Supplementary Information

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

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Supplementary materials and methods

Plasmid constructs.

Reporter plasmids. pGL4-1×MRE(GG)-MYC, pGL4-2×MRE(GG)-MYC, pGL4-3×MRE(GG)-MYC, pGL4-4×MRE(GG)-MYC, and pGL4-5×MRE(GG)-MYC were constructed in two steps. An oligo containing a sequence from human *MYC* P2 core promoter was first inserted into the *Kpn*I/*Sma*I site of pGL4 basic (a designed derivative of pGL3 basic (E1751; Promega)) where the MCS was changed by oligo-insertion to contain sites for *Mlu*I, *Xho*I, *Kpn*I, *Sma*I and *Bgl*II). Then oligos were designed to contain one, two, three, four or five Myb-responsive elements (MREs, TAACGG) with a spacing of four bp (TTTT) giving a phasing of ten bp between the MREs (i.e. MREs starting in position +1, +11, +21 etc; Fig 1A). Duplex oligos were subcloned between *Mlu*I and *Xho*I sites in pGL4 basic. Oligo insertions were verified by sequencing. In the same way, pGL4-4×MRE(GG)abab-MYC was constructed by inserting an oligo containing four MREs in which the phasing was 15 bp between the responsive elements. The reporter pGL4-3×MRE(GG)aab-MYC contains three MREs in which the phasing is 10 bp between the first two and 15 bp between the second and third MRE. The Myb-responsive pGL3-MYC reporter was made by amplifying the human *MYC* promoter from position -1213 to +285 relative to the transcription start site (acc. no. NM_002467), inserting it into the pGL3 basic vector between *Mlu*I and *Hind*III.

The *E1b* driven Gal4p responsive luciferase reporter, pG5E1bLuc, containing five binding sites for the yeast transcription factor Gal4p (Gal4p recognition elements; GRE) upstream of an Adenovirus E1b TATA-box and a luciferase gene, used in the Gal4 tethering assays is described (1). The pG1E1bLuc reporter containing only one GRE was made by removing the

5×GRE *Xho*I-*Sac*I fragment from pG5E1bLuc and replacing it with a shortened version of the excised fragment. This insert was generated by annealing of oligos, followed by Klenow fill-in and finally digestion by *Xho*I and *Sac*I. The pGL3b-5GRE-SNRPN is an *SNRPN*-driven Gal4p-responsive luciferase reporter, which has been described earlier (2).

Mammalian expression plasmids. The mammalian expression vectors pCIneo-hcM-HA and pCIneo-hcM-HA-2KR (encoding wild-type and sumoylation deficient c-Myb, respectively), pCIneo-hcM-HA-K503R and pCIneo-hcM-HA-K527R (single sumoylation sites mutated) have been described (3). c-Myb mutants pCIneoB-hcM-HA-E505A, pCIneoB-hcM-HA-E529A and pCIneoB-hcM-HA-E505/529R (abbreviated 2EA) were generated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) on a subfragment of human *MYB*, before subcloned into pCIneoB-hcM-HA. pCIneo-hcM-2KR-SUMO-1 was constructed by PCR amplification and modification of SUMO-1 from the appropriate IMAGE clone, followed by subcloning in-frame into pCIneo-hcM-HA-2KR (between *Psh*AI and *Sal*I), loosing the HA tag, but gaining SUMO-1 in the expressed product. pCIneo-hcM-VP16 was made by PCR amplification of the herpes simplex virus VP16 transactivation domain from pDBD11 (4). The VP16-TAD region was subcloned in-frame into pCIneo-hcM. pCIneo-hcM-VP16–2KR has been described (2).

