µl of TEV protease (Invitrogen) for 2 h at 19 °C. The eluate was added to Calmodulin agarose beads (Stratagene) resuspended in 3 ml of Calmodulin binding buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl2, 10% (v/v) glycerol, 0.1% (v/v) Tween-20 and 10 mM β-mercaptoethanol) and incubated at 4°C for 1 h. Beads were then washed with 30 ml of Calmodulin binding buffer and proteins were eluted with 400 µl of elution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, 10 % (v/v) glycerol, 0.1% (v/v) Tween-20 and 10 mM β -mercaptoethanol). Eluates were aliquoted and stored at -80°C or used directly for further analysis. Pep4p, Pep4p D294A and Prc1p proteases C-terminally tagged with 6xHis-HA-Prescission-Protein A were affinity purified from WCEs of the YGP151, YGP152, YGP153 strains, respectively. Cell pellets were resuspended in Buffer E with 500 mM NaCl instead of 150 mM without protease inhibitors, and protein extraction was performed as described previously (30). Protein A purification was carried out as described above. Beads were then collected and washed with 2 ml of prescission buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT). Proteases were eluted with 5 µl of PreScission protease (GE healthcare) in 400 µl of prescission buffer at 10°C for 4 h. 10 % (v/v) glycerol was added to the eluate and aliquots were stored at -80°C for further analysis. Purifications were analyzed on NuPage 4-12% Bis-Tris gradient gels with MOPS running buffer (Invitrogen) according to the manufactures' instructions. Subunits were identified by mass spectrometry (30). Molecular weight markers were obtained from New England Biolabs.

In vitro cleavage assay, SDS-PAGE and immunoblot analysis

The substrate for the *in vitro* cleavage assay was prepared as follows: FY2031 WCE was prepared as above to yield the SAGA preparations devoid of SLIK via purification of Spt7p via its C-terminal TAP tag. The *in vitro* cleavage assay included the following: 4 µl (or otherwise indicated) of yeast extract or purified protease, 10 µl substrate in a total volume of 15 µl. Incubation was for 30 min at 30°C unless otherwise stated. The reaction was stopped by addition of Laemmli buffer and incubation for 5 min at 95°C. Reactions were run on 10% SDS-PAGE gel and transferred to PVDF membrane. Molecular weight markers were obtained from New England Biolabs (cat. P7708S). Spt7p was detected using an HA antibody recognizing an N-terminally fused HA epitope. Inhibition of proteolysis was assayed by pre-incubation of the WCEs or purified proteases on ice for 30 min with the following protease inhibitors: 1 µg/ml Pepstatin (Sigma), 1 mM AESBF (Sigma), 10 µM E64 (Sigma), or 1% (v/v) broad-specificity protease inhibitor cocktail (P8250 Sigma). Antibodies used were: anti-HA antibody (3F10, Sigma), anti-Spt7p antibody (a gift from F. Winston), anti-Spt8p antibody (a gift from J. Workman), anti-TAP antibody (PAP, Sigma), and anti-tubulin (Immunologicals).

Results

An aspartic protease cleaves Spt7p^{SAGA} into Spt7p^{SLIK}.

In search of the protease responsible for Spt7p cleavage and SLIK protein complex formation, we developed an *in vitro* cleavage assay in which Spt7p present in SAGA (Spt7p^{SAGA}) is cleaved into Spt7p present in SLIK (Spt7p^{SLIK}). As shown previously, SLIK together with the SAGA complex can be purified using a shared subunit as bait (*SPT20-C-TAP*) (46). In this purification two forms

of Spt7p can be detected by Coomassie staining (Fig. 1A, left lane) or using an anti-Spt7p antibody (Supplemental Fig. S1A, lane 1): a full-length form related to the SAGA complex ($Spt7p^{SAGA}$) and a shorter form related to the SLIK complex ($Spt7p^{SLIK}$). Additionally, SAGA without SLIK (SAGA-only) can be purified using a C-terminal TAP tagged Spt7p (N-HA-SPT7-C-TAP). In this preparation, only the $Spt7p^{SAGA}$ form can be detected by Coomassie staining (Fig. 1A, right lane) or Spt7p immunoblot (Supplemental Fig. S1A, lane 2). The SAGA-only purification was used as substrate in the cleavage assay. Detection of the processed forms was performed by anti-Spt7p or anti-HA immunoblot according to the presence of both epitopes at the N-terminus of Spt7p. We first tested whether the protease activity is present within the SAGA complex itself. Incubation of the SAGA-only complex for 30 min at 30 °C failed to show any cleavage (Fig. 1B, lane 2 and Supplemental Fig. S1A, lane 3). The same was true for longer incubations (1 or 2 h; data not shown). This indicated that the protease activity was not part of the SAGA complex under the assay conditions tested. In contrast, incubation of SAGA-only with WCE for 30 min at 30 °C resulted in processing of $Spt7p^{SAGA}$ to $Spt7p^{SLIK}$ (Fig. 1B, lane 3). We used whole cell extract (WCE) of FY2031 as positive control for the size of $Spt7p^{SLIK}$. In this strain, $Spt7p^{SLIK}$ is produced from N-HA-SPT7-C-TAP *in vivo* and can be detected by the anti-HA antibody (Fig. 1B, lane 5). As a second positive control we used a protein A-purified Spt7p in which the C-terminal part of Spt7p was deleted mimicking $Spt7p^{SLIK}$ (46) (Fig. 1B, lane 4). To further characterize the activity responsible for $Spt7p^{SLIK}$ formation, we inactivated the WCE for 5 min at 95 °C or we added protease inhibitors. Fig. 1C shows that both heat treatment and addition of the aspartic protease inhibitor pepstatin inhibited cleavage of Spt7p, whereas AESBF and E64 treatment had no effect. This indicated that an aspartic protease activity is responsible for the generation of $Spt7p^{SLIK}$.

Knockout screen reveals that Pep4p protease is required for cleavage of $Spt7p^{SAGA}$ into $Spt7p^{SLIK}$. To identify the protease for Spt7p, we screened a yeast protease knockout collection for Spt7p cleavage activity. The *S. cerevisiae* genome encodes a total of 121 proteases with a viable knock out phenotype (merops.sanger.ac.uk). WCEs of 89 knock out strains (Open Biosystems) belonging to all protease classes were prepared and were tested using the *in vitro* cleavage assay described in Fig. 1B. Interestingly, 87 out of 89 WCEs tested contained similarly high activity towards processing of $Spt7p^{SAGA}$ into $Spt7p^{SLIK}$ (Supplemental Fig. S1B). Two WCEs, #2 and #35, which lack Prb1p and Pep4p respectively, showed moderately reduced (#2) or strongly reduced (#35) activity towards $Spt7p^{SAGA}$ processing. WCEs from these two candidates were re-tested for their cleavage activity on $Spt7p^{SAGA}$ as above and the WCE total protein content was monitored by anti-tubulin immunoblot analysis. Consistent with the results from the screen, no proteolytic activity was detected in *Δpep4* WCE, and a lower activity was present in the *Δprb1* WCE compared to wt WCE (Fig. 2A). In yeast, the serine protease Prb1p activates Pep4p by cleavage of the N-terminal inhibitory domain. Pep4p can also cleave itself in the absence of Prb1p resulting in an active but less stable conformation (reviewed in (32)). Therefore, the absence of Prb1p leads to a reduction of Pep4p activity in yeast cells. We tested whether the low proteolytic activity detected in the *Δprb1* WCE was related to reduced Pep4p protease activity. We employed the *in vitro* cleavage assay using $Spt7p^{SAGA}$ but now pre-incubated the *Δprb1* WCE on ice with inhibitors for the three major classes of protease inhibitors classes as indicated (Fig. 2B). Inhibition of aspartic (pepstatin), but not serine (AEBSF) and cysteine (E64) proteases reduced the cleavage of $Spt7p^{SAGA}$ (Fig. 2B). This is consistent with the hypothesis that the residual activity present in the *Δprb1* WCE may be caused by auto-activation of the aspartic protease Pep4p.

We next tested whether Pep4p is required for Spt7p cleavage *in vivo*. To test this, we used an N-terminal TAP tagged *SPT7* strain in which $Spt7p^{SAGA}$ and $Spt7p^{SLIK}$ can be detected by anti-TAP immunoblot analysis. WT cells indeed contain both SAGA and SLIK forms of Spt7p (Fig. 2C). Deletion of *PEP4* abolished the $Spt7p^{SLIK}$ formation whereas deletion of *PRB1* or *PRC1* had no effect on Spt7p cleavage (Fig. 2C). Taken together, these results indicate that Pep4p is required for cleavage of $Spt7p^{SAGA}$ into $Spt7p^{SLIK}$ both *in vivo* and *in vitro*.

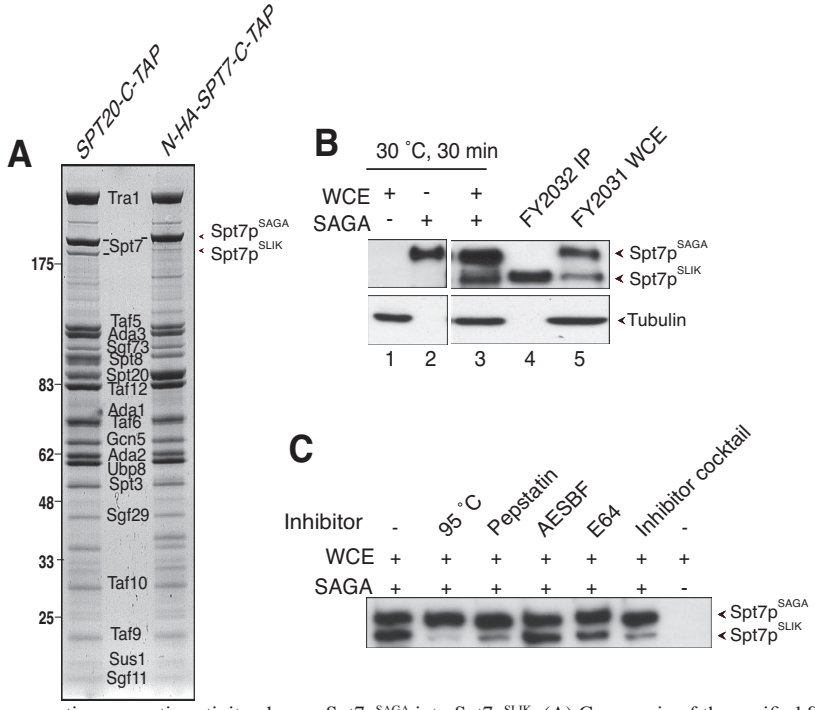


Figure 1. An aspartic enzymatic activity cleaves Spt7p^{SAGA} into Spt7p^{SLIK}. (A) Coomassie of the purified SAGA and SLIK complexes. *SPT20-C-TAP* was used to purify both SAGA and SLIK (left lane). *N-HA-SPT7-C-TAP* was used to purify SAGA-only (right lane). Positions of full length (Spt7p^{SAGA}) and C-terminally truncated (Spt7p^{SLIK}) forms of Spt7p as well as other SAGA/SLIK subunits are indicated. Identification of SAGA/SLIK subunits by mass spectrometry has been reported by us previously (30).(B) An activity present in yeast whole cell extract (WCE) cleaves Spt7p^{SAGA} into Spt7p^{SLIK}. Purified SAGA-only was incubated with yeast WCE for 30 min at 30 °C, and Spt7p processing was monitored by immunoblotting using an anti-HA antibody. Anti-tubulin antibody was used as a control for the amount of WCE added. FY2031 (*N-HA-SPT7-C-TAP*) WCE was used as positive control for *in vivo* generated Spt7p^{SLIK}. FY2032 (46) IP was prepared by TAP purification and represents a genetic mimic of Spt7p^{SLIK} (C). The Spt7p-processing activity present in WCE belongs to the aspartic protease family. *In vitro* cleavage assay as in (B), but after inactivation of the WCE at 95 °C or with addition of protease inhibitors as indicated.

Pep4p protease directly cleaves Spt7p within the SAGA complex

To further investigate the role of Pep4p in Spt7p^{SLIK} formation we expressed and purified tagged versions of Pep4p wt, a catalytically inactive Pep4p (mutation D294A) and the unrelated Prc1p protease from yeast overexpression strains (Supplemental Fig. S2). As shown previously, two bands were present in the Pep4p purification due to auto-activation: the upper one is related to the proteolytic inactive form and the lower one represents the active form. The Pep4p D294A catalytic dead mutant protease purification showed only the inactive form confirming the loss of proteolytic activity (32). These protease preparations were used in Spt7p cleavage assay and immunoblot analysis revealed that incubation with the wt Pep4p protease but not with Pep4p D294A or Prc1p results in Spt7p^{SLIK} formation (Fig. 3A). To further confirm that Pep4p was responsible for this activity we performed the assay as above but pre-incubated the protease with pepstatin for 30 min on ice. We observed that indeed the proteolytic activity of Pep4p on Spt7p^{SAGA} was reduced in the presence of pepstatin (Fig. 3B). We next asked whether the Pep4p proteolytic activity was acting specifically on the Spt7p subunit of SAGA or on other subunits as well. We performed the *in vitro* cleavage assay using purified SAGA as described above (without pepstatin pre-incubation) but now used Coomassie staining to visualize all SAGA subunits (Fig. 3C). The migration of other SAGA subunits except Spt7p was

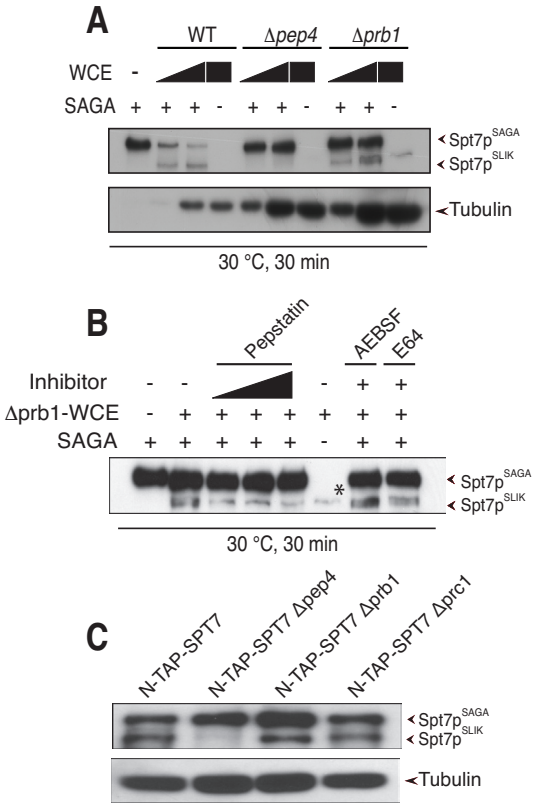


Figure 2. The aspartic protease Pep4p is required to cleave Spt7p^{SAGA}. (A) WCEs from *Δpep4* and *Δprb1* lack or have a reduced activity for generating Spt7p^{SLIK} from Spt7p^{SAGA} respectively. *WT*, *Δpep4*, or *Δprb1* yeast WCE was incubated with purified SAGA for 30 min at 30 °C and cleavage was monitored by anti-HA immunoblot analysis. Total protein content of WCE was monitored by anti-tubulin immunoblot (B). Residual protease activity in *Δprb1* WCE is inhibited by pepstatin A. *In vitro* cleavage assay was performed as in (A) but with a 30 min pre-incubation of WCE with aspartic (10-100-1000 ng/ml pepstatin), serine (1mM AESBF) or cysteine (10 µM E64) protease inhibitor (*: background band). (C) Pep4p is required for endogenous Spt7p^{SLIK} formation. *N-TAP-SPT7* wt or *Δpep4*, *Δprb1* or *Δprc1* WCEs were analyzed for the presence of Spt7p^{SAGA} and Spt7p^{SLIK}. Immunoblot detection was performed using anti-TAP antibody. Anti-tubulin antibody was used to monitor the total protein content.

not affected by incubation with Pep4p protease whereas Spt7p^{SAGA} was processed specifically to Spt7p^{SLIK}. This suggests that Pep4p specifically targets Spt7p within SAGA and does not cause a general proteolysis of SAGA subunits. Furthermore the Coomassie staining analysis indicates that a substoichiometric amount of Pep4p is sufficient to cleave Spt7p^{SAGA}. Taken together these results demonstrate that Pep4p directly cleaves Spt7p^{SAGA} into Spt7p^{SLIK} with high efficiency and specificity.

Spt8p interferes with Spt7p^{SLIK} formation

We previously showed that the cleavage at the C-terminus of Spt7p occurs between the residues 1141-1142 or 1142-1143 (30). The cleavage site resides in the truncation domain flanked by the Spt8p binding domain (46). This suggested the possibility that Spt8p binding to Spt7p may interfere with Spt7p cleavage. To test this we purified a SAGA-only complex via *SPT7-C-TAP* from a yeast strain with a deletion of SPT8 (SAGA complex w/o Spt8p) (Supplemental Fig. S3). Deletion of SPT8 does not disrupt SAGA (46) as illustrated by the co-purification of Gcn5p (Supplemental Fig. S3). We incubated SAGA complex w/o Spt8p or SAGA complex with a limiting amount of purified Pep4p to detect differential sensitivities, and found that the Spt7p^{SLIK} formation was more efficient in the absence of Spt8p (Fig. 4). This suggests that Spt8p reduces Pep4p-induced cleavage of Spt7p, possibly by steric hindrance in which the accessibility of the cleavage site in Spt7p for Pep4p is blocked by the nearby binding of Spt8p.

