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A SUMO-regulated activation function controls synergy of c-Myb through a repressor–activator switch leading to differential p300 recruitment

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ABSTRACT

Synergy between transcription factors operating together on complex promoters is a key aspect of gene activation. The ability of specific factors to synergize is restricted by sumoylation (synergy control, SC). Focusing on the haematopoietic transcription factor c-Myb, we found evidence for a strong SC linked to SUMO-conjugation in its negative regulatory domain (NRD), while AMV v-Myb has escaped this control. Mechanistic studies revealed a SUMO-dependent switch in the function of NRD. When NRD is sumoylated, the activity of c-Myb is reduced. When sumoylation is abolished, NRD switches into being activating, providing the factor with a second activation function (AF). Thus, c-Myb harbours two AFs, one that is constitutively active and one in the NRD being SUMO-regulated (SRAF). This double AF augments c-Myb synergy at compound natural promoters. A similar SUMO-dependent switch was observed in the regulatory domains of Sp3 and p53. We show that the change in synergy behaviour correlates with a SUMO-dependent differential recruitment of p300 and a corresponding local change in histone H3 and H4 acetylation. We therefore propose a general model for SUMO-mediated SC, where SUMO controls synergy by determining the number and strength of AFs associated with a promoter leading to differential chromatin signatures.

INTRODUCTION

Synergy between transcription factors is a well-known phenomenon. Several models have been proposed to explain this more-than-additive activity of multiple activators. Key concepts are multiplicity of contacts to the basal transcription apparatus (1,2) promoting assembly of the pre-initiation complex (PIC) (3), physical interactions between transcription factors (4) particularly emphasized in the enhanceosome model (5), co-activators harbouring distinct domains that interact simultaneously with different factors (6) and cooperative interactions of transcription factors with nucleosomal DNA (7). Activators able to stimulate distinct steps in the transcription process, such as initiation and elongation, may also contribute to concerted action and synergy (8). In general terms, synergy appears to be intrinsic to the transcription process, being related to the multiplicity of interactions necessary to assemble an active PIC at the transcription start site (TSS), triggering the productive elongation by RNA polymerase II. This makes synergy an ideal target for control of transcriptional output.

An interesting twist to the phenomenon of synergy was the finding of a specific negative control mechanism. Iniguez-Lluhi and Pearce (9) identified a short protein motif in the glucocorticoid receptor (GR) that mediated 'synergy control' (SC) by acting as a disruptor of synergy on promoters with multiple response elements. Mutations of this motif induced a strong synergistic behaviour of GR at compound, but not at single, response elements. It soon became apparent that the SC motif was a SUMO-conjugation site and that the disruption of synergy was caused by sumoylation of the factor at that site (10,11). This role of SUMO (small ubiquitin-related modifier) as a disruptor of synergy has been extended to other transcription factors such as SF-1, MITF and ZBP-89 (12–14). Still, compared to the rapidly expanding literature on SUMO only a tiny fraction of papers have addressed its synergy-controlling properties.

The SUMO family proteins function by becoming covalently linked to a variety of proteins, including many

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nuclear regulators of key processes such as transcription, nuclear transport, chromatin structure and DNA repair (15,16). The modification by SUMO is a highly dynamic process, controlled by the balance between a set of conjugation enzymes, analogous to those of the ubiquitin pathway and a set of SUMO-specific proteases.

The transcription factor c-Myb is a key regulator of stem and progenitor cells in the bone marrow, colonic crypts and a neurogenic region of the adult brain (17,18). c-Myb becomes sumoylated at two sites within its negative regulatory domain (NRD) leading to a severe drop in its activity (19–21). Interestingly, both SUMO-conjugation sites are deleted in the oncogenic variant AMV v-Myb (19). The molecular mechanism by which SUMO is controlling c-Myb activity is poorly understood. Interestingly, synergy is a well-documented aspect of c-Myb action. The factor has been reported to activate promoters in synergy with several other transcription factors such as Ets, C/EBPs, PU.1, Pax-5 and CBF (core binding factor) (22–28) and being assisted by co-activating factors such as p300, Mi-2α and FLASH (25,29–32). Consistently, many of the genes activated by c-Myb appear to be controlled by compound promoters harbouring multiple recognition sites for both c-Myb and other cooperating factors.

Given the role of SUMO as a disruptor of synergy for some specific transcription factors, we reasoned that studying its role in SC of c-Myb might lead to a better understanding of the mechanisms by which SUMO controls c-Myb action. In this work, we show that c-Myb is subject to a strong SC, tightly linked to its level of SUMO-conjugation, and that this control mechanism is abolished in AMV v-Myb. Chromatin-embedded target genes of c-Myb respond differentially to sumoylation in haematopoietic cells. We show that SUMO affects the synergistic behaviour of c-Myb by switching off a SUMO-regulated activation function (SRAF) in its NRD. This switch is reflected in a SUMO-dependent change in the efficiency of p300 recruitment to promoters. A similar SUMO-mediated switch in the inhibitory domain (ID) of Sp3 and in the C-terminal regulatory domain (CRD) of p53 indicates that this might be a general mechanism by which SUMO controls transcriptional synergy.

MATERIALS AND METHODS

Plasmid constructs

Plasmid constructs are outlined in Supplementary Data.

Cell culture, transfection and luciferase assays

CV-1, COS-1, HD11 and K562 cells were grown as previously described (31,32). HEK 293 cells, stably transfected with a 5×Gal4-luciferase reporter (33), were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and puromycin. Cells were kept at 37°C in a humidified atmosphere of 5% CO_2 in air. The three first cell lines were transiently transfected with the indicated plasmids (0.2 μg when nothing else is given) using FuGENE6 (Roche Applied Science) 24 h after seeding. For the luciferase assays, CV-1 cells were harvested 24 h after transfection and lysed in Passive Lysis Buffer (Promega). Luciferase assays were performed in triplicate (24-well trays, 2×10^4 cells/well) using Luciferase Assay Reagent (Promega). Data from at least three independent transfection experiments are presented.

Protein expression and GST pull-down

GST and GST-fusion proteins were expressed in *Escherichia coli* as previously described (31). The regions of human c-Myb expressed in the fusion proteins GST-TAD, GST-NRD and GST-NRD 2KR spanned amino acid residues 259–337 and 410–640, respectively. The GST-NRD-SUMO1 corresponded to GST-NRD with SUMO-1 (amino acid residues 1–96) fused in frame to its C-terminal. GST pull-down assays were performed as earlier described, using Frackelton-buffer [20 mM HEPES pH 7.4, 10% glycerol, 0.2% Triton X-100, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM Iodoacetate, 5 μM $ZnCl_2$, pH adjusted to 7.10, Protease Inhibitor cocktail (Roche)] as lysis, interaction and washing buffer (31). Electrophoretic mobility shift assay (EMSA) was performed as outlined in Supplementary Data.

Western blotting and antibodies

Cell lysates from transfected COS-1, CV-1, HD11, K562 and HEK 293 cells were subjected to western blotting. Mouse anti-Myb 5e11 (31), rabbit anti-c-Myb H141 (sc-7874, Santa Cruz), rabbit anti-GAL4 (DBD, sc-577, Santa Cruz), rabbit anti-HA (H6908 Sigma), mouse anti-human p300 (33-7600, Zymed), mouse anti-αTubulin (T9026, Sigma) and mouse anti-GAPDH (H86504M Biodesign International) were used as primary antibodies, while anti-mouse IgG-HRP (NA931, GE Healthcare) and anti-rabbit IgG-HRP (NA934, GE Healthcare) were used as secondary antibodies. For chromatin immunoprecipitation (ChIP) additional antibodies were used: anti-FLAG M2 antibody (F3165, Sigma), isotype control: anti-secretory Component (IgA) IgG1 (I6635, Sigma), anti-SETDB1 (ab12317-50, Abcam), anti-Histone H3 trimethylated at lysine 9 (pAb-056-050, Diagenode), anti-Mi2 (06-878, Millipore and sc-12547, Santa Cruz), anti-acetyl-Histone H4 (06-866, Millipore), anti-acetyl-Histone H3 (06-599, Millipore), anti-p300 (sc-585, Santa Cruz) and control IgG (ab46540, Abcam).

RNA isolation and quantitative RT–PCR

HD11 cells were transfected with 2 μg DNA (pCIneo-hcM-HA, pCIneo-hcM-2KR-HA or empty pCIneo vector) per well in 6-well trays. Total RNA was isolated and cDNA synthesized as previously described (31). The cDNA obtained was subjected to real-time PCR analysis (31) to determine the expression of two c-Myb target genes: *mim*-1 and *lysozyme*, using the LightCycler DNA MasterPlus SYBR Green Kit (Roche). The relative amounts of the mRNAs were normalized to the reference gene *HPRT1*. Primer sequences are available upon request.

Chromatin immunoprecipitation

Human leukaemic K562 cells were stably transfected with plasmids expressing 3×FLAG-c-Myb-HA wild-type and 2KR under the control of *EEF1A1* promoter using the Amaxa nucleofector kit. Single positive clones were expanded and maintained in IMDM (Invitrogen) supplemented with 10% FCS and 200 μg/ml G418 (Geneticin from Gibco BRL Life Technologies, Inc).

