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Identifizierung und funktionelle Charakterisierung des neuen MAR-bindenden Proteins, SATB2

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Erklärung

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Ehrenwörtliche Versicherung

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List of Abbreviations

A - ampere

A_{260}, A_{280}, A_{595} - absorbance at 260, 280 and 595 nm, respectively

APS - ammonium persulphate

ARID - AT-rich interaction domain

ATP - adenosine 5'-triphosphate

BES - N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid

b / bp - base(s) / base pair(s)

BSA - bovine serum albumin

BUR(s) - base unpairing region(s)

C_α - immunoglobulin alpha constant heavy chain

cDNA - complementary deoxyribonucleic acid

CDP - CCAAT displacement protein

CoIP - co-immunoprecipitation

CUE - core unwinding element

dH_2O - distilled water

D - dalton

dlmut - double sumoylation mutant (K233R/K350R) of SATB2

dNTP(s) - deoxynucleoside triphosphate(s)

DEPC - diethyl pyrocarbonate

DMSO - dimethyl sulfoxide

DNA - deoxyribonucleic acid

DTT - dithiothreitol

E. coli - *Escherichia coli*

EDTA - ethylenediamine tetraacetic acid

(E)GFP - (enhanced) green fluorescent protein

EGTA - ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EMSA - electrophoretic mobility shift assay

EST - expressed sequence tag

FBS = FCS - foetal bovine serum = foetal calf serum

Fig. - figure

g - gram

GST - glutathione-S-transferase

GTP - guanosine 5'-triphosphate

HEPES - N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

His - hexahistidine tag

h. - hour(s)

l - liter

M - molar (mol/l)

MAR(s) - matrix-attachment region(s)

min. - minute(s)

Mw - molecular weight

NM - nuclear matrix

OD - optical density

PBS - phosphate-buffered saline

PCR - polymerase chain reaction

PFA - paraformaldehyde

PIAS - protein inhibitor of activated STAT

PIPES - Piperazine-N,N′-bis(2-ethanesulfonic acid)

PML - promyelocytic leukemia (protein)

RanGAP - Ran GTPase-activating protein

RNA - ribonucleic acid

RT - room temperature

s. - second(s)

SATB1/2 - special AT-rich sequence binding protein 1/2

SDS - sodium dodecylsulphate

SDS PAGE - sodium dodecylsulphate polyacrylamide gel electrophoresis

SUMO - small ubiquitin-related modifier

SUSP(s) - SUMO-specific protease(s)

TBE - Tris/borate/EDTA (buffer)

TEMED - N,N,N’,N’-Tetramethylethylenediamine

Tris - Tris(hydroxymethyl)aminomethane

UE(s) - unwinding element(s)

V - volt(s)

W - watt(s)

wt - wild-type

Summary

The regulation of gene expression is governed in large part by transcription factors that bind to enhancers and promoters. The functions of transcription factors involve both the modulation of chromatin accessibility via the recruitment of histone-modifying enzymes or nucleosome-remodeling complexes, and the stimulation of RNA polymerase via an interaction with the mediator complex. In addition to enhancers and promoters, nuclear matrix attachment regions (MARs) have been implicated in the regulation of gene expression by altering the organization of eukaryotic chromosomes and augmenting the potential of enhancers to act over large distances. Although a lot is known about the function of MARs, the precise mechanism of their action is still obscure and probably diverse. One proposed model stipulates that their function is accomplished through the action of transcription factors, which are components of the nuclear matrix.

Here, we identify and characterize a novel cell-type specific MAR-binding protein, SATB2, which binds to the MARs of the endogenous immunoglobulin μ locus in pre-B cells and enhances gene expression. In contrast to the closely related, thymocyte-specific MAR-binding protein SATB1, SATB2 is not proteolytically cleaved by caspase 6, but is instead SUMO-modified at two lysine residues. This modification is specifically augmented by the SUMO E3 ligase PIAS1. Mutation of the sumoylation sites enhances the association of SATB2 with the immunoglobulin MARs, as well as its transactivation potential. Moreover, covalent attachment of SUMO1 and SUMO3 represses SATB2-dependent transcription, without affecting either the DNA binding or the dimerization capacity of SATB2. Interestingly, SUMO conjugation affects the subnuclear localization of SATB2 and is involved in its targeting to distinct nuclear speckles (bodies). Thus, our data indicate that the regulation of SATB2 function through sumoylation can be mediated by both altering its transcriptional activation potential and by sequestering it in specific nuclear locations.

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I. Introduction

1. Nuclear matrix

DNA within the eukaryotic nucleus is organized into higher-order looped domains ranging from 5 to 200 kb in length (Zlatanova and van Holde, 1992). The domain organization of the nucleus is thought to be mediated by a proteinacous intranuclear framework called "nuclear matrix" (Berezney and Coffey, 1974) or "nuclear scaffold" (Mirkoritch et al., 1984; Ivanchenko et al., 1992). The nuclear matrix is composed of two parts: a peripheral nuclear lamina and an internal filamentous network that transects the nuclear remnant (Luderus et al., 1992). This network is built up of branched core filaments, which provide a supporting structure for the formation of DNA loops and participate in diverse matrix-associated processes such as DNA replication, transcription, recombination, RNA processing and transport, as well as signal transduction and apoptotic events. The association of DNA with the nuclear matrix (skeleton) on the one hand serves to structurally define the borders of chromatin domains at the sites of attachment, and on the other hand participates in the regulation of transcription (Razin, 1987, 2001; Bode et al., 2000). The development of a method based on LIS (2,5-Lithium diiodosalicilate) detergent extraction of the nuclear matix allowed the isolation of nuclear matrix-associated DNA sequences (Mirkovitch et al., 1984). The scaffold-bound DNA fragments obtained by this extraction procedure were termed MARs (matrix attachment regions) or SARs (scaffold attached regions).

2. Matrix attachment (association) regions (MARs)

MARs are DNA sequences of at least 250 base pairs, which have more than 70% AT content and bind reversibly to isolated nuclear matrices *in vitro*. Studies, using single strand-specific enzymes or reagents have shown that these sequences can undergo spontaneous strand separation *in vitro* (Bode

et al., 1992; 1996). Although prokaryotes do not contain MAR sequences (as evidenced by the fact that prokaryotic DNA, even in large excess, does not interfere with MAR/nuclear matrix reassociation *in vitro*), some plasmids possess two intensively studied narrow unwinding elements (UEs) that flank the ampicillin resistance gene (Benham, 1997). These sites are insufficient to mediate matrix attachment, but they serve as convenient internal standards for measuring the properties of the eukaryotic MARs. Eukaryotic MARs form extended base unpairing regions (BURs), which usually consist of multiple UEs, that compete strongly with the above-mentioned standards. The UEs typically include a major core unwinding element (CUE), which is the nucleation center for base unpairing (Bode et al., 1996).

MARs are evolutionarily conserved and are often found at the borders of chromatin domains and in close association with certain enhancers or in introns (Gasser and Laemmli, 1986; Cockerill and Garrard, 1986a,b). MARs are located either in non-transcribed regions or within transcription units, but rarely if ever in coding regions (Bode et al., 2000).

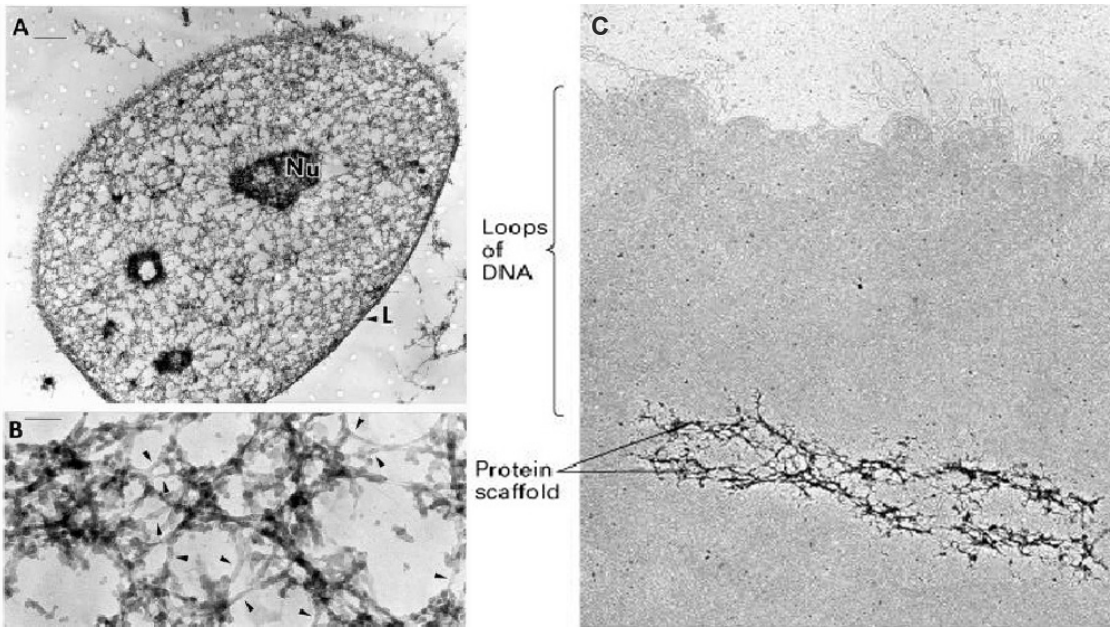


Figure 1. High-resolution structure of the nuclear matrix. The nuclear matrix was visualized by resinless section electron microscopy, following high salt extraction and DNase I digestion. The nuclear matrix of an interphase nucleus consists of two parts – the nuclear lamina (L) and a network of internal nuclear fibres (Nu - nucleoli, L - lamina). **(A)** Overview. ***(B)*** High magnification. The arrowheads point the 10 nm filaments. ***(C)*** Histone-depleted metaphase chromosome. Nonhistone proteins provide a structural scaffold for long chromatin loops. (From Nickerson, 2001.)

The best studied matrix attachment regions are the ones found in the genes for lymphocyte receptors. Our main interest at the onset of this work was the functional characterization of the immunoglobulin μ gene MARs. Therefore, the following section will focus on the current knowledge about the properties and functions of the matrix attachment regions from the immunoglobulin and other lymphocyte receptor genes.

2.1. MARs of the lymphocyte receptor genes

2.1.1. The MAR of the Igκ gene

The first identified MAR was in the Igκ receptor gene locus (Fig. 2) (Cockerill and Garrad, 1986b).

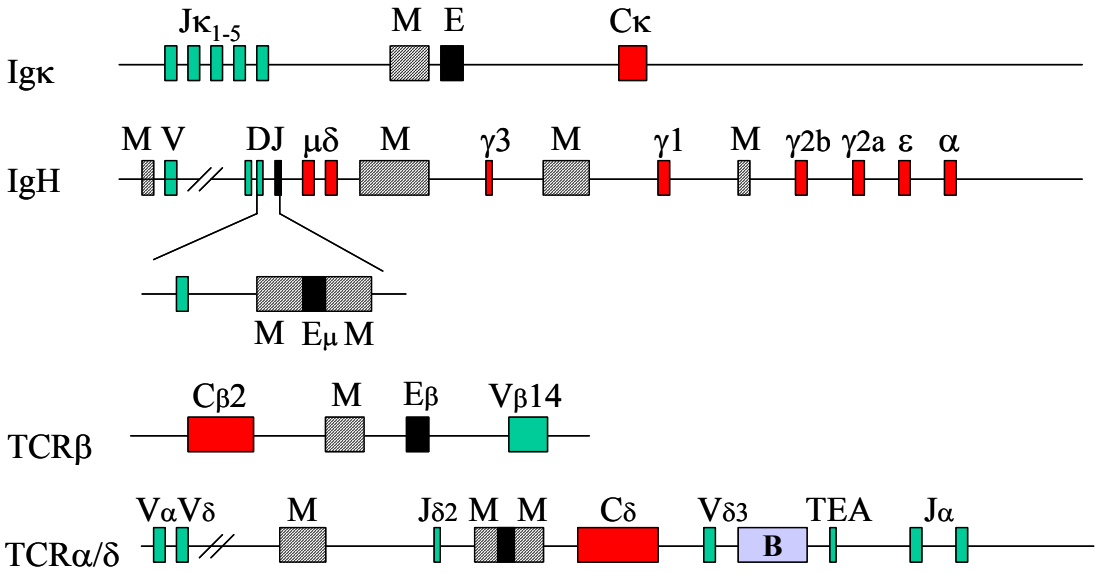


Figure 2. Physical location of MARs in lymphoid receptor genes. MARs are shown as cross-hatched boxes, exons in green; B stands for boundary element, TEA for T-early α. (Based on Scheuermann and Garrad, 1999.)

The MAR in the immunoglobulin κ gene locus (Igκ) has been shown to positively regulate the expression of a rearranged Igκ gene in both cell culture and transgenic mice studies (Blasquez et al., 1989; Goyenechea et al., 1997;

Xu et al., 1989). It has also been demonstrated that the Igκ MAR is required for the demethylation of *in vitro* methylated Igκ constructs, following their stable integration into plasmocytoma cell lines (Kirillov et al., 1996; Lichtenstein et al., 1994). In addition, the Igκ gene MAR is involved in regulating somatic hypermutation of Igκ transgenes, since transgenes lacking the MAR showed an impaired ability to serve as a substrate for hypermutation (Goyenechea et al., 1997). However, in mice with a targeted MAR deletion no significant defects in the levels of κ gene rearrangement, B cell populations or antibody production were detected (Yi et al., 1999). More detailed studies later demonstrated that in a fraction of B cells, Vκ-Jκ joining occurred earlier during development at MAR deleted alleles. Thus, it is possible that this MAR can act to insulate the enhancer activity in pro-B but not in pre-B cells. Supporting the transgenic mice studies, in ΔMAR mice the extent of somatic hypermutation in germinal centers is significantly reduced (Yi et al., 1999).

2.1.2. The IgH gene locus MARs

The immunoglobulin heavy chain (IgH) gene locus has multiple MARs. The most extensively studied ones are the 5' and 3' MARs flanking the Igμ gene intronic enhancer (see Fig. 2). Initial experiments involving transient transfection or stable integration of reporter constructs suggested that these MARs might play a role in repressing the Igμ gene intronic enhancer in non-B cells (Scheuermann and Chen, 1989). The IgH MARs are found to activate or repress transcription of reporter genes when the appropriate MAR-binding proteins are co-expressed in transient transfection experiments (Herrscher et al., 1995; Wang et al., 1999).

Detailed analysis found that the intragenic enhancer region is required for the regulated expression of rearranged μ transgenes at different chromosomal locations (Jenuwein et al., 1991) and that the flanking MAR regions play a positive role in regulating transcription (Bode et al., 1992). Moreover, they are absolutely required for the transcription of rearranged, ectopically integrated IgH genes in transgenic mice, although they were not required for the

expression of the same constructs in stably transfected tissue culture cells (Forrester et al., 1994). In addition, the transgene with deleted MARs is susceptible to chromosomal position effects and is expressed at low and variable levels, ranging between 0.1 and 3% relative to the level of the wild-type enhancer transgene (Forrester et al., 1994). The requirement for MAR function in transgenic animals, but not in cell lines or animals created from blastocyst fusions, suggests a function of MAR in chromatin remodeling during early development or passage through the germline (Sakai et al., 1999). In fact, methylation of the μ gene constructs *in vitro*, prior to stable transfection into murine B cells, abrogates the ability of the core enhancer alone to activate the Vh promoter over a distance of 1.2 kb (Forrester et al., 1999). In most of the clones the pre-methylated DNA templates were demethylated, which is in line with the findings of other groups that the presence of MARs in the immunoglobulin μ enhancer region correlates with the demethylation of the adjacent DNA (Lichtenstein et al., 1994; Kirillov et al., 1996). Furthermore, it was shown that MARs facilitate long-range chromatin accessibility (Jenuwein et al., 1997) and generate an extended domain of histone acetylation (Forrester et al., 1999). The effect of the MARs on the extended acetylation of histone H4 is independent of transcription and together with the finding that the H4 C-terminal tail interacts with the neighboring nucleosome (Luger and Richmod, 1998) implies that acetylation could result in disorganization of the higher order chromatin fibers and may account for the active transcription in wild-type μ transgenes.

2.1.3. The TCRβ gene locus MAR

The TCRβ MAR is required for reporter gene transactivation in transiently transfected T-cell lines (Chattopadhyay et al., 1998a). Deletion of the TCRβ gene MAR from the native locus has no effect on T-cell development and abundance, TCR synthesis, D-J rearrangement patterns, or allelic exclusion (Chattopadhyay et al., 1998b). These results suggest that redundant elements present in the normal locus may compensate for the loss of this MAR.

2.1.4. The TCR α/δ gene locus MARs

Recent studies led to the identification of three MARs in the TCR α/δ gene locus. Two of these flank the TCRδ transcriptional enhancer in a similar manner to the MARs in the IgH intronic enhancer (see Fig. 2). It was demonstrated that they are required for efficient VD to J recombination (Zhong et al., 1999).

Overall, the presence of MARs in close proximity to enhancers is a conserved feature of lymphoid receptor genes, arguing for the functional importance of these elements. MAR sequences are also found within the immunoglobulin V_H and V_κ loci at a much higher frequency than throughout the rest of the genome (Goebel et al., 2002). It has been proposed that variations in nuclear matrix factors binding to these MARs could potentially influence the extent of localized accessibility to V(D)J recombination and thus could play a role in the unequal rearrangement of individual V genes. The MAR sites could also contribute to the effective transcription of immunoglobulin genes in mature and activated B cells, bringing both the promoter and the enhancer regions into close proximity at the nuclear matrix (Goebel et al., 2002).

3. MAR-binding proteins

Following the initial description of the nuclear matrix as a general structure a number of specific proteins were identified as matrix components. They are referred to as MAR-binding proteins, due to their ability to associate with matrix attachment regions.

In addition to ubiquitous MAR-binding proteins, such as topoisomerase II, lamin B, HMGI(Y), SAF-A, Cux-CDP and MeCP2 (Cockerill and Garrad, 1986; Lundres et al., 1992; Romig et al., 1992; Scheuermann and Chen, 1989; Weitzel et al., 1997), two MAR-binding proteins, SATB1 and Bright, are expressed specifically in T cells and activated B cells, respectively (Dickinson et al., 1992; Herrscher et al., 1995) – see Table 1.

Factor	Expression pattern	Target MAR	Putative biochemical functions	Expression function
Cux/CDP	Ubiquitous	IgHE, Vh, TCRβ,CD8a, TCRα	Displaces MAR-BP1, Bright, binds SATB1, stimulates histone deacetylation	Transcriptional repression, antagonizes Bright transactivation
SATB1	Thymocytes	IgHE, Igκ, TCRβ, CD8a, TCRδ	Influences base unpairing	Transcriptional repression
Bright	Mature B and plasma cells	IgHE, Vh	Chromatin remodeling	Transcriptional activation
SMAR1	Ubiquitous, abundant in thymus	TCRβ (MARβ)	Unknown	Transcriptional modulation
HMGI(Y)	Ubiquitous	IgHE	Displaces histone H1	Derepression
MeCP2	Ubiquitous	IgHE, chicken lysozyme MAR	Recruits histone deacetylases	Transcriptional repression
CTCF	Ubiquitous	Unknown	Forms topologically independent chromatin loops that may support gene silencing.	Transcriptional repression
MAR-BP1	Ubiquitous	IgHE, Igκ	Stimulates nuclear matrix attachment	Unknown
hnRNP-A1/B1	Ubiquitous	IgHE	DNA helicase/RNA splicing	Antagonizes Cux/CDP repression
AP1 (Fos/Jun)	Ubiquitous	Igκ	Trans-activation	Transcriptional activation
Nucleolin	Ubiquitous	IgHE	Unknown	rRNA transcription and ribosome assembly

Table 1. Characteristics of selected MAR-binding proteins. (Based on Scheuermann and Garrad, 1999.)

3.1. Cux/CDP (CCAAT displacement protein)

Cux/CDP, also called NF-μNR, is expressed in various cell types but is not present in mature B cells. Cux is a member of a novel family of proteins that bind to DNA through repetitive domains termed CUT repeats. The family includes human CDP (Neufeld et al., 1992), canine Clox (Andres et al., 1992), and *Drosophila* Cut (Blochlinger et al., 1988). Each of these homologues contains three CUT repeats in addition to an atypical homeodomain (HOX)

located near the carboxy terminus. Separately, each of the CUT and the HOX domains are able to bind DNA with high AT-content (Harada et al., 1994; 1995). However, full-length Cux/CDP appears to recognize DNA in a unique way that depends more on tertiary DNA structure rather than the linear nucleotide sequence, because a consensus motif is not found.