Pep4p-mediated SLIK formation is involved in rapamycin resistance

Rapamycin is a widely used drug that is known to mimic nitrogen starvation by inhibiting the TOR complex. Responses to rapamycin treatment are numerous and include large changes in gene ex-

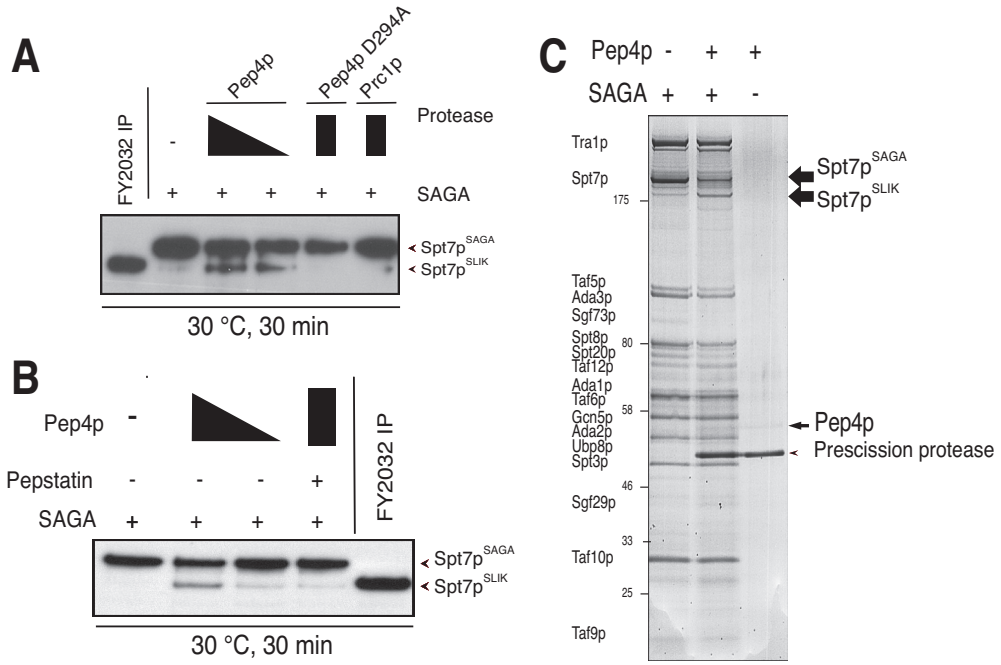


Figure 3. Purified Pep4p directly cleaves Spt7p^{SAGA} to Spt7p^{SLIK}. (A) Purified Pep4p cleaves Spt7p^{SAGA} into Spt7p^{SLIK}. Purified Pep4p along with control protease purifications (Pep4p D294A, Prc1p) were tested for *in vitro* cleavage of N-HA-Spt7p^{SAGA} for 30 min at 30 °C. Detection of cleavage was obtained using HA antibody. FY2032 IP was used as size control for the Spt7p^{SLIK} generated. (B) Protease activity of Pep4p on Spt7p^{SAGA} is pepstatin-sensitive. *In vitro* cleavage assay was carried out as in (A) but with a 30 min pre-incubation on ice with the aspartic protease inhibitor pepstatin. FY2032 IP was used as size control for the cleavage product. (C) Pep4p specifically cleaves Spt7p^{SAGA} within the SAGA complex. *In vitro* cleavage assay was performed as in (A) and the reaction was analyzed on 4-12% acrylamide gel by Coomassie staining.

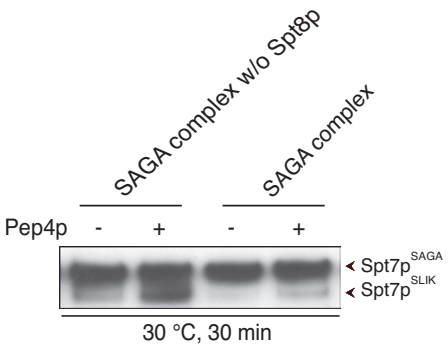
pression programs such as activation of the nitrogen discrimination pathway (NDP) and retrograde response (RTG) pathway and repression of genes involved in cell growth and proliferation (47). Among the genes activated by rapamycin is also *PEP4* (14). Yeast cells deleted for *PEP4* show increased sensitivity to rapamycin treatment (Fig. 5 and (14)), indicating that Pep4p is required for growth under rapamycin conditions. Interestingly, yeast cells in which forced presence of SLIK was mimicked, either by deletion of *SPT8* (Fig. 5 and (46)) or by deletion of the C-terminus of *SPT7* as in SLIK (Fig. 5, SLIK-only) showed increased resistance to rapamycin treatment. This suggests that SLIK formation by Pep4p is involved in the rapamycin response of yeast cells and confers resistance to this treatment. It is likely that SLIK functions as a co-activator to regulate the changes in gene expression programs in response to nitrogen starvation.

Discussion

Here we identify Pep4p as the protease responsible for Spt7p cleavage. Purified Pep4p specifically cleaves Spt7p^{SAGA} into Spt7p^{SLIK} within the SAGA complex *in vitro*, and genetic deletion of Pep4p completely abolishes Spt7p^{SLIK} formation *in vivo*. The Spt8p SAGA subunit reduces Spt7p cleavage and hence its release from the SAGA complex. Strains mimicking constitutive SLIK formation showed increased resistance to rapamycin treatment, suggesting a function for SLIK in the response to nutrient starvation.

The Pep4p protease belongs to the class of pepsin-like aspartic proteases (reviewed in (32)). It predominantly localizes to the vacuolar compartment (yeastgfp.yeastgenome.org), but translocation

Figure 4. Spt8p interferes with Spt7p^{SLIK} formation. Pep4p protease activity is more efficient on SAGA-only complex lacking Spt8p ('SAGA complex w/o Spt8p') compared to SAGA-only complex ('SAGA complex'). Purified complexes were incubated with purified Pep4p protease at 30 °C for 30 min and truncation of Spt7p^{SAGA} into Spt7p^{SLIK} was detected by HA immunoblot.



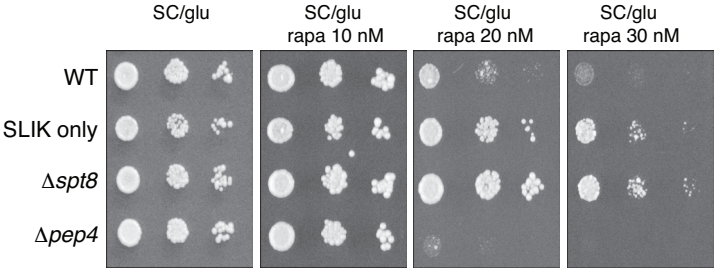


Figure 5. Pep4p-mediated SLIK formation is involved in rapamycin resistance. Rapamycin sensitivity of strains deleted for PEP4 or mimicking constitutive presence of SLIK using FY2032 (SLIK-only; lacking the C-terminus of Spt7p as in SLIK (46)) or by deletion of SPT8. Cells were spotted in a dilution series from 10^6 to 10^4 cells/ml.

of this protease from the vacuole to the cytosol has been described. The active enzyme migrates from the vacuole in H2O2-triggered apoptotic cells (29). Translocation is associated with an increase in vacuolar permeability. This correlates with the degradation of nucleoporins resulting in an increase of nuclear pore complex permeability. Pep4p shares 40-60% identity with the human lysosomal proteases Cathepsin D and Cathepsin E (www.yeastgenome.org). Cathepsin D can trigger lysosome-mediated apoptosis in mammalian cells (11). It migrates from the lysosome via an unknown mechanism and exerts its proteolytic function in a regulated manner (36). Interestingly, the cysteine protease Cathepsin L also migrates from the lysosomal compartment to exert site-specific proteolytic cleavage. Upon translocalization to the nucleus, Cathepsin L has been shown to be responsible for the processing of the transcription factor CDP/Cux (15) and for histone H3 clipping during embryonic stem cell differentiation (12). These results indicate that these vacuolar/lysosomal proteases do not only have degradative functions but that they are involved also in regulated protein processing as well.

A previous study found that the cleavage of Spt7p does not require its incorporation into SAGA (19). Our findings indicate that cleavage of Spt7p can occur in the context of SAGA using an *in vitro* cleavage assay. Two possible mechanisms can be envisioned for cleavage of Spt7p^{SAGA} into Spt7p^{SLIK} and in turn for SLIK protein complex formation. First, Spt7p may be truncated in the cytosol prior to assembly into SLIK complex. Second, SLIK formation may be subsequent to the truncation of Spt7p within the SAGA complex and this would most likely occur in the nucleus. In the latter case truncation of Spt7p would lead to the release of Spt8p from the SAGA complex. Both genetic and biochemical evidence indicates that Spt8p interacts with TBP (13, 38). To further investigate this point, we used Spt8p as bait in tandem affinity purification, and observed that substoichiometric

amounts of SAGA were co-purified, indicating that the majority of Spt8p in yeast cells is not in complex with SAGA (data not shown). This suggests that a pool of free Spt8p is present in yeast cells and that the binding to and release from its Spt7p partner within SAGA may be dynamically regulated. We showed that Spt8p interferes with Spt7p cleavage *in vitro* (Fig. 4). As a consequence, Spt8p may inhibit its own release from SAGA by reducing Pep4p-induced cleavage of Spt7p. This effect is likely to be sterical due to the close vicinity of the Spt8p binding domain to the cleavage region in Spt7p (46). Truncation of Spt7p *in vivo* may depend on a balance between cellular concentrations of Spt8p and Pep4p, which may be regulated by specific signals. Increased Pep4p levels upon nitrogen starvation or stress (9, 14, 28) may increase Spt7p cleavage and as a result Spt8p release. It is interesting to note that yeast strains deleted for the Ubp8p subunit display a reduced formation of SLIK, which suggests that Ubp8p can stimulate Pep4p-mediated cleavage of Spt7p (45).

Using rapamycin to mimic conditions of nitrogen starvation, we obtained evidence that Pep4p-mediated SLIK formation is important for the resistance to nitrogen starvation. Interestingly, deletion of several subunits shared between SAGA and SLIK has been shown to increase the sensitivity to rapamycin (18) and are in line with the idea that disruption of SLIK is involved in this phenotype. The deletions of *SPT8* or the C-terminus of *SPT7* used here specifically mimic SLIK formation and allow to specifically address the function of SLIK. Our results are consistent with the proposed function of SLIK in regulating genes involved in the RTG pathway upon nitrogen starvation (35). Rtg2p, a key transcription factor of the RTG pathway, has been reported to associate with SLIK (26). We hypothesize that increased Pep4p levels during nitrogen starvation induce Spt7p cleavage, Spt8p release from SAGA, and SLIK formation, which in turn regulates expression of genes important to respond properly to the changed nutritional status. The release of Spt8p is thought to change the TBP delivery function of SAGA. Future studies should determine the effect of Spt7p truncation by Pep4p on TBP recruitment and on the cellular responses to rapamycin.

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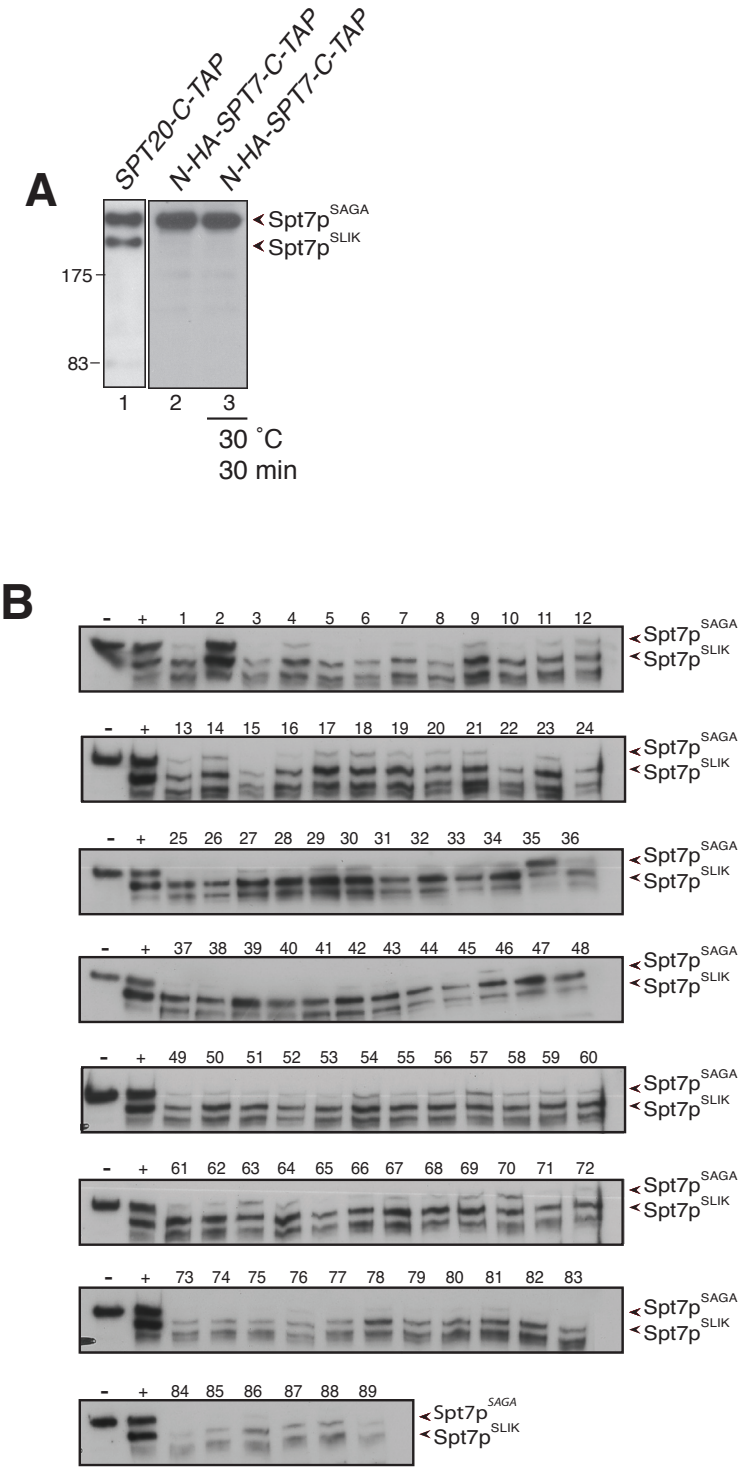
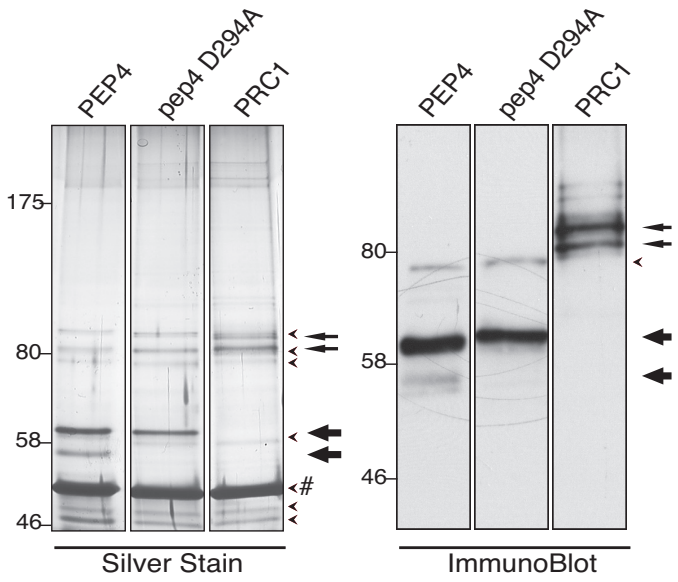
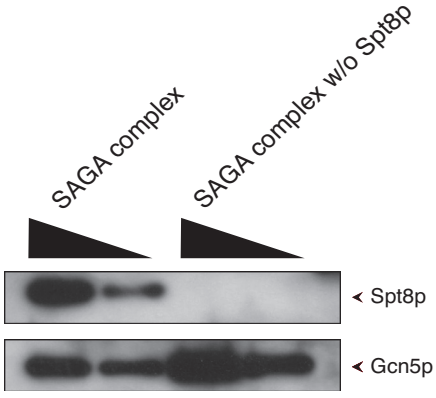
Supplemental Material



Supplemental Figure 1.
A) SAGA is devoid of protease activity.
Purified SAGA/SLIK (lane 1), SAGA only (lane 2) and SAGA-only incubated for 30 min at 30 °C (lane 3), were analyzed by SDS-PAGE and immunoblotting using an anti-Spt7p antibody.
B) Screening of protease knock out yeast strains identifies *PEP4* and *PRB1* to be required for cleavage of $Spt7p^{SAGA}$ into $Spt7p^{SLIK}$.
89 protease knock out yeast strains were cultured until saturation, and WCEs were prepared in buffer lacking protease inhibitors. WCE for each strain was tested for cleavage using TAP-purified SAGA as substrate. Incubation was for 30 min at 30˚C. Reactions were run on SDS-PAGE and analyzed by immunoblotting using an HA antibody. We used approximately 3 times higher concentrated WCEs for the knock out strains compared with the positive control [WT WCE (+)] in order to facilitate the identification of the protease for $Spt7p^{SAGA}$ cleavage. Under these conditions $Spt7p^{SAGA}$ was actively processed into $Spt7p^{SLIK}$ and further C-terminal truncation products. Strong reduction of this process was observed in *Δpep4* (#35) and *Δprb1* (#2) WCEs. [(-) Substrate without WCE, (+) Substrate with WT WCE].



Supplemental Figure 2.
Purification of the Pep4p and Prc1p proteases. WT Pep4p, catalytic dead Pep4p D294A or Prc1p containing the C-terminal tag HIS-HA-Prescission-Protein A were overexpressed from galactose-inducible plasmids in yeast, and proteases were purified using protein A affinity resin. Elution was carried out using Prescission protease. The protease preparations were analyzed by silver staining (left panel) and immunoblotting (right panel) using anti-HA antibody. Specific Pep4p (large arrows), Prc1p (small arrows) and non-specific (small arrowheads) bands are indicated (#: Prescission protease).



Supplemental Figure 3.
Analysis of a SAGA complex lacking Spt8p. FY2031 and YGP160 were used to TAP purify SAGA-only complex ('SAGA complex) and SAGA-only complex lacking Spt8p ('SAGA complex w/o Spt8p), respectively. The purified complexes were analyzed for Spt8p by immunoblotting using anti-Spt8p antibody. Gcn5p immunoblot was used as loading control.