The expression plasmids pEF1neo-3FLAG-hcM-HA and pEF1neo-3FLAG-hcM-HA-2KR, used for stable integration into K562 cells, were made by exchanging the CMV promoter in pCIneo with the human *EEF1A1* promoter (source: the pEBB vector). The region encoding three consecutive FLAG tags was from pCIneoB-3FLAG (2), and the cDNAs encoding hcM-HA or hcM-2KR-HA were subcloned from their corresponding pCIneo constructs. pCIneo-AMV encoding the AMV v-Myb protein (residue 72–440 in chicken c-Myb) is described in (5), while the pCIneo-hcM-ΔNRD-HA, encoding human c-Myb residue 1–443, has been described elsewhere (6).

The c-Myb expression constructs with the central TAD deleted (pCIneoB-hcM-ΔT1-HA and pCIneoB-hcM-ΔT1-HA-2KR) were made from a cDNA in Bluescript lacking an internal fragment (*Hpa*I to *Sma*I) encoding human c-Myb amino acids 229-325. The *Eco*RI-*Bgl*II fragment from pBS-ΔSE-hcM-ΔT1 was subcloned between the corresponding sites in pCIneoB-hcM-HA and pCIneoB-hcM-HA-2KR, respectively.

The mammalian expression vectors for Gal4p-DBD fused to HA-tagged human c-Myb NRD (pCIneoB-GBD2-NRD-HA; coding for amino acid residue 410 to 640) were made by PCR amplification of the corresponding sequence in pCIneo-hcM-HA and subcloning of this

fragment into pCIneoB-GBD2 (described earlier (2)) between *Sal*I and *Not*I. pCIneoB-GBD2-NRD-HA-2KR and -2EA were made by subcloning the *Bgl*II-*Not*I fragment from pCIneo-hcM-HA-2KR and -2EA, respectively, into pCIneoB-GBD2-NRD-HA. The Gal4p-DBD NRD-SUMO fusion proteins were made by the same strategy using pCIneo-hcM-2KR-SUMO-1 and pCIneo-hcM-SUMO-1 (2). The GBD-TAD fusion construct, covering amino acid residues 259 to 337 from human c-Myb, were made by PCR amplification using pCIneo-hcM-HA as template and subcloning the fragment into pCIneoB-GBD2 between *Sal*I and *Not*I. The same strategy was used for the pCIneo-GBD2-p53-CRD expression vector (encoding amino acid residue 300 to 393 of human p53) as well as for the sumoylation deficient pCIneo-GBD2-p53-CRD KR. The expression vectors pCIneoB-GBD2-hcM[233-640]-HA and pCIneoB-GBD2-hcM[233-640]-HA 2KR encode human c-Myb (amino acid residue 233-640; wild type and sumoylation-deficient) lacking its own DBD in fusion Gal4p-DBD. Both constructs were made by PCR amplification of the corresponding c-Myb cDNA and subcloning of the *Mlu*I-*Bgl*II fragment into pCIneoB-GBD2-hcM-NRD-HA and pCIneoB-GBD2-hcM-NRD-2KR-HA, respectively. pCIneoB-3FLAG-Mi2α, expressing full-length FLAG-tagged Mi-2α, has been described (2).

pSG424-Sp3 and pSG424-Sp3/kee (mutated I_{550}KEE -> I_{550}AAA), both encoding human Sp3 amino acid residue 81-613 (acc.no. NP_003102) in fusion with Gal4p DBD (7), were kind gifts from Prof. G. Suske. pCIneoB-GBD2-Sp3-ID-HA and pCIneoB-GBD2-Sp3-ID-HA/kee encoding the inhibitory domain of Sp3 (ID; amino acid residue 534-612) in fusion with Gal4p DBD, were made by PCR amplification of the corresponding cDNA from the pSG424-Sp3 constructs.

The SENP1 expression plasmids pFlag-CMV-SENP1 and pFlag-CMV-SENP1-mutant (R630L, K631M) were a kind gift from Dr. E.T. Yeh (8). The p300 expressing mammalian vector pCMVβ-NHA-p300 was a kind gift from Prof. D. Livingston and has been described previously (9). pCMV-T7-mPIASy (10) expressing PIASy was kindly provided by Prof. R. Grosschedl.