Further experiments have shown that Cux/CDP binds in a cooperative manner to multiple sites in the two MARs flanking the IgH intronic enhancer, suggesting that multiple DNA-binding subunits are involved in the formation of a higher-order complex (Scheuermann and Chen, 1989). Transactivation studies have revealed that it is involved in the negative regulation of IgH enhancer-mediated transcriptional activation (Zong et al., 1995; Wang et al., 1999).

3.2. Bright (B cell regulator of IgH transcription)

Bright has a restricted cell-type expression, being present predominantly in mature B cells, splenic B cells and a population of small pre-B cells in bone marrow. Its expression in splenic cells can be augmented following LPS stimulation (Webb et al., 1999). Bright is retained in the nucleus following nuclear matrix preparation and co-localize with PML bodies, which also associate with the nuclear matrix (Webb et al., 1999).

The DNA binding region of the protein is represented by an ARID domain (AT-rich interaction domain) (Kortschak et al., 2000), that has a homology to the *Drosophila* protein Dead ringer and the yeast Swi1, which are involved in chromatin remodeling. Further studies have demonstrated that Bright binds discrete sites in the Vh gene promoter region and in the MARs flanking the IgH intronic enhancer (Herrscher et al., 1995). Moreover, co-transfection experiments revealed that Bright augments IgH gene transcription. It was also suggested that its transcriptional activity is regulated by Cux/CDP, that binds to similar sites and displaces Bright, thereby antagonizing Bright-mediated transactivation (Wang et al., 1999).

3.3. SATB1 (special AT-rich sequence binding protein 1)

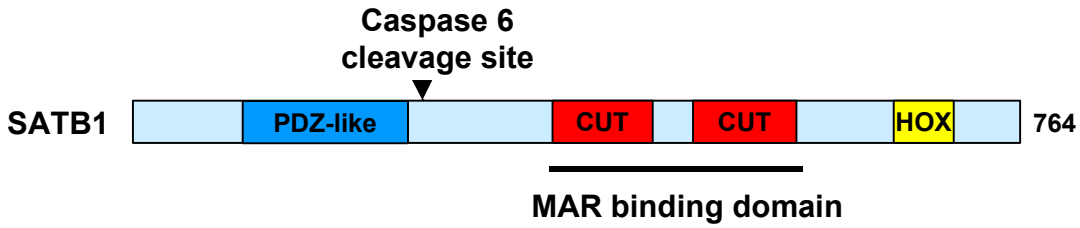


Figure 3. Domain organization of SATB1. (See the text for details.)

SATB1 is highly expressed in thymus and binds selectively to matrix-associated regions (MARs) of DNA. Specific mutations that diminish the unwinding potential of MAR sequences greatly reduce their binding affinity for SATB1 (Dickinson et al., 1992). The DNA-binding domain consists of two CUT repeats, also referred to as the MAR-binding domain (Dickinson et al., 1997), a homeodomain (HOX) and a dimerization (PDZ-like) domain (Galande et al., 2001; see Fig. 3). The isolated MAR-binding domain recognizes a certain DNA sequence context within MARs that is highly potentiated for base unpairing. Unlike the MAR-binding domain, the isolated homeodomain binds poorly and with low specificity to DNA. However, the combined action of the MAR-binding domain and the homeodomain allows SATB1 to specifically recognize the core-unwinding element within the base-unpairing region of MARs (Dickinson et al., 1997). The core-unwinding element is critical for MAR structure, since point mutations within it abolish the unwinding propensity of the MAR (Dickinson et al., 1997).

SATB1 has multiple binding sites in the genome and associates with chromatin as a dimer or multimer (Cai et al., 2003). Furthermore, SATB1 has a cage-like "network" distribution in thymocyte nuclei and selectively tethers specialized DNA sequences onto its network (Cai et al., 2003, see Fig. 4).

SATB1 is involved in the regulation of a number of T-cell specific genes. During T cell differentiation, the induction of DNase I hypersensitivity upstream of the transcriptional enhancer (Eβ – see Fig. 2) correlates with an increased association of this region with the two MAR-binding proteins –

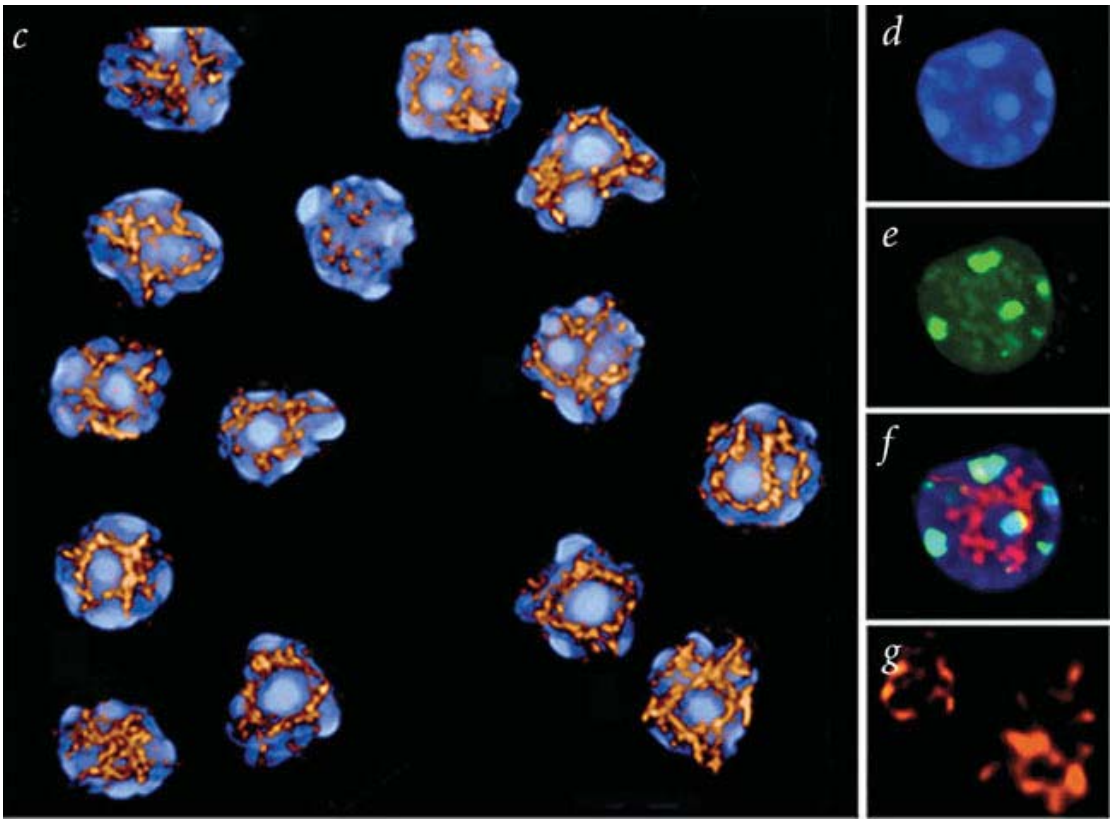


Figure 4. SATB1 forms a three-dimentional cage-like network, surrounding dense regions of chromatin. The image shows thymocyte nuclei, which were salt-extracted, digested with DNase I, and stained with DAPI (blue, *d*), with an antibody against the heterochromatin-associated protein M31 (green, *e*), and an antibody against SATB1 (red, *g*). *f* shows the merged image of *d* and *e* with the SATB1 staining (in red). *c* displays a 3-D reconstruction of the SATB1 network in several thymocyte nuclei. (From Cai et al, 2003.)

Cux/CDP and SATB1. The binding site was further shown to be a nuclear matrix attachment region, referred to as MARβ (See Fig. 2) (Chattopadhyay et al., 1998a). Binding sites for SATB1 are also found in the CD8B gene locus and are thought to be important for the epigenetic regulation of CD8 expression (Kieffer et al., 2002).

Transient transfection reporter assays revealed a role for SATB1 in modulating the expression of multiple genes. For example, SATB1 is a component of a large complex called γ–PE (for β-globin promoter and enhancer binding factor) that binds to five sites located 5' and 3' of the human γ-globin gene and participates in positive regulation of γ-globin expression (Case et al., 1999). However, several other examples demonstrated a negative role for SATB1 in the regulation of transcription of the gp91(phox)

gene and the MMTV long terminal repeat reporter gene (Hawkins et al., 2001; Liu et al., 1999). Furthermore, cells stably expressing SATB1 have lower activity of a reporter gene, containing multimerized binding sites in front of a minimal promoter, compared to cells that do not express SATB1 (Kohwi-Schigematsu et al., 1997). Further understanding of SATB1 function was gained through the functional inactivation of the protein by gene targeting. SATB1-null mice exhibited multiple defects at almost every stage of T-cell development. These defects included greatly reduced triple negative (TN – $CD3^{-}CD4^{-}CD8^{-}$) cells, an arrest mainly at the double positive (DP – $CD4^{+}CD8^{+}$) stage, inappropriate migration of DP thymocytes to lymph nodes, and apoptosis of peripheral CD4 single positive (SP) cells in response to activating stimuli. Dramatic reduction in mature T-cell subsets was observed since both CD4 and CD8 SP thymocytes and peripheral T cells were found at very low levels (Alvarez et al., 2000). Also consistent with an immature phenotype of SATB1-null thymocytes was the failure to up-regulate CD5. Since SATB1 is normally expressed in every thymocyte subpopulation - TN, DP, and SP cells, as well as in activated T cells (Beadling et al., 1993), it was proposed that the multiple defects observed were a consequence of disordered transcription of multiple genes due to SATB1 ablation (Alvarez et al., 2000). The ectopic expression of the IL-2Rα and IL-7Rα genes, as well as a dysregulation of many other genes (including an oncogene, a chemokine gene, apoptosis-related genes, tumor susceptibility genes, DNA-binding protein genes) and developmental surface markers was found in SATB1-null thymocytes and peripheral T cells. These results indicated that the spatial and temporal transcription of multiple genes is disordered in thymocytes and peripheral T cells in the absence of SATB1 and suggested that SATB1 is a cell type-specific global gene regulator (Alvarez et al., 2000). Further experiments led to a better understanding of the biochemical mechanisms of SATB1-mediated gene regulation, demonstrating that the binding of SATB1 to specific DNA sites creates a “landing platform” for the chromatin remodeling enzymes ACF1 and ISWI, subunits of ACF and CHRAC nucleosome mobilizing complexes, to specific sites, and regulates nucleosome positioning over long distances. SATB1 also recruits the histone deacetylase contained in

the NURD chromatin remodelling complex to a SATB1-binding site in the IL-2Rα locus, and mediates the specific deacetylation of histones in a large domain within the locus (Yasui et al., 2002). However, Cai et al., (2003) showed by histone-modification analyses across a gene-enriched genomic region of 70 kb that acetylation of histone 3 at Lys9 and Lys14 peaks at a SATB1-binding site and extends over a region of roughly 10 kb covering genes regulated by SATB1, and that this closely correlates with SATB1-mediated transcriptional augmentation. These controversial results could be explained by different associations of SATB1 with the histone modifying and chromatin remodeling machinery at different binding sites and will correlate with the observed opposing effects on the transcription of different genes. Thus, it was proposed that SATB1 is a new type of gene regulator with a novel nuclear architecture, providing sites for tissue-specific organization of DNA sequences and regulating region-specific histone modification (Yasui et al., 2002).

SATB1's transcriptional activity is tightly regulated. One common mechanism to regulate homeodomain protein function is through protein-protein interactions. It was shown that SATB1 associates with CDP and this leads to inhibition of their binding to specific MAR sites, suggesting that the SATB1-to-CDP ratio in different tissues is an important mechanism to control gene expression (Liu et al., 1999; Case et al., 1999). Another very important mechanism for the regulation of the SATB1 activity is proteolysis. It was demonstrated that during negative selection processes in the thymus, when the majority of thymocytes are eliminated by apoptosis, SATB1 is specifically cleaved by a caspase 6-like protease at amino acid position 254 (Galande et al., 2001). This cleavage separates the DNA-binding domains from the PDZ-like dimerization domain. The resulting 65-kD major fragment contains both the base-unpairing region (BUR)-binding domain and the homeodomain. However, since this SATB1 fragment is monomeric, it loses its BUR-binding activity, despite containing both its DNA-binding domains, and rapidly dissociates from chromatin *in vivo* (Galande et al., 2001).

In conclusion, it should be pointed out that although a lot has been learned about the function of MARs, the precise mechanism through which they influence gene expression is still obscure and probably diverse. One possible model involves the MAR-binding protein HMGI(Y) that could depelete histone H1 and cause chromatin opening. MARs also mediate the targeting of DNA to the nuclear matrix, which is enriched in RNA polymerases and splicing complexes (Berezney et al., 1995). Another mechanism could rely on the MAR-induced changes in DNA topology, since matrix attachment regions have been shown to interact with topoisomerase II and contain sequences, which unwind easily under superhelical tension (Bode et al., 1992; Benham et al., 1997). Yet another pathway may involve MeCP2 - a ubiquitously expressed protein that interacts with both methylated CpG dinucleotides and MAR sequences. MeCP2 associates with histone deacetylases and might therefore confer repression upon MAR-containing regions of chromatin. Finally, certain MAR sequences are also recognized by tissue-specific transcriptional regulators, such as Bight and SATB1, which can modulate transcription at the sites of binding.

4. Post-translational protein modifications. SUMO modification

4.1. SUMO (small ubiquitin-related modifier)

Post-translational modification of proteins is a fundamental mechanism of modulating their function, activity or localization after their synthesis has been completed. These modifications are usually accomplished via enzymatic reactions: acetylation, methylation, phosphorylation, ADP ribosylation, carboxylation, adenylation, glycosylation, prenylation, and ubiquitination. Modification with ubiquitin was the first identified case when the modifier itself is a small polypeptide. Ubiquitin is a 76-amino acid polypeptide that is highly conserved. The proteins ligated to multi-ubiquitin chains (usually formed through isopeptide bonds between lysine 48 of one ubiquitin and the C-terminal glycine residue of the neighboring ubiquitin) are degraded by the ATP-dependent 26S proteasome (Coux et al., 1996). More recently it was shown that ubiquitination has a number of additional functions. For example,

mono-ubiquitination, unlike poly-ubiquitination, is not involved in the protein degradation pathway, but plays a role in at least three distinct cellular processes, such as histone regulation, endocytosis, and budding of retroviruses from the plasma membrane (Terrell et al., 1998; Hofmann and Pickart, 1999; Deng et al., 2000; Hicke, 2001).

During the past few years, several proteins have been discovered that have sequence similarity to ubiquitin. These ubiquitin-like proteins form two separate classes: 'ubiquitin-like modifiers' (UBLs) and 'ubiquitin-domain proteins' (UDPs). UBLs function as modifiers in a manner analogous to that of ubiquitin. This class is comprised of SUMO (small ubiquitin-related modifier), Rub1 (also called Nedd8), Apg8 and Apg12. UDPs harbour domains that are homologous in sequence to ubiquitin but are otherwise unrelated to each other. In contrast to UBLs, these proteins are not conjugated to other proteins (Jentsch et al., 2000; Hochstrasser et al., 2000). This class includes parkin, RAD23 and DSK2.

SUMO (small ubiquitin-related modifier) is the best-characterized member of a growing family of UBLs and shows a high degree of conservation from yeast to humans. *Saccharomyces cerevisiae* contains only one SUMO homologue, Smt3p, which was originally discovered as a suppressor of mutations in the centromeric protein MIF2 (Meluh and Koshland, 1995). In contrast, the mammalian SUMO family consists of three members: SUMO-1, -2, and -3 (Kamitani et al., 1998; Saitoh and Hinchey, 2000; see Fig. 5). In humans, SUMO-1 is a 101 amino acid polypeptide that shares 48% identity with SUMO-2 and 46% identity with SUMO-3. SUMO-2 and -3 share 95% identity, and can be grouped into a subfamily distinct from SUMO-1. Human SUMO1 shares only 18% sequence identity with ubiquitin. Nevertheless, NMR structure analysis (Bayer et al., 1998) revealed that SUMO1 contains the characteristic $\beta\beta\alpha\beta\beta\alpha\beta$ ubiquitin fold common to ubiquitin-like proteins (Mayer et al., 1998). Similarly to ubiquitin, SUMO-1 is synthesized as a precursor with a C-terminal extension of several amino acids, which needs to be processed to make the C-terminal double-Gly motif available for conjugation. Interestingly, SUMO-1 has a long and highly flexible N-terminal extension that is absent in ubiquitin, but at present the functional importance of this

sequence is not known. SUMO2 and SUMO3, analogously to SUMO1, can also be conjugated to target proteins (Kamitani et al., 1998). Further experiments demonstrated that the target proteins for SUMO1 and SUMO2/3 are different and the conjugation of SUMO-2 and -3 can be promoted by stress-inducing stimuli, such as acute temperature shift and high osmolarity (Saitoh and Hinchey, 2000). Moreover, SUMO2/3 can form poly-SUMO chains (Tatham et al., 2001). It was shown that both SUMO-2 and -3, but not SUMO-1, contain a consensus motif, ψKXE (ψ stands for a hydrophobic amino acid and X for any amino acid), in their N-terminal regions that is used for sequential SUMO conjugation. Point mutation of the lysine residue in the above sequence abolishes the poly-SUMO chain formation. The yeast SUMO homologue, Smt3p, also contains a similar sequence motif, and can form poly-SUMO chains (Johnson and Gupta, 2001) but until now the functional significance of the poly-SUMO chain formation is unknown.

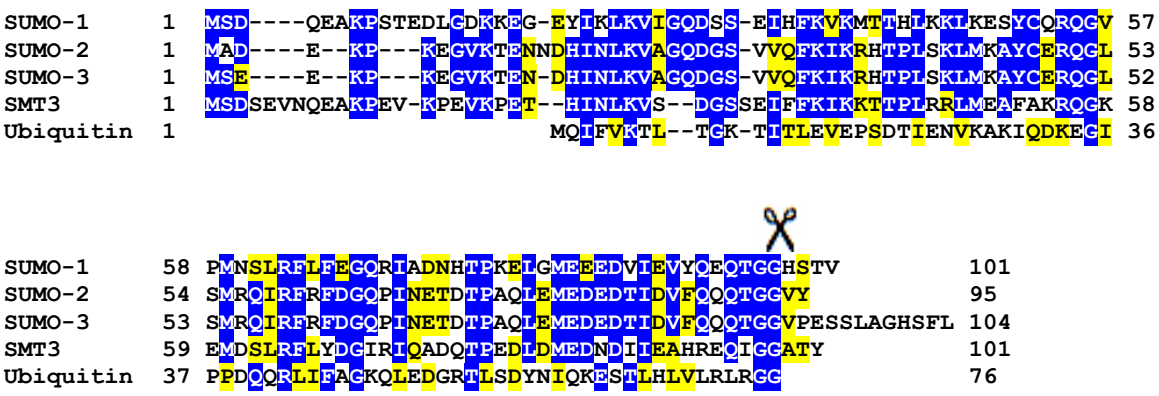


Figure 5. Sequence alignment of SUMO family members and ubiquitin. Sequence comparison of the human SUMO family members (SUMO-1, -2, and -3) to the yeast SUMO homologue SMT3 and to human ubiquitin. Identities are shown in blue, similarities in yellow. The scissors symbol indicates the sites of precursor processing, which occurs in the carboxy-terminus after the double-glycine motif. (Based on Müller et al., 2001.)