Supplemental Table 1. Yeast strains used in this study

Strain	Genotype	Source
FY3	*MATa ura3-52*	Ref 1
FY2031	*MATa HA-SPT7-TAP::TRP1 ura30 leu21 trp163 his4-917 lys2-173R2*	Ref 1
FY2032	*MATa HA-spt7-1125-TAP::TRP1 ura30 leu21 trp163 his4-917 lys2-173R2*	Ref 1
YGP160	Isogenic to FY2031 except *spt8::KAN*	This study
YGP161	Isogenic to FY2031 except *pep4::KAN*	This study
W303-1B	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Ref 3
YGP101	Isogenic to W303-1B except *TAP-SPT7*	Ref 2
YGP102	Isogenic to W303-1B except *TAP-spt7-1141::HIS3*	Ref 2
YGP103	Isogenic to YGP101 except *spt8::KAN*	This study
YGP104	Isogenic to YGP101 except *pep4::KAN*	This study
YGP151	Isogenic to W303-1B except (pOB-YPL154C::*URA3*)	This study
YGP152	Isogenic to W303-1B except (pOB-YPL154C D294A::*URA3*)	This study
YGP153	Isogenic to W303-1B except (pOB-YMR297W::*URA3*)	This study
COy142	Isogenic to W303-1B except *SPT20-TAP::TRP1*	Ref 3

Supplemental Table 2.
Protease expression plasmids. PEP4 and PRC1 expression plasmids were obtained from Open Biosystems. The pep4 D294A expression plasmid was generated by site-directed mutagenesis.

Plasmid	ORF	Marker
pOB-YPL154C	PEP4	*URA3*
pOB-YPL154C	pep4 D294A	*URA3*
pOB-YMR297W	PRC1	*URA3*

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Supplemental Table 3. Protease null strains used in this study

	Yeast strain	Gene KO	38	YDR125C	*ECM18*	76	YIR039C	*YPS6*
1	YER017C	*AFG3*	39	YDR069C	*DOA4*	77	YKR087C	*OMA1*
2	YEL060C	*PRB1*	40	YDL216C	*RRI1*	78	YER151C	*UBP3*
3	YMR035W	*IMP2*	41	YDL122W	*UBP1*	79	YMR062C	*ECM40*
4	YMR150C	*IMP1*	42	YDL104C	*QRI7*	80	YOL098C	*YOL098C*
5	YMR223W	*UBP8*	43	YCL057W	*PRD1*	81	YOL107W	*YOL107W*
6	YMR247C	*RCE1*	44	YBR139W	*YBR139W*	82	YER078C	*ICP55*
7	YMR297W	*PRC1*	45	YBR074W	*YBR074W*	83	YER098W	*UBP9*
8	YMR300C	*ADE4*	46	YBL067C	*UBP13*	84	YJR099W	*YUH1*
9	YMR304W	*UBP15*	47	YBL045C	*COR1*	85	YJR117W	*STE24*
10	YHR028C	*DAP2*	48	YBL022C	*PIM1*	86	YJR126C	*VPS70*
11	YPL246C	*RBD2*	49	YDR349C	*YPS7*	87	YJL172W	*CPS1*
12	YNL320W	*YNL320W*	50	YDR415C	*YDR415C*	88	YPR024W	*YME1*
13	YNL293W	*YCP1*	51	YDR430C	*CYM1*	89	YMR322C	*SNO4*
14	YJL197W	*UBP12*	52	YDR533C	*HSP31*			
15	YJL130C	*URA2*	53	YGL203C	*KEX1*			
16	YLL029W	*RUP2*	54	YGR101W	*PCP1*			
17	YOR330C	*MIP1*	55	YGR124W	*ASN2*			
18	YOL057W	*YOL057W*	56	YGR135W	*PRE9*			
19	YOR003W	*YSP3*	57	YKL094W	*YJU3*			
20	YHR047C	*AAP1*	58	YKL100C	*YKL100C*			
21	YHR113W	*YHR113W*	59	YKL103C	*LAP4*			
22	YHR132C	*ECM14*	60	YKL158W	*APE2*			
23	YNL239W	*LAP3*	61	YLR244C	*MAP1*			
24	YNL191W	*DUG3*	62	YLR299W	*ECM38*			
25	YNL123W	*NMA111*	63	YLR389C	*STE23*			
26	YPL072W	*UBP16*	64	YNL045W	*YNL045W*			
27	YLR121C	*YPS3*	65	YPR122W	*AXL1*			
28	YLR120C	*YAP3*	66	YPR145W	*ASN1*			
29	YLR099C	*ICT1*	67	YPR191W	*QCR2*			
30	YOR219C	*STE13*	68	YFR006W	*YFR006w*			
31	YOR197W	*MCA1*	69	YFR018C	*YFR018C*			
32	YOR124C	*UBP2*	70	YBL091C	*MAP2*			
33	YIL156W	*UBP7*	71	YBR281C	*DUG2*			
34	YIL137C	*TMA108*	72	YBR286W	*APE3*			
35	YPL154C	*PEP4*	73	YER144C	*UBP5*			
36	YPL176C	*TRE1*	74	YFR044C	*DUG1*			
37	YDR144C	*MKC7*	75	YIR027C	*DAL1*			

Chapter 5

Tight cooperation between Mot1p and NC2β in regulating genome-wide transcription, repression of transcription following heat shock induction, and genetic interaction with SAGA

Manuscript under revision

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TATA-binding protein (TBP) is central to the regulation of eukaryotic transcription initiation. Recruitment of TBP to target genes can be positively regulated by one of two basal transcription factor complexes: SAGA or TFIID. Negative regulation of TBP promoter association can be performed by Mot1p or the NC2 complex. Recent evidence suggests that Mot1p, NC2, and TBP form a DNA-dependent protein complex. Here, we compare the functions of Mot1p and NC2β during basal and activated transcription using the anchor-away technique for conditional nuclear depletion. Genome-wide expression analysis indicates that both proteins regulate a highly similar set of genes. Upregulated genes were enriched for SAGA occupancy, while downregulated genes preferred TFIID binding. Mot1p and NC2β depletion during heat shock resulted in failure to downregulate gene expression after initial activation, which was accompanied by increased TBP and RNA pol II promoter occupancies. Depletion of Mot1p or NC2β displayed preferential synthetic lethality with the TBP-interaction module of SAGA. Our results support the model that Mot1p and NC2β directly cooperate *in vivo* ***to regulate TBP function, and that they are involved in maintaining basal expression levels as well as in resetting gene expression after induction by stress.***

Introduction

Transcription initiation starts with the binding of TATA-box-binding protein (TBP) to gene promoters (7). This is followed by a cascade of protein-protein interactions during which the preinitiation complex (PIC) is formed, which ultimately leads to recruitment of RNA polymerase II and initiation of transcription (18, 32). In yeast, delivery of TBP to promoters and subsequent formation of an active PIC is mediated by two transcription factor complexes: SAGA and TFIID, depending on the promoter DNA sequence. Although SAGA and TFIID are partially redundant, promoters containing a TATA box prefer SAGA for TBP delivery, while promoters lacking a consensus TATA box are in general dominated by TFIID (3, 23, 46). It has become clear that SAGA-dominated and TFIID-dominated genes have a number of different properties. SAGA-dominated genes are lowly expressed, have high TBP turnover rates, and are critically involved in the response to various stresses including heat shock and nutrient limitations during diauxic shift. In contrast, TFIID-dominated genes include many housekeeping genes, which in general are expressed at high levels, and have lower TBP turnover rates (3, 43, 45).

Removal of TBP from promoters and/or inhibition of the formation of an active PIC can be mediated by two distinct repressors: Mot1p and NC2, which consists of a heterodimer between NC2α (also called Bur6p) and NC2β (also called Ydr1p). Mot1p is a major TBP interactor in cell extracts (30). It contains an ATPase domain of the SWI2 family, which it uses to remove or redistribute TBP from promoter DNA in an ATP-dependent manner (2). Various models have been proposed to explain how Mot1p can achieve this. These include changing the conformation of TBP (8), short-range ATP-driven translocation of Mot1p along the DNA (34) and the use of the N-terminal TAND domain of Mot1p as a wedge between TBP and DNA (29). ATP-independent mechanisms are also relevant as indicated by the finding that the binding of Mot1p to TBP in the absence of ATP already relaxes the binding specificity of TBP for the canonical TATA box sequence (17).

In contrast to Mot1p, the NC2 complex associates with TBP in a DNA-dependent manner. The two subunits, NC2α and NC2β, form, via their N-terminal histone fold domains, a heterodimer that structurally resembles the H2A-H2B heterodimer (15, 22). Biochemical and structural studies suggest that NC2 can inhibit TBP function by interfering with the binding of the PIC components TFIIA (by NC2α) and TFIIB (by NC2β) (16, 22). Binding of NC2 to DNA-bound TBP has also been shown to result in movement of TBP away from the TATA box, presumably by inducing a conformational change in TBP (31). Besides their established roles as transcriptional repressors, both Mot1p and NC2β have also been implicated in gene activation, although the mechanism involved is presently unclear (1, 6, 10-12, 27, 38).

Interestingly, both TBP delivery (SAGA and TFIID) and TBP removing (Mot1p and NC2) proteins are recruited to active genes *in vivo* (1, 6, 10-12, 38, 42). This is consistent with a model in which TBP dynamics plays an important role in the regulation of gene expression (35, 42). Recently, we purified a protein complex from yeast chromatin extracts that consists of Mot1p, both NC2 proteins, TBP, and 20-70 basepairs (bp) of DNA. Addition of a hydrolysable form of ATP resulted in disruption of the complex (42). The co-occurrance of Mot1p and NC2 in one protein complex raises the question to what extent these proteins cooperate to regulate TBP function and gene expression *in vivo*. Genome-wide expression studies of temperature-sensitive (ts) mutants of *MOT1* and *NC2α* have been reported in separate studies (6, 9, 11, 27, 43). In silico comparison reveals that Mot1p and NC2α regulate expression of overlapping sets of genes. However, a direct experimental comparison between profiles of MOT1 and NC2 has not been reported so far. In addition, genome-wide expression analysis of NC2β has not yet been performed because ts alleles for this protein are scarce.

Here we applied the recently published anchor-away technique for conditional depletion to Mot1p and NC2β (19). Genome-wide expression analysis indicates that Mot1p and NC2β regulate basal expression of highly similar sets of genes (R2=0.8). Upregulated genes were enriched for SAGA occupancy, while downregulated genes preferred TFIID binding. Depletion of Mot1p or NC2β re-

sulted in increased promoter occupancy of TBP and/or RNA pol II, and increased basal and induced transcription of the *HSP26* gene. Both Mot1p and NC2β preferentially interacted genetically with the TBP-binding module of SAGA. These data show that Mot1p and NC2β cooperate *in vivo* to regulate TBP function and gene expression.

Materials and methods

Strains, plasmids and primers

Yeast strains used in this study are derivatives of HHY168 (Euroscarf #Y40343), and are listed in Supplemental Table 1. *MOT1* and *NC2β* were C-terminally tagged with *FRB* and *FRB-GFP* as described (19). Details of the primers and plasmids used are listed in Supplemental Table 2 and Supplemental Table 3, respectively. For the complementation assay, strains were transformed with empty vector or with vector harboring the galactose-inducible *MOT1* or *NC2β* genes (Supplemental Table 3). Generation of full gene deletions was performed using standards methods (33).

Cell cultures, spot assay and growth curves

Cells were cultured in Complete Synthetic Medium (CSM) supplemented with 2% glucose unless stated otherwise. For mRNA and Chromatin Immunoprecipitation (ChIP) analyses overnight cultures were diluted in fresh medium to an A600 of 0.2-0.3, and grown until an A600 of 1. Cells were then exposed to 1 μg/ml rapamycin (LC laboratories) for 60 minutes, and subsequently transferred from 30°C to 38°C for heat shock. Samples were harvested at the indicated time points for both mRNA and ChIP analysis from the same experiment. For spot assays, cells at an A600 of 0.1 were spotted on CSM/2% glucose plates containing rapamycin at 1 μg/ml as indicated. The plates shown were incubated for three days at 30°C. For the complementation assay, cells were spotted on CSM/2% galactose with or without rapamycin. For liquid growth curves, the automated Infinite 200 incubator (Tecan) was used. Cells were diluted to an A600 of 0.1 per well in a 24-wells plate and incubated at 30°C with orbital shaking (87 rpm, 1 mm amplitude). A595 was measured every 10 minutes.

Subcellular localization of Mot1-FRB-GFPp and NC2β -FRB-GFPp

MOT1-FRB-GFP and *YDR1-FRB-GFP* strains were grown to an A600 of 1, treated with rapamycin at a final concentration of 1 μg/ml, and samples were taken at the indicated time points. Cells were fixed for microscopy analysis as described previously (37). A DeltaVision Instrument (Applied Precision) equipped with Olympus Objective 100X/1.40 was used for imaging. ImageJ was used for images analysis.

Genome-wide expression analysis

Genome-wide expression analysis was performed as described previously (40) with minor changes. Briefly, for each gene expression profile two independent colonies were inoculated in CSM with 2% glucose. Overnight cultures were diluted to an A600 of 0.3 in 50 ml medium and grown to an A600 of 1. Cultures were then grown for 60 minutes in the absence or presence of 1 μg/ml rapamycin. Cells were harvested by centrifugation at 4000 rpm for 3 minutes and were frozen in liquid nitrogen. The two independent cultures from each strain were hybridized in dye-swap against an untreated isogenic wt RNA for all hybridizations. The microarrays carried 60-mer oligonucleotide probes for all yeast genes, each spotted in duplicate, resulting in a total of four measurements for each gene. Data was normalized by Loess and dye-bias was removed by application of GASSCO (26). For each condition, average gene expression that differed between mutant and wild type reference ($p<0.05$, Limma) and with a higher than 1.7-fold change were considered significant and were used for further analysis.

RT-qPCR analysis
RNA extraction and purification was performed as described previously (40). cDNA was prepared using oligo-dT priming and Superscript III (Invitrogen). qPCR analysis was performed as described previously (41). For mRNA quantitation, ACT1 mRNA was used as reference for calculations of fold enrichment. The standard deviation (SD) for each sample was calculated from three technical repeats. Experiments were repeated at least twice.

Chromatin immunoprecipitation (ChIP)
ChIP was performed as described previously (43) with minor modifications. Antibodies (TBP, RNA pol II) were coupled to 25 µl protein A dynabeads (Dynal). Beads were washed and incubated with 500 µl of chromatin for 2 hr at room temperature. Beads were then washed three times with FA lysis buffer [50 mM Hepes-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-Deoxycholate, 0.1% SDS] and three times with FA lysis buffer containing 0.5 M NaCl. Cross links were reversed by incubation at 65°C overnight in 130 µl 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% SDS. Samples were then treated with Proteinase K, and DNA was purified using a PCR purification kit (Qiagen). Samples were analyzed by qPCR and are presented as fold enrichment over the HMR locus as described (42). The SD for each sample was calculated from three technical repeats. Experiments were repeated at least twice.

Immunoblot analysis
Whole cell extract preparation, SDS-PAGE and immunoblotting were performed as described (33). Antibodies used were anti-GFP (a gift from Dr. Geert Kops) and anti-tubulin (Immunologicals).

Microarray data accession numbers
Microarray data has been deposited in ArrayExpress with experiment number GS003 and accession numbers E-TABM-1177.

Results

Anchoring Mot1p or NC2β causes nuclear depletion resulting in growth delay
Analysis of the functions of Mot1p and the NC2 complex is complicated by the fact that the corresponding genes are essential for yeast viability. Previous results have depended on ts alleles for *MOT1* and *NC2*, which display (weak) growth phenotypes under permissive growth conditions (1, 6, 9, 11, 27, 43). To circumvent the use of ts alleles, we applied the anchor-away technique developed by Laemmli and colleagues (19). An anchor-away strategy was chosen that should translocate nuclear proteins of interest to the cytoplasm (19). To this end, the Mot1p or NC2β proteins were C-terminally tagged with FRB or FRB-GFP. Attempts to tag NC2α with FRB or FRB-GFP failed, suggesting that these tags interfered with the function of the protein. We used a strain carrying an FKBP12 tag to the ribosomal protein RPL13A that serves to anchor FRB-tagged proteins to the cytoplasm in response to rapamycin. It also contains a mutation of TOR1 and a deletion of FPR1, rendering it rapamycin-insensitive and FKBP12-sensitive (19).
We first tested the effect of anchoring Mot1p or NC2β on growth using a spot assay. Rapamycin treatment of strains carrying either *MOT1-FRB-GFP* or *NC2β-FRB-GFP* resulted in strongly reduced growth up to day 3 after spotting (Figure 1A). Growth was restored at day 4 (data not shown). Similar results were obtained when the FRB tag without GFP was used (Fig. S1A). Growth delay rather than lethality suggests that residual amounts of nuclear Mot1p and NC2β remain present upon anchoring. To verify that the observed growth defects were caused by anchoring Mot1p or NC2β, we complemented untagged versions of these proteins using galactose-inducible expression plasmids,