Cells were cross-linked with 1% formaldehyde in PBS at room temperature, K562 cells for 22 min and HEK 293 cells for 15 min. Cross-linking was performed with rotation at room temperature, and the reaction was stopped by addition of glycine to a final concentration of 125 mM. After two washes with PBS, cells were lysed in IP buffer {50 mM Tris–HCl [pH 7.5], 5 mM EDTA, 1% Triton, 0.5% NP-40, 150 mM NaCl, 0.1% SDS and Complete™ protease inhibitor cocktail tablets (Roche Diagnostics)}. Samples were sonicated to generate sheared DNA fragments around 400 base pairs (soluble chromatin fraction), and insoluble chromatin was discarded after centrifugation. Dynabeads™ ProteinG were washed with PBS and incubated with antibody at room temperature for 40 min followed by washing with PBS. The soluble chromatin fraction was then added followed by incubation overnight at 4°C with rotation. Chromatin equivalent to typical 350 000 cells was used per IP with 20 μl Dynabeads™ ProteinG and 2 μg antibody, in a total volume of 0.5 ml IP buffer. The immunoprecipitates were washed five times in IP buffer, before DNA was eluted with 1% SDS in 100 mM sodium carbonate at 65°C for 10 min. After treatment with RNAse A and proteinase K, cross-linking was reversed by incubation at 65°C for 6 h. DNA was purified using silica columns (Macherey-Nagel) and eluted in 50 μl 10 mM Tris–HCl [pH 7.5]. Eluted DNA was used as template in quantitative real-time PCR, in a 1:2 dilution in a total volume of 20 μl (LightCycler® 480 SYBR Green I Master, Roche Diagnostics). Standard curves of genomic DNA were run alongside the ChIP samples for each primer pair, and analyzed on a LightCycler® 480 (Roche Diagnostics). Input DNA was used to normalize values from ChIP samples.

RESULTS

Synergy behaviour of c-Myb and its relation to SUMO-conjugation

Previous studies have reported that the activity of c-Myb is significantly enhanced by removal of its two SUMO-conjugation sites (19,20). To better understand the mechanism of this enhancement, we asked whether c-Myb was subject to the phenomenon of SC. Systematic analysis of this type of behaviour requires a dedicated set of reporter constructs with defined changes in the multiplicity of response elements. Hence, we constructed a set of reporter plasmids based on the pGL3 backbone with identical core promoters (from *MYC* P2), activated by one to five copies of an optimal Myb-response element (MRE) each with a phasing of 10 bp (Figure 1A). Binding to differently spaced MREs was examined in a systematic EMSA study using recombinant c-Myb. Here, we observed that a phasing of 6 bp caused interference from one bound factor on the binding of a second, while a phasing of 10 bp did not (Figure 1B). When reporters with one to five MREs were compared, wild-type c-Myb activated the various reporters with only minor differences (all about 3-fold) suggesting a modest response to increasing multiplicity (Figure 1C). In contrast, the SUMO-conjugation negative mutant, c-Myb 2KR, showed a remarkable increase in activity when the number of MREs was augmented (Figure 1C). In order to quantify this difference in synergistic behaviour, we defined a relative 'synergy factor' (SF) as the ratio of the reporter activity with four MREs to the activity with one MRE, divided by four (the latter to obtain ratios independent on the number of MREs). A simple proportional increase in reporter activity with the number of MREs should then give an $SF = 1$, while a positive synergy will result in $SF > 1$. An $SF < 1$ would then imply a less than additive effect, which might be termed 'negative synergy' (illustrated in Figure 1D). We calculated the SF based on four MREs in this work, but very similar SFs were obtained when we calculated ratios based on three or more MREs (data not shown). It should be noticed that SF is a relative quantity showing little dependence on the expression level of the factor tested. Using this simple quantification scheme, we compared wild-type and mutant c-Myb (Figure 1E). In fact, wild-type c-Myb displayed a negative synergy ($SF = 0.26$), while the SUMO-negative c-Myb 2KR showed a strong positive synergy ($SF = 3.9$). Single mutants, retaining one of the two SUMO-conjugation sites, showed intermediate levels (K503R: $SF = 0.98$, K527R: $SF = 0.72$). By comparing the SF data for the SUMO mutants (Figure 1E, upper panel) with their modification pattern (Figure 1E, lower panel), it seems clear that SF is inversely related to the level of c-Myb sumoylation. It may be noticed that the multiple bands representing one (c-Myb 1S) or two (c-Myb 2S) conjugated SUMO-moieties appear to be caused by various states of phosphorylation (data not shown). Taken together, these results suggest that the synergistic behaviour of c-Myb is highly dependent on its ability to become sumoylated.

In the reporters used to calculate SF values, the phasing of the MREs causes the bound c-Myb activators to have the same helical orientation on the promoter (marked *cis* in Figure 1F). We designed a 4×MRE reporter with a phasing of 15 bp to give an alternating helical orientation (marked *trans* in Figure 1F). While the SF values obtained with the SUMO-negative c-Myb 2KR did not change with helical orientation, the lower values obtained with wild-type c-Myb did, in fact, change. The level of 'negative synergy' became more relaxed when the helical orientation became alternating ($SF = 0.83$) compared to when all elements were oriented on the same side of the DNA helix ($SF = 0.26$). This may reflect a different co-operation mechanism responsible for generating positive synergy as compared to the mechanism restricting synergy.