4.2. The SUMO conjugation pathway

The SUMO conjugation pathway (Fig. 6) is highly analogous to the ubiquitin pathway. The first step involves an E1 (SUMO-activating) enzyme that activates SUMO in an ATP-dependent reaction, which results in the formation

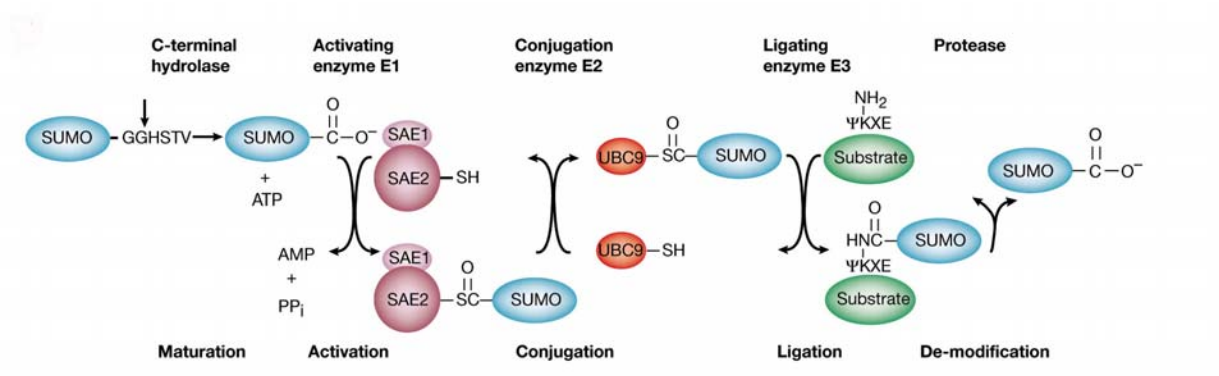


Figure 6. The SUMO conjugation pathway. The SUMO precursor is subjected to proteolysis to expose the C-terminal Gly residue to the E1 activating enzyme (a dimer of SAE1/SAE2 (Aos1/Uba2 in yeast)), which forms a highly reactive thioester bond with SUMO. SUMO is subsequently transferred to the E2 conjugating enzyme, Ubc9, and with the help of the substrate specific E3 ligase is conjugated to target proteins at ψKXE consensus motifs. The resulting isopeptide bond is stable and SUMO-deconjugating enzymes are required for hydrolysis. (From Verger et al., 2002.)

of a highly reactive thioester bond between the E1 enzyme and SUMO. In contrast to the ubiquitin-activating enzyme, the SUMO E1 is a heterodimer consisting of two subunits: Aos1 and Uba2. Interestingly, Aos1 shows sequence and structural similarity to the amino-terminal part of Uba1 - the E1 enzyme for ubiquitin, whereas Uba2 is related to the carboxy-terminal region of Uba1. The Uba2 subunit harbours the 'active site' cysteine residue required for the formation of SUMO–E1 enzyme thioesters; nevertheless both subunits are required for SUMO activation *in vitro* and *in vivo*.

The second reaction of the SUMO-conjugation pathway is the transfer of SUMO from the E1 activating enzyme to the E2 conjugating enzyme - Ubc9, a single polypeptide that is specific for SUMO and does not act on ubiquitin (Gong et al., 1997; Jonson et al., 1997; Schwarz et al., 1998). Structural analysis revealed that the feature that confers the specificity of Ubc9 versus the two known ubiquitin-specific E2 enzymes (Ubc4 and Ubc7) is the positively charged SUMO binding surface, which is highly complementary in its electrostatic potential and hydrophobicity to the negatively charged surface of SUMO. Ubiquitin cannot bind to Ubc9 because of the positive charges in this region (Giraud et al., 1998).

In vitro experiments demonstrated that the E1 activating and E2 conjugating enzymes (together with ATP and SUMO) are sufficient for a relatively robust modification of proteins at precisely the lysines that are preferred *in vivo*. In contrast, in the ubiquitin pathway an additional factor, called E3 ligase or ubiquitin-protein ligase, is almost always necessary for efficient substrate ubiquitination, both *in vitro* and *in vivo* (Hershko and Ciechanover, 1998).

4.3. SUMO protein ligases

Recently, several E3-like proteins for sumoylation have been identified from yeast and mammals. Using the yeast two-hybrid approach Siz1 was found to associate with both Ubc9 and Cdc3 (a member of the septin family), and to strongly stimulate the sumoylation of the septin. Another yeast E3-like protein, Siz2, also promotes sumoylation of protein substrates that are different from the substrates modified with Siz1 (Johnson and Gupta, 2001; Takahashi et al., 2001a,b). However, Siz1 and Siz2, unlike Aos1/Uba2 and Ubc9, are not essential for cell survival in yeast. The mammalian proteins to which Siz1 and Siz2 are most closely related are the PIAS (protein inhibitor of activated STAT) proteins, and indeed it was soon reported that PIAS1 is an E3-like protein for sumoylation of the p53 tumor suppressor in human cells (Kahyo et al., 2001). PIAS1 was isolated as a SUMO-1-binding protein by yeast two-hybrid screening, and shown to interact with both p53 and Ubc9. Furthermore, PIAS1 augmented the SUMO conjugation of p53 in U2OS cells, when p53, SUMO-1, and PIAS1 were co-transfected. Independently, it was shown that all members of the PIAS family can act like E3 ligases (Sachdev et al., 2001; Kotaja et al., 2002; Nishida et al., 2002). Although in the presence of high amounts of Aos1\Uba2 and Ubc9 both septins and and p53 can be SUMO-modified without Siz1 and PIAS1 *in vitro*, the amount of sumoylated proteins greatly increases when the E3-like ligases are also present in the system. Thus, the E3-like proteins may serve to specifically recognize the substrates, increase the affinity between them and the E2 conjugating enzyme (Ubc9), and by bringing them in close proximity in a catalytically favourable orientation, can stimulate the sumoylation to occur at a maximal rate.

The SUMO E3 ligases can be separated in two classes. The first class comprises Siz1, Siz2 and the PIAS family. One common feature in this class is the presence of a RING-like domain that is known to mediate interaction of E3 with E2 in the ubiquitin system (Freemont, 2000). Mutations in the RING-like domain of Siz1 and PIAS1 result in the loss of binding of the E3-like enzyme to Ubc9 (Takahashi et al., 2001a,b; Kahyo et al., 2001). Thus, the RING-like domain of the E3-like enzymes might function in a similar manner to the RING domain of E3 ubiquitin ligases. The second class consists of RanBP2 and Pc2, which have no significant homology to the RING-like ligases. RanBP2 (Ran binding protein 2) contains a RanGAP-binding domain, and repeats responsible for the nuclear transport receptor binding together with a cyclophilin homology region, but has no similarity to the other E3 SUMO ligases (Pichler et al., 2002). Pc2 is a member of the Polycomb group (PcG) proteins, which were first described in *Drosophila* as factors responsible for maintaining the transcriptionally repressed state of Hox/homeotic genes in a stable and heritable manner throughout development (Otte et al., 2003). The PcG complex forms unique nuclear structures, termed PcG bodies (Saurin et al., 1998). These domains vary in number and structure and it is currently unclear whether they are storage compartments or are directly involved in silencing (Spector, 2001).

4.4. SUMO-specific proteases (SUSPs)

Like ubiquitination, sumoylation is a dynamic, reversible process. Deubiquitination enzymes (DUBs) are thiol proteases that hydrolyze ester, thiol ester, and amide bonds formed by the carboxyl group of Gly76 of ubiquitin. They are involved in the processing of ubiquitin precursors, dissociation of polyubiquitin chains, recycling of ubiquitin from late proteolytic intermediates and regulation of the ubiquitination state of proteins (Chung and Baek, 1999; Wilkinson, 1997). Like ubiquitin, SUMO proteins are synthesized as precursors with an amino acid stretch after the double glycine motif, that needs to be released through proteolytic cleavage by C-terminal hydrolases, in order to become accessible for SUMO conjugation. Furthermore, SUMO-

protein conjugates are highly susceptible to deconjugation in cell extracts and different subcellular fractions, indicating the presence of several isopeptidases.

In yeast, two SUMO-specific proteases - Ulp1 and Ulp2 were identified (Li and Hochstrasser, 1999, 2000; Schwienhorst et al., 2000). *In vitro*, both Ulp1 and Ulp2 can catalyze the C-terminal processing of the SUMO precursor and both enzymes can remove SUMO from isopeptide-linked conjugates. Genetic studies have shown that like SUMO conjugation, SUMO de-conjugation is needed for viability in the budding yeast (Li and Hochstrasser, 1999, 2000; Schwienhorst et al., 2000). It was further demonstrated that Ulp1, but not Ulp2, is essential for viability. Analysis of an Ulp1 temperature sensitive mutant showed an arrest of the cells at the G2/M boundary when shifted to the restrictive temperature, suggesting that a SUMO-conjugated protein needs to be deconjugated in order that the cell could progress into mitosis. In addition, it is suggested that Ulp1 is involved in the processing of the SUMO precursor molecule, because the lethality of Ulp1 deletion can be partially overcome by expressing mature SUMO. Disruption of Ulp2, although not absolutely lethal, leads to several abnormalities including slow and thermosensitive growth, defects in cell morphology, chromosome instability and sporulation defects. Both Ulp1 and Ulp2 mutant strains revealed an increased accumulation of SUMO conjugates. Interestingly, the overall pattern of the SUMO conjugated proteins differs in each of the individual mutants, suggesting that the two enzymes act on distinct substrates. Surprisingly, however, inactivation of Ulp2 partially rescues the defects caused by Ulp1 deficiency, and the double mutant accumulates fewer SUMO conjugates than either of the single mutants. One suggested explanation is that Ulp1 and Ulp2 control the modification levels of proteins in opposing pathways (Li and Hochstrasser, 2000).

In humans, at least 7 SUSPs, with sizes ranging from 238 to 1112 amino acids, have been identified (Yeh et al., 2000; Gong et al., 2000; Kim et al., 2000). These enzymes have also been termed SENPs (for sentrin-specific proteases; sentrin is an alternative name for SUMO-1).

The sequence similarity between Ulp1, Ulp2, SUSP1 and SENP1 is restricted to a 200 amino acid sequence, termed ULP domain, which harbours the catalytically active center. Remarkably, no sequence or structural similarity exists between the SUSPs and the de-ubiquitinating enzymes, although both belong to the cysteine protease superfamily. Moreover, these proteins show similarity to the adenovirus L3 protease, suggesting that SUSPs may use a catalytic mechanism similar to that of viral proteases (Li and Hochstrasser, 1999; Andres et al., 2001).

4.5. Biological functions of SUMO modification

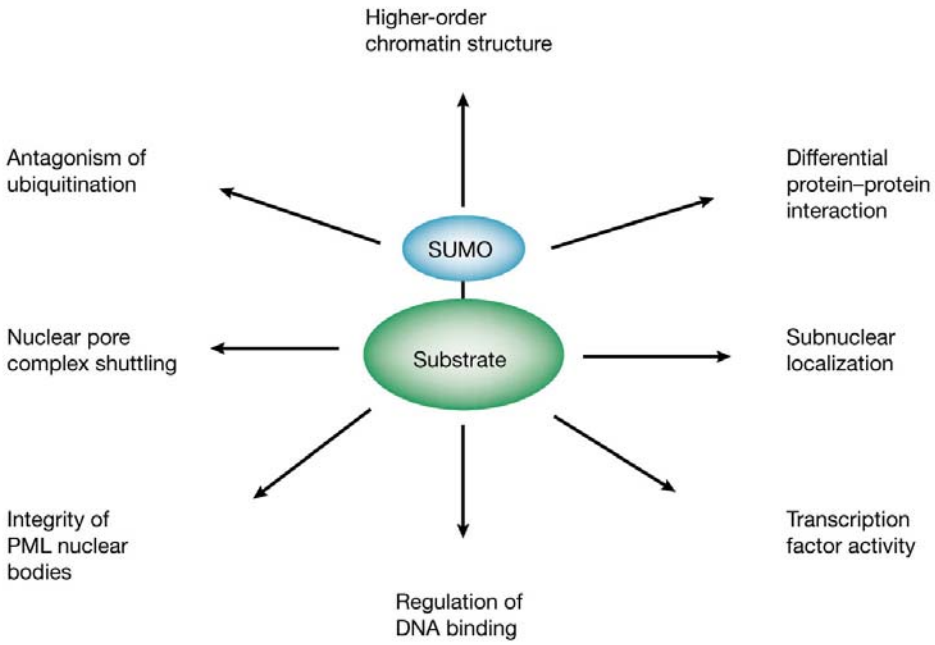


Figure 7. Functional significance of SUMO modification. (From Verger et al., 2002.)

4.5.1. Nuclear pore complex shuttling

The first identified substrate for SUMO1 modification was the mammalian GTPase-activating protein RanGAP1 (Matunis et al., 1996; Mahajan et al., 1997). The function of RanGAP1 includes the GTP-dependent activation of the small nuclear GTPase Ran, which was shown to be essential for transport across the nuclear pore complex (NPC) (Melchior et al., 1993; Moore and

Blobel, 1993). Sumoylation, almost exclusively with SUMO1, is required for the stable association of RanGAP1 and RanBP2. Since SUMO-1 itself cannot bind to RanBP2 (Mahajan et al., 1997), sumoylation most likely induces a conformational change to create a binding surface for RanBP2 in the C-terminal domain of RanGAP1. It was further demonstrated that RanBP2 is the E3 ligase for RanGAP1 (Pichler et al., 2002).

Indirect evidence for the role of SUMO in nuclear import processes comes from studies of *Drosophila melanogaster* harbouring a loss-of-function mutation in the *UBC9* gene (*semushi*) (Epps et al., 1998). In these mutants, nuclear import of the transcription factor BICOID is prevented, leading to defects in embryogenesis. However, it remains unclear whether sumoylation is indeed involved in the control of the nuclear protein import pathway.

4.5.2. Changes in subnuclear localization and targeting to PML bodies

PML (promyelocytic leukemia protein) is a RING-finger protein with a tumor suppressor activity. In the majority of the patients with acute promyelocytic leukemia, the PML gene is translocated to the retinoic acid receptor gene (RARα) and generates a chimeric PML/RARα protein (Kakizuka et al., 1991). PML consists of a RING domain, cysteine- and histidine-rich B1 and B2 boxes and a coiled-coil domain, which was shown to be responsible for the dimerization of the protein (Kastner et al., 1992). PML is enriched in discrete nuclear matrix-associated structures, termed PML nuclear bodies (sometimes also referred to as ND10 bodies, Kr bodies, or PML oncogenic domains) (Dyck et al., 1994; Koken et al., 1994; Weis et al., 1994). PML is one of the target proteins for SUMO-1 modification. Sumoylation of PML was shown to target the protein to nuclear bodies, whereas the unmodified form remains in the nucleoplasmic fraction (Boddy et al., 1996; Sternsdorf et al., 1997; Müller et al., 1998; Zhong et al., 2000). Sumoylation is also required for the nuclear body localization of several other proteins, including Sp100, Daxx, CBP, ISG20, HIPK2, TEL, LEF-1, TCF-1, and Sp3 (Sternsdorf et al., 1997; Kim et al., 1999; Chakrabarti et al., 2000; Sachdev et al., 2001; Ross et al., 2002). Recently, a role for SUMO-1 modification of PML in protein degradation was

suggested (Lallemand-Breitenbach et al., 2001). It was shown that sumoylation at a specific residue triggers the proteasome-dependent degradation of PML and that the mature nuclear bodies harbor 11S proteasome components, suggesting that these structures might be the site of intranuclear proteolysis.

4.5.3. Modulation of protein-protein interactions

Sp100 is an interferon-inducible protein, which was initially characterized as an antigen reactive with antibodies from patients with autoimmune disorders (Szostecki et al., 1990). SUMO-conjugation of Sp100 promotes its interaction with members of the HP1 (heterochromatin protein 1) family of non-histone chromosomal proteins, suggesting its regulatory role in chromatin organization and in the functional interplay between the nuclear bodies and chromatin (Lehming et al., 1998; Sternsdorf et al., 1999; Seeler et al., 1998, 2001).

4.5.4. Regulation of DNA binding

HSF1 and HSF2 (heat shock factors 1 and 2) are transcription factors that mediate the induction of heat shock protein gene expression under environmental stress conditions (Cotto and Morimoto, 1999). Electromobility shift assays demonstrated that SUMO modification enhances the DNA-binding ability of both proteins. Furthermore, mutation of the SUMO target lysine on HSF1 results in a significant decrease in stress-induced transcriptional activity of the protein *in vivo* (Goodson et al., 2001; Hong et al., 2001).

4.5.5. Modulation of the activity of transcription factors

TEL is a transcription factor specifically required for bone marrow hematopoiesis (Wang et al., 1998). It associates with histone deacetylases and can act as a transcriptional repressor (Fenrick et al., 1999; Lopez et al., 1999). Sumoylation of TEL modulates its transcriptional activity by recruiting

TEL to nuclear speckles in a cell cycle-specific manner (Chakrabarti et al., 2000). The transcriptional repressor Daxx can be recruited to nuclear bodies upon sumoylation of PML, thereby relieving the Daxx-mediated transcriptional repression of its target genes (Ishov et al., 1999; Lehembre et al., 2001). Sumoylation of PML is also responsible for the recruitment of p53 to the nuclear bodies and promotes the transcriptional and pro-apoptotic activities of p53 (Fogal et al., 2000). In addition, recruitment of p53 to the nuclear bodies has been suggested to trigger post-translational modifications (e.g. acetylation), which also stimulate the transcriptional activity of p53 (Giaccia and Kastan, 1998; Pearson et al., 2000). Later experiments revealed that p53 is also a target for SUMO-1 modification and that the SUMO conjugation is induced by UV irradiation (Gostissa et al., 1999; Rodriguez et al., 1999; Müller et al., 2000). The target lysine residue for sumoylation, however, is different from the one conjugated to ubiquitin. Therefore SUMO conjugation at Lys-386 does not affect the stability of p53. Furthermore, the apoptotic potential of a mutant form of p53, in which Lys-386 is replaced by arginine (K386R), is moderately impaired, implying that sumoylation of p53 is necessary for exerting its full apoptotic activity (Müller et al., 2000). In addition, SUMO modification is a common mechanism for regulating the transcriptional activity of the steroid receptor superfamily. Point mutations of lysine residues that are targets for SUMO conjugation in the androgen receptor enhance its transcriptional activity, suggesting that sumoylation negatively regulates the activity (Tian et al., 2002). SUMO modification was shown to be a common mechanism for repressing a variety of transcription activators (e.g. LEF1, Sp3 and CtBP) by altering their subnuclear localization and recruiting them to nuclear matrix-associated PML bodies (Sachdev et al., 2001; Ross et al., 2002; Kagey et al., 2003).

4.5.6. Antagonism of ubiquitination

IκB is an inhibitory protein that sequesters the transcriptional factor NF-κB (involved in a variety of processes such as immune function, inflammatory response, cell adhesion, and growth control) in an inactive complex (Baeuerle

and Henkel, 1994; Siebenlist et al., 1994; Beg and Baldwin, 1993; Verma et al., 1995). Upon stimulation with proinflammatory cytokines, phorbol esters, oxidants, or viral infection, IκBα is rapidly phosphorylated by a signal-inducible IκB kinase (IKK) complex (Mercurio et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). Once IκBα is phosphorylated, it is recognized by the ubiquitin-conjugating machinery, which leads to the formation of poly-ubiquitin chains that target the protein for rapid degradation by the 26S proteasome (Alkalay et al., 1995; DiDonato et al., 1996). This results in the liberation of NFκB, which then translocates from the cytosol to the nucleus to activate the transcription of target genes. Interestingly, SUMO can be conjugated to the same lysine residue as ubiquitin, thus leading to stabilization of IκBα against degradation by the 26S proteasome (Desterro et al., 1998). Sumoylated forms of IκBα have been detected in a number of cell types, and are resistant to TNFα-induced degradation. Another example of the antagonistic role of sumoylation against ubiquitination is Mdm2 (Buschmann et al., 2001). Mdm2 is an E3 ligase catalyzing the ubiquitination of p53, as well as of itself (Honda et al., 1997). In normal cells, most of the Mdm2 proteins are sumoylated at Lys-446, which is the same site as the one for self-ubiquitination (Buschmann et al., 2001). SUMO-conjugation protects Mdm2 from destabilization, but increases p53 ubiquitination and degradation. It is suggested that upon DNA damage Mdm2 is desumoylated and subsequently conjugated to ubiquitin, which targets the protein for degradation. This results in a dramatic increase of the activity of p53, leading to cell cycle arrest or apoptosis (Oren, 1999).

The work described in this thesis identified and characterized a novel cell-type specific MAR-binding protein, SATB2. We find that SATB2 binds to the MAR sequences of the endogenous immunoglobulin μ enhancer region and activates transcription. We also show that this protein is modified with SUMO in a PIAS1-dependent manner, and that this modification is crucial for the SATB2-mediated gene activation, MAR association and subnuclear localization.