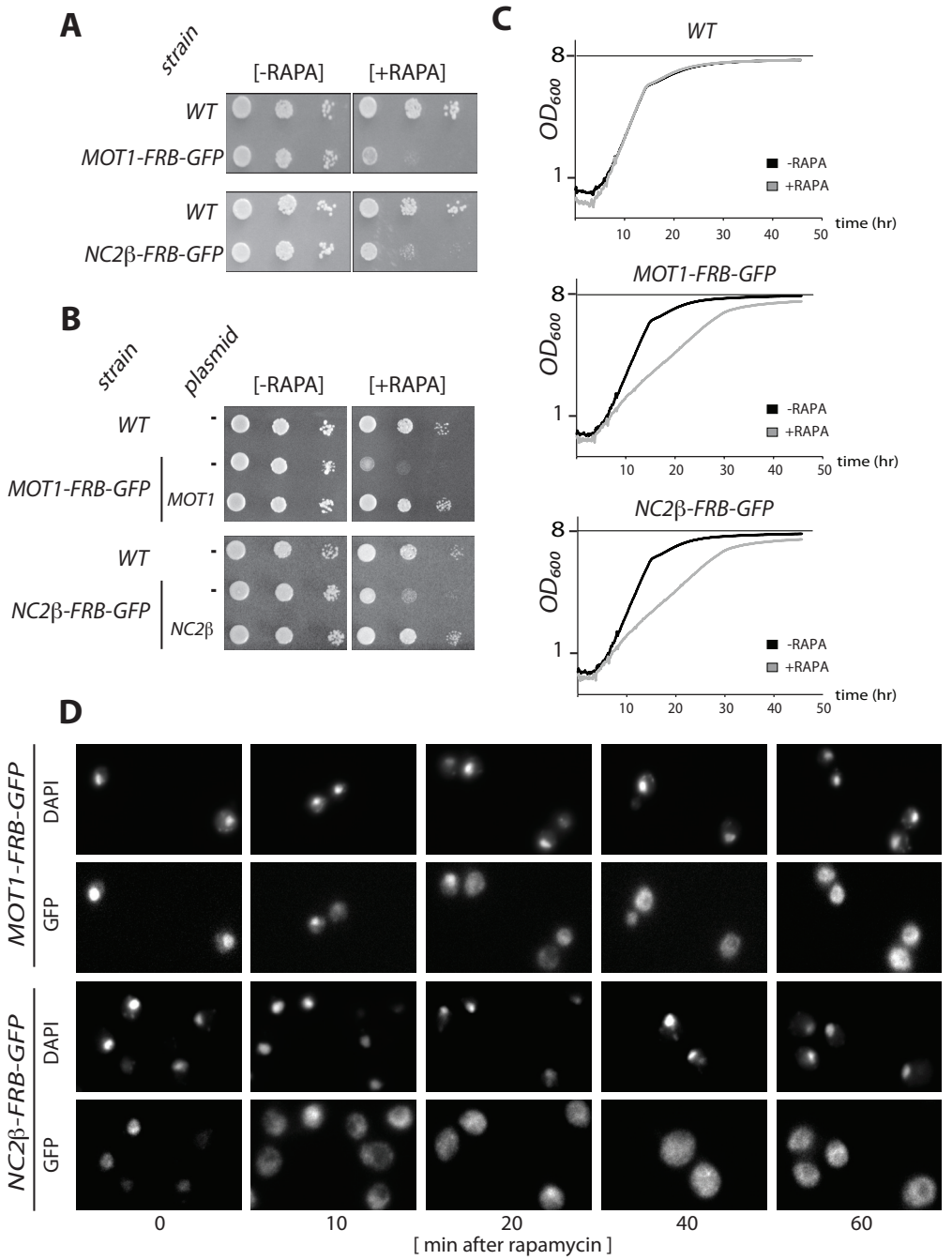


Figure 1. Anchoring Mot1p and NC2β causes growth delay. (A) Spot assay on SC plates in the absence or presence of 1 μg/ml rapamycin. The parental strain HHY168 was used as negative control (wt). Dilutions were 10-fold. (B) Complementation of *MOT1-FRB-GFP* or *NC2β-FRB-GFP* with untagged, galactose inducible MOT1 or NC2β, respectively. Spot assay as in (A) on SC galactose plates in the absence or presence of 1 μg/ml rapamycin. The parental strain HHY168 (wt) containing the empty vector pRS303 (-) was used as negative control. (C) Growth in liquid culture. Saturated cultures were diluted to an A600 of 0.1, and grown in CSM in the absence or presence of 1 μg/ml rapamycin for 2 days. Automatic cell counting was performed using an Infinite 200 incubator (Tecan). (D) Anchoring nuclear Mot1-FRB-GFPp and NC2β-FRB-GFPp to the cytoplasm. Cells were treated with 1 μg/ml rapamycin for the time points indicated. After fixation, GFP fluorescence was used to monitor localization of Mot1p and NC2β. DAPI was used to visualize nuclei.

and repeated the spot assay. Co-expression of Mot1p or NC2β in the *MOT1-FRB-GFP* or *NC2β-FRB-GFP* strain, respectively, resulted in complete rescue of rapamycin-induced growth delay (Fig. 1B). Similar results were obtained when the FRB tag without GFP was used (Fig. S1B). This confirmed that the anchor-away approach resulted in a conditional depletion of Mot1p and NC2β.
Next, we tested the effect of anchoring Mot1p or NC2β in liquid culture. Cultures were started at an A600 of 0.1, and rapamycin treatment was performed from this time point onwards. Similar to growth on plates, growth of *MOT1-FRB-GFP* or *NC2β-FRB-GFP* strains in liquid culture was sensitive to rapamycin (Fig. 1C). The effects were seen during the exponential and diauxic shift phases of the growth curve. After 2 days, when cells reached the saturation phase, both untreated and rapamycin-treated cultures had similar A600 values. Comparable results were obtained when the FRB tag without GFP was used (Fig. S1C, please note that rapamycin treatment was started later in this case, see arrowhead).
To confirm nuclear depletion of Mot1p or NC2β upon rapamycin treatment, we used the GFP moiety of the FRB-GFP tag to monitor Mot1-FRB-GFPp or NC2β-FRB-GFPp by fluorescence microscopy. In the absence of rapamycin, both Mot1-FRB-GFPp and NC2β-FRB-GFPp were exclusively present in nuclei, which were visualized using DAPI (Fig. 1D). Mot1-FRB-GFPp was present in the cytoplasm starting at 20 min after rapamycin treatment, while NC2β-FRB-GFPp showed cytoplasmic fluorescence starting at 10 minutes after rapamycin treatment. Immunoblot analysis indicated that the total cellular protein concentrations of Mot1p or NC2β were not affected by rapamycin treatment (data not shown). Taken together, these analyses indicate that the anchor-away approach can be applied to study the function of Mot1p or NC2β in yeast cells.

Genome-wide analysis of transcriptional defects in response to anchoring Mot1p or NC2β
To determine the effects of anchoring Mot1p or NC2β on transcription, we used yeast oligonucleotide arrays to monitor genome-wide mRNA levels 60 min after rapamycin treatment. Using 1.7 fold change and $p<0.05$ as criteria for significance, we found that nuclear depletion of either NC2β or Mot1p resulted in 500-600 genes with changed mRNA expression levels, respectively, of which in both cases the majority (80%) was upregulated, while a smaller proportion (20%) was downregulated (Fig. 2A, compare left panel (without rapamycin) with right panel (with rapamycin), Fig. 2B). The following analysis validated our experimental approach: first, scatter plot analysis of the untagged parental strain used in this study confirmed that this strain is rapamycin-insensitive (Fig. 2A, compare upper left and upper right plots). Second, in the absence of rapamycin, the Mot1p and Nc2β anchor-away strains exhibited expression profiles very similar to the untagged wild type strain (WT), with only a few transcript levels affected (Fig. 2A, compare the three plots on the left), confirming that the anchor-away technique was not leaky and that the tags used did not interfere with the function of the target proteins. Third, RT-qPCR analysis of *HSP26*, *HSP42*, and *ARO10* mRNAs confirmed that expression of these genes is upregulated by anchoring Mot1p and NC2β (Fig. S3). And finally, the profile of *MOT1-FRB* significantly overlapped with the *mot1-1* profile (24) generated at the permissive temperature ($R2=0.4$; $p<10\text{-}14$, hypergeometric test), despite the differences in experimental platforms and genetic backgrounds (Fig. S2B, C).
Venn diagram analysis revealed a high degree of overlap ($p<10\text{-}14$, hypergeometric test) between the upregulated genes in the *MOT1-FRB* and *NC2β-FRB* profiles (Fig. 2B). 70% of genes upregulated in *MOT1-FRB* overlapped with the genes upregulated in *NC2β-FRB*, while 80% of genes upregulated in NC2β-FRB overlapped with genes upregulated in *MOT1-FRB*. Downregulated genes also overlapped significantly ($p<10\text{-}14$), but to a lower extent: 40% of downregulated genes in *MOT1-FRB* overlapped with *NC2β-FRB*, while 60% of downregulated genes in NC2β-FRB overlapped with MOT1-FRB (Fig 2B). Correlation plot analysis also indicated a high level of correlation between *MOT1-FRB* and *NC2β-FRB* profiles ($R2=0.8$) (Fig. 2C). This comparison supports the model that Mot1p and the NC2 complex cooperate to regulate mRNA expression.

Regulation by Mot1p and NC2β depends on the promoter sequence

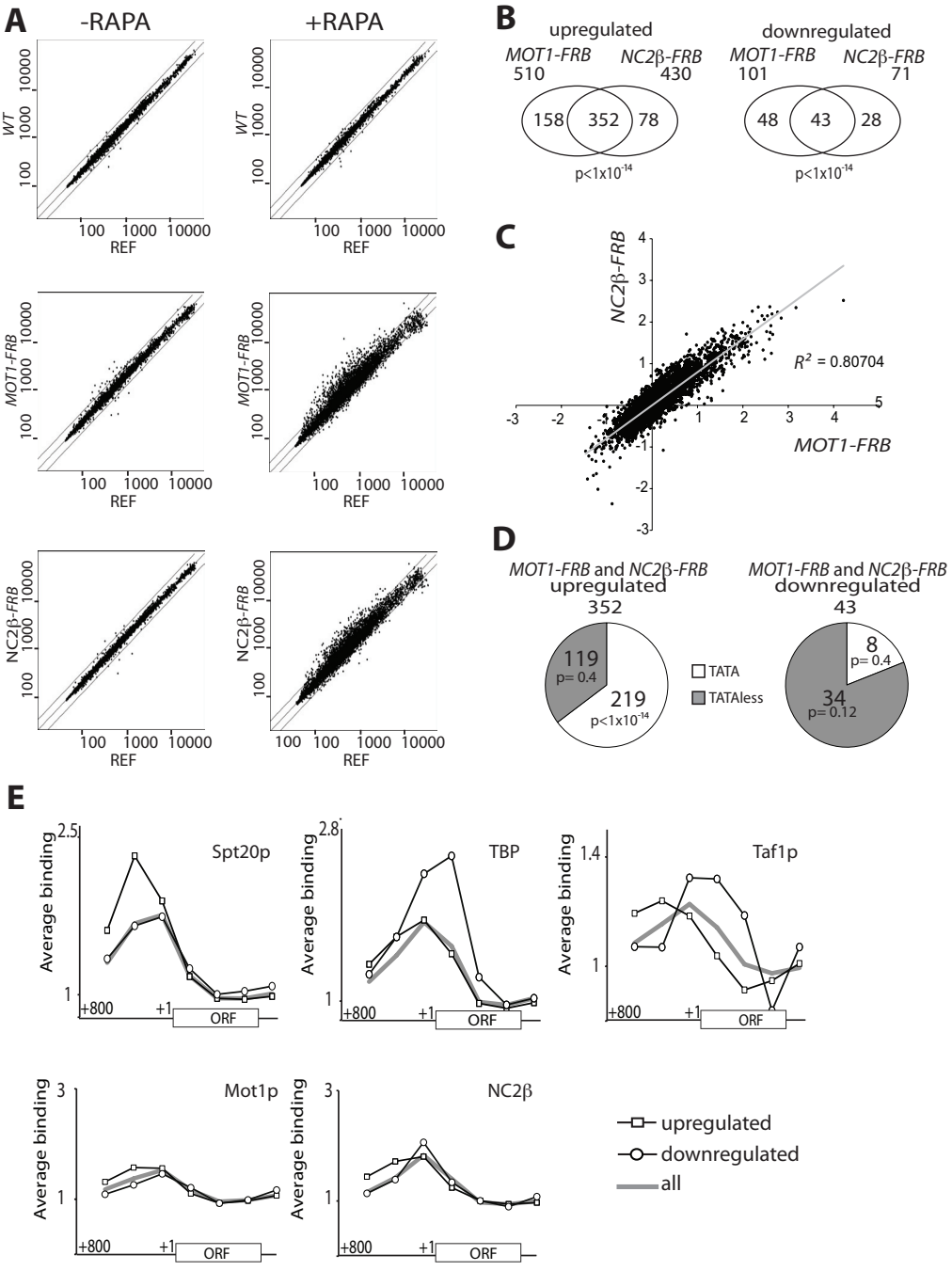
Genes that were upregulated in both the depletion of Mot1p and of NC2β were characterized by a significant ($p<10^{-14}$) enrichment for the presence of a TATA box in their promoters: 60% of upregulated genes contained a TATA box, whereas the average occurrence is 19% (Fig. 2D)(3). These genes were expressed at average levels (Fig. S2A). In contrast, genes that were significantly downregulated in response to both depletion of Mot1p and of NC2β were not enriched for the presence of a TATA box (19%, similar to the average of all genes). This relatively small gene group (43 genes) was dominated by the highly expressed ribosomal protein genes, explaining the high average expression level in this group (Fig. S2A).

Previous studies have shown that the presence of the TATA box at a gene promoter correlates with the binding of SAGA to the Upstream Activating Sequence (UAS) of that gene (3). To examine binding of SAGA and other basal transcription factors to the promoters of genes affected by Mot1p or NC2β depletion, we used our previously published dataset on the genome-wide DNA binding of basal transcription factors (42). As expected, binding of SAGA (using the Spt20p subunit) to the UAS was enriched in genes upregulated by depletion of Mot1p and NC2β (Fig. 2E). Downregulated genes did not show such enrichment. In contrast, promoter binding of TBP or TFIID (via the Taf1p subunit) was enriched in genes downregulated by depletion of Mot1p and NC2β, while upregulated genes showed no enrichment. Interestingly, both the upregulated and downregulated genes failed to show enrichment for binding of Mot1p or NC2β, indicating that the outcome of the transcriptional response to Mot1p or NC2β depletion is not determined by the degree of promoter binding of these factors. Rather, the outcome correlates with preferential binding of either SAGA or TFIID. Taken together, Mot1p and NC2β regulate highly overlapping sets of genes: 80% of these are repressed by Mot1p and NC2β, are enriched for TATA box DNA, and are preferentially SAGA-dominated, while 20% of genes are activated by Mot1p or NC2β, do not show enrichment for TATA box DNA, and are preferentially TFIID-dominated.

Mot1p and NC2β repress basal expression of HSP26 by inhibiting TBP and RNA pol II promoter binding

To investigate the repressive functions of Mot1p and NC2β in more detail, we analyzed the *HSP26* target gene. Basal expression of this gene is repressed by both Mot1p and NC2β, which can be detected 40-60 min after rapamycin treatment (Fig. 3A). Chromatin immunoprecipitation (ChIP) analysis of TBP binding to the TATA box present in the HSP26 promoter indicated that depletion of Mot1p resulted in higher TBP occupancy (Fig. 3B). Also, binding of RNA pol II to the TSS was increased after depletion of Mot1p, which is consistent with the observed increase in *HSP26* mRNA. Similar results were obtained for depletion of NC2β, except for TBP occupancy, which showed similar promoter occupancy in response to rapamycin treatment, perhaps due to the limited sensitivity of the TBP ChIP. This suggests that Mot1p and NC2β repress basal expression of *HSP26* by preventing promoter binding of TBP and/or RNA pol II.

Figure 2 *(next page)*. Anchoring Mot1p or NC2βp leads to highly overlapping changes in gene expression. Cells were treated for 60 min with rapamycin. Genome-wide mRNA expression analysis was performed using yeast oligonucleotide arrays (36). (A) Average expression levels from four measurements of each strain for each condition as indicated. Diagonal lines indicate 1.7 fold change. All hybridizations were performed against a reference sample of untreated wt cells (REF, X-axis). Expression values for the experimental strains are plotted on the Y-axes. (B) Venn diagrams showing genes with significant changes in gene expression relative to the reference pool (1.7 fold, $p<0.05$). Left panel: upregulated genes. Right panel: downregulated genes. P values of the overlap as tested by hypergeometric test are indicated. (C) Correlation plot showing the *MOT1-FRB* and *NC2β-FRB* experiments. Axes are in 2log scale. R2 value is indicated. (D) Analysis of enrichment for the presence or absence of a TATA box in the promoters of genes upregulated or downregulated in response to anchoring Mot1-FRBp and NC2β-FRBp. P values are indicated (hypergeometric test). (E) Analysis of Spt20p, TBP, Taf1p, Mot1p, and NC2β occupancy of genes upregulated or downregulated in response to anchoring Mot1-FRBp and NC2β-FRBp. Median binding profiles were taken from van Werven *et al* (26).


A
-RAPA
+RAPA
WT
MOT1-FRB
NC2β-FRB
REF
100
1000
10000
B
upregulated
downregulated
MOT1-FRB
NC2β-FRB
510
430
158
352
78
101
71
48
43
28
p<1x10-14
C
R² = 0.80704
D
MOT1-FRB and NC2β-FRB
upregulated
352
119
p= 0.4
219
p<1x10-14
TATA
TATAless
downregulated
43
8
p= 0.4
34
p= 0.12
E
Spt20p
TBP
Taf1p
Mot1p
NC2β
Average binding
+800
+1
ORF
upregulated
downregulated
all

Mot1p and NC2β repress *HSP26* expression during recovery from heat shock activation

To compare the functions of Mot1p and NC2β during activated transcription, we analyzed *HSP26* expression following heat shock induction under anchor-away conditions. In wild type yeast or anchor-away strains in the absence of rapamycin, the heat shock response of this gene showed a characteristic curve with initial increase ('activation') in expression to high levels (~75 fold induction), followed by downregulation ('recovery') of expression to moderately elevated levels compared to basal expression (Fig. 4A). Nuclear depletion of Mot1p or NC2β did not affect the timing or amplitude of the initial activation of expression (Fig. 4A). In contrast, depletion of Mot1p or NC2β prevented efficient downregulation of expression during the recovery phase, resulting in continuous high levels of HSP26 transcripts.

To determine how Mot1p and NC2β mediate transcriptional repression during heat shock activation, we performed ChIP-qPCR analysis of TBP and RNA pol II to the HSP26 promoter. In the absence of rapamycin, occupancies of TBP to the TATA box and RNA pol II to the TSS (Fig. 4B) were high during the activation phase, but were reduced 4-6 fold during the recovery phase, paralleling transcript levels (Fig. 4C). Anchoring of Mot1p or NC2β had only minor or no effects, respectively, on TBP and RNA pol II occupancies during the activation phase, while occupancies were increased during the recovery phase (Fig. 4C). This suggests that both Mot1p and NC2β repress HSP26 expression during the recovery phase of heat shock induction by removing TBP and thereby inhibiting promoter binding of RNA pol II.