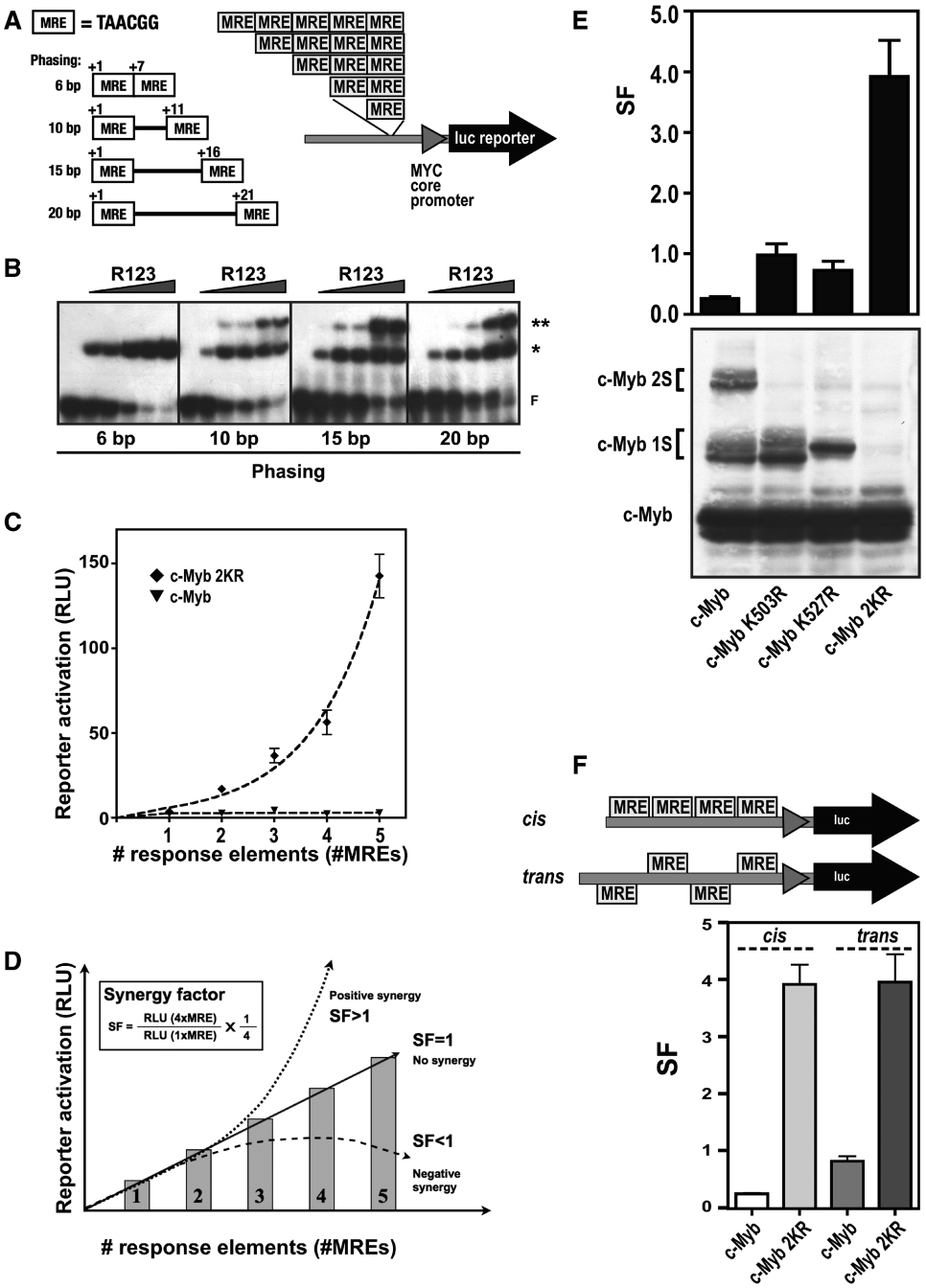

Figure 1. A SUMO-mediated SC is operating on c-Myb. (**A**) Schematic picture of the different EMSA probes used to identify the minimal phasing between the MREs, and the luciferase reporters used in this study. The reporter plasmids contained increasing numbers of MREs (1× to 5×, all with 10 bp phasing) upstream of a core promoter from human *MYC* driving the luciferase reporter gene. (**B**) Recombinant c-Myb DBD (R123: 0–25 fmol) was bound to 2×MRE(GG) oligonucleotides (20 fmol each) with different phasing (6, 10, 15 or 20 bp) and complexes were separated on EMSA gels. F, free probe; */**, one or two R123 molecule(s) in complex with the probe, respectively. (**C**) CV-1 cells were transfected with reporter plasmids containing increasing numbers of MREs (1× to 5×) as indicated and a plasmid encoding c-Myb wild-type (inverted filled triangle) or a SUMO-negative c-Myb 2KR (filled diamond). Reporter activation is presented as relative luciferase units (RLU) ± SEM. (**D**) The definition of the SF. (**E**) CV-1 cells were transfected with reporters containing one or four MREs [(1×MRE(GG)-MYC or 4×MRE(GG)-MYC] and plasmids encoding c-Myb wild-type, c-Myb with both SUMO-conjugation sites mutated from lysine to arginine (2KR) or single SUMO-conjugation sites mutated (K503R/K527R, upper panel). CV-1 cells were co-transfected with plasmids encoding the different c-Myb SUMO-mutants and PIASy (ratio 4:1) to visualize the sumoylation pattern. The cells were lysed directly in SDS–PAGE loading buffer to maintain the modifications (lower panel). c-Myb-S and c-Myb-2S: c-Myb modified with one or two SUMO moieties, respectively. (**F**) SFs were measured using wild-type c-Myb and c-Myb 2KR expression plasmids together with two variants of a luciferase reporter activated by four MREs, differing only in their helical phasing. In the *cis* reporter, pGL4-4×MRE(GG)-MYC, the MRE phasing is 10 bp, while in the *trans* reporter, pGL4-4×MRE(GG)-MYC abab, the MRE phasing is 15 bp. The results are presented as SF ± SEM.

To investigate the association between synergy and sumoylation further, we asked whether the strong synergy of c-Myb 2KR would be reduced by SUMO-1 covalently fused to its C-terminal. As shown in Figure 2A, the c-Myb-2KR-SUMO-1 fusion gave a strong reduction in synergy (SF = 0.95) when compared to the non-fused 2KR protein (SF = 3.9). This SF reduction was not due to alterations of the protein levels (Figure 2A, lower panel). The SF value of the SUMO-fusion is in fact very similar to the SF of the single mutants (K503R and K527R, Figure 1E) also harbouring one SUMO moiety in their conjugated state. We also studied the effect of alternative mutations (ΨKXE → ΨKXA) known to abolish SUMO-conjugation, while allowing other Lys-directed modifications to take place (34). The 2EA mutant (E505A+E529A) caused a strong increase in synergy (SF = 2.5, Figure 2B).

The oncogenic activated AMV v-Myb harbours both point mutations and N- and C-terminal deletions, the last one removing both SUMO-conjugation sites. We expected the latter deletion to affect the synergy behaviour of v-Myb. The cumulative effects of the other mutations, however, were less predictable in a synergy assay. As shown in Figure 2C, v-Myb in fact displayed the strongest synergy of all Myb-variants tested so far (SF = 4.8), which suggests that escaping synergy control

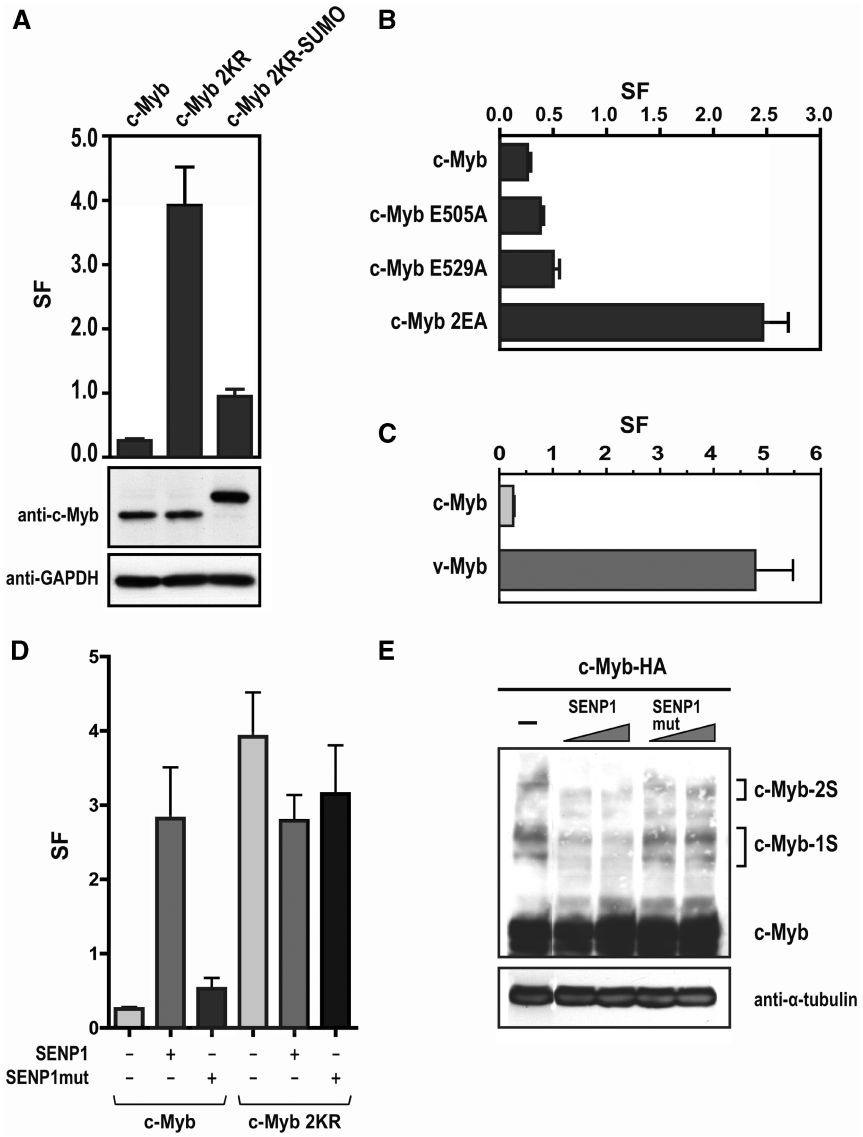

Figure 2. The level of synergy is highly dependent on the sumoylation status of c-Myb. CV-1 cells were transfected with reporters containing one or four MREs [1×MRE(GG)-MYC or 4×MRE(GG)-MYC] and plasmids encoding (**A**) c-Myb wild-type, c-Myb 2KR or a 2KR-mutant fused to SUMO-1, (**B**) c-Myb wild-type, c-Myb with individual SUMO-conjugation sites mutated from glutamate to alanine (E505A, E529A) or with both sites mutated (2EA), (**C**) c-Myb wild-type or the oncogenic version AMV v-Myb, or (**D**) c-Myb wild-type or a SUMO-negative c-Myb 2KR, with or without the SUMO-protease SENP1 or SENP1 mutant as indicated. The results are presented as SF ± SEM. Western controls shown in (A) were analyzed using anti-c-Myb (5E11) and anti-GAPDH antibodies. (**E**) CV-1 cells were co-transfected with a plasmid encoding c-Myb wild-type (1.0 μg) and either SENP1 or SENP1 mutant (0.25 or 0.50 μg) to visualize changes in the sumoylation pattern. The cells were lysed directly in SDS–PAGE loading buffer to maintain the modifications. c-Myb-S and c-Myb-2S: c-Myb modified with one or two SUMO proteins, respectively.

might be an important strategy during oncogenic activation.

Finally, the desumoylation of c-Myb induced by ectopic expression of the SUMO-protease SENP1 enhanced the synergy behaviour of wild-type c-Myb (SF increased from 0.26 to 2.8), while a protease-dead SENP1 had no effect (Figure 2D). As expected, the synergy behaviour of c-Myb 2KR was not influenced by SENP1. Co-expression of SENP-1 wild-type, but not the mutant, shifted the balance towards desumoylation of c-Myb (Figure 2E), confirming that SENP-1 is a relevant SUMO protease for c-Myb under these experimental conditions. Taken together, these data suggest that SUMO-conjugation is the major determinant of synergy behaviour of c-Myb as revealed by this MRE-multiplicity assay.

Relevance of SC to resident promoters

Most natural promoters are complex and often activated by a combination of transcription factors, several of which may be SUMO-conjugated. The activation of a natural promoter through desumoylation would be expected to involve removal of SUMO from various transcription factors. Unfortunately, this situation is not easy to mimic in an experimental setup. However, two Myb-responsive genes have been characterized in detail with respect to chromosomal organization of their Myb-responsive regulatory regions (Figure 3A). The *mim*-1 gene is activated by c-Myb through interaction with two distinct regions, a promoter and an enhancer region, both of which contain several MREs (35,36). The *lysozyme* gene has a similar organization but with fewer active MREs involved (36,37). Hence, we addressed the physiological relevance of the SUMO-mediated SC of c-Myb by comparing the activation of these two endogenous target genes by wild-type c-Myb and c-Myb 2KR, both expressed to similar levels (Figure 3B). We used c-Myb negative chicken macrophage HD11 cells since these cells express C/EBPβ known to cooperate with c-Myb in transcriptional activation of both *mim*-1 and *lysozyme* (24,38). As shown in Figure 3C, co-transfection of c-Myb wild-type or 2KR mutant resulted in a clear differential activation of *mim*-1, as expected if several MREs contribute to the activity of the chromatin embedded promoter. For the *lysozyme* gene (Figure 3D), the difference between c-Myb wild-type and 2KR was much less pronounced, consistent with fewer MREs being involved. We conclude that natural promoters differ significantly in their synergy behaviour when c-Myb is compared in the two states. We expect, however, that a larger difference would have been observed if we had been able to compare two or more cooperating factors in both SUMO-conjugated and non-conjugated states.

