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

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ABSTRACT

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 NC2βduring 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 (1). 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 (2,3). 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 (4–6). 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 (4,7,8).

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 (9). 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

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(10). Various models have been proposed to explain how Mot1p can achieve this. These include changing the conformation of TBP (11), short-range ATP-driven translocation of Mot1p along the DNA (12) and the use of the N-terminal TAND domain of Mot1p as a wedge between TBP and DNA (13). 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 (14).

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 (15,16). 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) (16,17). 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 (18). 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 (19–25).

Interestingly, both TBP delivery (SAGA and TFIID) and TBP removing (Mot1p and NC2) proteins are recruited to active genes *in vivo* (19–21,23,24,26,27). This is consistent with a model in which TBP dynamics plays an important role in the regulation of gene expression (26,28). Recently, we purified a protein complex from yeast chromatin extracts that consists of Mot1p, both NC2 proteins, TBP and 20–70 bp of DNA. Addition of a hydrolysable form of ATP resulted in disruption of the complex (26). The co-occurrence 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 (8,20,24,25,29). *In silico* comparison reveals that *MOT1* 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 (30). Genome-wide expression analysis indicates that *MOT1* and *NC2β* regulate basal expression of highly similar sets of genes ($R^2 = 0.8$). Upregulated genes were enriched for SAGA occupancy, while downregulated genes preferred TFIID binding. Depletion of Mot1p or NC2β resulted 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 Supplementary Table S1. *MOT1* and *NC2β* were C-terminally tagged with *FRB* and *FRB-GFP* as described (30). Details of the primers and plasmids used are listed in Supplementary Tables S2 and S3, respectively. For the complementation assay, strains were transformed with empty vector or with vector harboring the galactose-inducible *MOT1* or *NC2β* genes (Supplementary Table S3). Generation of full gene deletions was performed using standards methods (31).

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 A_{600} of 0.2–0.3, and grown until an A_{600} of 1. Cells were then exposed to 1 μg/ml rapamycin (LC laboratories) for 60 min, and subsequently transferred from 30 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 A_{600} of 0.1 were spotted on CSM/2% glucose plates containing rapamycin at 1 μg/ml as indicated. The plates shown were incubated for 3 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 toan A_{600} of 0.1 per well in a 24-wells plate and incubated at 30°C with orbital shaking (87 r.p.m., 1 mm amplitude). A_{595} was measured every 10 min.

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

MOT1-FRB-GFP and *YDR1-FRB-GFP* strains were grown to an A_{600} 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 (32). A DeltaVision Instrument (Applied Precision) equipped with Olympus Objective × 100/1.40 was used for imaging. ImageJ was used for images analysis.

Genome-wide expression analysis

Genome-wide expression analysis was performed as described previously (33) 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 A_{600} of 0.3 in 50 ml medium and grown to an A_{600} 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 r.p.m. for 3 min 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 (34). 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 (33). cDNA was prepared using oligo-dT priming and SuperscriptIII (Invitrogen). qPCR analysis was performed as described previously (35). 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.

ChIP

ChIP of TBP and Pol II was performed as described previously (8) 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 h at room temperature.ChIP for TFIID and TFIIH was performed as described by Ahn *et al.* (36) with minor modifications. Antibodies (Taf1, Tfb3) were coupled to 25 µl of Protein A/G Plus Agarose beads (Santa Cruz) for 1 h at room temperature. Beads were washed and incubated with chromatin overnight at 4°C. 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 (26). The SD for each sample was calculated from three technical repeats. Experiments were repeated at least twice.

Microarray data accession numbers

Microarray data has been deposited in ArrayExpress with the experiment name GS003 and the accession number 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 (8,20,23–25,29). To circumvent the use of ts alleles, we applied the anchor-away technique developed by Laemmli and collegues (30). An anchor-away strategy was chosen that should translocate nuclear proteins of interest to the cytoplasm (30). 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 (30).