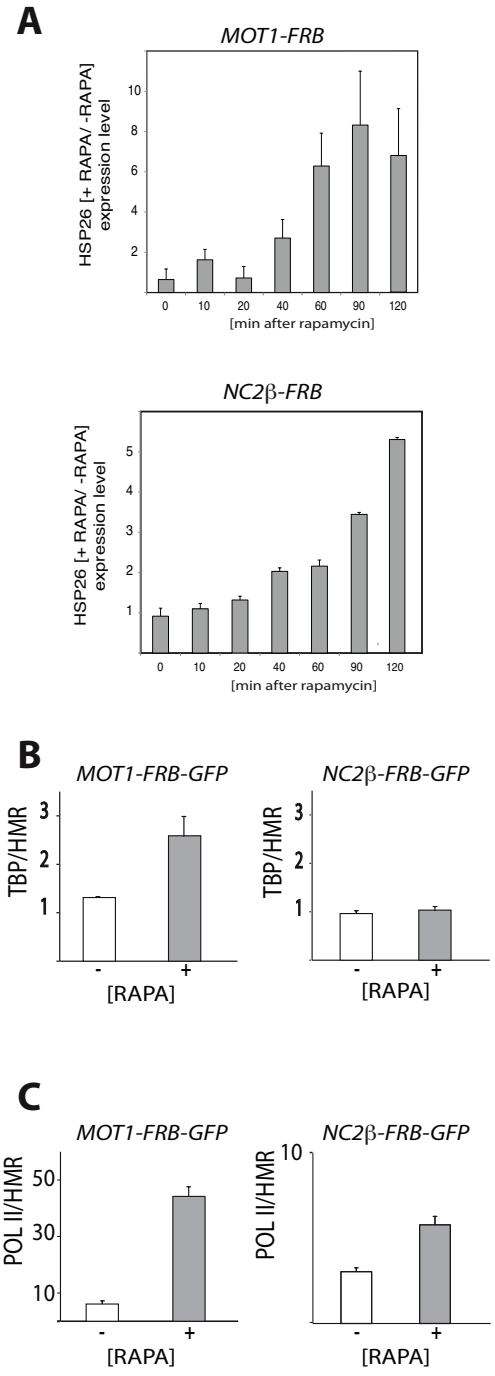


Figure 3. Effects of anchoring Mot1-FRBp or NC2β-FRBp on gene expression and promoter occupancy at the HSP26 gene under basal conditions. (A) Time course of the effect of rapamycin treatment on basal *HSP26* expression using RT-qPCR analysis. Data were normalized on *ACT1* transcript levels. The 60 min time point was used for the ChIP-qPCR analysis of Fig. 3B,C. (B) TBP occupancy was measured near the TATA box, which is located at nt -330/-210 relative to the open reading frame (primer B in Fig. 4B). (C) RNA pol II occupancy was measured at the transcription start site (primer C in Fig. 4B). Data were normalized to binding to the silent *HMR* locus.

Preferential genetic interaction between MOT1 or NC2β and the TBP-binding module of SAGA

Studies using *ts* alleles of *MOT1* have shown genetic interaction with the SAGA subunits *SPT3*, *SPT7*, *SPT8*, and *GCN5* (10, 25, 38). The multisubunit protein complex SAGA has dis-

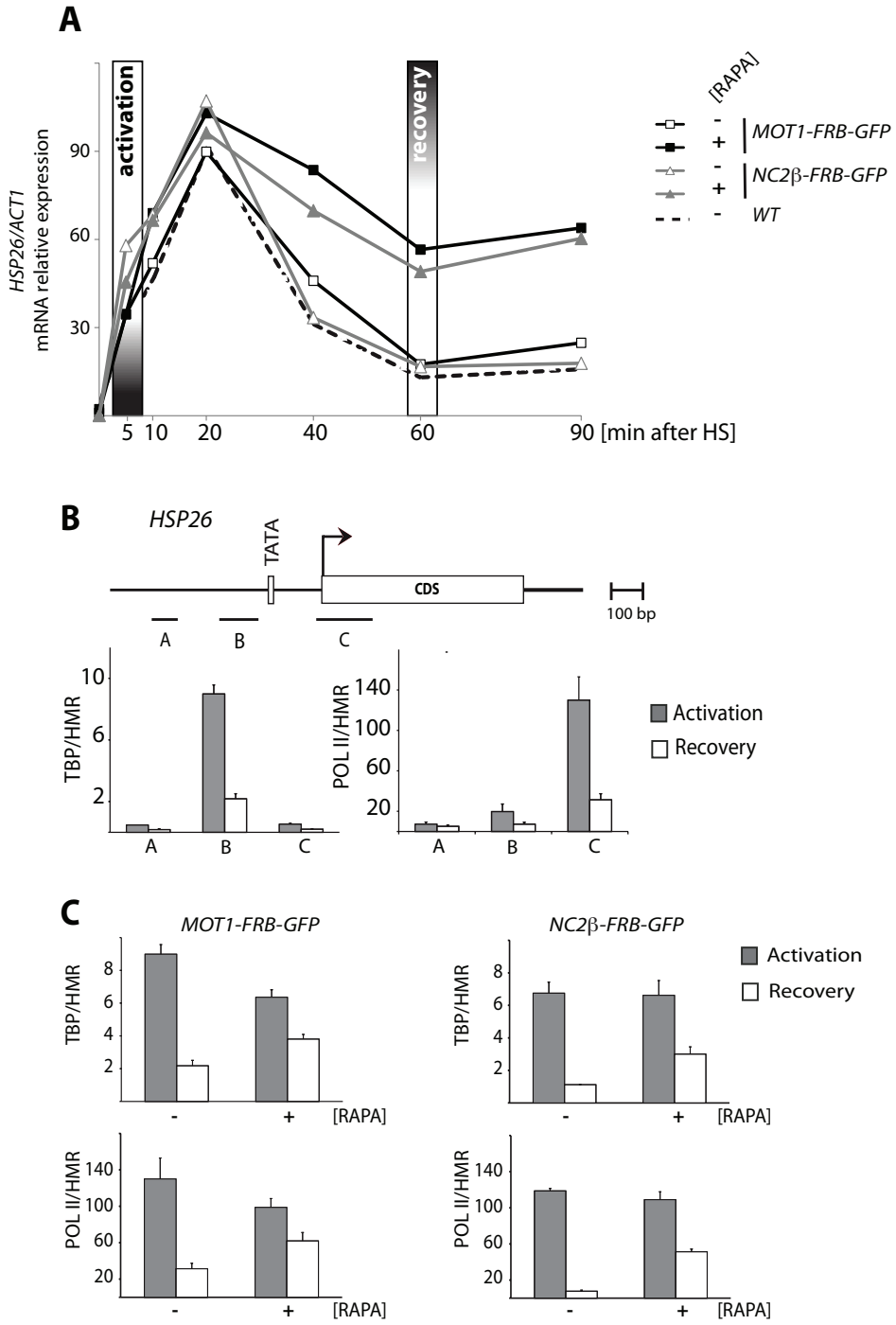


Figure 4. Effects of anchoring Mot1-FRBp or NC2β-FRBp on gene expression and promoter occupancy at the HSP26 gene under heat shock conditions. *MOT1-FRB* or *NC2B-FRB* strains were treated with rapamycin for 60 min, and subsequently heat shocked to 38°C. (A) RT-qPCR analysis of *HSP26* expression at the indicated time points after heat shock. Wild type (wt) yeast is shown for comparison. (B) ChIP-qPCR of TBP and RNA pol II at the HSP26 locus at the activation phase (5 min after heat shock) and the recovery phase (60 min after heat shock) in wt cells. Primer locations are indicated. (C) Effect of anchoring Mot1-FRBp or NC2β-FRBp on TBP and RNA Pol II occupancy.

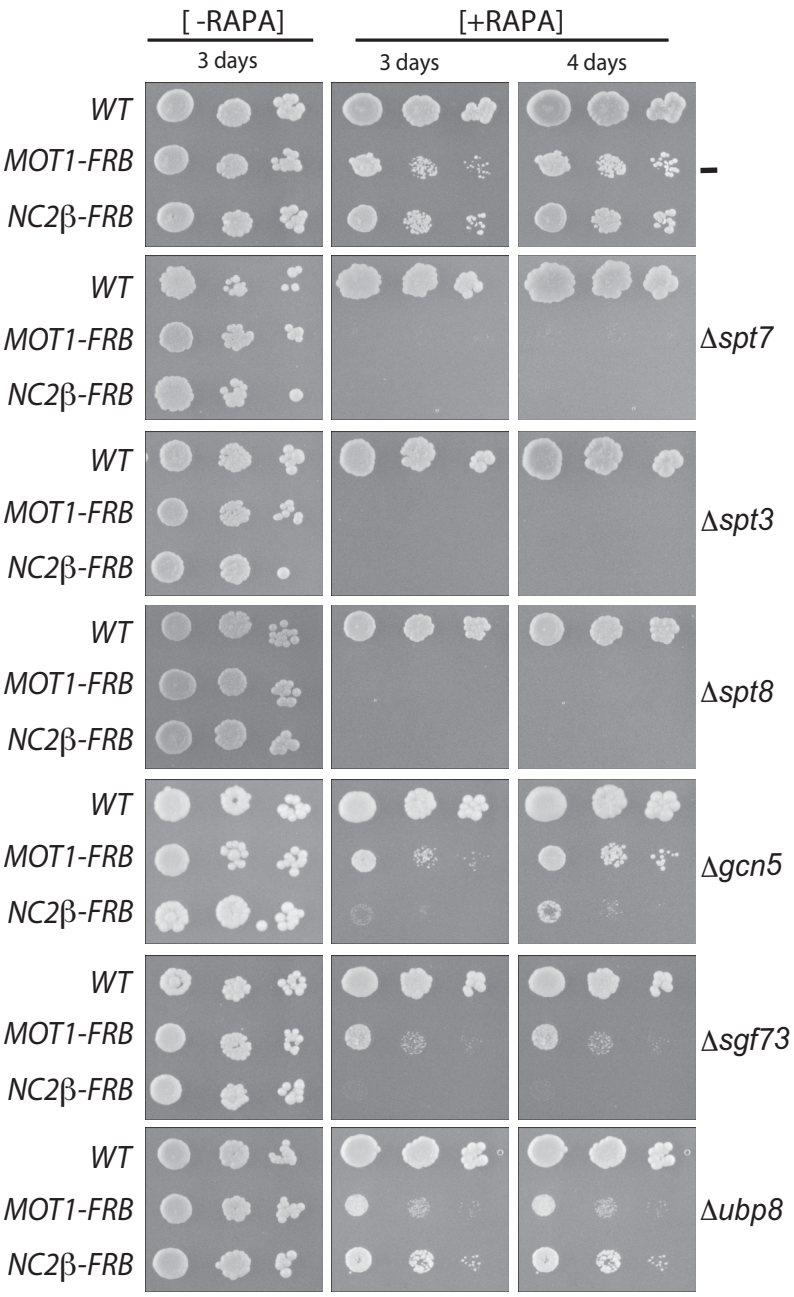


Figure 5. Both *MOT1-FRB* and *NC2β-FRB* display strong genetic interaction with the TBP binding module of SAGA. Spot assay on SC plates in the absence or presence of 1 μg/ml rapamycin. Genes encoding the indicated SAGA subunits were deleted in *MOT1-FRB, NC2β-FRB* or HHY168 parental strains. Dilutions were 10-fold. Plates were incubated at 30 °C for 4 days.

tinct functional modules, including a TBP-interaction module (Spt3p, Spt8p), a histone acetyltransferase (HAT) module (Gcn5p, Ada2p, Ada3p), a deubiquitination module (DUB) (Ubp8p, Sgf73p, Sgf11p), a structural module required for complex integrity (Spt20p, Spt7p), and a chromatin interaction module (Sgf73p) (5, 36). We were therefore interested to determine which SAGA module displays genetic interactions with *MOT1*. In addition, we tested the prediction that also *NC2β* genetically interacts with SAGA. To this end, deletion of subunits from different SAGA modules were made in *MOT1-FRB*, *NC2β-FRB* or the parental HHY168 strains (Fig. 5). Anchoring Mot1p or

NC2β in an Δspt7 background resulted in synthetic lethality, suggesting that both Mot1p and NC2β interact with the intact SAGA protein complex. Deletion of subunits of the TBP-interaction module (*SPT8* or *SPT3*) also resulted in synthetic lethality when combined with anchoring Mot1p or NC2β. Synthetic interactions with the HAT module (*GCN5*) or the DUB/chromatin interaction module (*UBP8* or *SGF73*) were also observed but were less severe. Within these modules, the severity of synthetic interactions differed between anchoring Mot1p and NC2β, suggesting that Mot1p and NC2β interact with SAGA in slightly distinct ways. Taken together, these results indicate that both *MOT1* and *NC2β* genetically interact with SAGA, and that they both preferentially interact with the TBP-interaction module.

Discussion

Here, we have applied the anchor-away technique to analyze the effects of conditional depletion of TBP regulators on gene expression. We find that Mot1p and NC2β regulate highly overlapping sets of genes. Regulation correlated with preferential binding of SAGA (for negative regulation) or TFIID (for positive regulation) to promoters. Analysis of the heat shock gene HSP26 indicated that Mot1p and NC2β repress both basal and induced expression by inhibiting promoter binding of TBP and RNA pol II. MOT1 and NC2β synthetically interacted with SAGA, and showed preference for the TBP interaction module. These data support the model that Mot1p and NC2β tightly cooperate to regulate TBP promoter binding and gene expression.

Application of the anchor-away technique to Mot1p and NC2β

In line with the original publication (19), we found that the anchor-away approach can be successfully applied to study the function of essential genes. Advantages of this technique included the fact that it is inducible, and that it avoids the use of heat shock, making it particular suitable for the study of stress-related events. This is well exemplified by the strong genetic interactions observed for *MOT1* and *NC2β* with *SPT7*, *SPT3*, and *SPT8* (Fig. 5). It is also one of the fastest techniques available for conditional functional interference. Anchor-away-mediated nuclear depletion of NC2β or Mot1p was induced 10 to 20 minutes after rapamycin treatment, respectively (Fig 1D). The difference in timing between NC2β and Mot1p may be explained as follows. A key feature in the anchor-away system is the abundance of the anchor protein compared to the targets (19). The numbers of copies per cell of Mot1p (6.56x103) and NC2β (2.95x103) are significantly lower than the anchor Rpl13Ap (1.33x105)(14). Nevertheless the nuclear concentration of target protein in the two strains is different which might affect their timing of nuclear depletion. In addition it has been shown that nuclear export efficiency correlates with the size of the cargo (44). The anchor trimeric complex (anchor-target-rapamycin) containing the 210 kD Mot1p is considerably larger than the complex containing the 17 kD NC2β, which may also explain why Mot1p delocalization is slower compared to NC2β. Unfortunately, NC2α proved to be incompatible with the anchor-away tag, suggesting that the tag interfered with the function of the essential NC2α protein.

We also noted that nuclear depletion of Mot1-FRB-GFPp and NC2β-FRB-GFPp was not complete as evidenced by residual nuclear GFP staining. This was the case even after 60 min of rapamycin treatment (Fig. 1D), while longer treatment showed similar results (data not shown). This is in line with the observation that cells survived the nuclear depletion of Mot1p and NC2β while these proteins are essential for viability. The delayed growth phenotype suggests that the nuclear concentrations of functional protein are significantly reduced, but that a minimal level remains to sustain cell growth.

Mot1p and NC2β regulate expression of similar sets of genes

Individual microarray profiles have been published for *MOT1* (9, 43) and *NC2 ts* alleles (6, 11, 27).

This has indicated that both Mot1p and NC2 are involved in transcriptional repression and to a lesser extent in transcriptional activation. In silico comparions have revealed significant overlap between the profiles of Mot1p and NC2α (3, 9). Here, we compare the NC2β and Mot1p profiles in identical experimental settings. This reveals that the degree of overlap between the two profiles is very high, and correlates with an R2 value of 0.8. All together, this argues that Mot1p, NC2α, and NC2β cooperate to regulate a common set of target genes.

Regulation by Mot1p and NC2β depends on the promoter sequence and SAGA versus TFIID occupancy

Previous analysis indicated that Mot1p and NC2α-repressed genes are enriched for the TATA box, while activated genes are not (3). Here we extend this with the conclusion that NC2β-repressed genes also have this property. Surprisingly, negative or positive regulation by Mot1p and NC2β did not depend on the strength of their promoter occupancies (as defined in van Werven *et al*. (42)), but rather depend on promoter occupancies of transcriptional activators: genes with high SAGA occupancy were preferentially repressed, while genes with high TFIID occupancy were preferentially activated by Mot1p and NC2β. In both types of genes, expression correlated with the binding of all basal transcription factors tested, including activators (TFIID, SAGA) and repressors (Mot1p, NC2)(42). We propose that regulation by Mot1p and NC2β is likely to be an intrinsic property of the promoter, and depends on which factors are involved in TBP recruitment: TFIID or SAGA. While repression of transcription by Mot1p and NC2 can be explained by their capacity to remove TBP from TATA box DNA and/or to block RNA pol II PIC assembly, activation of transcription as reported here and in a number of previous reports is more difficult to understand. Proposed mechanisms include recruitment of TBP to promoters (1), formation of an alternative PIC, in which Mot1p replaces TFIIA (13), formation of an alternative TBP complex (10), and relieve of inhibition by TBP on TATA box containing promoters in Drosophila (21). It will be interesting to further investigate the mechanisms involved in future experiments.

Mot1p and NC2β are required for basal and activated HSP26 expression

A more detailed analysis of one of the upregulated genes confirmed that basal expression of the HSP26 gene was upregulated upon Mot1p as well as upon NC2β depletion (Fig. 3A, Fig. S3). Fold inductions reached between 5 and 8 fold for NC2β or Mot1p depletion, respectively. Previous analysis of *HSP26* expression in *mot1-14* or *mot1-42* did not show effects on basal expression at the non-permissive temperature (10). Possibly, this is caused by differences in experimental approaches and/or genetic backgrounds. The increased basal expression of *HSP26* following Mot1p or NC2β depletion was accompanied by increased promoter occupancies of TBP and RNA pol II (Fig. 3B, C). This is in agreement with impaired removal of TBP and subsequent RNA pol II from the *HSP26* promoter upon Mot1p or NC2β depletion. As a result, the balance between TBP association and dissociation that normally operates during basal gene expression may be shifted towards TBP association, leading to a total increase in transcription.