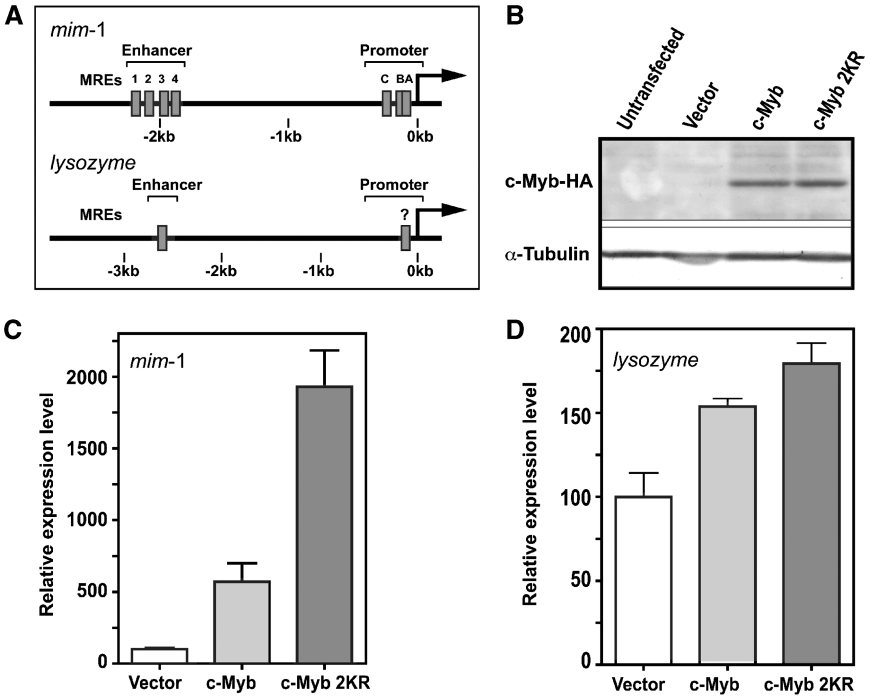

Figure 3. Different c-Myb-mediated synergy on natural chromatin-embedded promoters. (**A**) Schematic presentation of the regulatory elements of the *mim*-1 and *lysozyme* genes according to (35–37). Both genes contain functional MREs in enhancer elements as well as in promoters. Question mark indicates functionality uncertain. HD11 cells were transfected with plasmids expressing c-Myb-HA or the sumoylation-negative mutant c-Myb-2KR-HA. (**B**) A western blot performed with anti-HA antibody, using lysates from the same cultures used for activity measurements. The α-tubulin was used as loading control. Activation of the endogenous Myb-target genes (**C**) *mim*-1 and (**D**) *lysozyme* was measured by quantitative real-time PCR. Target gene expression data were normalized by the relative expression of the housekeeping gene *HPRT1* and represented as relative to empty vector-transfected cells, which were set to 100. The results represent the mean ± SEM of three independent biological assays, each analysed in duplicate for expression levels.

SUMO does not alter the DNA binding of c-Myb

Being confident that the synergy property of c-Myb is controlled by SUMO, we asked what mechanism might be involved. First, we explored SUMO-induced changes in intrinsic properties of c-Myb, i.e. whether SUMO-conjugation converts c-Myb into a less synergizing factor by causing either reduced specific DNA binding or impaired transactivation properties. Secondly, we explored altered interactions, i.e. whether SUMO-conjugation might, by adding a new interaction surface, change the recruitment of co-factors to c-Myb.

An EMSA analysis showed that the specific DNA binding observed was similar for c-Myb wild-type and 2KR as well as for the SUMO-fusion protein (Supplementary Figure S1), indicating that SUMO moieties conjugated or fused to the C-terminal of c-Myb have no significant influence on the activity of the DNA binding domain localized in the N-terminal of the protein. To monitor the DNA binding *in vivo*, we analysed c-Myb occupancy on the endogenous *MYC* promoter, an established target gene of c-Myb, harbouring multiple MREs [(39) and references therein]. For this analysis, we generated stable cell lines derived from K562 with integrated double-tagged c-Myb constructs in both wild-type and 2KR mutant versions. The levels of expressions of c-Myb in these cells are very close to the endogenous gene product and thus represent physiological levels of c-Myb (see western-insert in Figure 4). The ChIP signals were highly similar for the two versions of c-Myb, suggesting no significant difference in chromatin occupancy between wild-type and 2KR c-Myb (Figure 4). We also tested the same *MYC* promoter region in a reporter assay and found c-Myb 2KR to activate this promoter significantly stronger than wild-type c-Myb did, confirming the relevance of this promoter for SC (Supplementary Figure S2). Taken together, these data do not support an explanation of the SUMO-induced change in synergizing properties of c-Myb based on changes in DNA binding properties, neither *in vitro* nor *in vivo*.

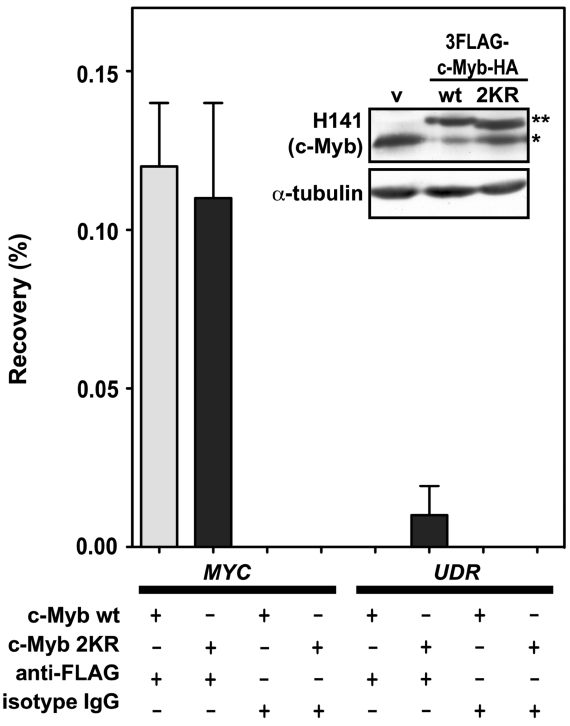

Figure 4. c-Myb occupancy on the *MYC* promoter is SUMO-independent. ChIP were performed with K562 cells to assess occupancy of c-Myb wild-type and SUMO-negative 2KR-mutant on the human *MYC* promoter. K562 cells were stably transfected with 3×FLAG- and HA-tagged c-Myb wild-type and 2KR, and clones were picked based on immunoblotting (inserted panel) showing similar expression of the integrated and endogenous c-Myb. ChIP was performed using anti-FLAG antibody, while an isotype IgG antibody was used as negative control. Occupancy was analysed by amplifying the *MYC* promoter by real-time PCR after reversal of the cross-linking. An unrelated DNA region (UDR) was used as negative control. The UDR was chosen from the gene desert region (53), the exact location is: chr2: 22153688+22153788. The results are calculated from triplicates of real-time PCR reactions and are expressed as percentage of recovery ± SD relative to the input. v, stable cell lines with integrated empty vector pEF1neo; asterisk, endogenous c-Myb; double asterisk, stably integrated, double-tagged c-Myb.

SUMO controls an activator-to-repressor switch in c-Myb NRD

The NRD of c-Myb obtained its name from the early observation that a C-terminal deletion in mouse or chicken c-Myb (similar to that found in AMV v-Myb) created a more active factor (40,41). The same is true for human c-Myb. We found human c-Myb-ΔNRD to be much more active than the full-length protein (bar 3 versus bar 1 in Figure 5A), probably due to the loss of the SUMO-conjugation sites in NRD. However, we noticed that the same deletion, when compared to the de-repressed SUMO-negative 2KR-version of c-Myb (bar 3 versus bar 2 in Figure 5A), created a less active factor suggesting that NRD in the absence of SUMO-conjugation might harbour an activation function (AF). If so, the c-Myb 2KR has not only lost its internal repressor function but also behaves like a protein with an added AF.