Electrophoretic Mobility Shift Assay (EMSA)

Expression of Myb proteins for EMSA. The human c-Myb DNA-binding domain, covering Myb repeats 1-3 ("R123"; amino acid residues 38-192) was expressed in *E. coli* and purified as earlier described (11-13). COS-1 cells (1×10^6/100-mm plates) were transfected 24 h after seeding with 5 μg DNA (pCIneo-hcM-HA, pCIneo-hcM-2KR-HA or pCIneo-hcM-2KR-SUMO-1) using FuGENE6 (Roche Applied Science). 24h after transfection the cells were

washed twice in 1×PBS on ice before lysis in 500 µl modified F-buffer (10 mM Tris-HCl [pH 7.05], 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 µM $ZnCl_2$, 100 µM Na_3VO_4, 1% Triton X-100, 1 mM PMSF and 1 mM DTT) supplemented with Complete Protease Inhibitor™ (Roche Applied Science) (14). The lysates were then centrifuged for 30 min at 4 °C, before aliquots of the supernatant were frozen in liquid N_2 and stored at -80 °C.

EMSA. DNA binding was monitored by EMSA as previously described (12). The oligonucleotide used for monitoring Myb binding is based on the MRE A site in the *mim-1* promoter (15): 5-GCATTATAACGGTTTTTTAGCGC-3′. The oligonucleotides used for monitoring interference from multiple bound c-Myb proteins were:
5′-TATTATAACGGTAACGGTCTTTAGCGCCTGG-3′ (6 bp phasing),
5′-TATTATAACGGTCTTTAACGGTCTTTAGCGCCTGG-3′ (10 bp phasing),
5′-TATTATAACGGTCTTTATTATAACGGTCTTTAGCGCCTGG-3′ (15 bp phasing) and 5′-TATTATAACGGTCTTTGCAACATTATAACGGTCTTTAGCGCCTGG-3′ (20 bp phasing). The probes were labelled with γ-^{32}P-ATP and purified as described in (16). For the experiments with recombinant c-Myb DBD, R123 (0-25 fmol) was incubated with duplex labelled MRE oligonucleotide probes (20 fmol) in CG-buffer (20 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 10% glycerol, 0,05% TritonX-100, 1 mM DTT and 50 mM NaCl. COS-1 cell lysates (1-9 µl) with the different Myb proteins were adjusted to equal volumes with F-buffer, before incubation with labelled probe (20 fmol) as described above. This was done in a final concentration of 75 mM NaCl and 1 µg poly[dI-dC]. In both cases, the binding reactions (total volumes 20 µl) were incubated for 10 min at 25 °C before electrophoresis. All binding reactions were run on 6% 0.5×TBE, 5% glycerol PA-gels at 4 °C.

Supplementary results

Synergy control is not caused by weakening of the DNA-binding by SUMO.

To monitor DNA-binding properties we expressed the three Myb variants: c-Myb wild-type, c-Myb 2KR, and c-Myb-2KR-SUMO1 in COS-1 cells. We evaluated the amount of protein by Western analysis (Fig. S1, middle and lower panel) and performed EMSA. With equal input of c-Myb proteins, the specific DNA-binding observed was similar for c-Myb wild-type and 2KR, as well as for the SUMO-fusion protein (Fig. S1 upper panel), suggesting that SUMO moieties conjugated (Fig. S1; lower panel) 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.