A similar mechanism may apply during heat shock induction of *HSP26* expression. Induction of this gene peaks strongly at 20 min, after which this gene is downregulated (Fig. 4A: 'recovery'). While Mot1p or NC2β depletion did not affect the activation, it inhibited the subsequent downregulation of transcription (Fig. 4A). A concomitant increase in TBP and RNA pol II occupancy was seen during this recovery phase, suggesting that the effects of Mot1p/NC2β depletion were mediated on PIC assembly and not on mRNA stability. Previous analysis of *HSP26* expression using *mot1-14* or *mot1-42* strains yielded a similar conclusion as presented here, but found in addition that Mot1p is also required for the initial activation (10). This may be explained by the use of different genetic backgrounds (ts alleles versus anchor-away strains) and technical procedures to induce heat shock. In our induction, heat shock was performed at 38°C, whereas in Dasgupta *et al*. (10), 35°C was used.

Using mot1-1 cells, promoter occupancies of TBP and NC2 were also increased during repression of the *HXT2* gene after activation by glucose shift (42), suggesting a more general role for Mot1p/NC2 in gene repression following activation.

Genetic interactions of MOT1 and NC2β with SAGA modules

Depletion of Mot1p or NC2β resulted in preferential synthetic lethality with deletions of the TBP-interaction module of SAGA, which consists of Spt3p and Spt8p (28). This is in line with previous reports on the genetic interaction between *SPT3* or *SPT8* with *MOT1 ts* mutant alleles (10, 25, 39). To our knowledge, this is the first demonstration that *NC2β* behaves like *MOT1* in this respect. We extended the analysis of genetic interactions to address the requirement for an intact SAGA complex, and to test interactions with other SAGA modules including those involved in histone acetylation, histone deubiquitination, and chromatin interaction. The strong synthetic lethality of *MOT1-FRB* and *NC2β-FRB* with *Δspt7* indicates that the intact SAGA complex is required, since this subunit is required for complex integrity (4). The synthetic interaction of *MOT1-FRB* and *NC2β-FRB* with GCN5, in agreement with previous reports using MOT1 ts mutants (10, 38), suggests that SAGA-mediated histone acetylation is important for both Mot1p and NC2β function. *NC2β-FRB* and *MOT1-FRB* also showed synthetic lethality with *Δsgf73*. This subunit has recently been shown to bind H2A/H2B heterodimers (5). The stronger synthetic interaction of NC2β-FRB compared to *MOT1-FRB* with two SAGA subunits involved in chromatin acetylation (*GCN5*) and binding (*SGF73*) suggest that NC2β may be critically involved in this aspect of gene regulation. In contrast, *MOT1-FRB* displayed a slightly stronger interaction with *UBP8* compared to *NC2β-FRB*. This subunit provides the enzymatic activity of the histone deubiquitination (DUB) module of SAGA (20), suggesting that DUB activity is required for Mot1p function. In conclusion, both *MOT1-FRB* and *NC2β-FRB* show synthetic lethality with distinct functional modules of SAGA, of which the TBP interaction module is the most critical module required. It will be interesting to determine the molecular mechanism of the interplay between Mot1p, TBP, and NC2 and the various functional modules of SAGA in gene regulation.

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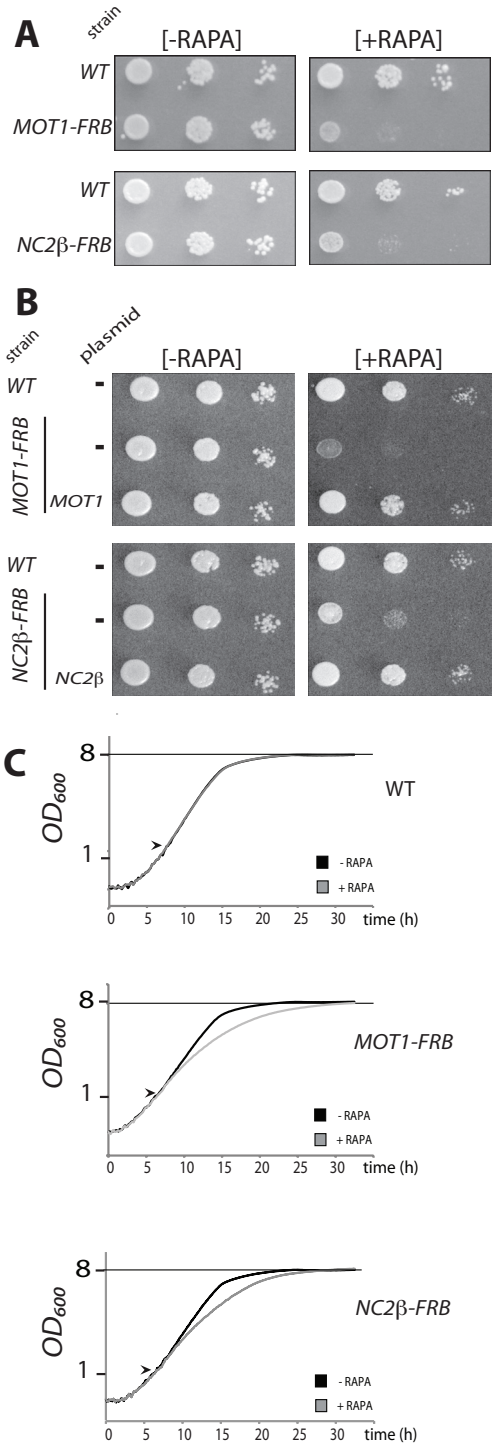
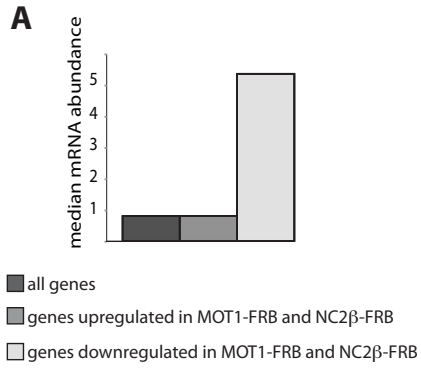
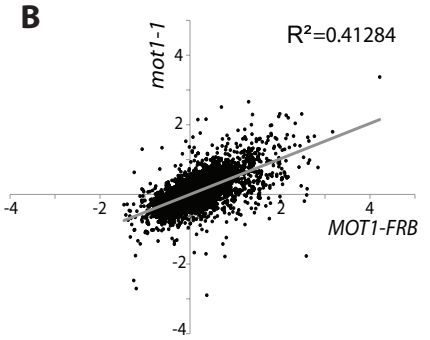
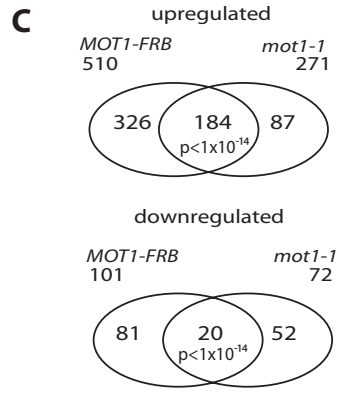
Supplemental Material



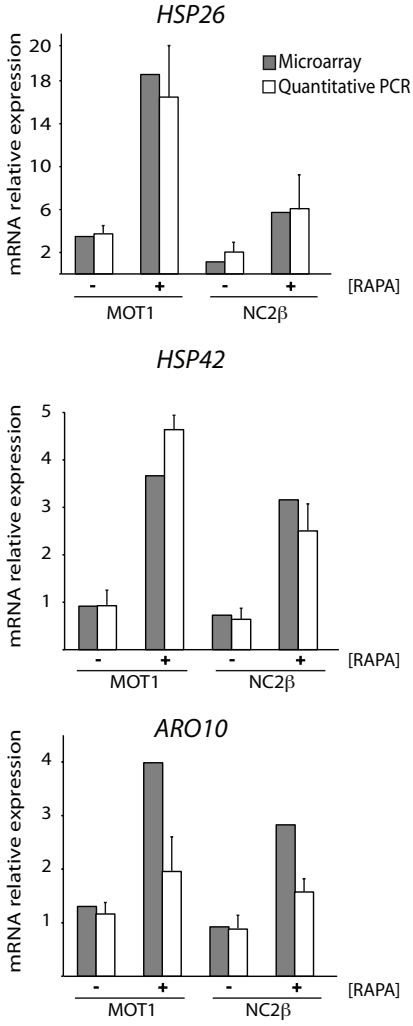
Supplemental Figure 1. Anchoring Mot1p or NC2β using FRB tagging without GFP. (A) Spot assay on SC plates in the absence or presence of 1 μg/ml rapamycin. The parental strain HHY168 was used as negative control (wt). Dilutions were 10- fold. (B) Complementation of *MOT1-FRB* or *NC2β-FRB* with untagged, galactose inducible *MOT1* or *NC2β*, respectively. Spot assay as in (A) on SC plates in the absence or presence of 1 μg/ml rapamycin. The parental strain HHY168 containing the empty vector pRS303 was used as negative control. (C) Growth in liquid culture. Note that the timing of rapamycin addition was different from that in Fig. 1C. In Fig 1C, rapamycin was added from the beginning of the growth curve. Here, rapamycin was added when cells reached 1 OD600 (arrow head). In both cases, the growth curve was started from an overnight, saturated culture diluted in CSM to 0.1 OD600. Growth was monitored for 2 days by automatic cell counting using Infinite 200 incubator.







Supplemental Figure 2. Genome-wide changes in gene expression by anchoring Mot1p or NC2β. (A) Genes downregulated by anchoring Mot1p and Nc2β are highly expressed, while upregulated genes have average expression levels. Median expression levels were taken from Holstege *et al* (1). (B) Correlation plot for the *MOT1-FRB* and previously published *mot1-1* datasets (2). Axes are in 2log scale. R2 value is indicated. (C) Venn diagrams of genes with significant changes in gene expression (1.7 fold, p= 0.05) in *MOT1-FRB* and *mot1-1* datasets. Left panel: upregulated genes. Right panel: downregulated genes.



Supplemental Figure 3. Validation of microarray data. Microarray data were taken from the experiment shown in Figure 2, and represent expression levels relative to the wt reference. RT-qPCR is expressed as fold change over ACT1 mRNA, and was performed on three independent biological replicas. Error bars indicate S.D.

Supplemental Table 1.

name	genotype	source
W303-1B	Matα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15	
HHY168	Isogenic to W303-1B except tor1-1 fpr1::NAT rpl13A-2×FKBP12::TRP1	Euroscarf
YGP200	Isogenic to HHY168 except *MOT1-FRB::HIS3*	This study
YGP201	Isogenic to HHY168 except *NC2β-FRB::HIS3*	This study
YGP202	Isogenic to HHY168 except *MOTt1-FRB-GFP::HIS3*	This study
YGP203	Isogenic to HHY168 except *NC2β-FRB-GFP::HIS3*	This study
YGP204	Isogenic to YGP202 except pRS303	This study
YGP205	Isogenic to YGP202 except pMAC-81 *MOT1*	This study
YGP206	Isogenic to YGP203 except pRS303	This study
YGP207	Isogenic to YGP203 except pYES2-*YDR1*	This study
YMK100	Isogenic to HHY168 except *Δspt7::KAN*	This study
YMK101	Isogenic to HHY168 except *Δspt3::KAN*	This study
YMK102	Isogenic to HHY168 except *Δspt8::KAN*	This study
YMK103	Isogenic to HHY168 except *Δgcn5::KAN*	This study
YMK104	Isogenic to HHY168 except *Δubp8::KAN*	This study
YMK105	Isogenic to HHY168 except *Δsgf73::KAN*	This study
YMK106	Isogenic to YGP200 except *Δspt7::KAN*	This study
YMK107	Isogenic to YGP200 except *Δspt3::KAN*	This study
YMK108	Isogenic to YGP200 except *Δspt8::KAN*	This study
YMK109	Isogenic to YGP200 except *Δgcn5::KAN*	This study
YMK110	Isogenic to YGP200 except *Δubp8::KAN*	This study
YMK111	Isogenic to YGP200 except *Δsgf73::KAN*	This study
YMK112	Isogenic to YGP201 except *Δspt7::KAN*	This study
YMK113	Isogenic to YGP201 except *Δspt3::KAN*	This study
YMK114	Isogenic to YGP201 except *Δspt8::KAN*	This study
YMK115	Isogenic to YGP201 except *Δgcn5::KAN*	This study
YMK116	Isogenic to YGP201 except *Δubp8::KAN*	This study
YMK117	Isogenic to YGP201 except *Δsgf73::KAN*	This study

Supplemental Table 2.

Primer name	sequence 5'-3'	Description
MOT1-Fw	CTCAATACGAGGAGGAGTATAATTTAGACACCTTCAT-CAAAACTTTACGA CGGATCCCCGGGTTAATTAA	FRB/FRB-GFP tagging
MOT1-Rv	ATAAAACAAAAATGACCTTGTATACGCGTCATTCCAAT-GCAAGAATTTGTGAATTCGAGCTCGTTTAAAC	FRB/FRB-GFP tagging
YDR1-Fw	GATTACACCACAATAGTGTATCTGATCCGGTTAAGTCG-GAGGATTCTTCT CGGATCCCCGGGTTAATT AA	FRB/FRB-GFP tagging
YDR1-Rv	CCATGTGGTTTATTTTCGTATATAGCATTACTTTGTTTA-GAAGCTTCTATGAATTCGAGCTCGTTTAAAC	FRB/FRB-GFP tagging
HSP26-A Fw	CCGTGTGTACCCCTAACTCC	qPCR-ChIP
HSP26-A Rv	GACGGTTACTATTCCGATCCA	qPCR-ChIP
HSP26-B Fw	TGGTTATTCCCTCCCCCTTA	qPCR-ChIP
HSP26-B Rv	GCATAGATCCGCAAGGACAT	qPCR-ChIP
HSP26-C Fw	AAGAGGCTACGCACCAAGAC	qPCR-ChIP
HSP26-C Rv	ATCATAAAGAGCGCCAGCAT	qPCR-ChIP
HMRF-RT	ACGATCCCCGTCCAAGTTATG	qPCR-ChIP
HMRR-RT	CTTCAAAAGGAGTCTTAATTTCCCTG	qPCR-ChIP
ACT1-F-RT	TACCACCATGTTCCCAGGTATTG	qPCR-RT
ACT1-R-RT	AGCCAAGATAGAACCACCAATCC	qPCR-RT
HSP26-F-RT	GTCATCACTTTGCCAGACTACCC	qPCR-RT
HSP26-R-RT	TGGTTCTTACCATCCTTCTGAGG	qPCR-RT

Supplemental Table 3.

plasmid	source	Description
pFA6a-FRB-His3MX6	Euroscarf	FRB tagging
pFA6a-FRB-GFP-His3MX6	Euroscarf	FRB-GFP tagging
pRS306	This study	Empty Vector / Complementation Assay
pMAC81-MOT1	This study	MOT1 vector /Complementation Assay
pYES2-YDR1	This study	YDR1 vector / Complementation Assay

Supplemental references

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2. Lenstra, T.L., Benschop, J.J., Kim, T., Schulze, J.M., Brabers, N.A., Margaritis, T., van de Pasch, L.A., van Heesch, S.A., Brok, M.O., Groot Koerkamp, M.J. *et al*. The specificity and topology of chromatin interaction pathways in yeast. Mol Cell, 42, 536-549.

Chapter 6

Discussion

Biochemical studies have shown that the recruitment of TBP to the promoter represents the first rate-limiting step in transcription initiation. TBP binding to DNA is essential for PIC nucleation and RNA pol II recruitment. In this light the TBP delivering and removing activities, which are present in TBP-binding complexes, execute a prominent role in the transcriptional process. In this thesis, we described a biochemical and genetic characterization of TBP-binding complexes. Here, the possible implications of these findings on the regulation of TBP activity and on transcriptional control are discussed.

Regulation of transcription function by posttranslational modification of SAGA subunits

TFIID and SAGA: assembling a core

TFIID and SAGA share a similar transcriptional function and nuclear organization (10, 13, 15, 19, 32). My interest has been to focus on possible mechanisms of interplay between the two complexes in gene regulation. A central question in the assembly of the multi-subunits TFIID and SAGA is whether the two complexes originate from the same structural core. Therefore, the decision to assemble one complex rather than the other might be mediated by posttranslational modification either on the core or peripheral subunits. This has been investigated by performing a PTM profiling of the SAGA and TFIID complexes (Chapter 3).

We found that shared subunits of the complexes are extensively posttranslationally modified. Particularly interesting, the shared Taf5p subunit was found to be differentially phosphorylated and acetylated when present in TFIID compared to SAGA: the S411, S414 and S415 residues were uniquely phosphorylated in SAGA-bound Taf5p, whereas the K103 residue was acetylated in TFIID-bound Taf5p. It is important to note that the pseudosymmetrical shape of the core structure of the two complexes is orchestrated by a Taf5p N-terminal dimerization domain (32, 37). In contrast, the C-terminal WD40 propeller structure of Taf5p interacts with the HFD-mediated heterodimers between Taf6p and Taf9p that are present in two copies in both TFIID and SAGA complexes (31). Interestingly two TAFs have different dimer partners in TFIID versus SAGA (Taf10p and Taf12p), suggesting a mechanism of complex assembly by interaction with specific subunits (21, 32, 37). We observed that the differentially phosphorylated residues of Taf5p localize next to the WD40 repeats at the C-terminus of the protein. In this context the Taf5p modification might represent a mechanism to prevent or facilitate the recruitment of a specific HFD pair and thus the formation of TFIID or SAGA.

Is Taf5p phosphorylation involved in SAGA structure/function?