The classical way of identifying an AF is by fusing the domain under investigation to the Gal4p DBD and monitoring the induced activation of a Gal4p-responsive reporter. When we tested the NRD region of c-Myb (amino acids 410–640) in such a system, we saw that the wild-type version of NRD had a weak repressive effect (Figure 6A). However, the SUMO-negative 2KR version of NRD was no longer acting negatively, but had switched into behaving as an activator (Figure 6A). To make sure that this notable observation was not some peculiarity related to the minimal *E1B* promoter in the pG5E1bLuc reporter used, we repeated the experiment with an *SNRPN*-driven Gal4p-responsive luciferase reporter and observed an equal effect (data not shown). We also tested out the E→A mutants instead of the K→R mutants and observed the same changes in the properties of the NRD becoming an activating domain upon reduced sumoylation (Figure 6A). The fusion of SUMO-1 to the active 2KR-version of the NRD abolished its AF totally. All Gal4p-DBD fusion constructs showed equal expression levels as evaluated by western (data not shown)

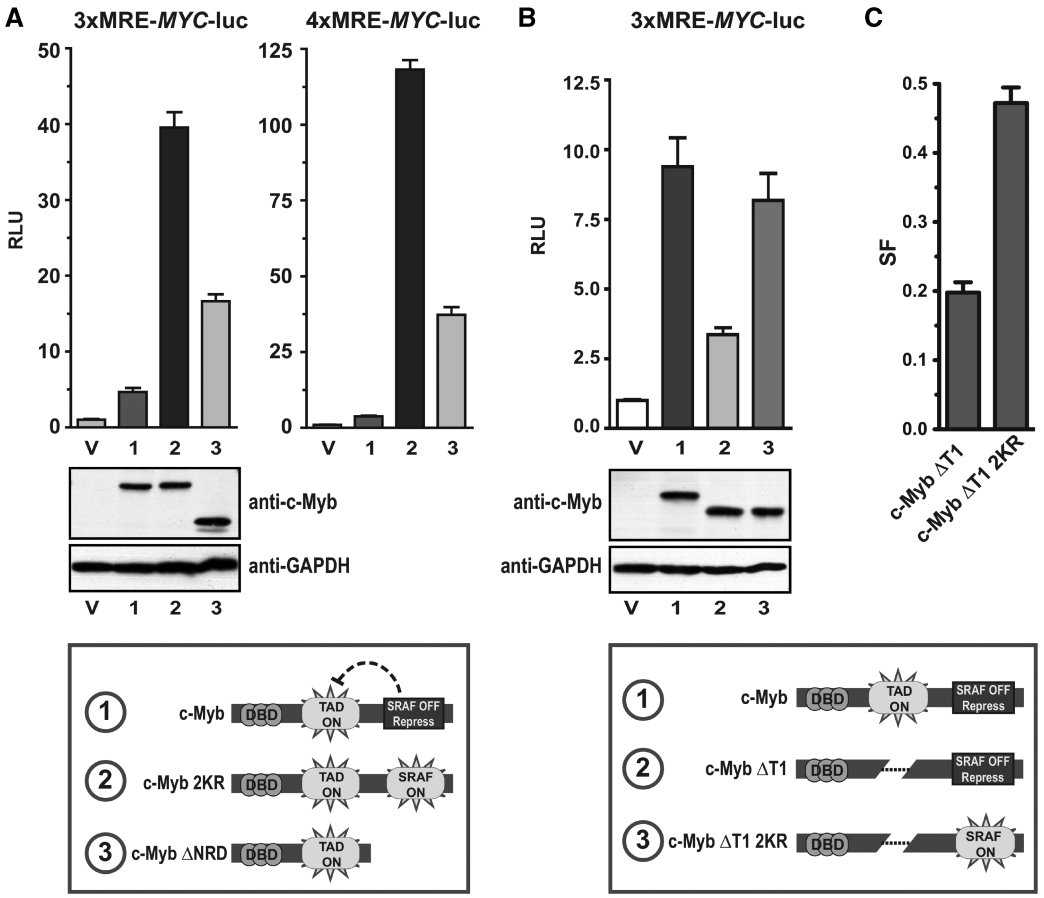

Figure 5. Evidence for two AFs in c-Myb. CV-1 cells were transfected with a Myb-responsive reporter plasmid containing three or four MREs (0.2 μg) as indicated and (**A**) 0.4 μg of plasmids encoding either full-length c-Myb, its SUMO-conjugation negative mutant c-Myb-2KR, or a deletion variant c-Myb ΔNRD, or (**B**) full-length c-Myb, a variant with the central transactivation domain deleted (c-Myb-ΔT1) and SUMO-negative c-Myb with TAD deleted (c-Myb-ΔT1-2KR). The results are presented as RLU ± SEM. Western controls shown in (A) and (B) were analysed using anti-c-Myb (5E11) and anti-GAPDH antibodies. The numbers under each lane/bar correspond to the constructs shown in the lower boxes, V: empty vector. (**C**) CV-1 cells were transfected with reporters containing one or four Myb responsive elements [1×MRE(GG)-MYC or 4×MRE(GG)-MYC] and plasmids encoding c-Myb-ΔT1 or c-Myb-ΔT1-2KR. The results are presented as SF ± SEM.

analysis. It is noteworthy that this gain of AF paralleled a strong increase in synergy properties as observed when SF-values were measured for all the Gal4p-fusions using a 1×GRE reporter in addition to the 5×GRE reporter (Figure 6B).

Finally, we analyzed Gal-NRD fusion proteins in the presence of overexpressed SENP1. As shown in Figure 6C, this SUMO-protease was able to induce the functional switch of the NRD from being a weak repressor to a positive activator. While the wild-type NRD changed 50-fold in activity upon overexpression of SENP1, the already derepressed 2KR version did not increase more than the background. As expected, this functional switch was accompanied by a large change in SF-values (data not shown).

The model implicated from these findings is that c-Myb has two AFs, one constitutively active, centrally located in the protein (TAD) and a second SRAF in the NRD region, functioning as an internal repressor in the wild-type protein (illustrated in Figure 5A). To test this model further, we reasoned that if the NRD contains a SRAF, which becomes active upon removal of SUMO-conjugation, we should be able to rescue a defect introduced in c-Myb caused by deletion of its constitutive central transactivation domain, simply by activating its silenced SRAF in NRD. In Figure 5B, we have compared c-Myb wild-type, c-Myb with its central TAD deleted and c-Myb with the TAD-deletion in combination with the 2KR mutation. Clearly, the expected rescue is observed showing that also in the full-length protein the NRD can be activated providing a second AF, which will contribute to the overall transactivation potential of the c-Myb protein. In parallel with this rescue, we observed an increase in synergy properties (Figure 5C).

Activators with two AFs are known from the literature, the steroid hormone receptor family being one prominent example. In fact, SC was first found in members of this family (9). To test whether the SUMO-dependent repressor-to-activator switch is a more general phenomenon, we chose to study the Sp3 transcription factor because this factor has been reported to activate or repress transcription depending on the promoter context (42). Moreover, the repressive function of Sp3 is mediated

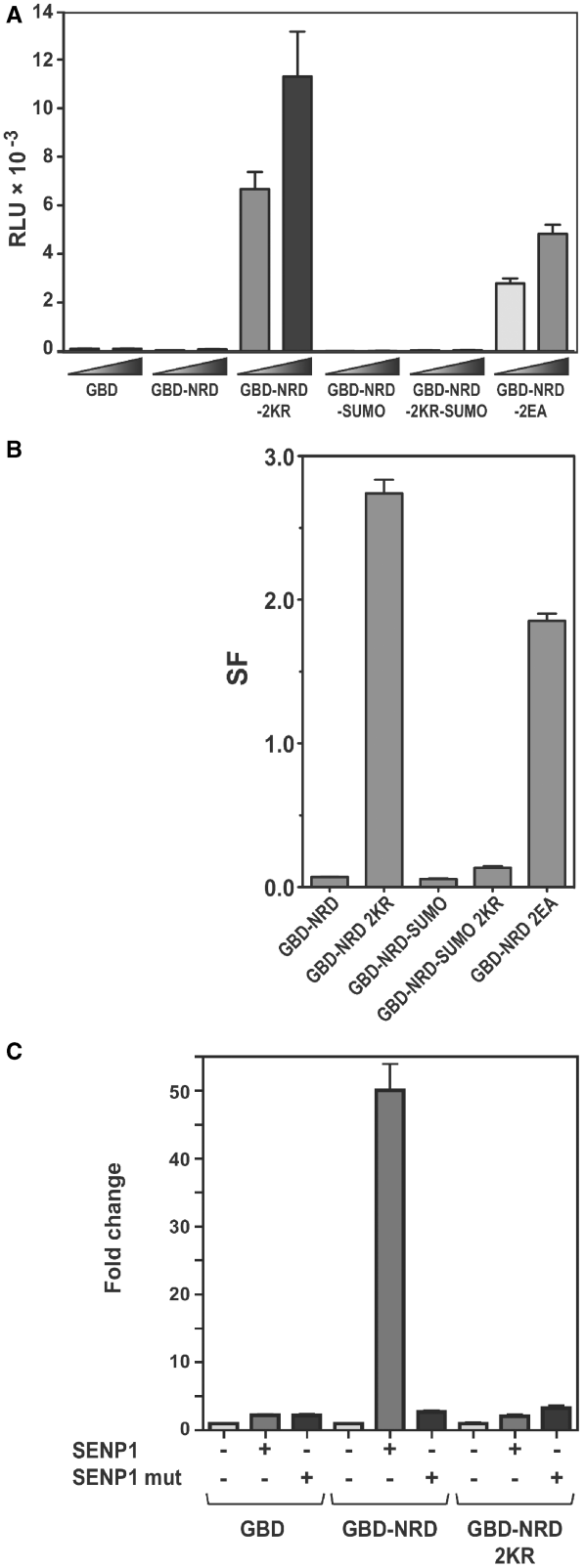

Figure 6. The NRD region of c-Myb harbours a SRAF that can switch from being repressive to be activating. (**A**) CV-1 cells were transfected with 0.2 or 0.4 μg of plasmids expressing Gal4p-DBD (GBD) fused to c-Myb NRD (amino acid residues 410–640) wild-type, 2KR, WT-SUMO-1, 2KR-SUMO-1 or 2EA. The reporter output from the *E1b*-driven Gal4p-responsive reporter plasmid (5×GRE, 0.2 μg) was normalized to the effect of Gal4p-DBD (0.2 or 0.4 μg), which was set to 100. The results are presented as RLU ± SEM. (**B**) Based on parallel transfections, using an 1×GRE-E1b-Luc reporter plasmid (0.2 μg), the SFs of the constructs assayed in (A) were calculated. The results are presented as SF ± SEM. (**C**) CV-1 cells were transfected with plasmids expressing GBD, GBD-NRD or GBD-NRD-2KR together with the SUMO-protease SENP1 or a protease-dead SENP1 mutant. The results are presented as fold change ± SEM, relative to the activity levels of the GBD constructs in the absence of the protease.