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 (Supplementary Figure 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, 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 (Figure 1B). Similar results were obtained when the FRB tag without GFP was used (Supplementary Figure 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 A_{600} 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 (Figure 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 A_{600} values. Comparable results were obtained when the FRB tag without GFP was used (Supplementary Figure 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 (Figure 1D). Mot1-FRB-GFPp was present in the cytoplasm

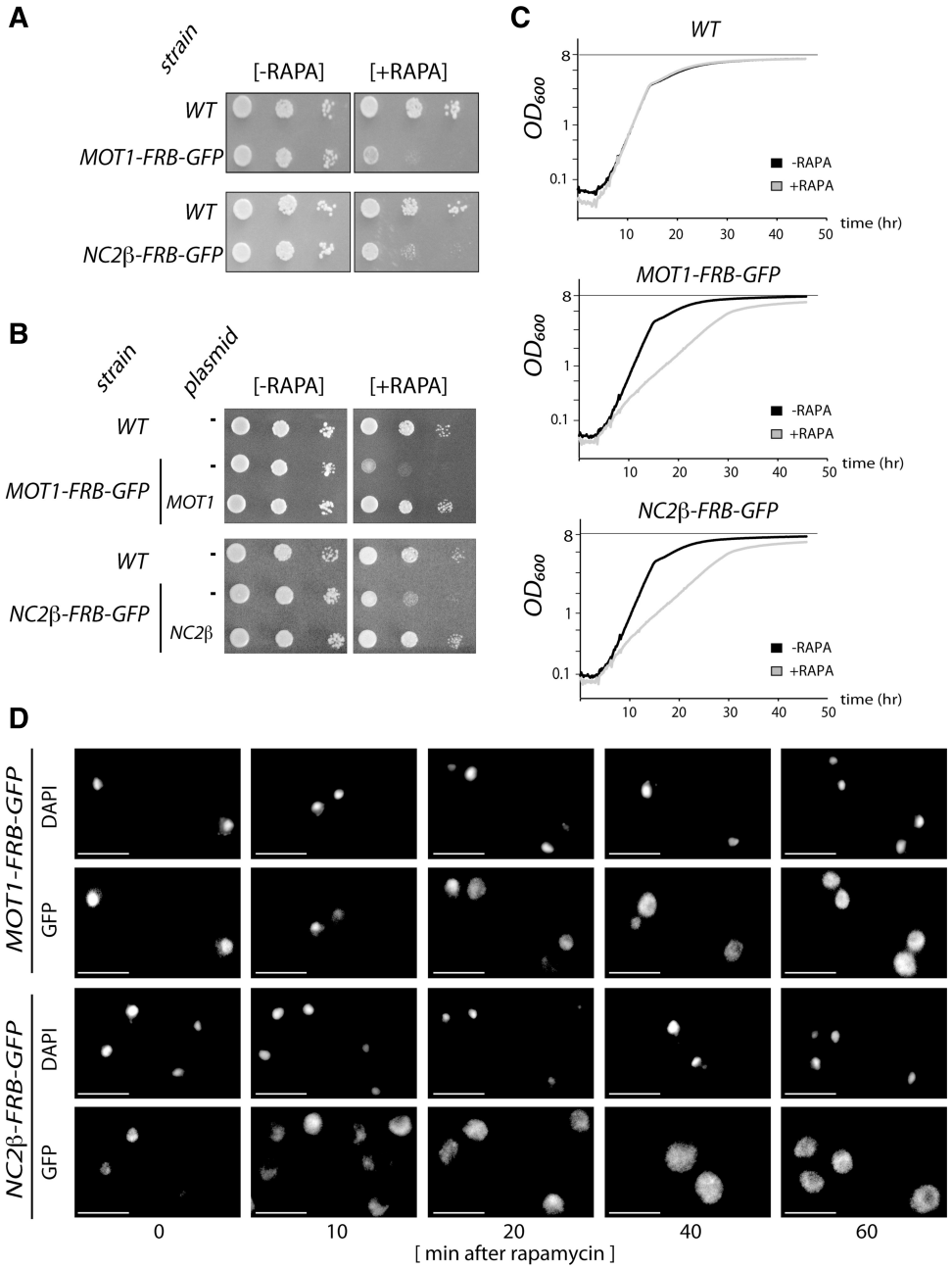

Figure 1. Anchoring Mot1p and NC2β causes growth delay. (**A**) Spot assay on SC plates in the absence or presence of 1 μg/ml rapamycin. The parental strain HHY168 was used as negative control (wt). Dilutions were 10-fold. (**B**) Complementation of *MOT1-FRB-GFP* or *NC2β-FRB-GFP* with untagged, galactose 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 A_{600} 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. At least 100 cells were analyzed per condition. Size bars correspond to 10 μm. Images were taken using a × 100 objective.