II. Results

1. Synthetic multimerized MAR-binding sites mimic the natural MARs in augmenting the transcription of the IgH gene

Previous experiments indicated that the nuclear matrix-binding regions (MARs) that flank the intragenic μ enhancer augment markedly the expression of a rearranged μ gene in pre-B cells derived from transgenic mice (Forrester et al., 1994). Furthermore, stable cell lines established with μ gene constructs, premethylated at all CpG dinucleotides, revealed that the MARs are responsible for the generation of an extended domain of histone acetylation, which could account for the long-range function of the μ enhancer in combination with MARs. Since the 5' and 3' MAR regions are fairly long (approximately 500 bp for the 5' MAR and 300 bp for the 3' MAR regions) and contain consensus sites for binding of general transcription factors, we decided to examine whether shorter, synthetic multimerized MAR consensus sites could mimic the natural MARs in augmenting IgH transcription. To answer that question, a construct - pμΔMAR-SBS_7, in which the core immunoglobulin enhancer is surrounded by 7 copies of the MAR consensus sequences (Dickinson et al., 1992) was generated (Fig. 8A). pμ, pμΔMAR or pμΔMAR-SBS_7 plasmid DNAs were subjected to *in vitro* CpG methylation with *SssI* DNA methylase (Fig. 8B) and were transfected in J558L cells. Twelve stable cell lines for each of the transfections were established and DNA and RNA were isolated in parallel (four representative clones are shown). The stable integration of the constructs was analyzed by Southern blotting with a probe specific for the rearranged VDJ region of the μ gene construct (Grosschedl et al., 1984; Fig. 8D). The transcriptional activity was monitored by Northern blot analysis. As shown previously, the transcriptional activity of the pre-methylated pμΔMAR construct was dramatically decreased (Fig. 8C, *middle panel*). However, when multimerized MAR-binding sites were introduced in place of the natural MARs, IgH transcription was restored (Fig. 8C, *lower panel*), suggesting that the AT-rich sequences in the 5' and 3' MAR regions are absolutely necessary for their function and implying that a MAR-

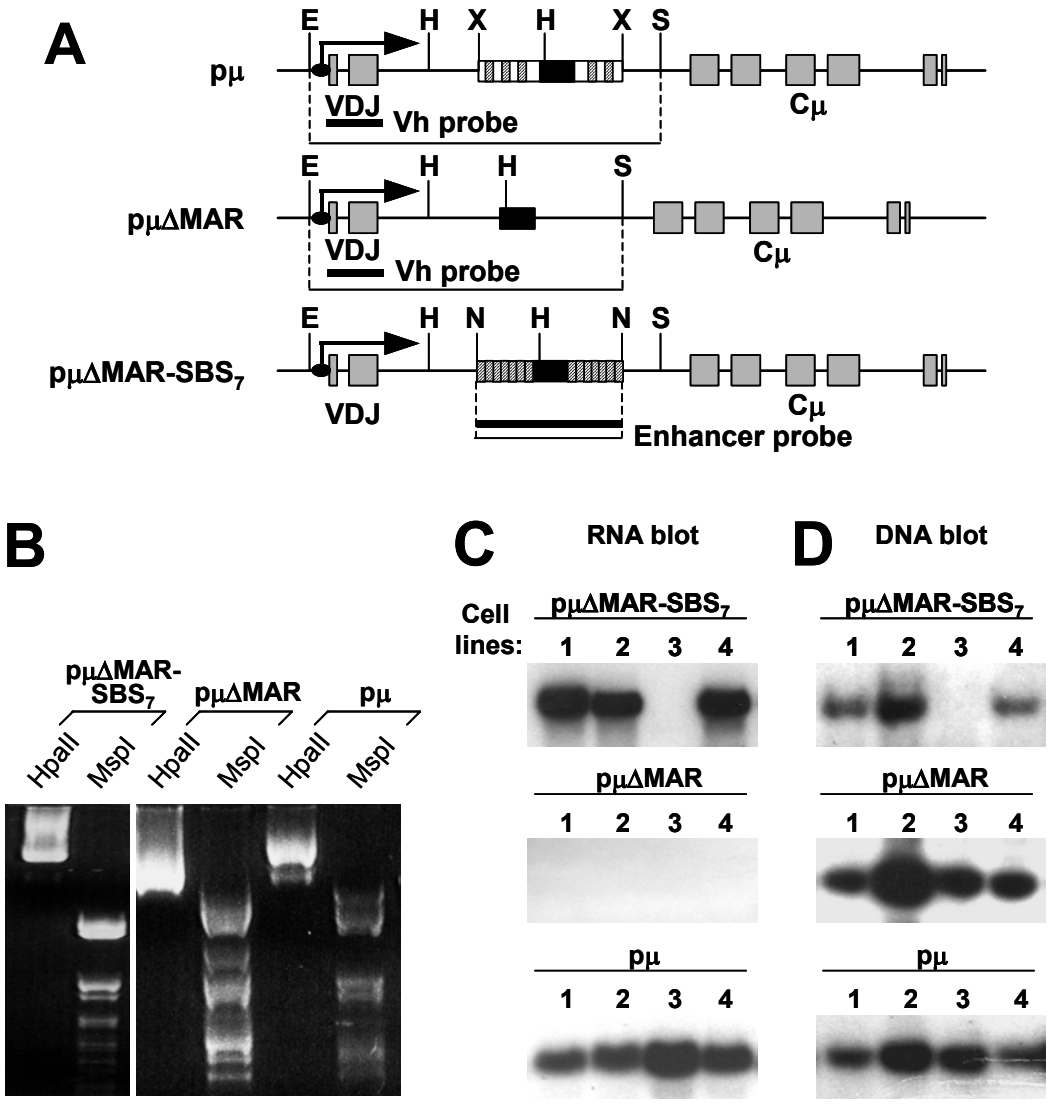


Figure 8. Synthetic multimerized MAR sites mimic the natural MARs in augmenting the transcription of the IgH gene. ***(A)*** Schematic diagram of the rearranged pμ wild-type, pμΔMAR and pμΔMAR-SBS_7 constructs. The intragenic locus control region (LCR), contains the μ enhancer (black bar), flanked by matrix attachment regions (MARs - hatched bars). The exons are shown as gray boxes, and the transcription start site of the *Vh* promoter is indicated by an arrow. Relevant restriction sites: (S) *Sac I*; (E) *EcoRV*; (H) *Hpa*II/*Msp*; (N) *NotI* sites, used to introduce the artificial enhancer in the vector pμΔ2, lacking the 1 kb *XbaI* fragment of the intragenic LCR; (X) XbaI sites, flanking the intragenic LCR. ***(B)*** Analysis of the methylation pattern of the SssI pre-methylated μ genes. The pre-methylated DNA was digested to completion with either *Msp* (M) or *Hpa*II (H) and run on a 1% agarose gel. ***(C)*** Northern blot analysis of total RNA isolated from stable cell clones in J558L cells, transfected with premethylated DNA of pμ, pμΔMAR and pμΔMAR-SBS_7 genes. Numbers represent individual cell clones. For the Northern blot 10 μg of total RNA was used to detect the μ transcripts. The equal loading was controlled by the intensity of the ribosomal bands visualized under UV light (data not shown). ***(D)*** Southern blot analysis. To check whether the constructs were integrated stably in the genome, genomic DNA isolated from the established cell lines was digested to completion with *EcoRV, SacI* for pμ, and pμΔMAR, and with *NotI*, for pμΔMAR-SBS_7. The blots were hybridized with a radiolabeled probe as shown in ***A***.

binding protein might be involved in the regulation of IgH transcription in B cells.

2. A homologue of the MAR-binding protein SATB1 is expressed in pre-B cells

Our data revealed that multimerized MAR consensus sites mimic the function of the natural MARs and suggested that MAR-binding proteins might be involved in the regulation of immunoglobulin transcription. Although two previously cloned MAR-binding proteins, SATB1 and Bright, were shown to interact with the same set of five binding sites in the μ MARs *in vitro* (Dickinson et al., 1992; Herrscher et al., 1995), neither of these proteins are expressed in early B cells, in which the enhancer and MARs collaborate to augment Vh promoter activity. In particular, SATB1 is predominantly expressed in T lymphocytes, whereas Bright is expressed in activated and terminally differentiated B cells. Therefore, we began to search for additional MAR-binding proteins that are expressed in pre-B cells.

To identify MAR-binding proteins that are expressed predominantly in early stages of the B cell lineage, we searched the murine and human databases for sequences that show homologies to known MAR-binding proteins and examined their expression in pre-B cells. We identified two clones that displayed 71% and 70% overall sequence homology with SATB1 and Bright, respectively (Fig. 9 and data not shown). Analysis of the expression patterns of these clones revealed a ubiquitous expression for the Bright homologue and a predominantly pre-B cell specific pattern for the SATB1 homologue, which we termed SATB2 (Fig. 11 and data not shown).

2.1. SATB2 has a high homology and a similar domain organization to SATB1

SATB2 was PCR amplified from a cDNA pool obtained from Abelson murine leukemia virus (AMuLV)-transformed pre-B cells. The 2002 bp open reading frame (ORF) of the SATB2 cDNA codes for a protein of 733 amino acids. The predicted molecular weight (Mw) of the full-length SATB2 protein is 82,5 kD.

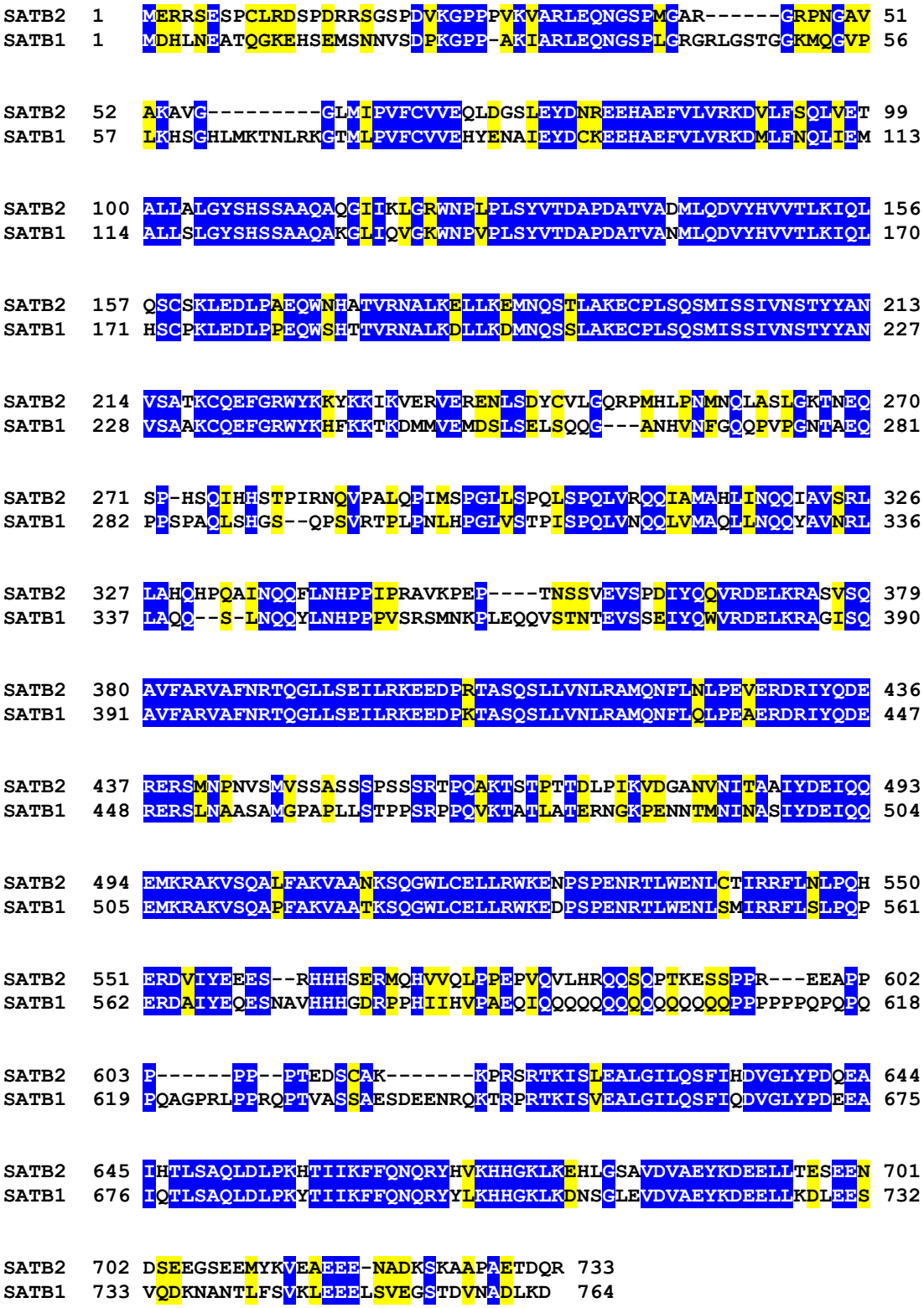


Figure 9. Alignment of SATB2 and SATB1. Mouse SATB1 and SATB2 were aligned using the Clustal algorithm. Identities are shown in blue, similarities in yellow.

The alignment of murine SATB1 and SATB2 shows that the two proteins are 59% identical and 71% similar (Fig. 9). Sequence analysis of SATB2 revealed the presence of two CUT domains and a Hox domain, which are highly conserved with the corresponding DNA-binding domains of SATB1 (Fig. 10; Nakagomi et al.,1994; Dickinson et al., 1997). A unique feature of the protein is a 23 amino acid stretch in the N-terminus of the protein, which is not homologous to any sequence in SATB1, suggesting that this portion may confer some specific properties on the molecule.

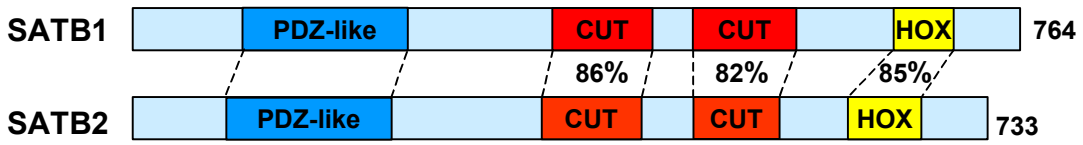


Figure 10. Domain organization of SATB2. Schematic linear representation of the domain organization of SATB1 and SATB2 (drawn to scale). The two CUT repeats (black boxes) represent the MAR-binding domain; HOX (yellow boxes) - homeodomain; PDZ-like dimerization domain (red boxes). The numbers indicate the percentage of identity between the respective domains.

2.2. Expression pattern of SATB2

To examine the tissue and cellular distribution of SATB2, RNA from different mouse organs and cell lines was isolated and a Northern blot was performed, using the full-length cDNA as a probe. The membrane was subsequently re-hybridized with a probe recognizing the transcript of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a control for loading and transfer.

RNA blot analysis of total RNA from different lymphoid and non-lymphoid cell lines indicated that SATB2 transcripts with sizes of ~6.3 and ~5.4 kb can be detected most abundantly in cell lines representing pre-B cells or B cells, and at lower levels in cells representing T cells (Fig. 11A). In tissue Northern blot, SATB2 transcripts could be detected predominantly in brain and kidney,

although differently sized transcripts were detected at a lower abundance in thymus and testis (Fig. 11B).

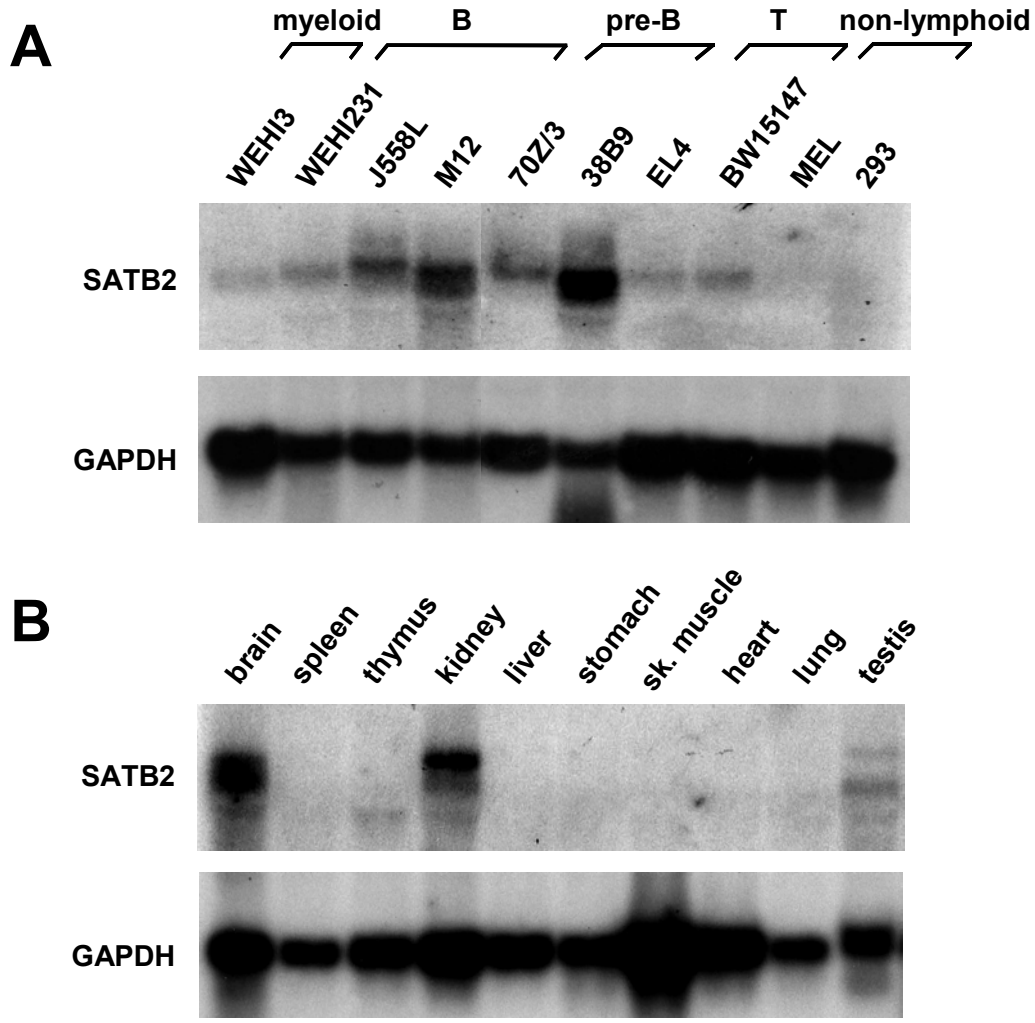


Figure 11. SATB2 has a cell type and tissue-specific distribution. Full-length SATB2 coding sequence was used in Northern blot to probe RNA isolated from various cell lines ***(A)*** and mouse organs ***(B)***. Abundant expression of ~6.3 and ~5.4 kb transcripts that hybridize with a ^{32}P-labeled SATB2 DNA probe can be detected in B cells, brain and kidney (***A***, ***B***; *top panels*). The quality and quantity of RNA was confirmed by hybridization with a GAPDH probe (***A***, ***B***; *bottom panels*).

2.3. SATB2 is a component of the nuclear matrix

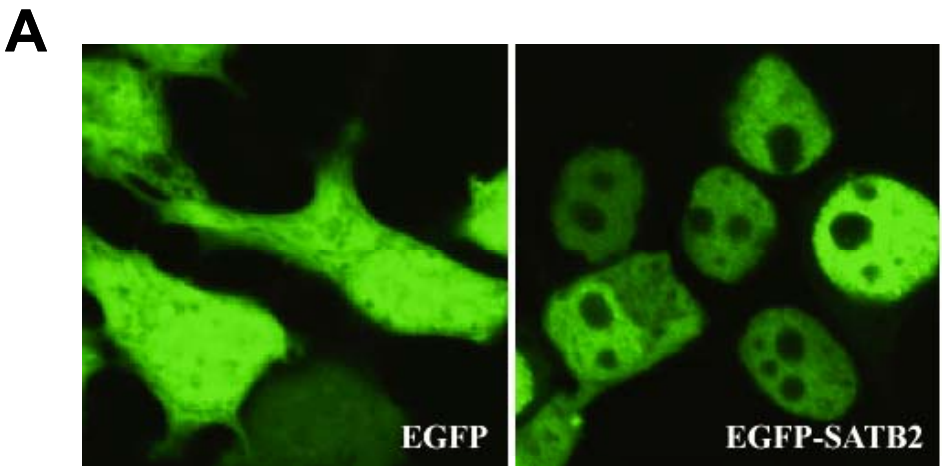
In order to obtain information about the intracellular localization of SATB2, an EGFP-SATB2 construct was generated and transfected in 293T cells, and its subcellular distribution was examined by direct immunofluorescence. Upon

transfection, EGFP-SATB2 and all other SATB2 constructs (FLAG-SATB2 and SATB2-myc, tested by indirect immunofluorescence) localized to the nucleus (Fig. 12A).