We hypothesized that phosphorylation of TAF residues is important for the assembly of the SAGA complex. To test this, a genetic analysis of phospho-preventing mutants at the S411, S414, S415 residues of Taf5p was performed. As read out, the viability of the mutant strains on selective media was used. This genetic test is commonly used to detect SAGA-phenotypes. Unfortunately, the selected phosphomutants did not show any differences in viability. This result does not exclude that the Taf5p PTM pattern is important for a more specific structural role. It is possible that the PTM pattern in SAGA recruits a peripheral subunit and that it is important for the assembly of one of the functional modules of the complex. In this case, changes in the complex composition would not be observed using our genetic read out. For instance, changes in the recruitment of the HAT or DUB module would be silent with the method used. In order to test, further experiments would be needed. A proteomic approach would for instance clarify whether the prevention of phopsphorylation at S411, S414, and S415 residues is important for the binding of subunits to the complex. Purification of SAGA from phospho-preventing mutants coupled to mass spectrometry analysis could identify changes in the subunit composition of the complex. An alternative scenario is that the PTM pattern forms a platform for the interaction with an activator or other transcriptional factors on a promoter. Since a gross population of the SAGA complex has been found to be phosphorylated on Taf5p, we presume that a possible interactor might have more general functions in transcriptional regulation. This might be identified by gene expression profile analysis and correlation analysis with a published deleteome dataset.

Could gene expression reprogramming affect the TFIID/SAGA balance or vice versa?

The differential PTM pattern on shared subunits (Chapter 3) has suggested alternative models of

transcription regulation. Although never investigated, it is possible for instance that during the SAGA-mediated transcriptional response, a shift in concentration from TFIID to SAGA might take place. This might occur not by de novo synthesis of all the necessary subunits but by a disassembly/re-assembly process. In this case the core structure of the TFIID complex could be used to assembly SAGA. The balance mechanism might be controlled by protein degradation or posttranslational modifications. In the first case the modulation of the concentration of specific subunits of one of the two complexes might be the limiting step in complex formation. Alternatively, the control might occur via posttranslational modification of the subunits of the complexes, which would allow the assembly of one complex rather than the other. It has been shown in yeast and mammals that conditional destabilization/degradation of a TAF core subunit of TFIID leads to the degradation of the other subunits of the core complex (28, 36). However knock down of the TAF1 subunit does not interfere with the protein half life of the core subunits (36). In yeast the deletion or overexpression of the specific SAGA subunit *SPT7* leads to growth defect (16). The deletion causes the disruption of the SAGA complex and therefore the misregulation of 10% of the genes in the yeast genome, and the phenotypic effect.
On the other hand the effect of overexpression might be mediated by a toxic unbalance of SAGA versus TFIID concentrations. The reduction of TFIID would indeed lead to severe transcription effect and then to a slow growth phenotype. The study of the stability of core or specific subunits of SAGA or TFIID using conditional degradation would be interesting to pursue. Moreover, it might be possible that specific posttranslational modifications are responsible for protein stability of the TFIID or SAGA subunits. Therefore, it would be interesting to explore the assembly and disassembly of TFIID/SAGA subunits using phosphomimicking or preventing mutations.

Does lysine acetylation of large stretches within Sgf73p and Spt7p mediate interaction of SAGA with chromatin?
In Chapter 3 we described that Sgf73p and Spt7p are acetylated at multiple K residues organized in stretches. It is interesting to note that these stretches localize to domains of the proteins that mediate (or might mediate) the interaction between SAGA and chromatin. Specifically, the stretch K171-K300 in Sgf73p is partially overlapping with the SCA7 domain of Sgf73p, which has been shown to be involved in nucleosome interaction (2). Furthermore, the K584-K680 stretch of Spt7p resides in a pseudobromo domain (38). Although the function of this domain has not been established with certainty, it might be involved in the interaction with acetylated nucleosomes. It is easy to imagine that acetylation of Sgf73p and Spt7p might have an important role in the transcriptional function of SAGA. However, strong functional data on the interaction of Spt7p and Sgf73p with nucleosomes is lacking, and therefore the modification of these domains might change the affinity of the complex for chromatin. For instance, acetylation of the K stretches could be a mechanism to release the complex from chromatin. Such a scenario could occur in a dynamic fashion in which SAGA is recruited to and released continuously from promoter DNA in order to open up chromatin structure and to allow PIC assembly. It would be interesting to identify the HAT activity responsible for acetylation of the stretches. One possible candidate is Gcn5p. This could be tested by mass spec analysis of SAGA from a *GCN5* null mutant. However, it cannot be excluded that acetylation of the stretches does not have a specific functional role.

Specificity of the Spt7p C-terminal truncation
In Chapter 3 and Chapter 4 another posttranslational modification occurring on the SAGA complex has been extensively described and characterized: the Spt7p truncation. The mapping of the truncation site in the C-terminus of Spt7p protein occurs in a region previously described, namely truncation region (38).
The biochemical approach described in Chapter 4 has allowed to identify the aspartic protease

Pep4p to be responsible for the Spt7p truncation. We show that purified Pep4p is able to specifically and uniquely cleave Spt7p within the SAGA complex. This result confirmed the high specificity of the protease for this truncation process. Although Pep4p does not recognize a specific sequence, analysis of the cleavage sites on known substrates shows a preference for hydrophobic residues in the cleavage region. Using an in silico analysis, a significant match with the residues in the mapped Spt7p cleavage site was not found (data not show). We were then wondering how such specificity might be achieved. In fact, since Pep4p is a degradative enzyme in the yeast vacuole, we might expect that the protease might cleave polypeptides non-specifically. How then can this specificity be explained?

First, although Pep4p is a degradative protease, the nuclear pH of the reactions is not at the optimum for Pep4p. Assuming that Spt7p cleavage occurs in the nucleus, this means that the protease activity is far less than in the vacuole. Such a mechanism to use suboptimal pH reaction conditions *in vivo* could be used to better control substrate processing. Although the protease might have a lower activity in the nucleus, this does not fully explain the target specificity.

Possibly the Pep4p protease cleaves Spt7p because this is one of the largest subunits in the complex. According to this argument, a truncation of the far larger Tra1p subunit should be observed. However, migration and integrity of Tra1p seems not to change upon incubation of SAGA with the Pep4p protease.

A related argument might be that Tra1p might have a globular structure whereas Spt7p C-terminus could be more unstructured and protruding from the complex. To address this point, more structural information about the proteins would be needed. However, it is possible that the tertiary structure organization plays a more important role in the protease specificity/recognition than the primary amino acid sequence of the Spt7p C-terminus. It is known that the truncation region localizes between an N-terminal part with an important structural function for the SAGA complex and a C-terminal region containing the Spt8p binding domain. A deletion of 50 aa has been shown to abolish the cleavage of Spt7p. However, smaller regions (20 or 10 aa) around the mapped truncation site did not abolish the cleavage (data not shown). A confirmation of such a protection mechanism might be provided by the experimental result presented in Chapter 4. The modulation of the activity of the Pep4p protease on Spt7p possibly by steric hindrance of Spt8p is shown. Based on this, I propose that a large unstructured accessible region (50 aa) of Spt7p localizes between two protected regions (N-terminal and C-terminal). Only when the complete region is removed the protected parts of Spt7p remain, thus preventing the targeting of the protease.

Possible mechanisms of SLIK complex formation

We suggest in Chapter 4 that the SLIK complex is formed by interaction of SAGA with Pep4p. As discussed, the high specificity of Spt7p cleavage results in loss of the C-terminus and of the Spt8p subunit from SAGA. It is likely that this happens in the nucleus? In order to achieve that, the protease Pep4p has to be imported in the nucleus from the vacuole or cytosol. Although there are no known mechanisms of protease import from the cytosol to the nucleus, there are supporting data for this hypothesis. For instance, in mammals the lysosomal Cathepsin L has been shown to have a role in nucleosome tail clipping, whereas the Cathepsin D is implicated in the response to apoptosis (7, 8, 11). The protease should be actively imported to the nucleus, for instance by binding to a functional partner.

Alternatively, it might also be possible that the protease reaches the nucleus by a passive diffusion mechanism from the vacuole, which implies that the Pep4p might be released from the vacuole (22). The vacuolar release might be active or passive. It is possible that a mechanism of membrane externalization exists, or that the protease might diffuse to the cytosol upon vacuolar leakage. The latest mechanism has been hypothesized already for Cathepsin D (27). In this context, a feedback mechanism could be imagined: a stress or environmental condition could trigger the vacuolar leak-

age; this would allow Pep4p to diffuse to cytosol and nucleus; Pep4p would affect SAGA transcriptional function thus activating/repressing genes; the new transcriptional program would be used by the cell to respond to the initial stimulus. The feedback mechanism model could be supported by knowing the SLIK-dependent transcriptional program. Unfortunately, no relevant information exists on this matter so far.
An alternative mechanism for SLIK formation has been hypothesized (12). The authors argue that Spt7p truncation occurs also in the absence of an integral SAGA complex by identifying Spt7p-cleaved forms in *Δada1* and *Δspt20* yeast extracts. Thus, they propose that Spt7p is first cleaved and then assembled in the SLIK complex. Even though this seems to be a possible mechanism, the supporting data are not sufficient. Moreover, although the null mutations of *ADA1* and *SPT20* result in SAGA complex disruption, Spt7p forms a partial complex with Spt8p and other subunits (38), which might still be recognized by Pep4p. It would be interesting in the future to explore the mechanisms of SLIK formation. A possible approach would be to study Pep4p localization and the SAGA-Pep4 interaction by microscopy. Pep4p recruitment to gene promoters could be studied by Chromatin Immunoprecipitation.

Does SLIK exist in humans?
The characterization of the TFIID and SAGA transcriptional complexes in humans is not as extensive as in *S. cerevisiae*. This is obviously linked to the complexity of the transcriptional processes in the two model organisms. However, it would be interesting in the future to investigate the existence and function of the SLIK complex in human cells. Support for this interest comes from the fact that the human hSPT7 subunit of hSAGA contains a C-terminal Spt8p-binding domain, and that the human Cathepsin D protease is highly homologous to the scPep4p protease (38). As mentioned before, indirect evidence indicates that the cathepsin protease is imported in the nucleus in human cells. This would support the speculation that SLIK formation occurs by a conserved mechanism.

A functional landscape for SLIK formation
It is known that the truncation of Spt7p leads to loss of the Spt8p from the TBP module of SAGA (38). This functional module of the complex is of great interest. Recent genetic and biochemical results strongly suggest that SAGA has a main role in TBP-delivery at promoters (17, 23, 30).
Apart from the chromatin-modifying features of the complex, several mechanisms of action of SAGA can be suggested in relation to the TBP-delivery function. The formation of SLIK might play a relevant role herein. It has been recently shown that SAGA and TFIID are recruited sequentially at specific promoters during the stress response. SAGA is tethered first, allowing a change of the chromatin structure and a fast transcriptional response. Second, TFIID is recruited and is needed for the establishment of the maintenance phase of transcription. In this scenario one could imagine that TBP has to be released from SAGA and to be available for the second step of the transcriptional response (10). This could be achieved by truncation of Spt7p and release of Spt8p-TBP from SAGA. Thus, SLIK formation would be needed to inactivate the SAGA response and allow the TFIID-mediated maintenance.
Another speculative scenario comes from integrating our results with those from Berger *et al*., (1). They found that Spt8p inhibits gene expression *in vivo* at certain promoters. This result is supported by biochemical evidence for competition of Spt8p with the DNA for TBP binding (30).
They propose that SAGA is recruited to the promoter; the truncation of Spt7p releases TBP, which will nucleate the PIC; the newly formed SLIK is then translocated to the gene body where its chromatin-modifying features are needed for optimal transcription. It is possible that this mechanism involves the controlled cleavage of Spt7p at the promoter by Pep4p. However, the mentioned mechanism would apply only for specific genes and cannot be a general mechanism of SAGA function. The finding that supports this genetically is that SLIK-mimicking mutations do not result in the

spt- phenotype that has been described for *SPT7* or *SPT20* null mutations. Therefore, the function of SLIK should be analyzed at particular promoters identified by genome-wide studies, or by phenotypic analysis upon stress stimulation.

Could SLIK be formed during post-lysis experimental procedures?

Many pieces of the final picture of SLIK transcriptional function are lacking. In this context, many hypotheses have been suggested, but not any of these has been supported and followed up by experimental data. Examples are: SLIK as a complex with overlapping function with SAGA (38); SLIK as the inhibited version of SAGA (1); SLIK as a complex involved in the retrograde response via interaction with Rtg2p (26). The finding that Pep4p is the protease responsible for SLIK formation has posed some doubts on the existence of the complex itself. This rises from the fact that Pep4p is well known as a degradative protease.

Could SLIK formation happen during/after the lysis of cells as part of the extraction procedure? It might indeed happen that the contact of the nuclear and vacuolar protein contents would lead to the truncation of Spt7p by Pep4p. We have tested this by pre-incubation of the cells with aspartic or cocktail protease inhibitors prior to the harvesting and lysis. The results showed that in this condition the Spt7p truncation was not abolished, suggesting that the Pep4p activity on SAGA was exerted prior to the start of the cell extraction protocol (data not shown). This suggests that Pep4p-mediated truncation of Spt7p occurs *in vivo*.

Mechanisms for modulating TBP activity at promoters

Merging the function of two different TBP regulators

In Chapter 5 we have shown that Mot1p and NC2 cooperate *in vivo* in regulating TBP activity and gene expression. The repressive functions of Mot1p and NC2 on TBP activity have been shown genetically and biochemically (14, 40).

However, from the latest work of van Werven *et al*., (35) it has become clear that in order to understand the functional role of Mot1p and NC2, it is necessary to integrate them in the larger picture of the regulation of TBP activity at promoters. The overlapping functions of Mot1p and NC2 can be taken as a starting point for this analysis.

A plethora of genetic and biochemical data have been produced about the Mot1 and NC2 proteins. All together, the main resulting model is confusing and some times contradicting. Possibly, this is linked to the complexity of the process studied and the insufficient methodology to explore it. This is, for instance, the case for the genome-wide profiling of Mot1p and NC2 (5, 9). So far, temperature sensitive (ts) mutants have been used to characterize the Mot1p and NC2α/NC2β essential proteins. However, as will be discussed later, it is likely the Mot1/NC2 together with SAGA regulate a class of genes that is linked to fast reprogramming and the stress response. Therefore, the use of ts mutants, together with the intrinsic variability among ts mutants of the same protein might have led to contradictory results. The genome-wide mRNA profiling of Mot1p in two studies has led to the conclusion that the protein exerts mainly a repressive role in one case and that Mot1p acts equally as repressor and activator in the other case (5, 9). In Chapter 5, we showed that Mot1p and NC2 regulate nearly the same set of genes, and that they have mainly a repressive role in gene expression. Furthermore, we showed that the transcriptional role of the two proteins is strictly linked and correlates with co-activator dominancy and the presence of a TATA box element on the core promoter. In the light of our new findings, we suggest that the current molecular mechanism modeled for Mot1p and NC2 should be merged and integrated.

So far, two main molecular mechanisms of action have been proposed for Mot1p and one for NC2 on their TBP removing activity. These are the ‘tracking’ and ‘spring-loading’ models for Mot1p and

the 'sliding' model for NC2 (4, 5, 25, 29). In all these models the possible influence and interaction between the two proteins has not been considered. It is remarkable for instance, that NC2, which does not contain enzymatic activities, is able to slide *in vitro* TBP from its original position. How could this mechanism influence Mot1p activity? It could be for instance that NC2-mediated prevention of DNA bending by TBP allows Mot1p to bind TBP on the DNA. An alternative scenario is that the binding of NC2 to TBP is essential for the recognition and tethering of Mot1p to the DNA. In our opinion, these models should be tested and possibly integrated with new structural studies on the Mot1/TBP/NC2 complex, which has been shown to be stable on DNA. Although this complex has been isolated and remains stable *in vitro* (34), we suggest that this complex is transient and occurs only on the DNA, and that the stability *in vitro* depends on the depletion of ATP during the extraction of the cell lysate. This is supported by the observation that exposure to ATP triggers complex disassembly *in vitro* (34).

Could basal transcription be a balance between two opposing activities? A case for SAGA-dominated promoters…

The functional perturbation of Mot1p and NC2 results mainly in a genome-wide increase of transcription and in the reduced expression of a minor group of genes. We have linked the two different transcriptional outputs with two different gene classes: SAGA-dominated and TFIID-dominated. It is obvious that the functional mechanisms of Mot1p and NC2 differ between the two classes, but how?

It has been shown by others and confirmed and extended by us that Mot1/NC2 interacts with SAGA, in particular with its TBP module (6, 33). We imagined that at SAGA-dominated genes, at least two opposing events could take place in a dynamic fashion. SAGA delivers TBP at the promoter, whereas Mot1p/NC2 removes it. Thus, by depleting Mot1/NC2 (by anchor away), TBP occupancy increases at the promoter leading to upregulation of the gene, as observed. We do expect then that depleting SAGA leads to downregulation of the same set of genes. When we performed a correlation analysis between mRNA profiles of Mot1p/NC2 and null mutations of SAGA subunits (20), we neither find a significant overlap nor the expected anti-correlation of the genes upregulated in the Mot1p/NC2 anchor away profile. However, when depleting both SAGA and Mot1/NC2, the strain is not viable anymore (Chapter 5), indicating that both activities are required for gene expression.

Unfortunately, how SAGA and Mot1p/NC2 interact remains obscure so far, and with the information available it is difficult to imagine which mechanism is most likely occurring *in vivo*. In light of the genetic results of Chapter 5, we suggest that the SAGA-Mot1p/NC2 interaction occurs via TBP. In particular, the TBP module of SAGA (Spt3p and Spt8p) gives the strongest genetic interaction with Mot1p and NC2. Spt3p and Spt8p exert a similar transcriptional role (20), suggesting a possible redundant role within the SAGA complex. However, in this case the null mutation of one or the other gene should not lead to synthetic lethality when combined with the anchored Mot1p or NC2.

If we assume that Spt3p-Spt8p form a TBP-module in which the presence of both proteins is essential to deliver TBP, deletion of one or the other gene would lead to a semi-functional module, which under normal growth conditions does not give a phenotype. However, the fully functional module may be required to deliver TBP at the promoter, and somehow this condition might be amplified by the anchoring of Mot1p/NC2. Under this condition the silent *Δspt3* or *Δspt8* phenotype becomes lethal. It would be interesting to explore this possible mechanism from a dynamic point of view. Data on TBP turnover in *Δspt3* or *Δspt8* conditions together with Mot1p/NC2 anchoring could help to answer our questions. Finally, we cannot exclude that the synthetic interaction observed is the effect of an indirect mechanism. It might well be that the anchoring of Mot1p/NC2 sets a stress state to which cells are unable to respond when *SPT3* and *SPT8* are deleted. Therefore, a combination of the two genetic states will lead to synthetic lethality. This possibility might be excluded by performing ChIP experiments of SAGA on genes that are upregulated during anchoring of Mot1p/NC2.