starting at 20 min after rapamycin treatment, while NC2β-FRB-GFPp showed cytoplasmic fluorescence starting at 10 min 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 (Figure 2A, compare left panel (without rapamycin) with right panel (with rapamycin), Figure 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 (Figure 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 (Figure 2A, compare the three plots on the left),

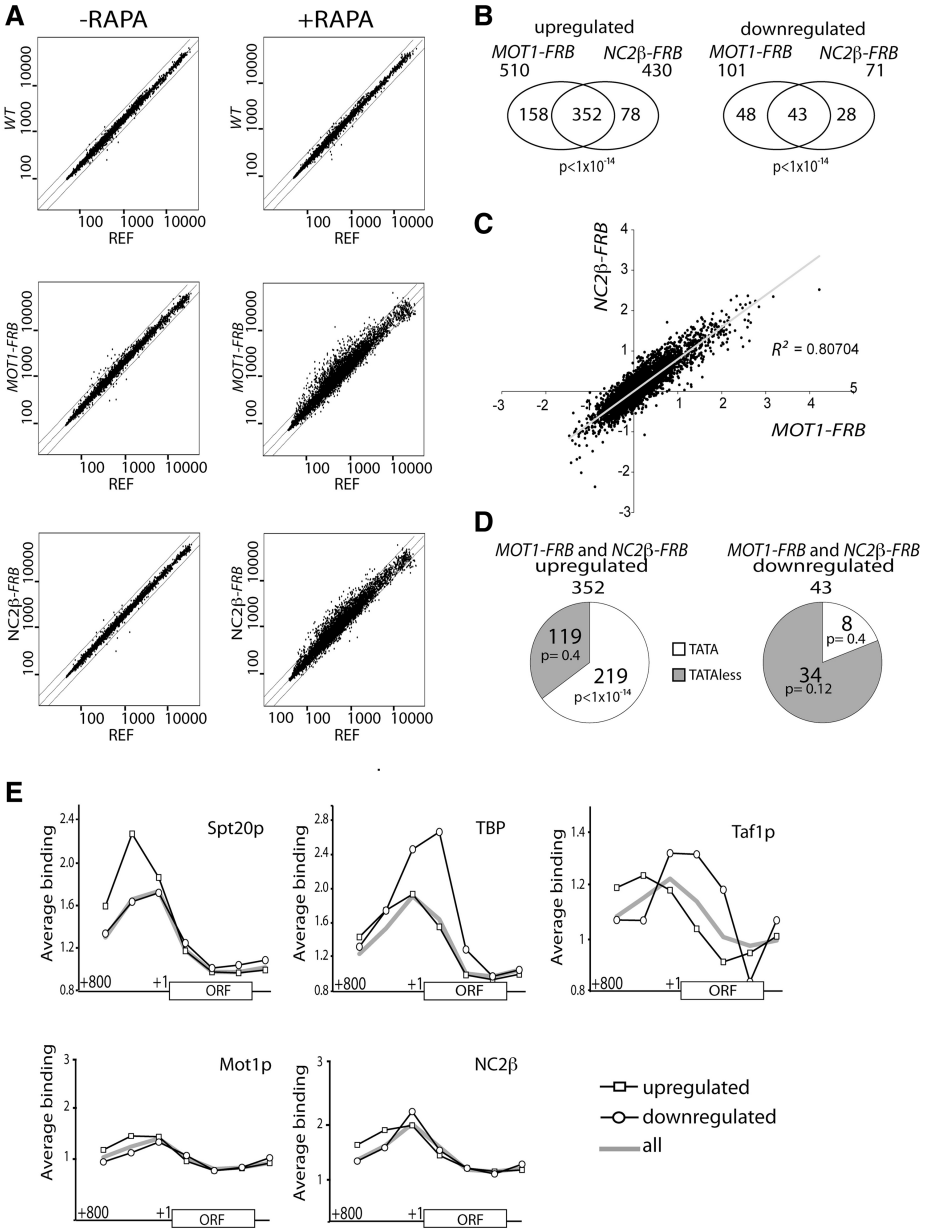

Figure 2. Anchoring Mot1p or NC2βp leads to highly overlapping changes in gene expression. Cells were treated for 60 min with rapamycin. Genome-wide mRNA expression analysis was performed using yeast oligonucleotide arrays (37). (**A**) Average expression levels from four measurements of each strain 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 testare indicated. (**C**) Correlation plot showing the *MOT1-FRB* and *NC2β-FR*B experiments. Axes are in ^{2}log scale. R^2 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).

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 confirmed that *HSP26*, *HSP42* and *ARO10* expression is upregulated and *RPS3* expression is downregulated by anchoring Mot1p and NC2β (Supplementary Figure S3). And finally, the profile of *MOT1-FRB* significantly overlapped with the *mot1-1* profile (37) generated at the permissive temperature ($R^2 = 0.4$; $P < 10^{-14}$, hypergeometric test), despite the differences in experimental platforms and genetic backgrounds (Supplementary Figure S2B and C).

Venn diagram analysis revealed a high degree of overlap ($P < 10^{-14}$, hypergeometric test) between the upregulated genes in the *MOT1-FRB* and *NC2β-FRB* profiles (Figure 2B). Around 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 ($P < 10^{-14}$), but to a lower extent: 40% of downregulated genes in *MOT1-FRB* overlapped with *NC2-FRB*, while 60% of downregulated genes in *NC2β-FRB* overlapped with *MOT1-FRB* (Figure 2B). Correlation plot analysis also indicated a high level of correlation between *MOT1-FRB* and *NC2β-FRB* profiles ($R^2 = 0.8$) (Figure 2C). A comparison of the *NC2β-FRB* profile with a published *NC2α* ts [*bur6-1*; (24)] profile showed a high degree of overlap (Supplementary Figure S2), suggesting that most effects of NC2β depletion are mediated by the NC2α/β complex rather than by NC2β alone. Taken together, these results 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 [as defined in (4)] in their promoters: 60% of upregulated genes contained a TATA box, whereas the average occurrence is 19% (Figure 2D) (4). These genes were expressed at average levels (Supplementary Figure 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 (Supplementary Figure 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 (4). 