Previous studies have shown that SATB1 is a component of the nuclear matrix (de Belle et al., 1998; Cai et al., 1999). To examine whether SATB2 can also associate with the nuclear matrix, we transfected 293T cells with plasmids encoding either an EGFP-SATB2 fusion protein or an EGFP-NLS control protein. 36 h. post transfection the cells were directly fixed or a nuclear matrix preparation was performed. In brief, nuclear matrices were prepared by removing the membrane and soluble proteins with detergent treatment, followed by DNaseI digestion and high salt extraction of the chromatin fraction (Fig. 12B). As shown in Fig. 12C, when cells were directly fixed, both EGFP-SATB2 and EGFP-NLS were localized in the nucleus; however, following high salt extraction SATB2 remained associated with the nuclear matrix, in contrast to EGFP-NLS, which was no longer found in the nucleus.

2.4. SATB2 binds to the core-unwinding element of the IgH enhancer MARs *in vitro*

While no primary consensus has been found for matrix attachment regions, except that they are generally AT-rich, MARs typically contain a small region of more than 150-200 bp that is highly potentiated for base unpairing, when examined under negative superhelical strain (Kohwi-Shigematsu and Kohwi, 1992). Within the base-unpairing region (BUR), a core-unwinding element exists and its mutation abolishes the base unpairing properties within the MAR (Kohwi-Shigematsu and Kohwi, 1992). The high unwinding capacity of MARs is important for conferring high-affinity binding to the nuclear matrix. With the use of a specific sequence containing the core-unwinding element derived from the IgH enhancer MARs, SATB1 was cloned and shown to bind specifically AT-rich sequences that are highly potentiated to unwind (Dickinson et al., 1992). Therefore we decided to test whether SATB2 could also specifically associate with the core-unwinding element from the IgH enhancer MARs - termed wtMAR.



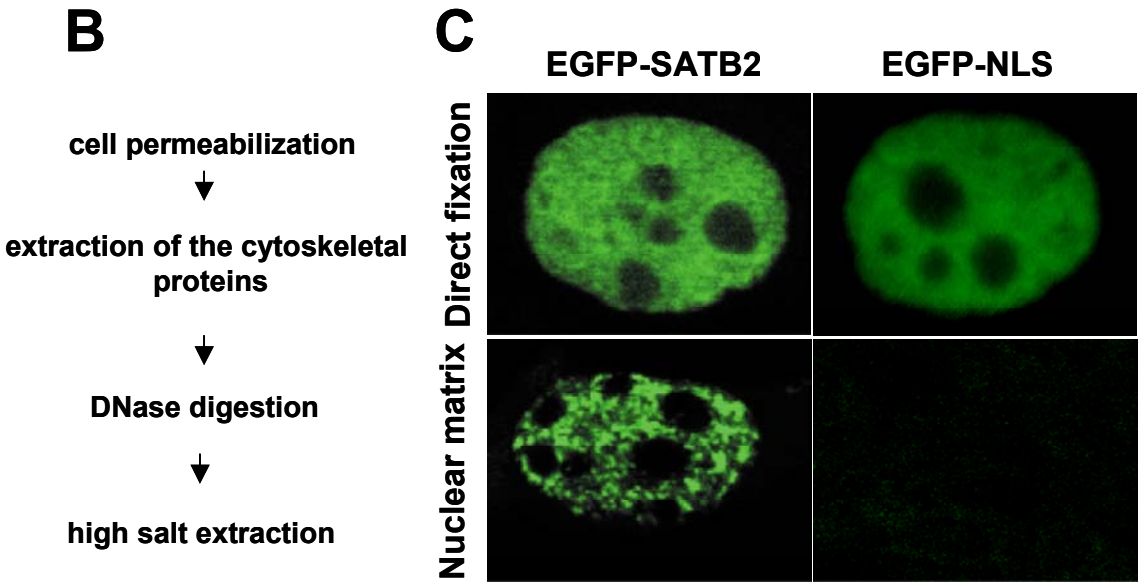


Figure 12. SATB2 is a component of the nuclear matrix. ***(A)*** SATB2 localizes in the nucleus. EGFP and EGFP-SATB2 were transfected in 293T cells and visualized by the EGFP fluorescence. SATB2 showed clear staining in the nucleus in contrast to EGFP, which showed diffuse distribution throughout the whole cell. ***(B)*** Nuclear matrices were prepared by removing the membrane and soluble proteins with detergent, followed by DNaseI digestion and high salt extraction of the chromatin fraction. ***(C)*** SATB2 is a component of the nuclear matrix. 293T cells were transiently transfected with expression vectors encoding EGFP-NLS or EGFP-SATB2. 48 h. post transfection the cells were either immediately fixed (*upper panels*) or processed for nuclear matrix preparations before fixation (*lower panels*) and visualized by the EGFP fluorescence.

To this end, we examined the DNA binding ability of SATB2 by electrophoretic mobility shift assays with purified recombinant SATB2 protein. Efficient binding was detected with the wild-type MAR consensus sequence (Dickinson et al., 1992), but not with a mutant oligonucleotide (Fig. 13; lane 2 and 12). We confirmed the specificity of DNA binding by the addition of excess unlabelled competitor DNA, which impaired DNA binding in a dose-dependent manner (Fig.13; lane 3-6). In contrast, the binding could not be competed with increasing amounts of nonspecific competitor (lane 7-10). Thus, SATB2 resembles SATB1 in its ability to bind the wt MAR consensus sequence, derived from the core unwinding element of the IgH enhancer MARs.

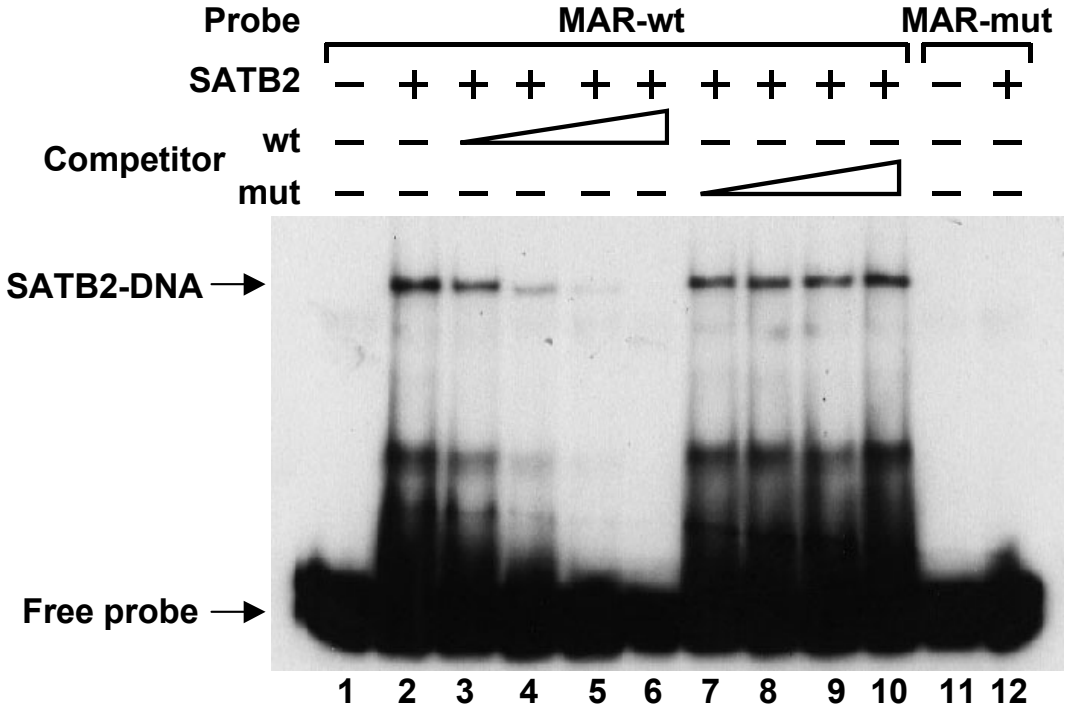


Figure 13. SATB2 binds to nuclear matrix attachment region (MAR) DNA. Recombinant SATB2 was tested for its ability to bind to a ^{32}P- labeled wild-type (wt) or mutated (mut) MAR (BUR) consensus sequence by electrophoretic mobility shift assays, as indicated. The binding reactions were performed as described in Materials and Methods. SATB2 binding to the wt MAR probe (lane 2) could be specifically competed with wt cold competitor (lanes 3-6) but not with the cold mutated sequence (lanes 7-10; see Materials and Methods for the sequences of the probes). The positions of the SATB2-DNA complex and of the free probe are indicated on the left.

2.5. SATB2 binds to the MARs of the endogenous μ locus

Our *in vitro* data demonstrated that SATB2 binds to the core-unwinding element of the IgH enhancer MARs. This raised the question whether the same is also true *in vivo.* To address this issue, we performed chromatin immunoprecipitations (ChIP). Due to the lack of antibodies that immunoprecipitate SATB2, we generated a pre-B cell line that had been stably transfected with a SATB2-TAPtag gene construct. After a two-step affinity purification (Rigaut et al., 1999) of SATB2-TAPtag protein that had been cross-linked to DNA *in vivo*, the immunoprecipitated DNA was amplified in serial dilutions by polymerase chain reactions with primers specific for the 5' MAR region of the intronic μ enhancer (Fig. 14, lanes 1-6), or with primers specific for β-globin gene sequences as a control (Fig. 14, lanes 7-12). Significant enrichment (approximately 100-fold) of μ 5' MAR sequences was detected with immunoprecipitated DNA from the SATB2-TAPtag-expressing cell line (lanes 1-6, *top panels*), but not with the immunoprecipitated DNA from the parental pre-B cell line (lanes 1-6, *bottom panels*). No enrichment of β-globin sequences was detected (lanes 7-12, *top panels*), indicating that SATB2 is specifically bound to the MAR flanking the intragenic enhancer of the endogenous μ gene.

2.6. SATB2 is a B cell-specific transcriptional activator

2.6.1. SATB2 mediates transcriptional activation under multimerized MAR consensus sites

Having demonstrated that SATB2 is bound to the μ enhancer *in vivo* we next wanted to address the possible role of this protein. Previous analysis of the functional activity of SATB1 in transfection assays and in knockout experiments had indicated that SATB1 represses transcription of several genes in T cells (Kohwi-Shigematsu et al., 1997; Alvarez et al., 2000).

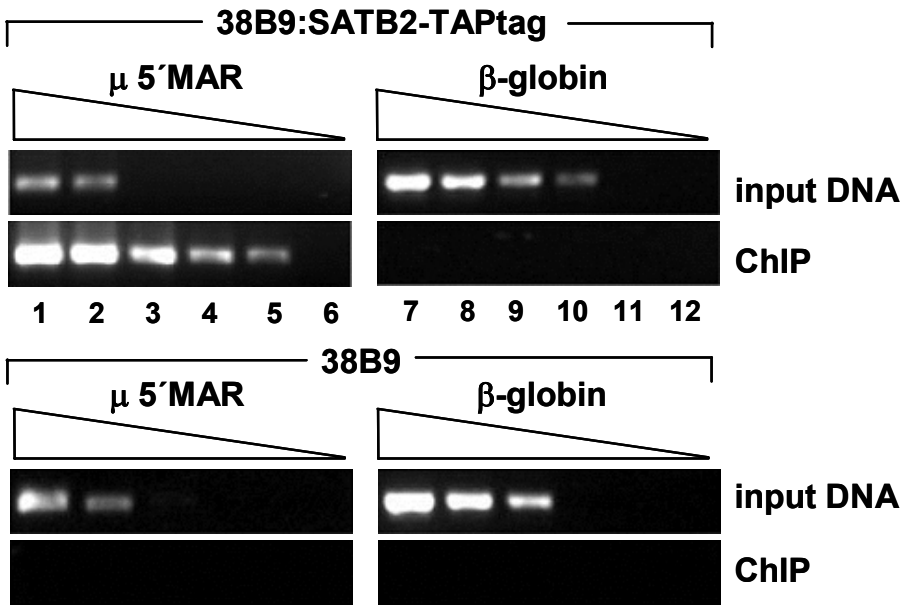


Figure 14. SATB2 binds to the MAR of the endogenous μ locus. Chromatin immunoprecipitations (ChIP) of extracts from stably transfected 38B9 cells, carrying a SATB2-TAPtag expression plasmid, and from control 38B9 cells were performed by affinity-purifying chromatin fragments that have been cross-linked to SATB2-TAPtag. The immunoprecipitated DNA was subsequently analyzed by semi-quantitative PCR amplification with primers located in the 5' MAR region of the immunoglobulin μ enhancer (lanes 1-6) or in the β-globin locus (lanes 7-12). The levels of enrichment in the immunoprecipitations were estimated by comparison with the amplification products of DNA isolated from the bulk chromatin extracts (input DNA). Template DNA was used in a linear dilution (3, 1, 0.3, 0.1, 0.03, 0.01 ng) to allow for a semi-quantitative determination in the PCR assays.

To examine the functional activity of SATB2, we transfected a fos-luciferase reporter construct containing multimerized wild-type or mutated SATB2-binding sites, together with a β-galactosidase control plasmid and increasing amounts of a SATB2 expression plasmid into J558L plasmacytoma cells (Fig. 15A). 48 h. post transfection the cells were collected and luciferase and β-galactosidase assay were performed. The results were presented as a relative luciferase number, normalized to the β-galactosidase values.

Transfection of increasing amounts of SATB2 in 293T cells did not have any effect on the transcription of the luciferase reporter construct (Fig. 15B). In contrast, when the experiment was performed in J558L plasma cells a dramatic transcriptional activation of around 140 fold was observed (Fig. 15B). A slight increase was observed for the construct containing the multimerized

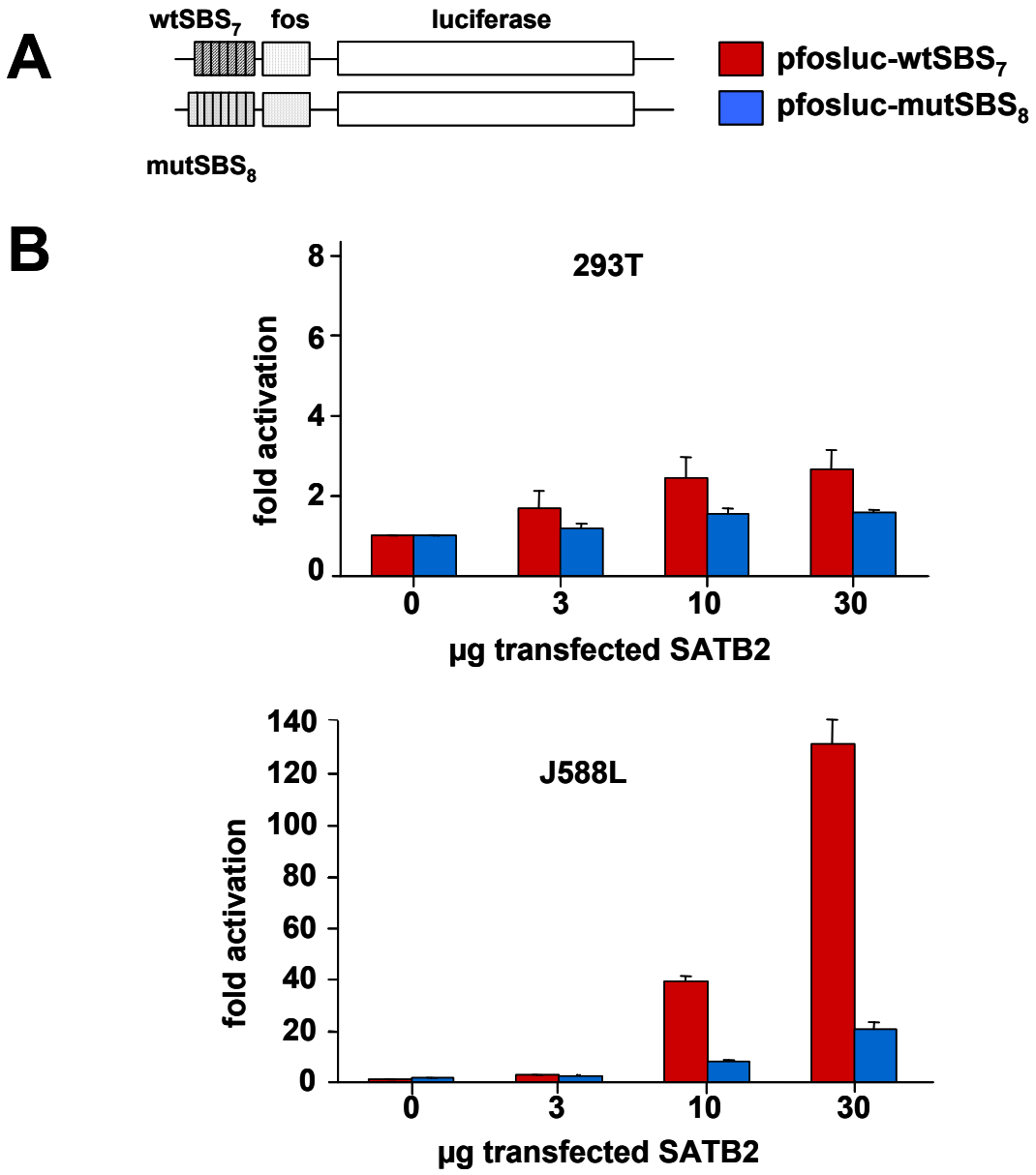


Figure 15. SATB2 mediates transcriptional activation under the multimerized MAR consensus sites. ***(A)*** Schematic linear representation of the reporter constructs used for the experiments in ***(B)***. Seven copies of the wt MAR consensus sequence were multimerized and cloned in front of a minimal fos promoter, driving the expression of a luciferase gene. ***(B)*** SATB2 mediates transcriptional activation in B cells via synthetic MAR sites. 293T (*top panel*) or J558L (*bottom panel*) cells were transfected with 5 μg of a MAR luciferase reporter construct containing multimerized wild-type (wt) or mutant (mut) MAR-binding sites together with expression constructs encoding for β-galactosidase (1 μg - for normalization) and increasing amounts of SATB2 - 3, 10 and 30 μg, as indicated. For this and subsequent experiments, the levels of luciferase activity were normalized to the β-galactosidase activity and expressed as fold activation relative to the level of luciferase from cells transfected with the reporter construct alone.

mutated MAR sequences, which correlates with the results from EMSA experiments indicating that SATB2 could also bind, albeit with significantly lower efficiency, to the mutant MAR sequences, when they are multimerized (data not shown).

2.7.2. SATB2 mediates transcriptional activation under the natural MARs of the immunoglobulin enhancer

Next we wanted to examine whether SATB2 could also mediate transcriptional activation via the natural MARs of the immunoglobulin enhancer. Co-transfection experiments using luciferase reporter constructs, carrying either the μ enhancer or the core enhancer, lacking the MAR regions (pfosluc-μΔMAR) in front of the fos promoter (Fig. 16A) and increasing amounts of SATB2 in Jurkat and J558L cells were performed. Transfection of increasing amounts of SATB2 in Jurkat cells revealed a slight activation of the transcription of the luciferase reporter construct (Fig. 16B). In contrast, when the experiment was performed in J558L plasma cells a huge transcriptional activation of around 450 fold was observed (Fig. 16C), implying that a B cell-specific co-activator may be involved in SATB2-mediated gene activation.