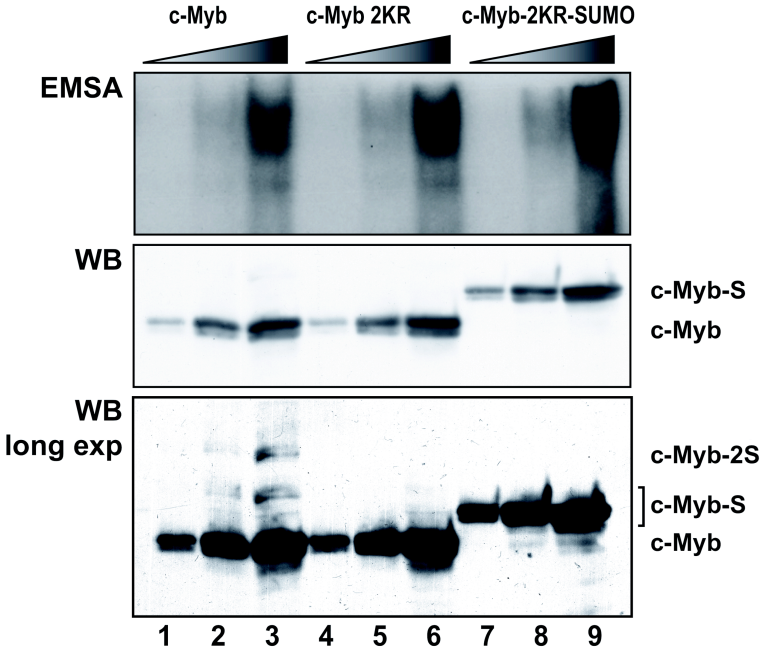

Figure S1. Increasing volumes of lysates from COS-1 cell transfected with plasmids coding for c-Myb wild-type (1, 3, 9 µl, lanes 1-3), SUMO-negative c-Myb 2KR (1, 3, 9 µl, lanes 4-6) or c-Myb 2KR fused to SUMO-1 (1, 3, 9 µl, lanes 7-9) were bound to 1×MRE [γ-^{32}P]-labelled probe (20 fmol). Complexes were incubated at 25°C for 10 min before analysis with EMSA (upper panel). A western blot was also performed. The amounts of lysate were adjusted to equal concentrations of proteins by western blot with anti-c-Myb (5e11) antibody (middle panel). A long exposure on x-ray film is included to show the SUMO modification of c-Myb wild-type (lower panel). c-Myb-S and c-Myb-2S: c-Myb modified with one or two SUMO proteins, respectively.

c-Myb-dependent activation of the *MYC* promoter is highly SUMO-dependent.

To examine how SUMO-modification influenced the c-Myb responsiveness of the *MYC* promoter (analyzed by ChIP in Fig. 4), we 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 that synergy control is operating on the *MYC* promoter (Fig. S2).

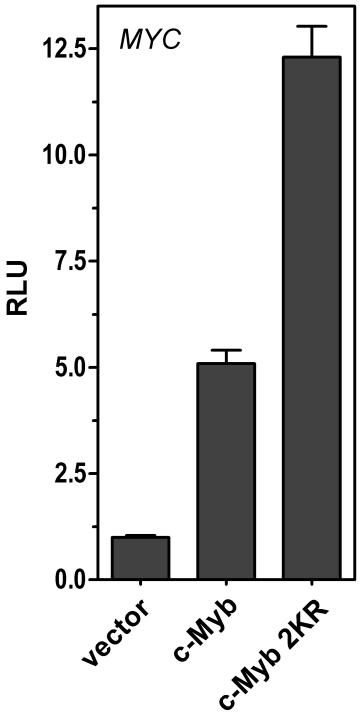

Figure S2. CV-1 cells were transfected with the Myb-responsive reporter pGL3-MYC (covering the human *MYC* promoter from position -1213 to +285 relative to the transcription start site (acc.no. NM_002467)) and 0.4 μg of plasmids expressing c-Myb wild-type or the SUMO-conjugation negative mutant c-Myb-2KR. The results are presented as relative luciferase units (RLU) ± SEM.

The C-terminal regulatory domain in p53 harbours a SRAF.

The p53 transcription factor has an N-terminal TAD and a C-terminal regulatory domain (CRD) in which p53 is sumoylated at Lys386 (17,18). Using the Gal4p tethering assay we compared CRD wild-type and the sumoylation deficient K386R mutant. Interestingly, and as seen for c-Myb NRD and Sp3 ID, wild-type p53 CRD had a weak repressive effect, while the KR-version was activating (Fig. S3), suggesting the presence of a SRAF in p53. Given that p53 tetramerizes on its natural promoters, the total contribution from four active CRDs in combination with four TADs is likely to result in an appreciable activation.