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 (26). As expected, binding of SAGA (using the Spt20p subunit) to the UAS was enriched in genes upregulated bydepletion of Mot1p and NC2β (Figure 2E). Downregulated genes did not show such enrichment. In contrast, promoter binding of TBP or TFIID (via the TAF1 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.

Additional analysis of transcriptional factor enrichment was performed on the two groups of genes (Supplementary Table S4). We used the ChIPcodis web-based tool (http://chipcodis.dacya.ucm.es/) to analyze genes that were up-or down-regulated in response to Mot1p and NC2β depletion for enrichment of promoter occupancy of transcription factors. We found that the upregulated genes were enriched for binding of transcriptional factors involved in the stress response including Yap4p, Phd1p and Hap1p. In contrast, downregulated genes were enriched for binding of transcriptional factors involved in ribosomal gene regulation including Fhl1 and Rap1.

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, are preferentially SAGA-dominated, and are targets of stress related transcription factors, while 20% of genes are activated by Mot1p or NC2β, do not show enrichment for TATA box DNA, are preferentially TFIID-dominated, and are targets of transcription factors regulating housekeeping genes.

Mot1p and NC2β regulate basal gene expression by modulating PIC assembly 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 (Figure 3A). This gene was most sensitive to Mot1p depletion, while NC2β depletion showed a milder phenotype. ChIP analysis of TBP binding to the TATA box present in the *HSP26* promoter indicated that depletion of Mot1p resulted in higher TBP occupancy (Figure 3B). This correlated with increased occupancy of additional PIC members TFIIH (via the Tfb3p subunit) and TFIID (via the Taf1p subunit). Promoter binding of RNA pol II was increased after depletion of Mot1p, which is consistent with the observed increase in *HSP26* mRNA. NC2β depletion also showed increased promoter occupancies of RNA pol II, TFIIH and TFIID, but to a lower extent compared to Mot1p depletion. TBP occupancy did not change after 60 min of rapamycin treatment (Figure 3B), but modestly increased 120 min after rapamycin treatment (data not shown), when the effect on transcript levels was maximal (Figure 3A). Analysis of the *ARO10* gene, which was