2.7.3. SATB2 augments immunoglobulin transcription

Following the demonstration that SATB2 can activate transcription we tested in co-transfection experiments whether SATB2 could augment the transcription of a rearranged μ gene, where the enhancer is more than 1 kb away from the Vh promoter. J558L cells were transfected with the rearranged μ genes - pμ and pμΔMAR (Fig. 17A) and increasing amounts of SATB2. 48 h. post transfection RNA was isolated and Northern blot was performed, using probe annealing specifically to the rearranged VDJ region. The membrane was then stripped and re-hybridized with a probe recognizing the transcript of

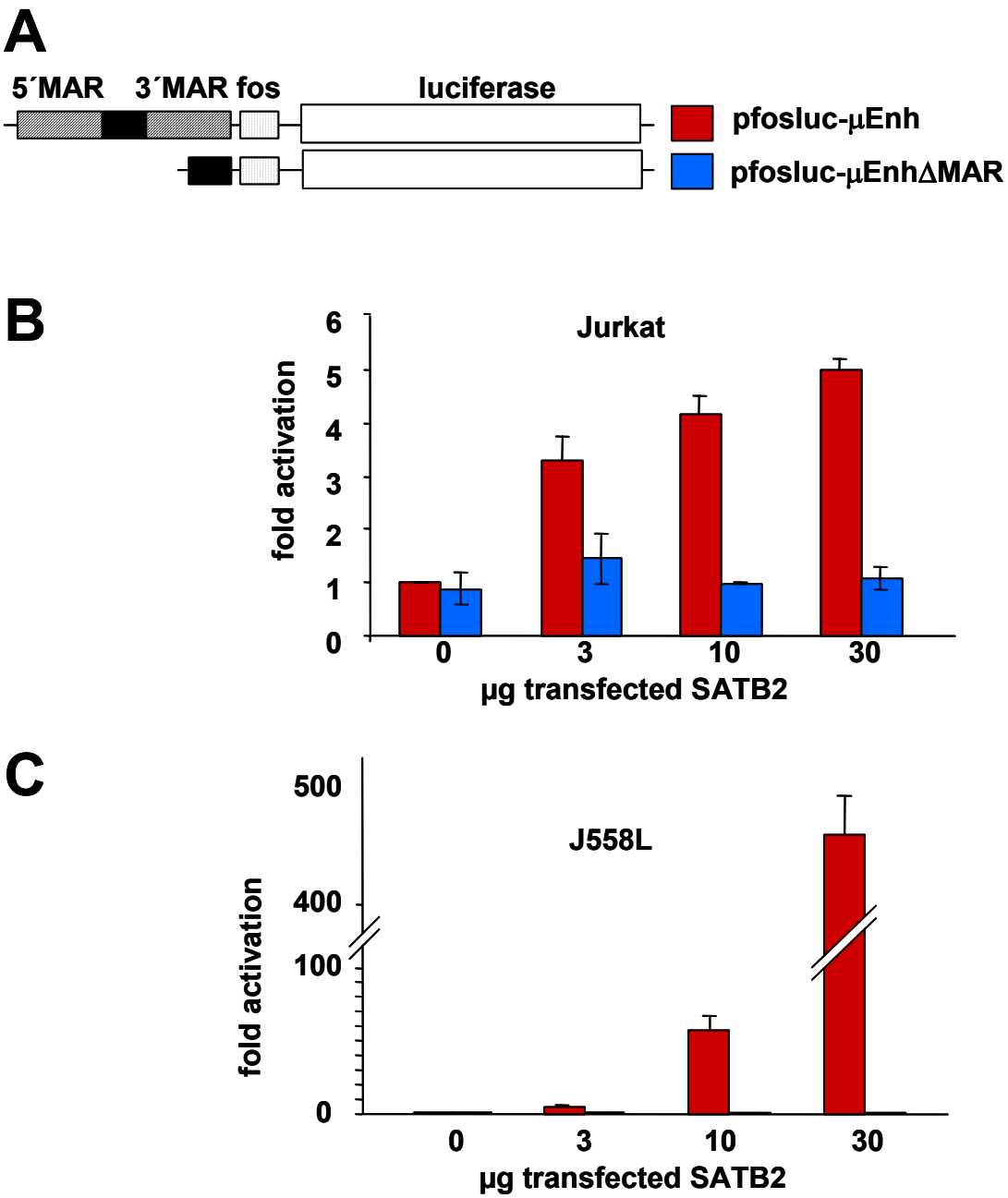


Figure 16. SATB2 mediates transcriptional activation under the natural MARs of the immunoglobulin enhancer. ***(A)*** Schematic linear representation of the reporter constructs used for the experiments in ***(B)*** and ***(C)***. The XbaI fragment of the μ enhancer derived from pμ and the enhancer fragment lacking both MAR regions from pμΔMAR (see Fig. 8) were cloned in front of a minimal fos promoter, driving the expression of a luciferase gene. ***(B)*** and ***(C)*** SATB2 mediates transcriptional activation in B cells via the natural MARs in the μ enhancer. Jurkat ***(B)*** or J558L ***(C)*** cells were transfected with 5 μg of a MAR luciferase reporter construct containing the μ enhancer or the μ enhancer with deleted MAR regions, together with 1 μg expression constructs encoding for β-galactosidase and increasing amounts of SATB2 - 3, 10 and 30 μg, as indicated.

the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as a control for loading and transfer. Indeed, consistent with the previous experiments, SATB2 augmented IgH transcription in a dose dependent manner, whereas no significant effect was observed with the ΔMAR μ gene (Fig. 17B).

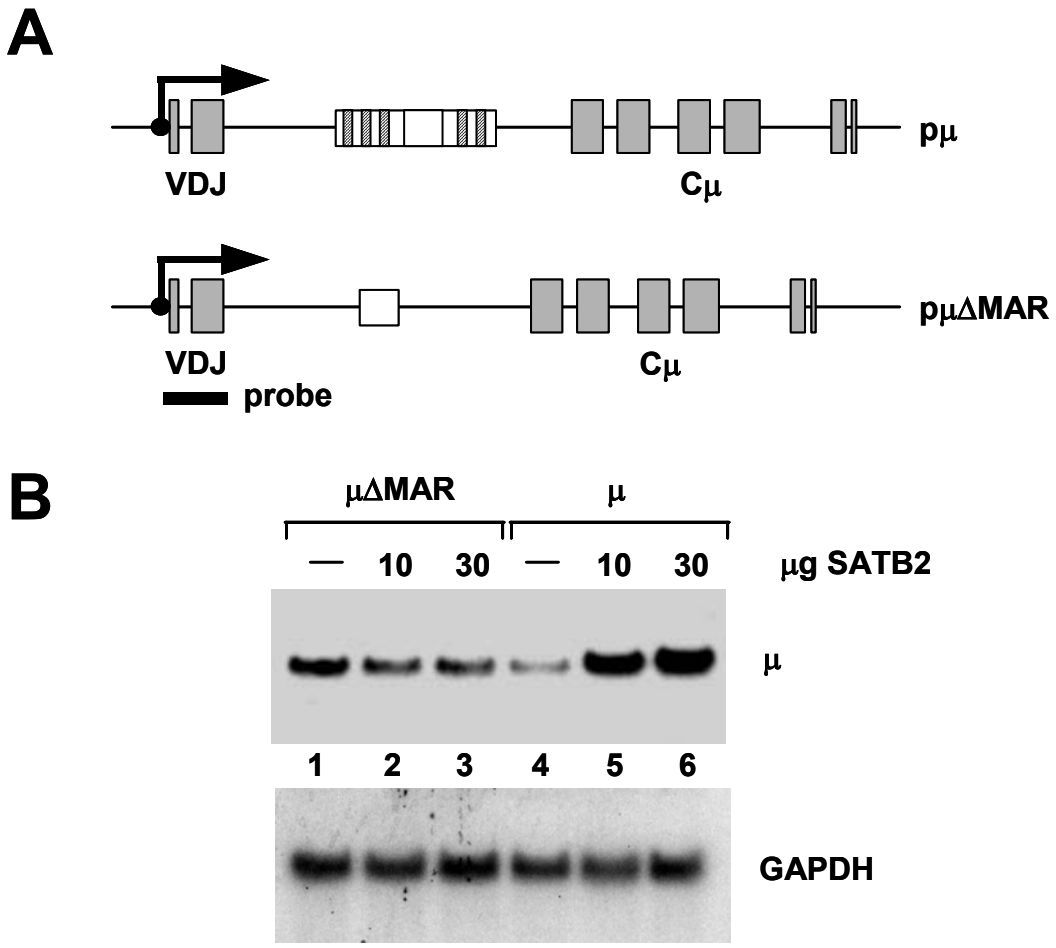


Figure 17. SATB2 augments the transcription of the immunoglobulin gene. ***(A)*** Schematic diagram of the rearranged μ wild-type and μΔMAR gene. The position of the probe against Vh17.2.25 ("VDJ probe", see Materials and Methods), used in the Northern blot is indicated. ***(B)*** SATB2 augments immunoglobulin μ expression. J558L cells were transfected with 5 μg of rearranged μ wild-type or μΔMAR genes and increasing amounts of SATB2 (10, 30 μg) as indicated. Northern blot using the probe against the VDJ region or against GAPDH for loading control, was performed.

3. SATB2 is posttranslationally modified

3.1. SATB2 is SUMO-modified

In an immunoblot analysis of lysates from cells transfected with a myc-tagged SATB2 expression plasmid, we detected SATB2 migrating at ~105 kD and two minor bands migrating at ~135-140 kD (Fig. 18A). One possible explanation of this observation was that the protein is posttranslationally modified. Various posttranslational modifications are known, including phosphorylation, acetylation, ubiquitination, methylation and sumoylation. Since most posttranslational modification do not lead to significant changes in the molecular weight of the protein, the large shift in the slower migrating SATB2 forms pointed in the direction of ubiquitination or sumoylation (see Introduction – 4.1).

SUMO is a *s*mall ubiquitin-related modifier that is covalently attached to lysine residues in target proteins via an isopeptide linkage in a multi-step process that is analogous to ubiquitination (Fig. 18B; see also Fig. 6 and Introduction - 4.2).

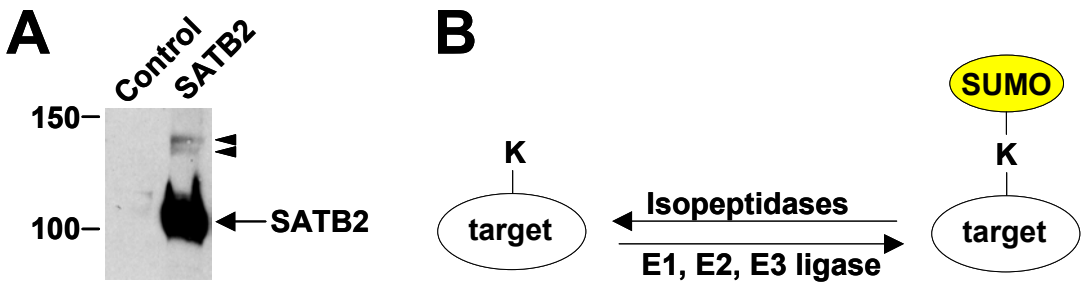


Figure 18. SATB2 is posttranslationally modified. ***(A)*** Immunoblot analysis of total protein extracts from 293T cells, transfected transiently with a myc-tagged SATB2 expression plasmid or a control vector. In addition to SATB2, migrating at ~105 kD, two forms of SATB2, migrating at ~135 kD and ~140 kD can be detected with an anti-myc antibody. ***(B)*** The SUMO conjugation pathway. After SUMO is proteolytically processed by C-terminal hydrolases, it serves as the substrate for an isopeptide bond formation between the free carboxyl group of the C-terminal glycine in SUMO and the ε-amino group of a lysine (K) in the acceptor protein. The catalytic reaction is mediated by the E1 activating enzyme: Aos1/Uba2, the E2 conjugating enzyme Ubc9, and an E3 ligase, which confers the substrate specificity. The cleavage of the isopeptide bond is mediated by isopeptidases.

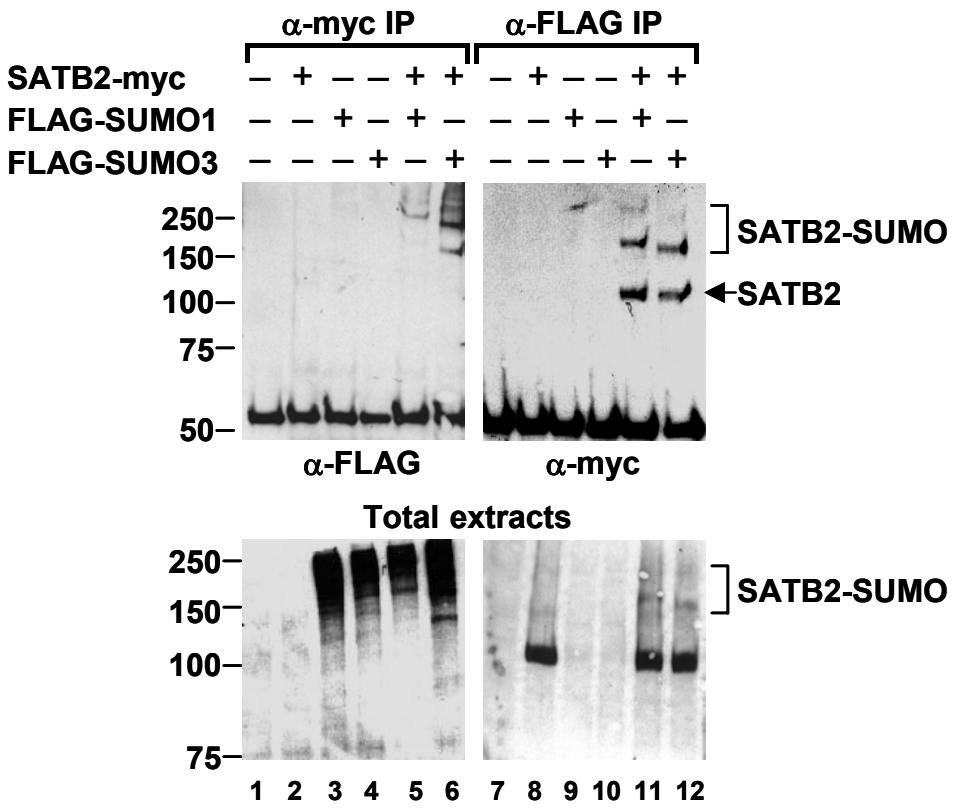


Figure 19. SATB2 is modified by SUMO conjugation. Myc epitope-tagged SATB2 and FLAG epitope-tagged SUMO1 or SUMO3 were transiently expressed in 293T cells. 24 h. post transfection the cells were lysed and equivalent amounts of total cellular protein were immunoprecipitated with anti-myc (lanes 1-6, *top panel*) or anti-FLAG monoclonal antibodies (lanes 7-12; *top panel*). Immunoprecipitated proteins that have been modified with FLAG-SUMO1 or FLAG-SUMO3 were detected with an anti-FLAG antibody (lanes 1-6); SATB2 and SUMO-modified forms of SATB2 were detected with an anti-myc antibody (lanes 7-12; *top panels*). Similar expression of SATB2-myc, as well as of FLAG-SUMO1 and FLAG-SUMO3 was confirmed by immunoblot analysis of total cell extracts (*bottom panels*).

To examine whether the two slower-migrating proteins represent covalent conjugations of SATB2 with the 12 kD *s*mall *u*biquitin-like *mo*difier (SUMO), we transfected the myc-tagged SATB2 expression plasmid, together with a FLAG-SUMO1 or FLAG-SUMO3 expression plasmid, into 293T cells. Co-immunoprecipitation of proteins with an anti-myc antibody and subsequent immunoblot analysis with an anti-FLAG antibody detected the slower migrating forms of SATB2 (Fig. 19, lanes 5 and 6). Likewise, we detected these slower migrating forms of SATB2 in a reciprocal co-immunoprecipitation

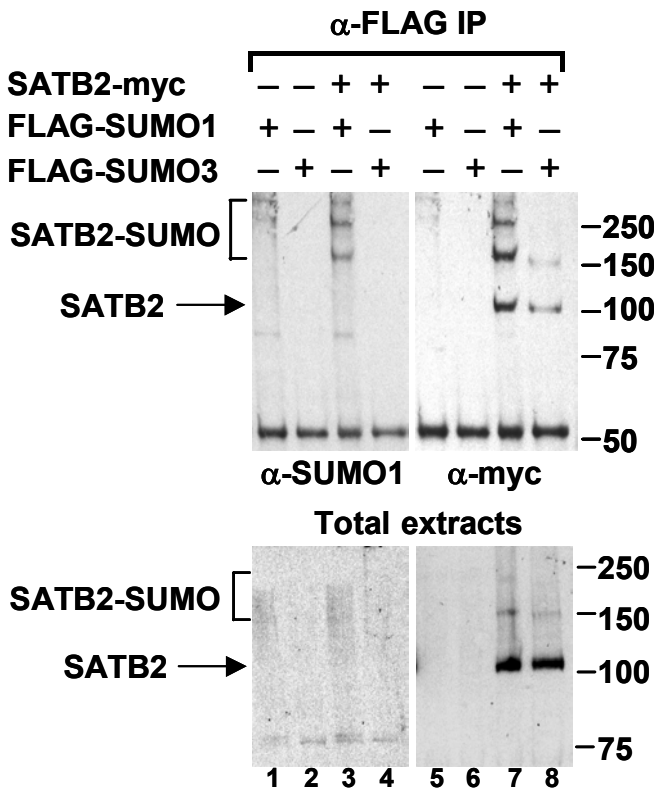


Figure 20. The 105 kD isoform of SATB2 is not SUMO-modified. SATB2-myc and FLAG epitope-tagged SUMO1 or SUMO3 were transiently expressed in 293T cells. Equivalent amounts of total cellular protein were immunoprecipitated with an anti-FLAG antibody (lanes 1-8, *top panels*) and the immunoprecipitated proteins that have been modified with FLAG-SUMO1 or FLAG-SUMO3 were detected with an anti-SUMO1 antibody (lanes 1-4). After stripping the blot, SATB2 and the SUMO-modified forms of SATB2 were detected with an anti-myc antibody (lanes 5-8, *top panels*). Similar expression of SATB2-myc and SUMO1-modified proteins was confirmed by immunoblot analysis of total cell lysates (*bottom panels*).

and immunoblot analysis with an anti-myc antibody (Fig. 19, lanes 11 and 12). In this experiment, we also detected unmodified SATB2, which could reflect a dimerization of the transfected myc-tagged SATB2 with the sumoylated SATB2. Previous experiments have shown that SATB1 can dimerize through a PDZ domain that is located upstream of the CUT domains (Galande et al., 2001). To confirm that the slower migrating forms of SATB2 correspond to a covalent modification with SUMO, we performed a co-immunoprecipitation with the anti-FLAG antibody and probed the immunoblot with an anti-SUMO1 antibody (Fig. 20, lanes 1-4), and, after stripping - with an anti-myc antibody (Fig. 20, lanes 5-8). The slower migrating forms of SATB2, but not the 105 kD

form of SATB2, could be detected with the anti-SUMO1 antiserum. Taken together, these data indicate that SATB2 can be modified by conjugation with SUMO1 and SUMO3.

3.1.1. SATB2 is SUMO-conjugated at two SUMO-consensus sites

Our data showed that SATB2 could be posttranslationally modified by exogenously expressed FLAG-SUMO1 and FLAG-SUMO3. SUMO acceptor sites have a minimal consensus sequence, ψKXE, in which ψ is a large hydrophobic residue and K is the lysine to which SUMO is added (Rodriguez et al., 2001). Inspection of the amino acid sequence of SATB2 revealed two consensus sites at lysine residues 233 and 350 (Fig. 21A). To determine whether the putative SUMO conjugation sites in SATB2 are the actual sites of modification, we mutated the lysines at positions 233 and 350, individually (K233R and K350R) or together (K233R/K350R). We examined the effects of these mutations on the sumoylation of SATB2 by co-transfecting wild-type or mutant FLAG-SATB2 expression plasmids together with SUMO1 or SUMO3 plasmids into 293T cells. Immunoblot analysis of total cell lysates indicated that the slower migrating, ~140 kD band corresponds to a sumoylation at position K233, whereas the faster migrating band at ~135 kD corresponds to a SUMO modification at position K350 (Fig. 21B; lanes 5, 6 and 8, 9). Consistent with the modification of SATB2 at both lysines, the ~135 kD and ~140 kD bands are both missing in the lanes containing cell lysates of the double mutant form of SATB2 (lanes 11, 12). Thus, the lysines at positions 233 and 350 are required for the SUMO-modification of SATB2.