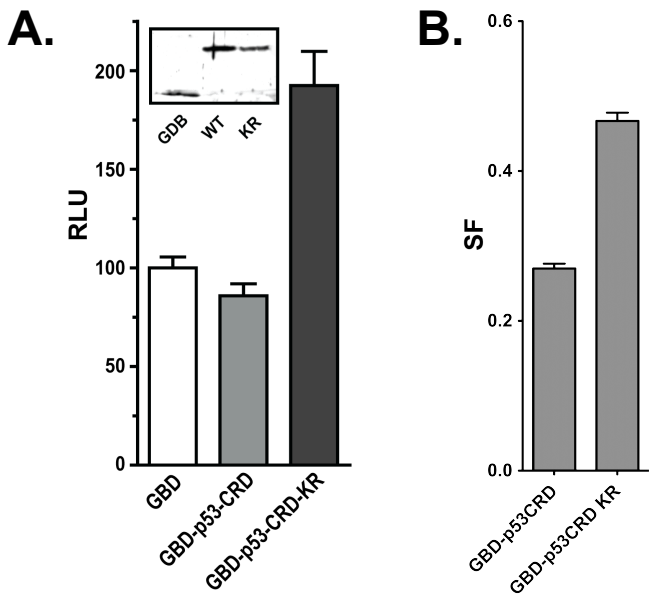

Figure S3. (**A**) CV-1 cells were transfected with 0.2 μg of plasmids expressing Gal4p-DBD fused to p53 CRD (amino acid residues 300-393) wt or K386R. The reporter output from the *E1b*-driven Gal4p-responsive reporter plasmid (5×GRE; 0.2 μg) was normalized to the effect of Gal4p-DBD (0.2 μg) and set to 100. The results are presented as relative luciferase units (RLU)±SEM. A western blot performed with anti-GAL4 (DBD) antibody, using the same lysates, is included. (**B**) Based on corresponding transfections using a 1×GRE-E1b-Luc reporter plasmid (0.2 μg) we calculated the synergy factors of the constructs assayed in (A). The results are presented as SF ± SEM.

The activated SRAF in c-Myb is p300 responsive.

The central TAD in c-Myb is known to bind p300 (19-21), and it is believed that much of its transactivation function is mediated through this recruitment. To further analyze the SRAF in c-Myb NRD, we examined whether the NRD-2KR is co-activated by p300. When GBD-TAD, GBD-NRD (SRAF OFF) and GBD-NRD-2KR (SRAF ON) were co-transfected with p300, the classical central TAD was activated by p300 (Fig. S4A, right panel), as earlier reported. In contrast GBD-NRD seemed to be unresponsive to p300. However, GBD-NRD-2KR (SRAF ON) was clearly p300-responsive (Fig. S4A, left panel, or RLU-plot Fig. S4B, right panel). This opens the possibility that the two activation functions in c-Myb cooperate in recruiting p300. Still, when we examined the interaction between p300 and the two AFs, only the classical TAD was able to pull-down full-length p300 from a COS-1 lysate (Fig S4C). This implies that if c-Myb NRD participates in p300 recruitment, the interaction is most probably indirect.