We originally assumed that these genes were upregulated as a result of a stabilization of the PIC on the core promoter. In an alternative scenario, the increased occupancy of the PIC components might have derived from a semi-activated state that can be identified by increased occupancy of the upstream activator and co-activator (SAGA).

....and for the TFIID-dominated promoters

In Chapter 5, we show that conditional depletion of Mot1p or NC2β results in a downregulation of TFIID-dominated gene transcription. This result contrasts with that of the SAGA-dominated genes, and is not of immediate understanding. Obviously, this finding, which is supported by previous data (3, 9, 24, 29), cannot be merely linked to the transcriptional function of Mot1p or NC2, but must be the sum up of activities whose balance is different on the TFIID-dominated promoter compared to the SAGA-dominated promoter.

We postulated the idea that the TBP turnover rate might have a role in the difference observed. Van Werven *et al*., showed that at pol II promoters, high TBP turnover correlates with SAGA occupancy, whereas low turnover correlates with TFIID occupancy (35). Moreover, TBP turnover at the promoter and mRNA expression of the corresponding gene anti-correlate. An extension of this concept leads to the hypothesis that the absence of turnover would then result in the highest transcription of the gene. Therefore, removal of Mot1p/NC2 from the nucleus (by anchoring), should lead to an increase in the expression of these genes. This might be true assuming that, as hypothesized by others, TBP, once assembled in the PIC, would be part of a stable scaffold that would remain for a long time on the promoter (39).

The occurrence of the scaffold structure at certain promoters cannot be excluded; certainly it cannot be a general transcriptional mechanism since it does not occur on the TFIID-dominated genes examined. In the light of new insight on transcription dynamics by use of single-molecules studies (18), the TFIID result can also be interpreted differently.

It has been shown that transcription is a stochastic event, in which 'bursts' (transcript synthesis) occur randomly in the population and are linked to the 'searching time' of transcriptional factors. Moreover, it has been confirmed using this new technology that initiation and repression are driven by a 'limiting-step' (which might well be the recruitment of TBP and Mot1p/NC2 respectively). In addition, re-initiation is not a favored mechanism but requires the re-occurrence of the initial 'limiting step' (TBP recruitment) (18).

Therefore, it can be hypothesized that a block of the PIC would result in a stable unproductive complex that does not allow re-initiation of transcription, leading to a drop of transcript levels, and to the observed downregulation of TFIID-dominated genes. This would not happen for the SAGA-dominated genes. These genes are indeed expressed at basal levels, possibly as products of stochastic transcriptional events. The stabilization of the PIC, derived by the Mot1p/NC2 anchoring might even lead to one burst of transcription and possibly does not allow a subsequent transcription-reinitiation. However, this semi-activated state would be measured by a slight increase in transcripts levels, as indeed observed experimentally in Chapter 5.

It is obvious that the observation of transcript reduction for TFIID-dominated genes depends on transcript stability and the initial abundance of transcript levels. However, the genes examined have the highest mRNA levels genome-wide. It would be interesting to perform the same analysis as in Chapter 5 in a time course and for longer times of nuclear depletion of Mot1p and NC2 to test whether this effect is reproducible for the rest of the TFIID-dominated genes transcribed at lower levels.

Gene activation as a result of balance loss

The transcriptional landscape described for SAGA-dominated genes during basal transcription goes to a radical transformation during gene activation. We investigated gene activation at the *HSP26* promoter during heat shock. In the current model of gene activation, an activator binds strongly the

UAS-recruiting SAGA. The co-activator is responsible through its multitask functions to mediate a re-arrangement of the promoter structure, to deliver TBP, and to settle the interactions for PIC assembly and stabilization. Despite the basal transcription, it might be that during the activated state, formation of a scaffold complex on the promoter occurs. Formation of a scaffold in order to facilitate re-initiation has been excluded by single-molecule studies for stably transcribed genes (18). However this does not exclude the possibility that a scaffold complex is assembled during gene activation in order to facilitate a fast re-initiation and an increase in gene transcripts. The assumption of formation of a scaffold complex might explain our results on gene activation.

We found that during the first 20 minutes of heat shock response, the HSP26 transcript increased in a Mot1p/NC2-independent fashion. This suggests that disassembly/re-assembly of the PIC complex in response to Mot1p/NC2 activity is not required to maintain the response. This effect might be mediated by the scaffold. Furthermore, the scaffold complex itself might exclude Mot1p/NC2 from the promoter, or alternatively it might inhibit the enzymatic activity of Mot1p, thus preventing its own disassembly. However, during the recovery phase the transcript level of *HSP26* decreases and reaches a state similar to the initial basal levels. This part of the response is Mot1p/NC2-dependent. It might be that the heat shock signaling reaches saturation, or goes to an autoinhibition. This would in turn inhibit activator and SAGA recruitment to the UAS. The lack of signaling would then destabilize the scaffold complex, which would now be attacked by Mot1p/NC2 activity. In the case that Mot1p/NC2 are depleted, the scaffold is not disassembled thus offering a platform for re-initiation. In this case, transcript levels would remain higher during the recovery phase, as experimentally observed. Taken together, we suggest that the effect observed during gene activation is caused by a change in the balance between SAGA-activating activity and Mot1p/NC2-repressing activity. During basal transcription, the two activities are equal, allowing stochastic transcriptional events resulting in low-level transcript levels. However, during activation, an initial SAGA-dominated state is followed by a Mot1p/NC2 dominated state allowing correct timing and amplitude of gene expression. However, this model needs to be thoroughly tested. ChIP analysis of Mot1p, NC2 and SAGA components are needed during the activation curve. In addition, it might be interesting to investigate: whether TBP is stably associated with the presumed scaffold complex; or whether it is dynamically exchanged during the first 20 minutes of activation; and whether Mot1p/NC2 activity during the recovery phase is linked to a re-establishment of the initial state by mediating increased TBP turnover.

Taken together, the understanding of cross-talk between TBP turnover and the activities of TBP and its protein complexes during gene activation should offer insight in the delicate mechanisms of switching from basal to activated gene expression and vice versa.

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Summary
Samenvatting
Riassunto

Summary

The regulation of transcription initiation is a key feature of gene expression control. RNA polymerase II is recruited to the DNA upon assembly of the Pre-Initiation Complex (PIC), which is in turn nucleated by the binding of TATA-binding protein (TBP) to the core promoter. The modulation of TBP activity occurs in a dynamic fashion by controlling the TBP delivery and removal rates. TFIID and SAGA have positive role on TBP activity whereas MOT1p and NC2 have negative roles. The interplay between the TBP-binding complexes TFIID, SAGA, MOT1 and NC2 regulates transcriptional output.

In this thesis, a biochemical and functional analysis of TBP-binding complexes has been performed to decipher their roles in transcription initiation. Posttranslational modifications of the TBP-binding complexes TFIID and SAGA were identified by mass spectrometry. This revealed that many residues on shared or specific subunits of the two complexes were subject to modification by phosphorylation or acetylation. The shared subunit Taf5p was phosphorylated when present in SAGA, but not when present in TFIID. The SAGA-specific subunits Spt7p and Sgf73p were hyperacetylated in chromatin binding domains.

The SAGA complex is also subject to proteolytic cleavage of the Spt7p subunit resulting in formation of the related SLIK protein complex. Using mass spectrometry, we identified this cleavage site. A knock out screen combined with an *in vitro* protease cleavage assay resulted in the identification of the Pep4p protease to be responsible for cleavage of Spt7p and formation of SLIK. The proteolytic processing of the Spt7p subunit affects the composition of the TBP-module of SAGA by removing the binding domain for the Spt8p subunit. We have reviewed the current literature on SAGA in yeast, flies and human, and addressed how yeast SAGA has diverged into two related but distinct protein complexes in metazoans: SAGA and ATAC.

Next, the genome-wide regulation of gene expression by Mot1p and NC2 has been studied using a new approach for conditional protein depletion in living yeast cells. This indicated that Mot1p and NC2 coregulate gene expression to a large extent. Both Mot1p and NC2 were required for maintaining basal gene expression and for the shut down of gene expression following induction by heat shock and acted by regulating promoter occupancy of TBP

Taken together, the results presented in this thesis have uncovered how the SAGA complex is converted to the SLIK complex; identified a large number of post-translational modifications on SAGA and TFIID; and provided further insight in the mechanism of gene repression by Mot1p and NC2. These findings should aid in our understanding of TBP function in the regulation of gene expression.

Samenvatting in het Nederlands

Dit proefschrift onderzoekt welke mechanismen een rol spelen bij genexpressie. Centraal staat het eiwit TATA-binding protein (TBP), dat een cruciale rol speelt bij het opstarten van dit proces. De functie van TBP wordt gereguleerd door een aantal eiwitcomplexen die uit meerdere subeenheden bestaan. Gebleken is dat een van die complexen, genaamd SAGA, door een protease (een enzym dat eiwitten splitst) gemodificeerd wordt waardoor een nieuw eiwitcomplex genaamd SLIK gevormd wordt. Het is met name de TBP-bindingscapaciteit van SAGA die in SLIK is veranderd. Door het testen van een groot aantal giststammen met elk een deletie voor een van de bekende proteases kon de identiteit van het protease worden vastgesteld. Met behulp van massa spectrometrie kon de precieze plaats van splitsing bepaald worden. Ook konden vele chemische variaties worden gemeten van subeenheden zoals fosforylaties en acetylaties. Deze modificaties spelen waarschijnlijk een belangrijke rol bij de functies van deze eiwitcomplexen. Een nieuwe techniek is toegepast die

essentiele eiwitten, zoals TBP regulatoren, kan verwijderen van hun normale locatie in levende cellen na het geven van een stimulus. De techniek is toegepast op Mot1p en NC2β, twee eiwitten die de functie van TBP remmen. Gebleken is dat deze eiwitten samenwerken in het reguleren van een specifieke groep genen, en dat ze werken door de DNA binding van TBP te verhinderen. Fundamentele kennis van TBP-regulerende eiwitten is belangrijk om in de toekomst te begrijpen hoe de identiteit van cellen, zoals spier of hersencellen, wordt bepaald door het aanzetten van specifieke genexpressie programma's.

Riassunto in italiano

Il diverso assortimento dei geni e il controllo della loro espressione stanno alla base della diversificazione biologica, determina, cioè, la differenza tra uomo e topo, cellule del cuoio capelluto e del sangue e cosi via. L'espressione genica è regolata nel tempo e nello spazio. Geni espressi durante lo sviluppo embrionale, ad esempio, possono essere repressi durante lo stadio adulto. E ancora, un gene specifico per la formazione del cervello è espresso nei neuroni ma non nelle cellule muscolari. La complessa e specifica regolazione dell'espressione genica avviene attraverso una miriade di proteine e complessi proteici, tra i quali anche i complessi trascrizionali. Dal lievito all'uomo, la proteina TBP rappresenta una delle proteine fondamentali nella regolazione dell'espressione genica. TBP si lega alle regioni promotrici di un gene e recluta il macchinario trascrizionale necessario per la trascrizione del gene stesso.
Ancora piu' importanti sono i complessi proteici che alterano e modificano la funzione trascrizionale di TBP. Tra questi, TFIID e SAGA operano in maniera positiva su TBP mentre Mot1p e NC2 in maniera negativa. I primi, infatti, facilitano il legame di TBP alle regioni promotrici mentre i secondi, al contrario, ne inducono il suo rilascio. Queste proteine e complessi proteici, la loro struttura e i loro rapporti funzionali sono molto conservati nella scala evoluzionistica. In questa tesi presento lo studio sulla caratterizzazione biochimica e funzionale dei complessi proteici in grado di alterare il ruolo trascrizionale di TBP. I dati scientifici presentati in questa tesi, sono stati ottenuti usando il lievito Saccharomyces cerevisiae come modello biologico usato.
Dopo il capitolo a carattere introduttivo, nel capitolo due è presentata una sintesi dello stato dell'arte delle ricerche svolte sul complesso trascrizionale SAGA. Il complesso trascrizionale SAGA è conservato a livello strutturale e funzionale dal lievito all'uomo. Recentemente, ATAC, un complesso trascrizionale simile a SAGA è stato isolato nell'uomo e nel moscerino della frutta. E' interessante notare che ATAC svolge, negli organismi superiori, funzioni che, negli organismi inferiori, sono svolte da SAGA. E' quindi facile supporre che il complesso ATAC si sia evoluto e dal complesso SAGA, acquisendone alcune delle funzioni. Nel capitolo 2, viene mostrato come la composizione delle subunita' dei complessi SAGA e ATAC e il loro coinvolgimento in particolari cascate di trasduzione del segnale supportino questa ipotesi evoluzionistica.
Nel capitolo 3 viene presentato lo studio proteomico dei complessi trascrizionali SAGA e TFIID. Lo studio si focalizza sulle modifiche chimiche che le subunita' dei due complessi possono subire nella cellula e che stanno alla base di possibili modulazioni funzionali degli stessi complessi. E' stato, cosi, individuato un gruppo di modificazioni chimiche che avvengono solo a carico di subunita' del complesso SAGA ma non del complesso TFIID e viceversa. E' interessante notare come alcune subunita' si trovino in entrambi i complessi. Nel nostro studio abbiamo appurato che queste subunita' possono essere modificate quando sono incorporate in un complesso piuttosto che nell'altro. Nello stesso capitolo viene presentata anche l'analisi dei possibili effetti funzionali della diversa modificazione chimica dei due complessi . Il complesso trascrizionale SAGA oltre alle modificazioni biochimiche, subisce anche un taglio proteolitico in una delle proteine che lo compongono: Spt7p. Il taglio proteolitico di questa subunità ha effetti sulla funzione dell'intero complesso.

Nel capitolo 4, viene presentato lo studio sull'isolamento della proteasi responsabile del taglio della proteina Spt7p. Successive caratterizzazioni di questa modifica a carico del complesso trascrizionale sono presentate: per esempio gli effetti sterici di subunità vicine sul taglio proteolitico di Spt7p o ancora gli effetti fenotipici in cellule prive della proteasi o con la subunità Spt7p in una forma naturale 'gia tagliata'. In questo studio viene anche dimostrato come gli effetti del taglio della proteina Spt7p si ripercuotono sulla funzione del complesso SAGA nel reclutare il fattore TBP a livello delle sequenze promotrici. Il capitolo 5 si focalizza invece sulla coppia di proteine, Mot1p e NC2, che influenzano negativamente l'attività del fattore TBP. In questo studio abbiamo applicato il metodo 'anchor away' per estrarre le due proteine dal loro sito naturale di funzionamento e studiarne gli effetti a livello genico e fenotipico. Lo studio dimostra come le due proteine collaborino nel far rilasciare il fattore TBP dalle sequenze promotrici e che questa funzione sia necessaria non solo in condizioni normali, ma anche e soprattutto in condizioni in cui la cellula deve rispondere velocemente a cambiamenti nell'ambiente esterno. Inoltre, vengono presentati alcuni dati sulla possible interazione tra I fattori proteici Mot1p, NC2 e il complesso SAGA. Questi ultimi dati suggeriscono che nella regolazione dell'attivita' trascrizionale di TBP è implicata una rete di connessioni funzionali tra i complessi proteici che legano TBP regolandone finemente la funzione.
Nel capitolo 6, infine, vengono discussi i dati ottenuti dalle nostre ricerche e le loro possibili implicazioni nella comprensione della complessa struttura e del funzionamento del macchinario trascrizionale nella cellula.

Curriculum Vitae

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Educational History

1994-1999	Pre-University education Scientific Liceum, Gangi, Italy
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2004-2006	Master of Molecular and Cellular Biology University of Palermo, Palermo, Italy Thesis title: *Pre-functional analysis of CLN8 protein, involved on Neuronal Ceroid Lipofuscinosis, throughout the Split-Ubiquitin Two-Hybrid Assay in yeast.* Advisors: A. Puglia, P. Guarneri and R. Passantino
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List of publications

Mischerikow N, **Spedale G**, Altelaar A.F, Timmers H.Th, Pijnappel W.W and Heck A.J.
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Manuscript under revision

Spedale G, Timmers H. Th. M and Pijnappel W.W.M. Diversification of the SAGA transcriptional coactivator in higher eukaryotes: interplay with signaling pathways.
Manuscript in preparation

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gianpiero

Abbreviations

ADA	adaptor
AT	acetyltransferase
AT	transcriptional activator
ATAC	ada-two-A-containing complex
Bp	basepair
CDS	coding sequence
ChIP	chromatin immunoprecipitation
CTD	pol II carbossi-terminal domain
CUT	cryptic unstable transcript
DUB	deubiquitination (module of SAGA)
FRAP	fluorescence recovery after photobleaching
FRB	FKBP12-Rapamycin Binding
FRET	forster resonance energy transfer
GCN5	general control Nonderepressible
GNAT	GCN5-related-N-acetyltransferases (family)
HAT	histone acetyltransferase
HFD	histone fold domain
HSP26	heat shock protein 26
ISWI	imitation swi
Mot1p	modifier of transcription
mRNA	messenger RNA
Nc2	negative cofactor 2
PCAF	p300/CBP-associated factor
PIC	pre-initiation complex
Pol II	RNA polymerase II
PTMs	post-translational modifications
SAGA	Spt-Ada-Gcn5-Acetyltransferase
SLIK	SAGA-like
Spt	suppressor of Ty
STAGA	SPT3-TAF(II)31-GCN5L acetylase
SUT	stable untraslated transcript
Swi2p/	switching 2 protein
Snf2p	sucrose non fermenting 2 protein
SWR1	swi/snf related protein 1
TAP	tandem affinity purification
TBP	TATA-binding protein
TF	transcriptional factor
TFIID	transcriptional factor (class II) D
TFTC	TATA-bindin protein (TBP)-free TAF containing complex
Ts	temperature sensitive
TSS	transcrptional start site
TTS	transcriptional termination site
UAS	upstream activating sequence
URS	upstream repressing sequence