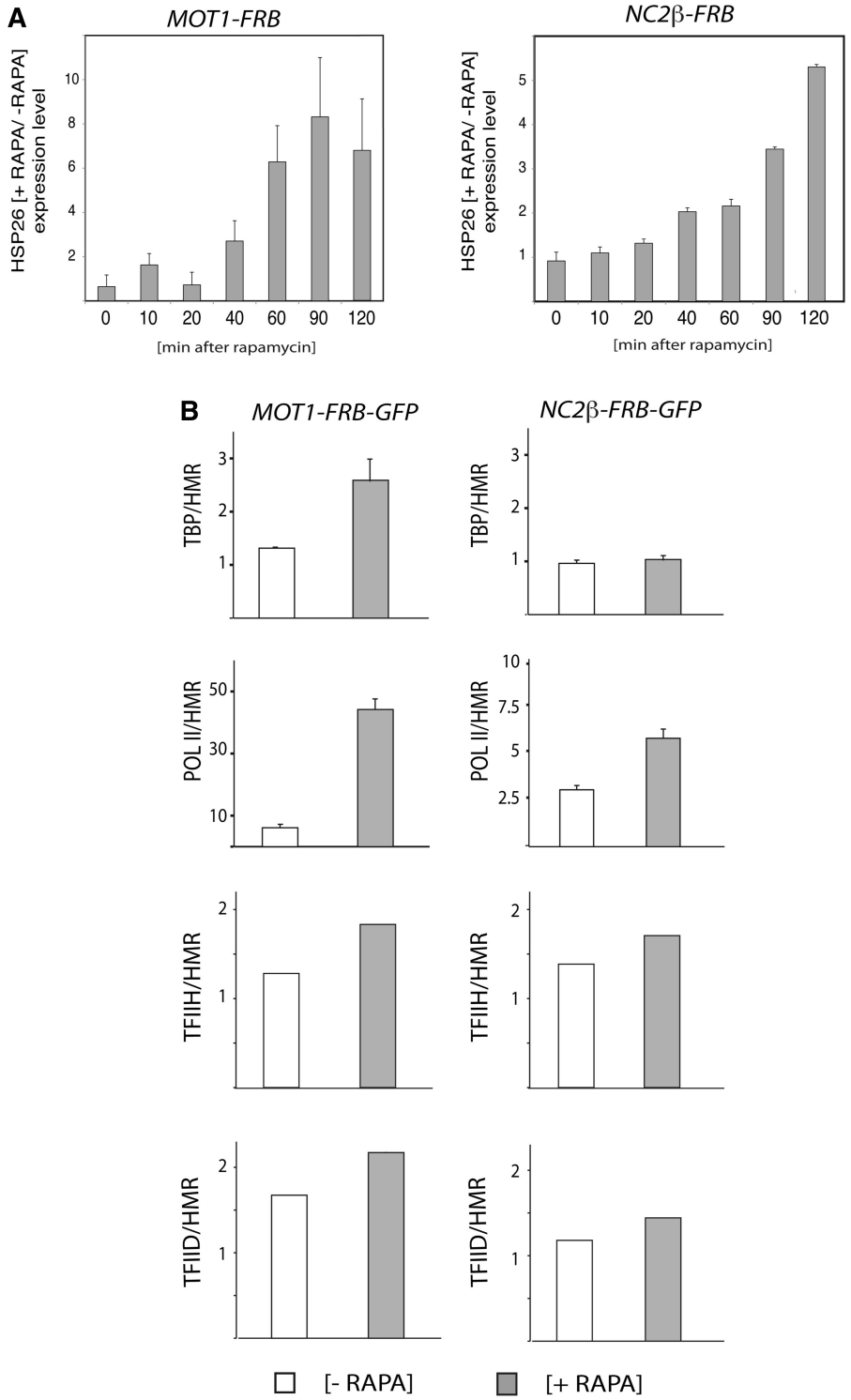

Figure 3. Effects of anchoring Mot1-FRBp or NC2β-FRBp on gene expression and promoter occupancy at the *HSP26* gene under basal conditions. (**A**) Time course of the effect of rapamycin treatment on basal HSP26 expression using RT–qPCR analysis. Data were normalized on ACT1 levels. The 60 min time point was used for the ChIP–qPCR analysis of Figure 3B and C. (**B**) TBP, TFIIH (via the Tf3bp subunit) and TFIID (via the Taf1p subunit) occupancy was measured near the TATA box, which is located at nuclotides −330/−210 relative to the open reading frame (primer B in Figure 4B). RNA polII occupancy was measured at the transcription start site (primer C in Figure 4B). Data were normalized to binding to the silent HMR locus, and represent the average to three technical replicates ± S.D. Experiments were performed at least twice with similar results.

repressed upon Mot1p or NC2β depletion (Supplementary Figure S3), also revealed decreased PIC formation and RNA pol II recruitment in Mot1p and NC2β depleted cells (Supplementary Figure S4A). In this case, all components displayed increased promoter occupancy except TFIIH, suggesting different requirements for active PIC formation compared to the *HSP26* gene (Supplementary Figure S4B). The *RPS3* gene served as an example of a gene whose basal expression is positively regulated by both Mot1p and NC2β (Supplementary Figure S3). Promoter occupancies of all PIC components tested, including TBP, pol II, TFIIH and TFIID decreased upon Mot1p or NC2β depletion. This suggests that Mot1p and NC2β have similar functions in both positive and negative regulation of basal gene expression via modulating assembly of the PIC.

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 expressionto moderately elevated levels compared to basal expression (Figure 4A). Nuclear depletion of Mot1p or NC2β did not affect the timing or amplitude of the initial activation of expression (Figure 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 (Figure 4B) were high during the activation phase, but were reduced 4–6-fold during the recovery phase, paralleling transcript levels (Figure 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 (Figure 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.

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* (19,22,38). The multi subunit protein complex SAGA has distinct 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 (Sgf73) (31,32). 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 (Figure 5). Anchoring Mot1p or NC2β in an Δ*spt7* background resulted in synthetic lethality, suggesting that both Mot1p and NC2*β* 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 *MOT1* and *NC2β*

In line with the original publication (30), 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* (Figure 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 10–20 min after rapamycin treatment, respectively (Figure 1D). The difference in timing betweenNC2β 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 (30). The numbers of