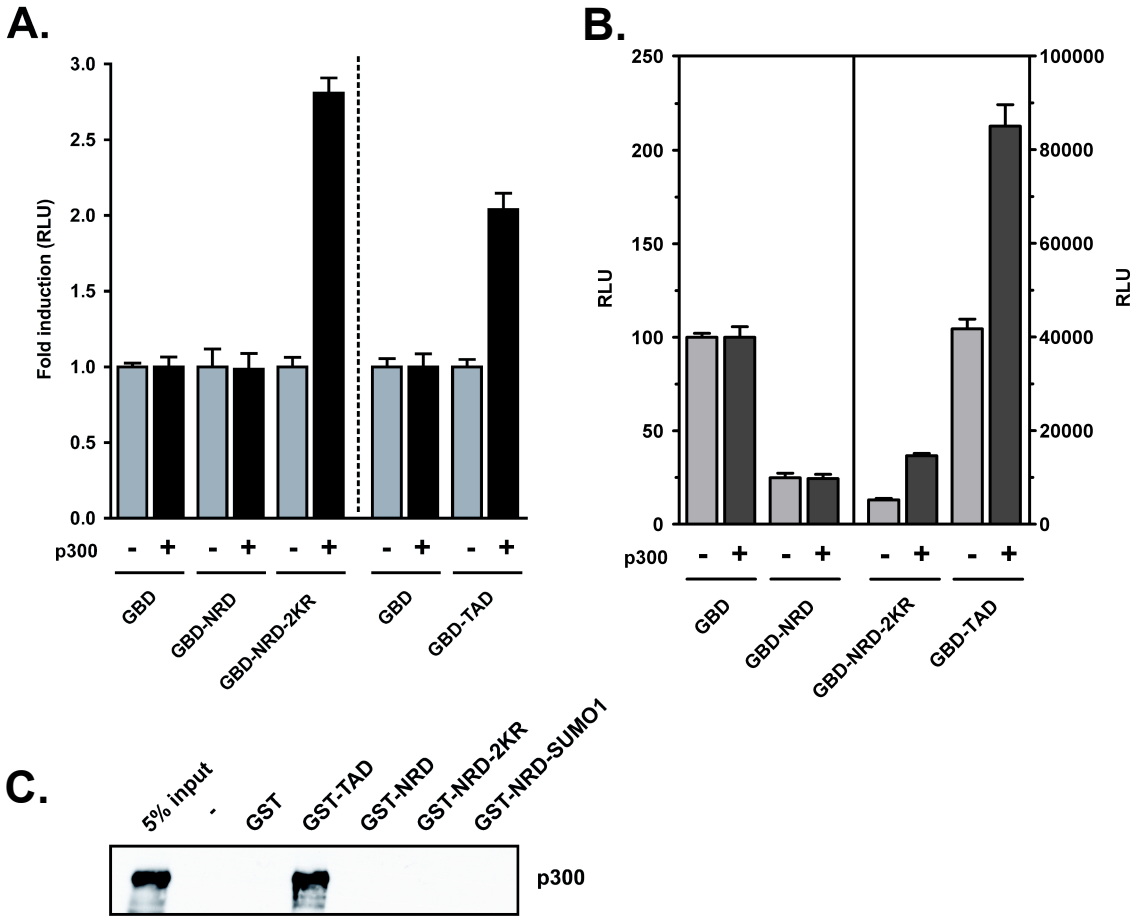

Fig S4. (**A**) CV-1 cells were transfected with 0.1 μg of plasmids expressing Gal4p-DBD fused to c-Myb NRD (amino acid residues 410-640) wild type or 2KR in the presence or absence of full-length p300 (0.3 μg, left panel). As a control Gal4p-DBD fused to c-Myb TAD (amino acid residues 259-337, 0.01 μg) was transfected with or without p300 (0.3 μg, right panel). The reporter output from the *E1b*-driven Gal4p-responsive reporter plasmid (0.2 μg) was normalized to the effect of Gal4p-DBD (0.1 or 0.01 μg) and set to 1. The results are presented as fold-induction of relative luciferase units (RLU)±SEM. (**B**) The results are also presented as relative luciferase units (RLU)±SEM. Here the effect of Gal4p-DBD (0.1 or 0.01 μg) was set to 100. (**C**) COS-1 cells were transfected with a plasmid expressing p300, and lysates were incubated with comparable amounts of recombinant GST, GST-TAD, GST-NRD, GST-NRD 2KR, and GST-NRD-SUMO1. The bound proteins were analyzed by SDS-PAGE and immunoblot analysis was performed using anti-p300 antibodies. 5% of the input (total cell extract) used for the pull-down was loaded as reference.

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