by SUMO-conjugation of K551 located in an ID. First, we confirmed using our Gal4-responsive reporter that mutation of its SUMO-conjugation site turned a Gal4p-Sp3 fusion protein into an activator (Figure 7A) as reported (42). This was accompanied by a large increase in SF value indicating a parallel change in synergy properties (Figure 7B). Next, we asked whether the small ID region harbouring the sumoylation site when removed from its two glutamine-rich activation domains would show SRAF behaviour. As seen for c-Myb NRD, wild-type Sp3-ID had a weak repressive effect by itself, while the SUMO-negative version was clearly activating (Figure 7C), showing the presence of a SRAF in Sp3. In parallel, we measured a large increase in SF value for SUMO-negative Sp3-ID (Figure 7D), linking the functional switch to changes in synergy behaviour. We also extended our analysis to the sumoylated CRD in p53. Again, we observed a similar change, but with more modest alterations (Supplementary Figure S3).

Synergy and number of AFs

Given that SUMO-conjugation suppresses a second AF in c-Myb, it appears quite probable that derepression of this SRAF upon removal of SUMO makes an important contribution to the enhanced synergy of c-Myb 2KR. An obvious model, combining the synergy properties of the c-Myb variants and the switch-property of NRD, would be that the efficiency of synergy as measured by the SF, relates to the number and strength of independent AFs brought to a promoter through the bound transcription factors. If this is correct, we should be able to measure increasingly larger SFs for c-Myb variants harbouring increasing number (and strengths) of AFs. To test this hypothesis, we measured SFs for two additional c-Myb variants: a c-Myb-VP16 fusion protein (harbouring two AFs linked to one repressive NRD function), and c-Myb-2KR-VP16 (harbouring three AFs). These were compared with wild-type c-Myb (harbouring one partially repressed AF), c-Myb ΔNRD (harbouring one AF) and c-Myb 2KR (harbouring two AFs). As shown in Figure 8, we observed the expected SF increase when number and strengths of AFs were increased. The deletion c-Myb ΔNRD, with one AF relieved from the repressive influence of NRD, gave a higher SF than both wild-type and c-Myb-VP16, but still well below that of c-Myb 2KR harbouring two AFs. The c-Myb-2KR-VP16 fusion protein, harbouring three AFs, showed an extremely large SF of 35. This variant was in fact 140-fold more active on a promoter with four MREs than on a promoter with a single MRE.

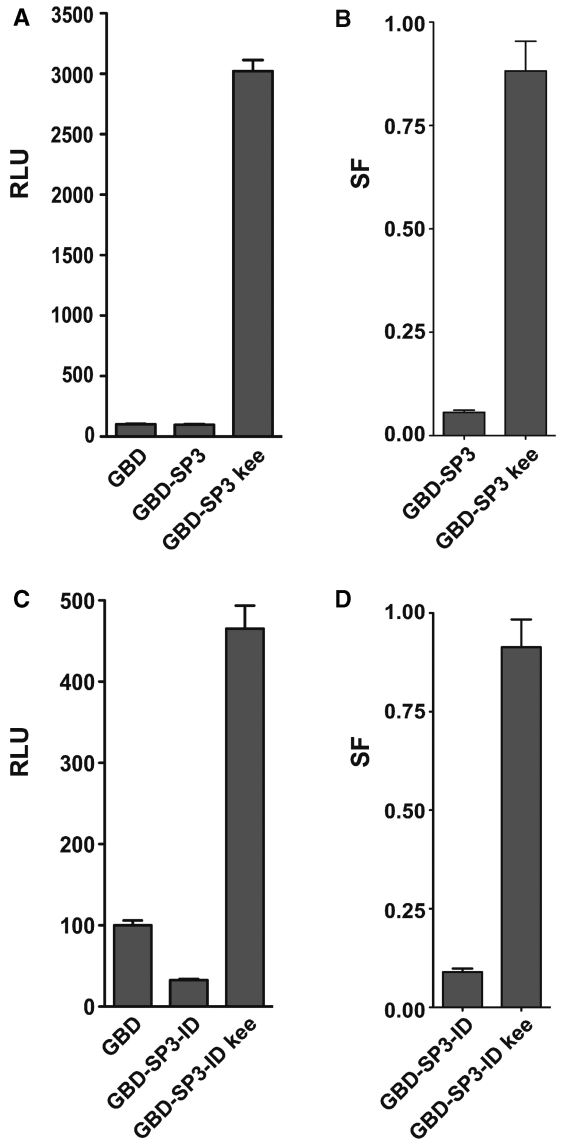

Figure 7. The ID in Sp3 harbours a SRAF. CV-1 cells were transfected with 0.2 μg of plasmids expressing Gal4p-DBD fused to (**A**) Sp3 (amino acid residues 81–613) wild-type or the kee SUMO-negative mutant, or (**C**) Sp3 ID (amino acid residues 534–612) wild-type and kee mutant. The reporter output from the *E1b*-driven Gal4p-responsive reporter plasmid (5×GRE, 0.2 μg) was normalized to the effect of Gal4p-DBD (0.2 μg) and set to 100. The results are presented as RLU ± SEM. (**B** and **D**) Based on parallel transfections, using an 1×GRE-E1b-Luc reporter plasmid (0.2 μg), the SFs for the GBD-Sp3 and GBD-Sp3-ID constructs assayed in (A) and (C) were calculated. The results are presented as SF ± SEM.

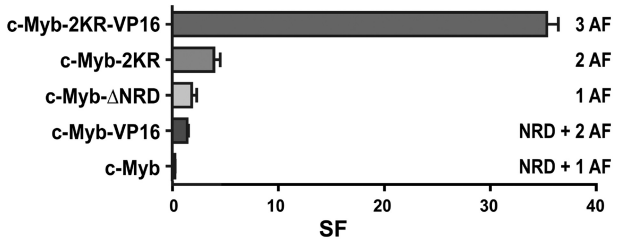

Figure 8. The importance of the number of AFs for level of synergy. CV-1 cells were transfected with reporter plasmids containing one or four MREs (1×MRE(GG)-MYC or 4×MRE(GG)-MYC) and an effector plasmid encoding one of the following c-Myb variants: c-Myb, c-Myb-ΔNRD encoding amino acids 1–443, the SUMO-conjugation negative mutant c-Myb-2KR, as well as c-Myb and c-Myb-2KR fused C-terminally to the herpes simplex virus VP16 transactivation domain. The results are presented as SF ± SEM.

SUMO-dependent recruitment to chromatin

Having explored how SUMO-conjugation affects intrinsic properties of c-Myb, we next investigated SUMO-modulated interactions. To assess interactions in the context of multiple SUMO-conjugated c-Myb proteins bound to a chromatinized promoter, we took advantage of the model system developed by Suske and co-workers (33), in which an array of Gal4-responsive elements (5×GRE) in front of a luciferase reporter is integrated in the genome of HEK 293 cells (Figure 9A). First, we made Gal4-c-Myb fusion constructs of the same design as used previously for Sp3 (33), with the c-Myb DBD replaced by Gal4-DBD (Figure 9A). Reporter assays showed a dramatic SUMO-dependent switch in activation in this system, with wild-type c-Myb showing quite modest activation and the SUMO-negative c-Myb 2KR behaving as a potent activator of the integrated reporter, being about 900-fold more active than wild-type c-Myb (Figure 9B, lower panel). Both forms of c-Myb were equally expressed (Figure 9B, upper panel). For comparison the sumoylation-deficient Gal4-Sp3-KEE mutant versus wild-type Gal4-Sp3 also showed a SUMO-dependent shift, but only about 10-fold (Figure 9C). Notably, the level of activation observed with sumoylation-competent Gal-Myb was comparable to that seen with sumoylation-deficient Gal4-Sp3-KEE (Figure 9C). ChIP analysis of c-Myb occupancy confirmed equal binding of both forms of c-Myb to the promoter region (Figure 9D). This is consistent with what we observed in the EMSA studies (Figure 1B and Supplementary Figure S1) and on the *MYC* promoter (Figure 4), confirming that SUMO-status does not affect the promoter recruitment of c-Myb.