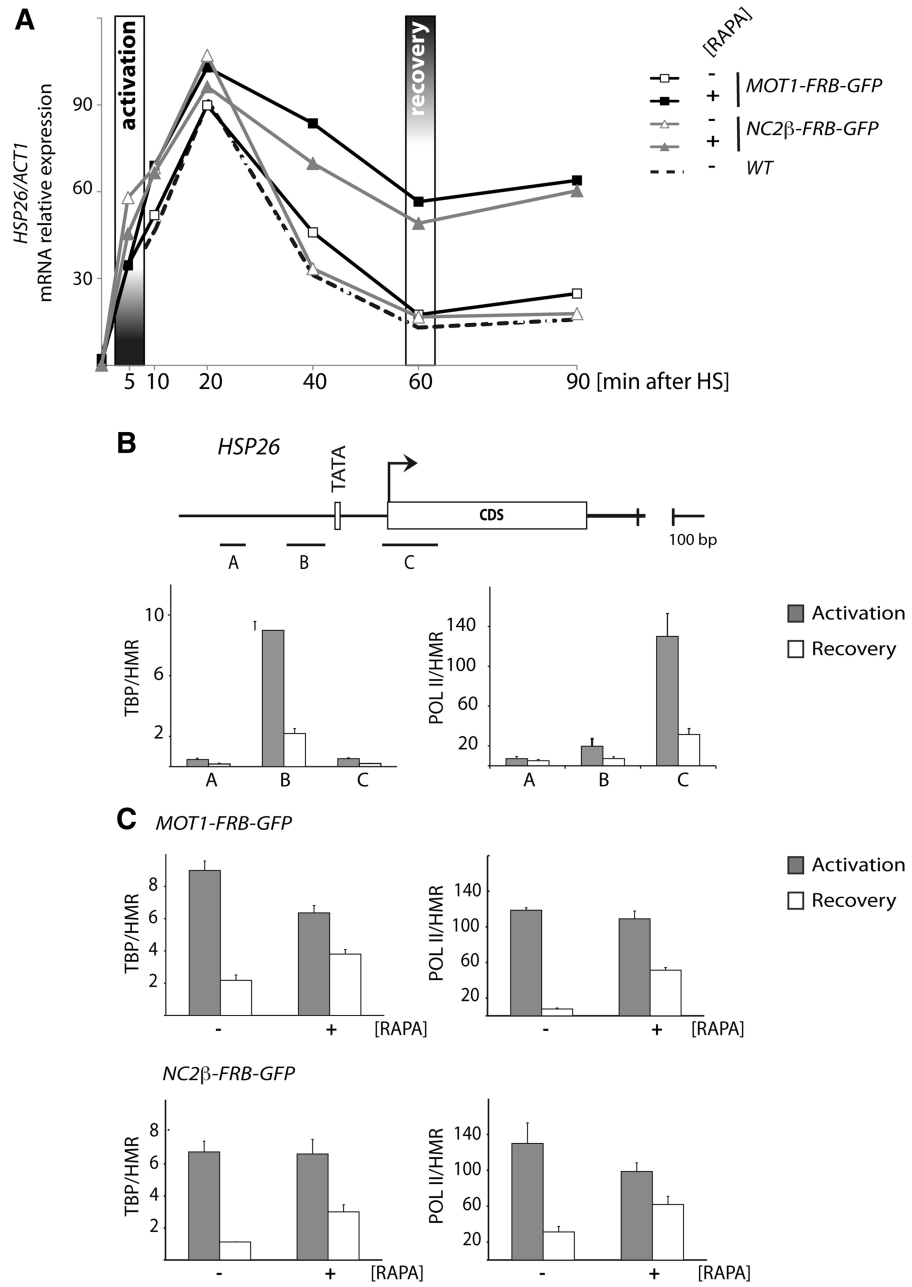

Figure 4. Effects of anchoring Mot1-FRBp or NC2β-FRBp on gene expression and promoter occupancy at the *HSP26* gene under heat shock conditions.*MOT1-FRB* or *NC2B-FRB* strains were treated with rapamycin for 60 min, and subsequently heat shocked to 38°C. (**A**) RT–qPCR analysis of HSP26 expression at the indicated time points after heat shock. Wild type (wt) yeast is shown for comparison. (**B**) ChIP–qPCR of TBP and RNA polII 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 PolII occupancy.

copies per cell of Mot1p (6.56×10^3) and NC2β (2.95×10^3) are significantly lower than the anchor RPL13A (1.33×10^5) (39). 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 (40). The anchor trimeric complex (anchor-target-rapamycin) containing the 210 kDa Mot1p is considerably larger than the complex containing the 17 kDa 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 (Figure 1D), while longer treatment showed similar results (data not shown). This is in line with the observation that