3.1.2. SATB1 is not SUMO-modified

Sequence alignment revealed that the corresponding sequences in SATB1 do not conform to the consensus SUMO modification motif (Fig. 22A). To test whether SATB1 is not SUMO-modified *in vivo*, 293T cells were co-transfected with constructs expressing myc-SATB1 or myc-SATB2 (as a positive control)

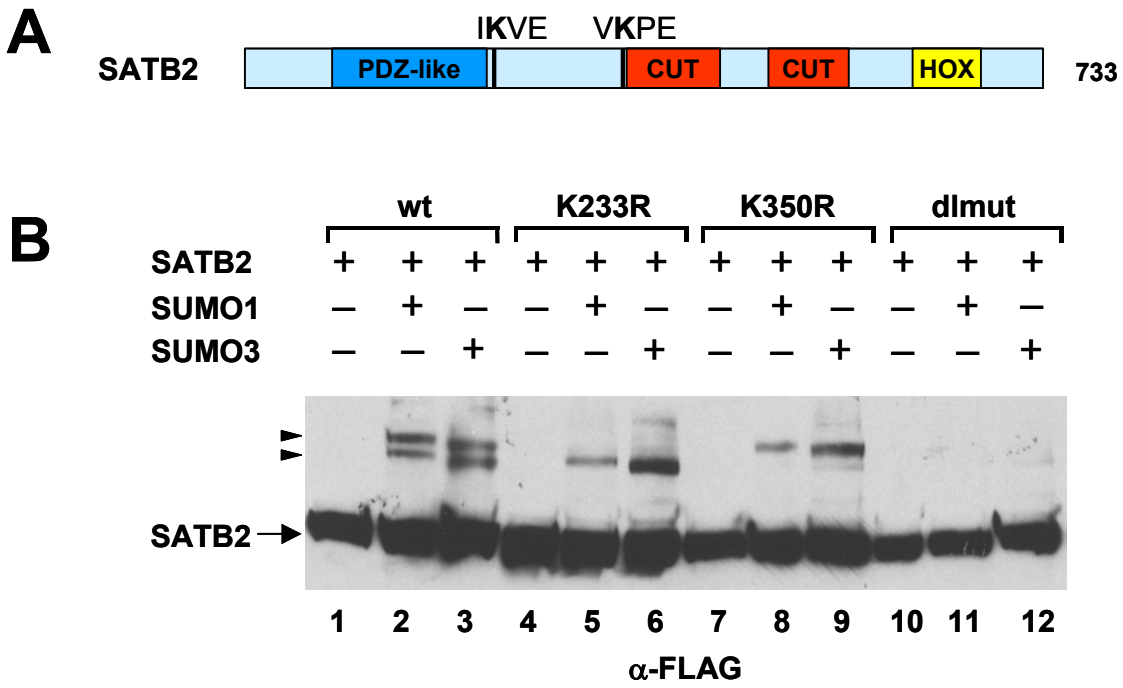


Figure 21. SATB2 is SUMO conjugated at two SUMO consensus sites. ***(A)*** Schematic linear representation of SATB2. The two CUT motifs, homeodomain (HOX), and putative SUMO acceptor sites at positions 233 (I**K**VE) and 350 (V**K**PE) of SATB2 that match the consensus SUMO acceptor site ΨKXE (Rodriguez et al., 2001) are shown. ***(B)*** Identification of K233 and K350 of SATB2 as the acceptor sites for sumoylation. Total protein extracts from cells transfected with expression plasmids of FLAG-SATB2 (lanes 1-3) or various FLAG-tagged SATB2 mutants: K233R (lanes 4-6), K350R (lanes 7-9) or the K233R/K350R double mutant (dlmut, lanes 10-12), alone or together with SUMO1 or SUMO3 expression plasmids, were analyzed by an immunoblot with an anti-FLAG antibody. Mutation of either sumoylation site interfered with the appearance of one of the two modified forms of SATB2, whereas the double mutation abrogated SUMO modification completely. The differences in the migration of the two modified forms of SATB2 are most likely due to the different branching position of the sumoylated polypeptides (Hoege et al., 2002).

and FLAG-SUMO-1 or FLAG-SUMO3. Western blot analysis with an anti-myc antibody did not detect any additional slower migrating forms for SATB1 (Fig. 22B, lane 2 and 3), whereas SATB2 was sumoylated with both SUMO1 and SUMO3 (Fig. 22B; lane 5 and 6).

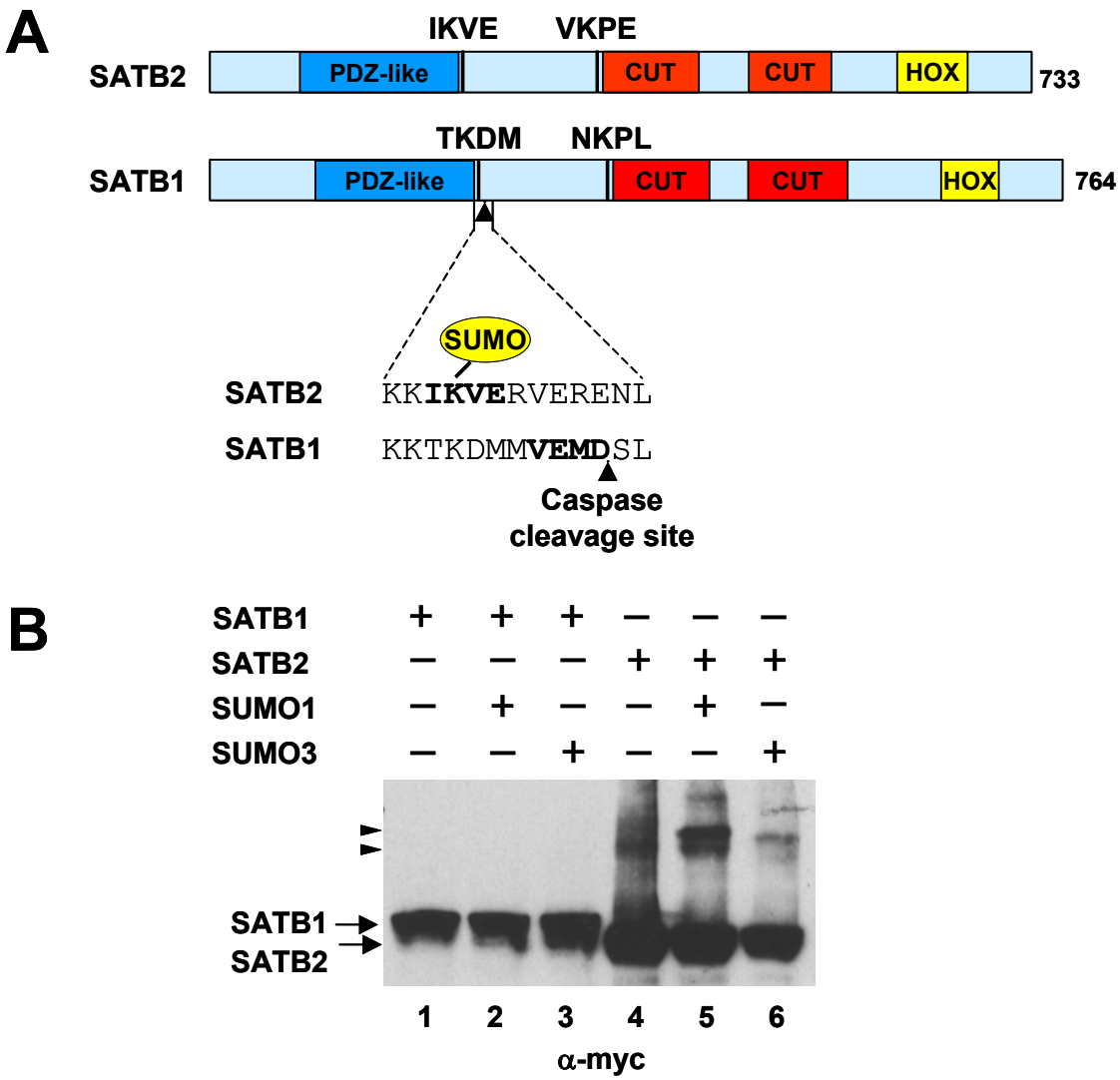


Figure 22. SATB1 is not conjugated to SUMO. ***(A)*** Linear diagrams and sequence alignment showing that the SUMO conjugation consensus sequences of SATB2 are missing from the homologous regions of SATB1. ***(B)*** SATB2, but not SATB1, can be modified with SUMO1 and SUMO3. Myc-tagged SATB1 (lanes 1-3) and myc-tagged SATB2 (lanes 4-6) were detected in total cell lysates of transfected cells by an anti-myc immunoblot analysis. In cells transfected with SATB2-myc and SUMO1 or SUMO3 expression plasmids, SUMO-modified forms of SATB2, but not of SATB1, can be detected (marked by arrowheads, lanes 5 and 6).

3.1.3 SATB2 is not proteolytically cleaved by caspase 6

The transcriptional activity of SATB1 is tightly regulated. One known mechanism involves proteolytic cleavage by caspase 6, that separates the DNA-binding domain from the dimerization domain, during thymocyte and T-

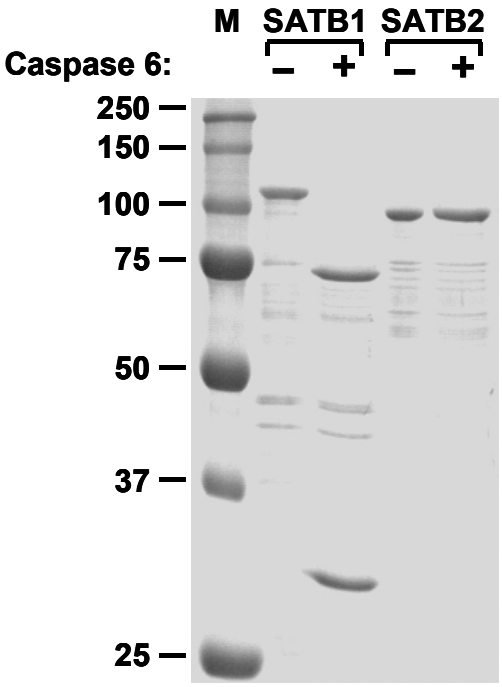


Figure 23. SATB2 is not proteolytically cleaved by caspase 6. Purified recombinant SATB1 and SATB2 were incubated with or without recombinant caspase 6 for 1 h. at 37 °C in caspase 6 cleavage buffer. The proteins were then resolved on a 10% polyacrylamide gel and stained with Commassie. M - molecular weight markers. The experiment was done with the assistance of Magdalena Strzelecka.

cell apoptosis. The resulting SATB1 monomers lose their BUR-binding activity, despite containing both DNA-binding domains, and rapidly dissociate from chromatin *in vivo* (Galande et al., 2001). Sequence alignment using the Clustal algorithm, revealed that the caspase 6 cleavage site is not conserved between SATB1 and SATB2, suggesting that SATB2 is not proteolysed by this enzyme. To confirm this observation experimentally we performed an *in vitro* cleavage assay, using purified recombinant SATB1-His, SATB2-His and caspase 6. Following a 1 h. incubation at 37 °C the proteins were resolved by SDS-PAGE and Commassie staining was performed. The results shown in Fig. 23 revealed that SATB2 indeed is not proteolytically cleaved in contrast to SATB1, which was 100% digested to two fragments of 75 kD and 35 kD.

3.2. PIAS1 is the E3 ligase for SATB2

3.2.1. SATB2 interacts specifically with PIAS1

PIAS proteins have been shown to act as SUMO E3 ligases that augment the sumoylation of proteins and have been proposed to confer the substrate specificity upon the E2 conjugating enzyme (Johnson et al., 2001; Sachdev et al., 2001; Kotaja et al., 2002; see Introduction – 4.3). To determine whether SATB2 can interact specifically with one of the members of the PIAS family of SUMO E3 ligases, we transfected FLAG-tagged gene constructs of PIAS1, PIAS3, PIASxα, PIASxβ and PIASy into 293T cells, alone or together with a myc-tagged SATB2 expression plasmid. Co-immunoprecipitation with an anti-FLAG antibody and subsequent immunoblot analysis with an anti-myc antibody indicated that PIAS1 was efficiently co-immunoprecipitated (Fig. 24A, lane 7). A weak association was also detected with PIASy (Fig. 24C; *middle panel*) but not with the other PIAS proteins. The association between SATB2 and PIAS1 was also detected by the reciprocal co-immunoprecipitation with an anti-myc antibody and immunoblot analysis with an anti-FLAG antibody (Fig. 24B, lane 7). Similar levels of expression of the PIAS proteins and SATB2 were confirmed by a parallel immunoblot analysis of total cell lysates (Fig. 24A and B, *lower panels*). Thus, SATB2 and PIAS1 can specifically interact in cells expressing both proteins.

3.2.2. PIAS1 stimulates SUMO conjugation to SATB2 in vivo

To examine whether PIAS1 can act as a specific SUMO E3 ligase for SATB2, we co-transfected the expression plasmids for wild-type SATB2 or the SATB2-K233RK350R double mutant (dlmut) together with an expression plasmid for each of the five PIAS family members. SUMO-modified forms of SATB2 were detected by an anti-myc immunoblot analysis specifically in extracts from cells expressing PIAS1 (Fig. 25, lane 2). As expected, the modified forms of SATB2 were not detected in extracts from cells expressing the SATB2 double mutant (Fig. 25, lane 8). Similar levels of expression of the FLAG-tagged PIAS proteins were confirmed by a parallel immunoblot analysis with an anti-FLAG antibody (Fig. 25, *lower panel*).

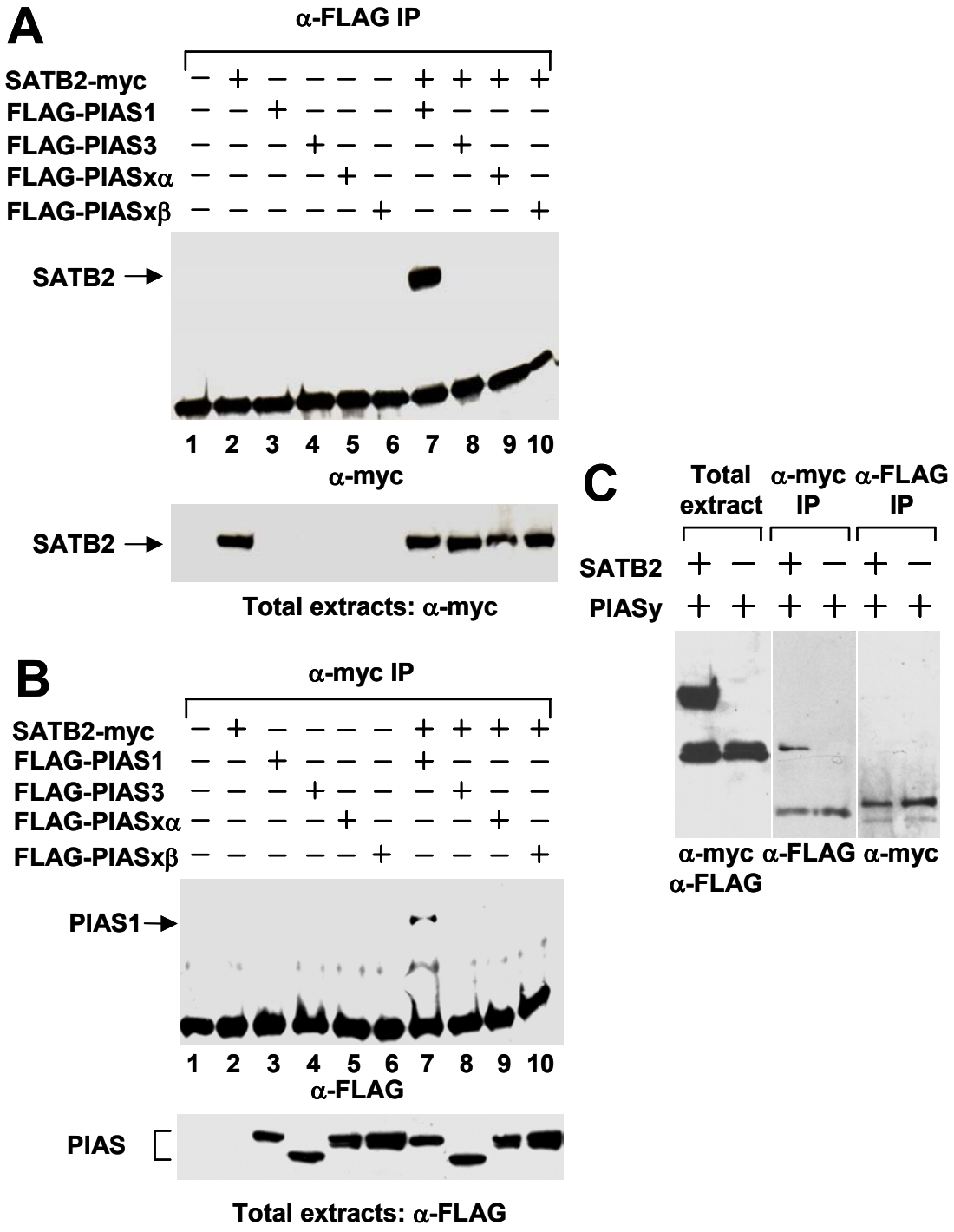


Figure 24. PIAS1 interacts with SATB2 *in vivo*. Myc-tagged SATB2 and FLAG-tagged forms of PIAS1, PIAS3, PIASxα, PIASxβ and PIASy were transiently expressed in 293T cells. Equivalent amounts of total cellular protein were immunoprecipitated with an anti-FLAG antibody (***A*** - lanes 1-10, *top panel*; ***C*** - *right panel*) or an anti-myc antibody (***B*** - lanes 1-10, *top panel*; ***C*** - *middle panel*) and the coimmunoprecipitated PIAS proteins or SATB2 were detected by immunoblot analysis with an anti-myc antibody (***A*** - lanes 1-10, *top panel*; ***C*** - *right panel*) or an anti-FLAG antibody (***B*** - lanes 1-10, *top panel*; ***C*** - *middle panel*), respectively. Similar expression of SATB2 and the PIAS proteins was confirmed by immunoblot analysis of total cell extracts (***A*** and ***B*** - *bottom panels*; ***C*** - *left panel*).

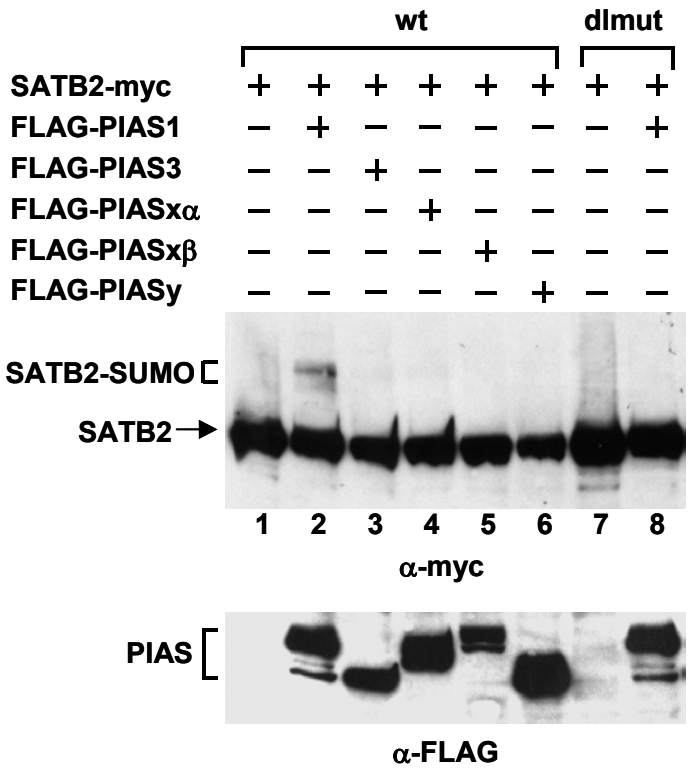


Figure 25. PIAS1 stimulates SUMO conjugation to SATB2 *in vivo*. Myc-tagged SATB2 or the SATB2-K233R/K350R double mutant (dlmut) were expressed alone, or together with FLAG-tagged PIAS proteins in 293T cells. SATB2 and SUMO-modified SATB2 were detected in total cell lysates by immunoblot analysis with an anti-myc antibody. SUMO-modified SATB2 can be detected only in cells expressing PIAS1 (lane 2).

3.2.3. PIAS1 is an E3 ligase for SATB2

The stimulatory effect of PIAS1 on the sumoylation of SATB2 raises the question whether PIAS1 is directly involved in this enzymatic process. To address this issue, an *in vitro* reconstituted system, consisting of purified proteins, was utilized (Pichler et al., 2002). Incubation of recombinant SATB2 with SUMO1, the E1 activating enzyme Aos1/Uba2, the E2 conjugating enzyme Ubc9, and ATP did not result in significant SUMO conjugation of SATB2 (Fig. 26A, lane 2). However, addition of bacterially expressed GST-PIAS1 led to efficient multiple conjugation of SUMO1 to SATB2, in a dose dependent manner (Fig. 26A, lanes 3-5). Taken together these data show that PIAS1 is indeed an E3 ligase for SATB2 (Fig. 26B).