Since the sumoylation-competent Gal-Sp3 repressor in the reporter cell line studied here has been shown to induce a local repressive chromatin state through recruitment of factors associated with heterochromatin formation (33), we addressed whether the same factors were differentially recruited by the two forms of c-Myb tested in this system. However, consistent with the activation profiles, neither form of c-Myb appears to behave like Gal-Sp3, but rather like the activating Gal-Sp3-KEE. We were not able to detect c-Myb-mediated recruitment of SETDB1, nor observe significantly different levels of H3K9me3 associated with the promoter (results not shown). We performed a ChIP-analysis of endogenous Mi-2, but could not detect any enrichment at the promoter (data not shown). Assaying transfected Mi-2α, we observed a c-Myb-dependent recruitment to the promoter, but no differential SUMO-dependent recruitment (Figure 9E). This is consistent with our previous observation that Mi-2α has affinity for c-Myb (31), which in this case may override a SUMO-mediated recruitment.

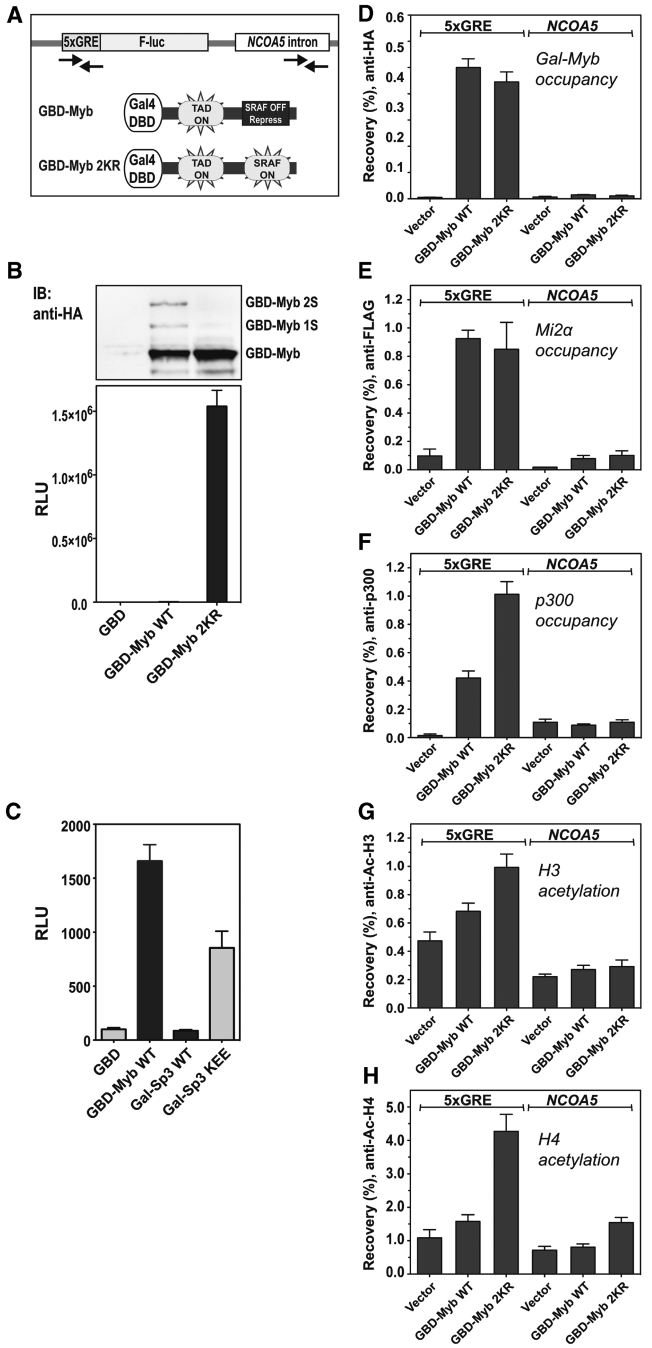

Figure 9. SUMO-dependent recruitment to chromatin. (**A**) ChIPs were performed in a HEK 293 reporter cell line (33) harbouring an integrated transgene as illustrated [drawing based on Fig.2 in ref. (33)]. The reporter cell line was transfected with Gal-fusion derivatives of c-Myb as illustrated. Occupancy of factors and presence of chromatin marks were assessed on the luciferase promoter 5×Gal4 responsive elements (5×GRE) and on an intronic element in the neighbouring *NCOA5* gene. Arrows indicate the regions amplified during analysis of ChIP samples by quantitative real-time PCR (ChIP–qPCR). (**B**) HEK 293 cells were transfected with plasmids expressing Gal4p-DBD (GBD) fused to c-Myb (amino acid residues 233–640) wild-type or 2KR. The output from the integrated luciferase was normalized to the effect of GBD alone, which was set to 100. The results are presented as RLU ± SEM. Immunoblotting was performed to control protein expression. Cells were lysed directly in SDS–PAGE loading buffer to maintain the modifications, and analysed using anti-HA antibody. GBD-Myb 1S and 2S: GBD-c-Myb modified with one or two SUMO proteins, respectively. (**C**) HEK 293 cells were transfected with plasmids expressing Gal4p-DBD fused to c-Myb wild-type, Sp3 wild-type or the kee SUMO-negative mutant of Sp3. The luciferase output was treated and presented as in (B). Occupancies of (**D**) Gal-Myb, (**E**) Mi-2α, (**F**) p300, (**G**) acetylated histone H3 and (**H**) acetylated histone H4 on the 5×GRE promoter and on the *NCOA5* intron were analysed using ChIP–qPCR with cells transfected with GBD-Myb wild-type, 2KR or empty vector. In (E) Gal-Myb was co-transfected with FLAG-tagged Mi-2α.

Taking into consideration the activation profile of the Gal-Myb proteins relative to the Gal-Sp3 proteins (Figure 9C), we turned to analysis of co-activator recruitment. Interestingly, the endogenous p300 co-activator showed a clear differential recruitment, as revealed by a significantly higher occupancy in the presence of the sumoylation-deficient Gal-Myb 2KR compared to wild-type Gal-Myb (Figure 9F). This behaviour is consistent with the observation that Gal-NRD 2KR is p300-responsive while Gal-NRD is not (Supplementary Figure S4A and B). We therefore assume that the SUMO-negative NRD (SRAF in ON-state) contributes to the observed differential recruitment of p300, although its contribution may be indirect involving additional co-activators (Supplementary Figure S4C). A ChIP analysis of the acetylation status of the promoter showed a parallel increase in histone H3 and H4 acetylation when sumoylation was abolished and p300 occupancy enhanced (Figure 9G and H), consistent with a p300-induced acetylation pattern (43).

We conclude that the SC of c-Myb is strongly coupled to SUMO-conjugation of c-Myb. When c-Myb is being sumoylated, its recruitment to promoters is not affected, but its SUMO-status will obstruct synergy and hinder its full transactivation potential to be unleashed. Removal of SUMO-conjugation triggers the potential of c-Myb to synergize on compound promoters. One important contribution to this increased synergy appears to be the derepression of a silenced AF in the NRD of c-Myb becoming active upon removal of SUMO-conjugation. This leads to a more efficient recruitment of the co-activator p300 and increased histone H3/H4 acetylation. In this manner, the SRAF switch makes an important contribution to the large differences observed between wild-type and SUMO-negative c-Myb on complex promoters.

DISCUSSION

Synergy is an inherent property of the transcription system being related to the multiplicity of interactions necessary to assemble an active PIC at the TSS to allow the productive elongation by RNA polymerase II. Any modulations of the many weak interactions may cause a large change in output. Thus, the phenomenon of synergy appears to be an ideal target for transcriptional control. We have in this work shown that c-Myb, which appears to be a rather weak activator, has an inherent potential to become a quite strong one. This property is revealed by three phenomena studied here. First, removal of SUMO-conjugation in c-Myb unleashes the potential of c-Myb to synergize on compound promoters. The larger the number of MREs, the larger the difference in

Myb-induced reporter activation between wild-type c-Myb and its SUMO-conjugation negative mutant. All experimental modulations that changed the level of SUMO-conjugation led to the expected changes in synergy behaviour, firmly linking the ability of c-Myb to synergize with its sumoylation status. We further show that a major contribution to the increased synergy caused by SUMO-removal is the derepression of a silenced AF in the NRD of c-Myb. This domain is able to switch from functioning as an internal repressor into functioning as an activator upon removal of SUMO-conjugation. Our data suggest a new model for c-Myb in which the factor has two AFs, one in the established central TAD acting in a constitutive fashion and a second inducible AF, designated SRAF, being repressed by SUMO-conjugation. The latter provide c-Myb with an inherent potential to change from a rather weak activator into a potent transactivating factor harbouring a double set of AFs. The dual function of the C-terminal region of c-Myb merits a change in designation from NRD to CRD. Finally, we show that this switch leads to a more efficient recruitment of the p300 co-activator to compound chromatin-embedded promoters, changing the local signature of histone modifications.

The link between the ability of a transcription factor to synergize on complex promoters and the factor being relieved from SUMO-conjugation was first observed in studies of the GR (9,10) and later extended to other factors such as C/EBPα, SF-1, MITF, and recently ZBP-89 (12–14,44). We show in this work that c-Myb may be added to the growing list of synergy-controlled transcription factors.