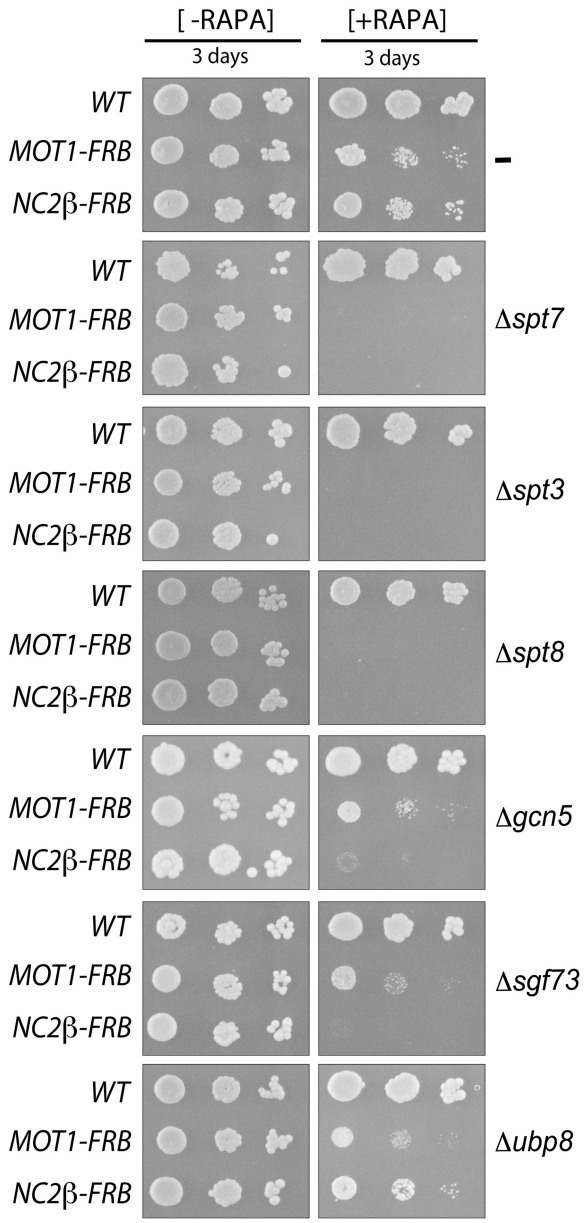

Figure 5. Both *MOT1-FRB* and *NC2β-FRB* display strong genetic interaction with the TBP binding module of SAGA. Spot assay on SC plates in the absence or presence of 1 μg/ml rapamycin. Genes encoding the indicated SAGA subunits were deleted in *MOT1-FRB*, *NC2β-FRB* or HHY168 parental strains. Dilutions were 10-fold. Plates were incubated at 30°C for 3 days.

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* (8,29) and *NC2* ts alleles (20,24,25). This has indicated that both Mot1p and NC2are involved in transcriptional repression and to a lesser extent in transcriptional activation. *In silico* comparisons have revealed significant overlap between the profiles of Mot1p and NC2α (29,41). 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 R^2 value of 0.8. Comparison of profiles obtained after anchor away of NC2β (this study) and using a *ts* strain of NC2α [bur6-1;(24)] revealed a significant overlap for both upregulated and downregulated genes.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 (41). 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.* (26)], but rather depend on promoter occupancies of transcriptional activators: genes with high promoter occupancy of SAGA and transcription factors involved in the stress response were preferentially repressed, while genes with high promoter occupancy of TFIID and transcription factors regulating housekeeping genes 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) (26)]. 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 (23), formation of an alternative PIC, in which Mot1p replaces TFIIA (42), formation of an alternative TBP complex (19), and relieve of inhibition by TBP on TATA box containing promoters in Drosophila (43). Our results on the *RPS3* gene suggest that Mot1p and NC2β can function as positive regulators of gene expression by stimulating rather than directly inhibiting formation of an active PIC. 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 the upregulated genes *HSP26* and *ARO10* confirmed that basal expression of these genes was upregulated upon Mot1p as well as upon NC2β

depletion (Figure 3A, Supplementary Figure S3). Fold inductions for the *HSP26* gene 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 (19). 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 (Figure 3B and 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 (Figure 4A: 'recovery'). While Mot1p or NC2β depletion did not affect the activation, it inhibited the subsequent downregulation of transcription (Figure 4A). A concomitant increase in TBP and RNA pol II occupancy was seen during this recovery phase, suggesting that the effects of Mot1p/NC2βdepletion 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 (19). 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*. (19), 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 (26), 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 (44). This is in line with previous reports on the genetic interaction between SPT3 or SPT8 with *MOT1* ts mutant alleles (19,38,45). 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 Δ*spt7* indicates that the intact SAGA complex is required, since this subunit is required for complex integrity (46). The synthetic interaction of *MOT1-FRB* and *NC2β-FRB* with *GCN5*, in agreement withprevious reports using *MOT1* ts mutants (19,22), suggests that SAGA-mediated histone acetylation is important for both Mot1p and NC2β function. *NC2β-FRB* and *MOT1-FRB* also showed synthetic lethality with Δ*sgf73*. This subunit has recently been shown to bind H2A/H2B heterodimers (47). 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 (48), 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.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures S1–S4, Supplementary Tables S1–S4 and Supplementary References [37,49].

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