Concerning synergy control mechanisms, several possibilities have been proposed, such as SUMO-mediated recruitment of co-repressors (9,10) to explain the repressive aspects. A significant advance in our understanding here comes from the recent works from Suske and co-workers (33,45) showing that a set of identified repressor proteins acts together to set up a local heterochromatin structure dependent on SUMO. However, there may be a larger diversity of mechanisms here than revealed by the action of Sp3 as examplified by the SUMO-dependent recruitment of HDACs (46,47), CoREST1 (48) and KAP1 (49). Still, the common theme appears to be that sumoylation plays a key role in coordinating histone modifications and chromatin structure, which explains its effect on transcription and on synergy. In the present work, we add three elements to a model that may explain the phenomenon of SUMO-mediated SC. First, we emphasize that the synergy behaviour observed points to *multiplicity* as an important aspect that has to be incorporated into the mechanistic model. Second, we show that sumoylated domains may be repressed activation domains that switch function upon desumoylation. Finally, we present evidence for a SUMO-dependent differential recruitment of co-activators leading to a change in the histone modification pattern.

Figure 10 illustrates some key elements in a model of transcriptional synergy. In the sumoylated state, synergy is restricted either because of a SUMO-induced blocking of

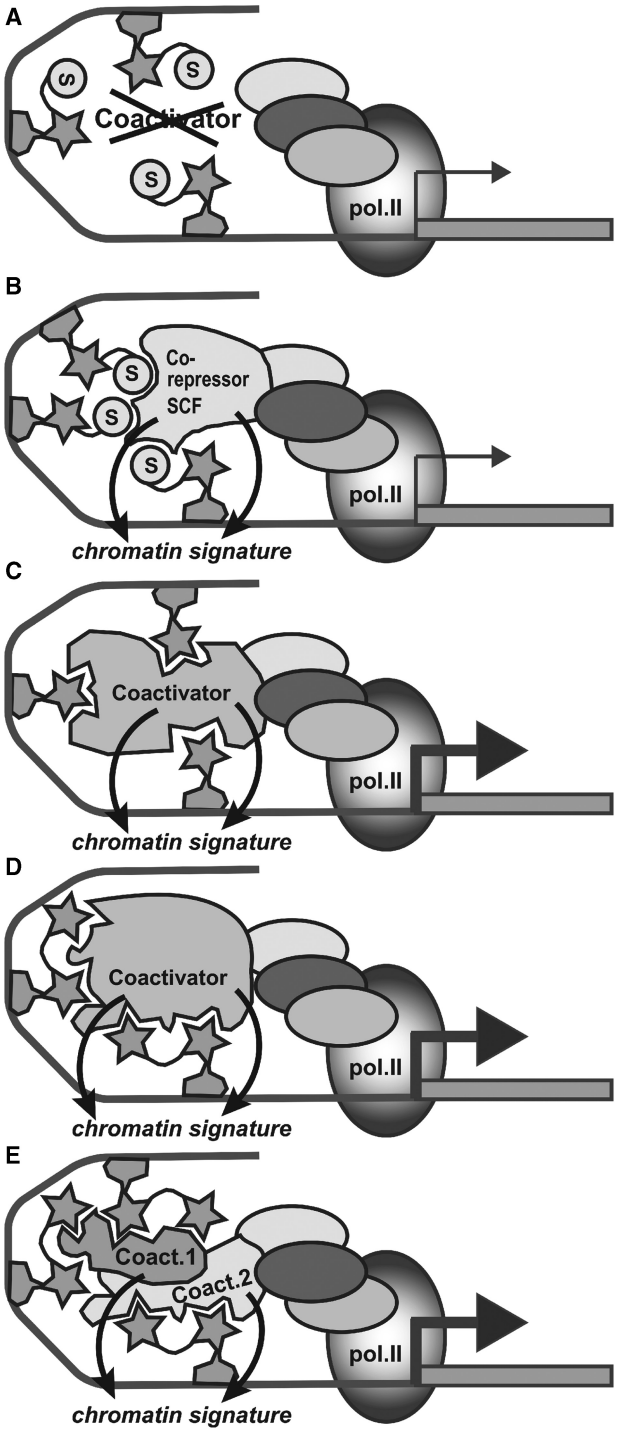

Figure 10. A model for SRAF and SC. (**A**) A model for the repressed state, where SUMO-conjugation disrupts the interaction with the co-activator. (**B**) A model for the repressed state where multiple SUMO-conjugated factors recruit a multivalent co-repressor; SCF, synergy control factor (9). (**C**) Traditional concept of synergy mediated by joint recruitment of a multivalent co-activator. (**D**) Current model for the activated state where multiple AFs (depicted as stars) per transcription factor lead to more efficient recruitment of a multivalent co-activator. (**E**) A variant of the model above (D) where different AFs may interact with different co-activators.

productive interactions with co-activators (Figure 10A) or a SUMO-induced generation of repressive interactions (Figure 10B). A combination of both is also quite likely. The recruitment of repressors to chromatin fits very well with the model of SUMO-mediated heterochromatin formation (33), as well as with other repressor studies (50). Although not emphasized in the model, the work on SUMO-governed hetrochromatic formation (33) was performed with the same integrated, multimerized Gal4-responsive reporter gene as in this study. Thus, we would not be surprised if some of the co-repressors reported in Suske's study turn out to have a preference for arrays of SUMO-conjugated factors. The active synergistic state would require removal of SUMO, which may be either a stochastic process caused by the unstable sumoylated state or a more directed process through signal-induced recruitment of SUMO-proteases.

Our identification of the SRAF phenomenon, where the NRD region of c-Myb as well as the ID of Sp3 and possibly the CRD of p53 have the particular ability to switch from acting negatively to acting positively depending on sumoylation status is a novel element that adds to our mechanistic understanding. We propose that positive synergy is determined by the number and strength of independent AFs associated with a promoter through the bound transcription factors. Under conditions of normal sumoylation, c-Myb contains one AF (in TAD) partially repressed by the sumoylated NRD and the level of synergy is rather low (SF = 0.26). Upon reduced sumoylation, as mimicked in the 2KR mutant or by ectopic expression of SENP1, the second AF is turned on and the central TAD is relieved of its repression. Now, c-Myb will operate with two AFs and show higher synergy (SF = 3.9 and 2.8, respectively). In this model, the number of AFs is a critical determinant of synergy.

How the SRAF mechanism may work is illustrated in Figure 10D and E. A promoter-centred view is appropriate here. When two or three transcription factors cooperate, this means that they provide a promoter with two or three AFs resulting in a gradually increased efficiency of co-activator recruitment (Figure 10C), which is the classical text-book model. A single factor presenting one or two AFs would in a similar fashion provide the promoter with one or two AFs each contributing to an increased efficiency of co-activator recruitment (Figure 10D and E). From the point of view of the promoter, it must be the total number and strengths of the associated AFs that together determine its level of activation. With multiple AFs per transcription factor, efficient synergistic activation may be achieved with a more limited number of factors than if each factor only carries single AFs. Whether each of the AFs recruits the same (Figure 10D) or different co-activators (Figure 10E) might be promoter dependent. For c-Myb, the full range of co-activators differentially recruited remains to be determined. In all variants of the model, diffential recruitment of co-repressors or co-actviators leads to a differential chromatin signature.

We were not able to demonstrate a SUMO-dependent co-repressor recruitment to wild-type c-Myb, despite its moderate activity compared to the SUMO-deficient 2KR mutant. Still, there might be factors, not assayed in this work, that show such a dependency also in a Myb context. What is obvious, is that c-Myb does not mimic the behaviour of Sp3 in this system. We did however observe a clear SUMO-dependent co-activator recruitment by showing a significantly higher p300 occupancy on a promoter to which a SUMO-deficient c-Myb is bound, compared to the wild-type reference (Figure 9F). This differential recruitment was mirrored in the expected change in the local histone acetylation pattern (Figure 9G and H). We believe this differential recruitment is linked to the switch property of the SRAF in NRD. Exactly, how the SUMO-negative NRD (SRAF in ON-state) contributes to more efficient p300 recruitment, remains to be determined. A simple GST-pull-down experiment did not reveal direct binding to p300, in a similar fashion as the central TAD region (Supplementary Figure S4). However, a Gal-NRD 2KR construct was found to be p300 responsive in contrast to the corresponding wild-type construct, consistent with a change in p300 recruitment (Supplementary Figure S4). Our working hypothesis is that the switch generates a novel interaction with an unknown protein that also binds to p300, thus leading to the observed differential p300 recruitment. Efforts are in progress to identify this factor.

A question worth asking is whether the SRAF model of synergy is specific for c-Myb or reflecting a more general mechanism. An argument for the latter is our observation of a robust SRAF in the ID region of Sp3 and a more moderate one in the CRD region of p53. This model may well be relevant also for nuclear receptors (NRs). Many NRs have at least two activation domains, the ligand-independent AF, AF1, which resides in the N-terminal domain, and the ligand-dependent, AF2, which is localized in the C-terminal domain. Taking GR as example, the AF1 region (amino acids 108 to 317) is sumoylated (K298 and K313) and is subjected to SC (9). Assuming then that AF1 may be switched on or off depending sumoylation status, while AF2 is turned on upon ligand binding, this represents an interesting equivalent to c-Myb with the potential to act with different numbers of AFs in a SUMO-controlled manner. In fact, both the GR AF1 and the AR AF1 have been shown to be switched on when desumoylated (51,52).

In this study of how SUMO-conjugation restricts the synergy behaviour of c-Myb, we have discovered a novel switchable function (SRAF) associated with NRD, providing c-Myb with a potential strong regulatory switch. This switch is associated with a SUMO-dependent co-activator recruitment changing the local chromatin signature.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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