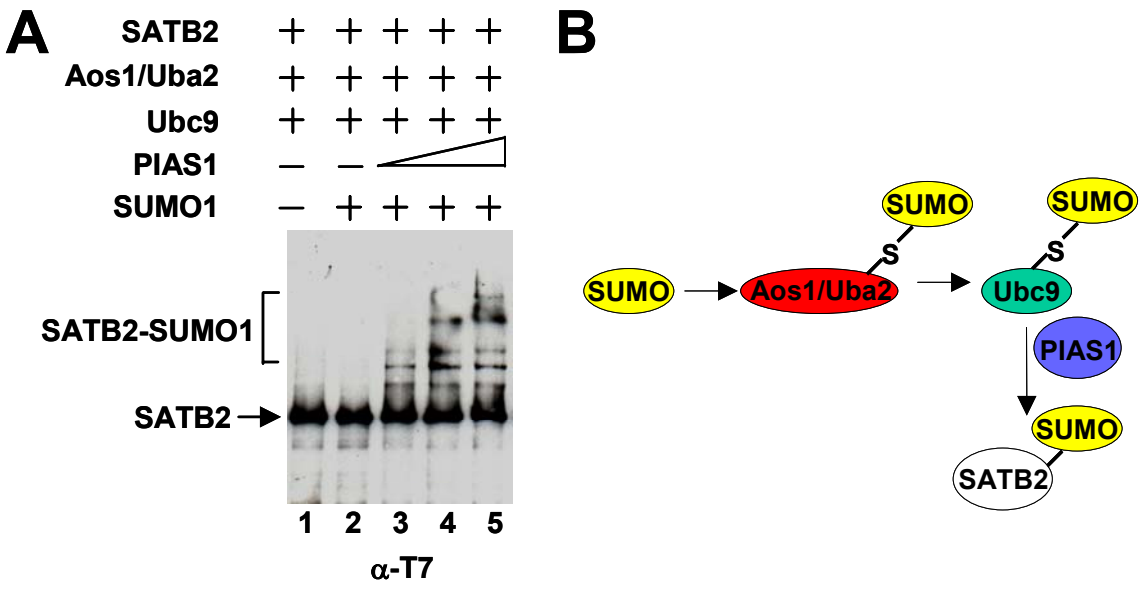


Figure 26. PIAS1 stimulates SUMO conjugation to SATB2 *in vitro*. *(A)* Purified His- and T7-tagged SATB2 was subjected to *in vitro* SUMO1 modification in the presence of GST-PIAS1: 300 ng purified His- and T7-double-tagged SATB2 protein was incubated for 30 min with 250 ng purified E1 enzyme (Aos1/Uba2 heterodimer), 250 ng E2 enzyme (Ubc9) and 1 μg SUMO1 alone or with increasing amounts GST-PIAS1. SATB2 proteins were detected by anti-T7 immunoblot analysis. ***(B)*** Schematic representation of the conjugation pathway leading to SUMO modification of SATB2. PIAS1 acts as an E3 ligase for the sumoylation of SATB2.

3.3. SUMO conjugation antagonizes SATB2-mediated gene activation

3.3.1. Mutations of the sumoylation sites of SATB2 augment its activation potential

SUMO-modification has been found to antagonize the activation potential of several transcription factors (Müller et al., 2000; Sachdev et al., 2001; Ross et al., 2002; Chun et al., 2003). To examine the effects of sumoylation on the transcriptional activation by SATB2, we transfected J558L cells with expression plasmids encoding wild-type or mutant forms of FLAG-tagged SATB2, together with a fos-luciferase reporter construct containing multimerized SATB2-binding sites (Fig. 27A; see also Fig. 16A). Wild-type SATB2 augmented reporter gene expression by a factor of up to ten, whereas mutations of both sumoylation sites of SATB2 (dlmut) further increased the

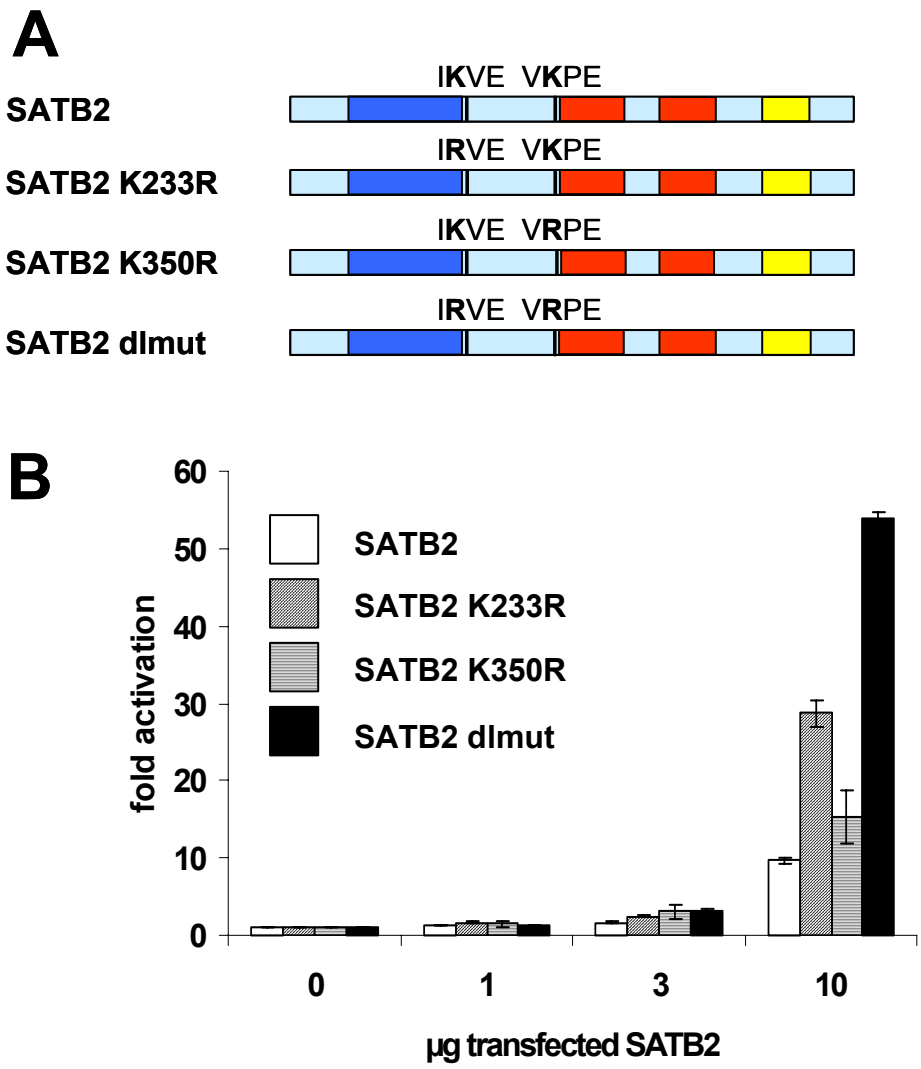


Figure 27. Sumoylation antagonizes SATB2-mediated transcriptional activation. ***(A)*** Schematic representation of wild-type SATB2 and mutant SATB2 proteins containing mutations in the sumoylation sites, individually (K233R, K350R) or in combination (K233R/K350R - dlmut). ***(B)*** Mutation of both sites of SATB2 augments transcriptional activation. J558L cells were transiently transfected by electroporation with 5 μg MAR-luciferase reporter construct containing multimerized SATB2-binding sites, alone or together with increasing amounts (1, 3 and 10 μg) of expression plasmids encoding wild-type SATB2 or mutated SATB2 proteins (K233R, K350R or the K233R/K350R double mutant - dlmut). For the normalization of luciferase activities, the activity of a co-transfected β-galactosidase expression plasmid (1 μg) was determined for each sample. The normalized levels of luciferase activity are expressed as fold-activation relative to the level of luciferase activity from cells transfected with the reporter construct alone.

level of reporter gene expression by a factor of approximately five (Fig. 27B). Mutation of the sumoylation site at position 233 alone resulted in a three-fold increase of reporter gene expression relative to the stimulation of expression by wild-type SATB2.

3.3.2. Covalent attachment of SUMO1 and SUMO3 antagonizes SATB2-dependent transcription

Our results indicate that SUMO1 modification represses the transcriptional activation mediated by SATB2. To examine whether the effects of the mutations of the lysines at positions 233 and 350 are due to a lack of SUMO modification, rather than a lack of other modifications such as acetylation or methylation (which also occur at lysine residues), we generated gene constructs in which SUMO1 or SUMO3 are fused to the amino-terminus of SATB2-dlmut (Fig. 28A). The C-terminal 5 amino acids of full-length SUMO1 and SUMO3, including the double glycine motif found at the C-terminus of mature SUMO1, were not included in these constructs in order to prevent cleavage of the fusion proteins by C-terminal SUMO hydrolases (see Fig. 6 and Introduction – 4.2). Transfection experiments in J558L cells showed that both SUMO1-SATB2-dlmut and SUMO3-SATB2-dlmut activate reporter gene expression at levels five-to six-fold lower than those observed with SATB2-dlmut (Fig. 28B). Immunoblot analysis with an anti-FLAG antibody indicated that the various SATB2 proteins are expressed at similar levels (data not shown). Taken together, these results indicate that the sumoylation of SATB2 antagonizes its transcriptional activation potential.

3.3.3. Wild-type and SATB2-dlmut, but not the SUMO-SATB2 fusions, augment immunoglobulin Cα gene transcription

To examine the activation potential of wild-type SATB2 and the mutant SATB2 proteins in a more physiological context, we generated J558L plasmocytoma

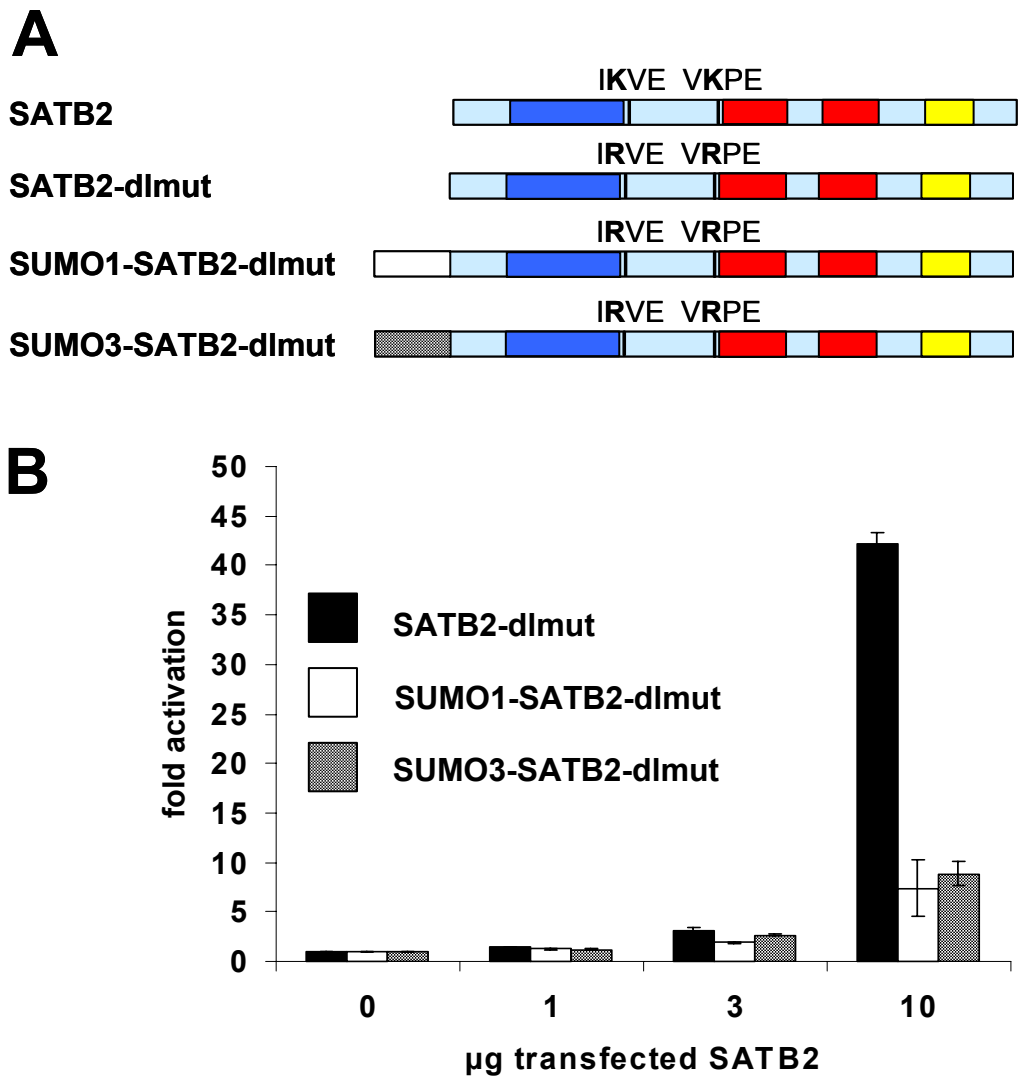


Figure 28. Covalent attachment of SUMO1 and SUMO3 antagonizes SATB2-dependent transcription. ***(A)*** Schematic linear representation of SATB2 and N-terminal SUMO1- and SUMO3-SATB2 fusions. ***(B)*** N-terminal SUMO1- and SUMO3-SATB2 fusions antagonize SATB2-mediated transcriptional activation. J558L cells were transfected with 5 µg of a MAR luciferase reporter construct together with expression constructs encoding β-galactosidase (1 µg - for normalization) and increasing amounts of SATB2 double mutant, SUMO1-SATB2-dImut or SUMO3-SATB2-dImut (1, 3, 10 µg), as indicated.

cell lines, stably transfected with FLAG-tagged SATB2 constructs, and examined the expression of the endogenous immunoglobulin α heavy chain gene (Cα). For this experiment, we chose clones that express the exogenous SATB2 proteins at similar, moderate levels (Fig. 29). RNA blot analysis

indicated that SATB2 and SATB2-dlmut augmented Cα expression, relative to the expression in the parental J558L cells, by a factor of two and three, respectively (Fig. 29, lanes 1-3 and lane 8). In contrast, no activation of Cα expression was observed with the covalent SUMO-SATB2-dlmut fusion proteins (Fig. 29, lanes 4-7).

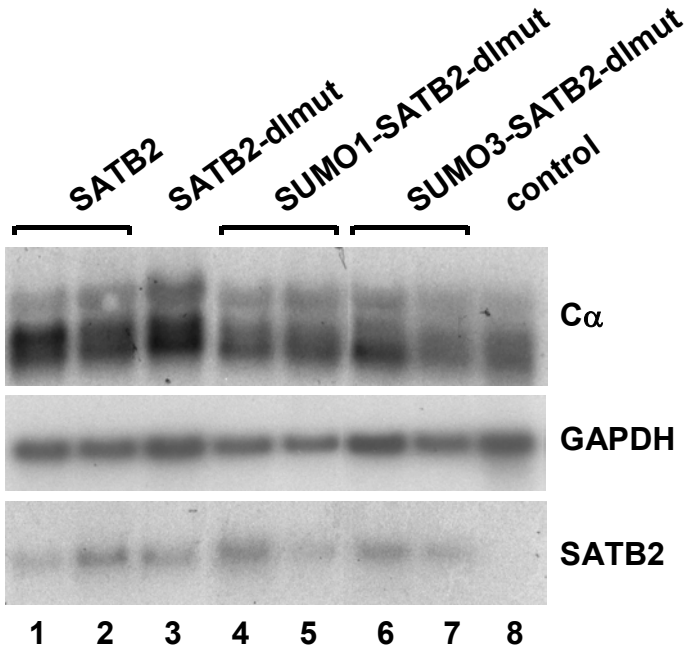


Figure 29. Wild-type and SATB2-dlmut augment immunoglobulin Cα gene transcription. RNA blot analysis of J558L clones, stably transfected with various SATB2 constructs, as indicated. 5 μg total RNA was hybridized with probes that detect the endogenous Cα (*top panel*) and GAPDH (*middle panel*) transcripts, as well as the transfected SATB2 mRNA (*bottom panel*). The intensities of the bands were quantified using the Image Quant 5.1. Software on a Storm™ Scanner.

3.4. Mutations of the sumoylation sites of SATB2 augment the association with MAR sequences of the endogenous immunoglobulin heavy chain locus.

One explanation for the lower activation potential of the sumoylation-competent SATB2 protein, relative to the SUMO-deficient SATB2-double mutant, could be a sumoylation-dependent decrease in the association of SATB2 with chromatin. To address this issue and more specifically - the binding of the different SATB2 mutants to the immunoglobulin enhancer MAR regions, we established stable cell lines expressing FLAG-tagged SATB2 or

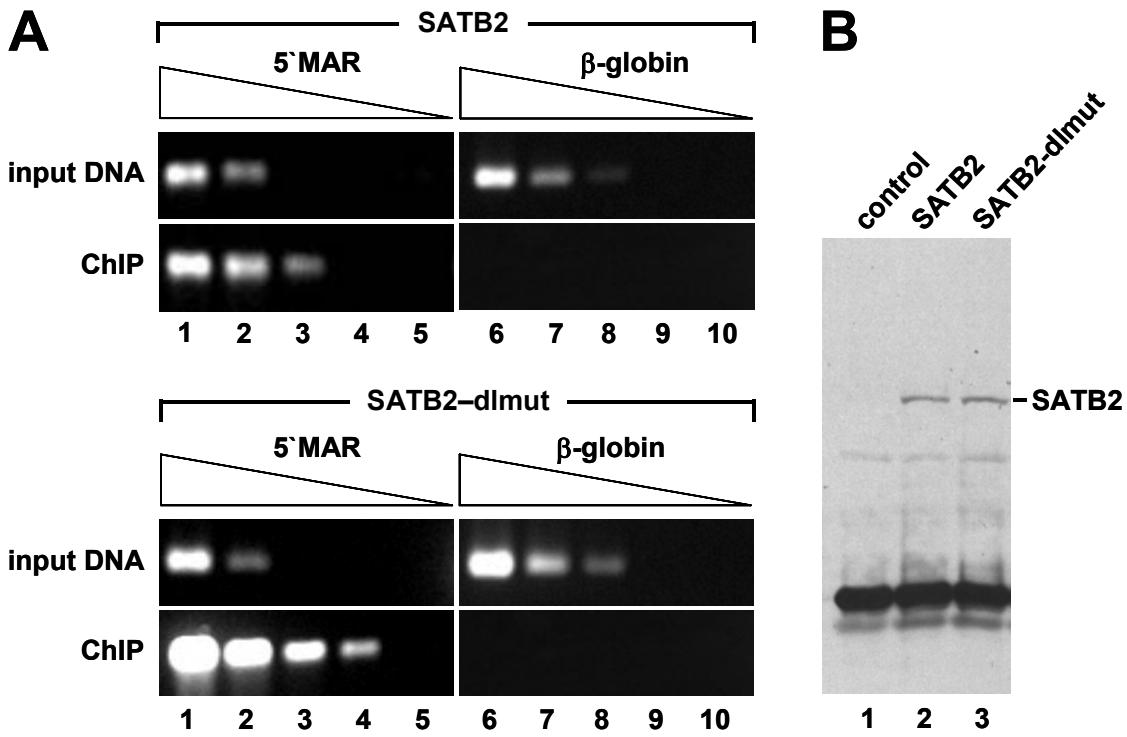


Figure 30. The SATB2 double mutant has a stronger association with the immunoglobulin intronic enhancer MARs. ***(A)*** Chromatin immunoprecipitation (ChIP) from stable cell lines expressing equal amounts of FLAG-SATB2 (*top panel*) or SATB2 double mutant (*bottom panel*) using an anti-FLAG antibody was performed. 20% of the immunoprecipitated material was used to purify the DNA, which was subsequently analyzed by PCR, using primers located in the 5` MAR region of the immunoglobulin μ enhancer *(right)* or in the β-globin locus (*left*). Template DNA was used in a linear five-fold dilution (starting from 5 ng) to allow for a semi-quantitative determination of the immunoprecipitated amount. ***(B)*** 80% of the immunoprecipitated material was used for an anti-FLAG Western blot analysis to detect SATB2 proteins that have been immunoprecipitated under ChIP conditions, to control for equal fractionation and recovery of SATB2 and SATB2-dlmut.

SATB2 double mutant and performed chromatin immunoprecipitation from clones expressing equal amounts of SATB2, using an anti-FLAG antibody. The immunoprecipitated DNA was amplified using specific primers for the 5' MAR region of the intronic enhancer and a region of the β-globin locus, as a specificity control. The results revealed that the SATB2 double mutant is associated with the 5' MAR of the intragenic μ enhancer region at least five times more efficiently than the wild-type SATB2 protein (Fig. 30A). This effect was not due to a more efficient immunoprecipitation of the SATB2-dlmut

relative to the wild-type protein, as confirmed by an anti-FLAG Western blot of the immunoprecipitated proteins (Fig. 30B).

3.5. Covalent fusion of SUMO1 and SUMO3 to SATB2 does not affect its DNA binding *in vitro*

Our data revealed that SUMO conjugation, as well as covalent attachment of SUMO by gene fusion, antagonize the transcriptional activation mediated by SATB2 and this closely correlates with its association with chromatin. The next question we wanted to answer was whether SUMO conjugation interferes with the DNA binding of SATB2. To address this issue, equal amounts of recombinant His-SATB2, His-SUMO1-SATB2-dlmut or His-SUMO3-SATB2-dlmut were tested for their ability to bind a wild-type MAR consensus probe in

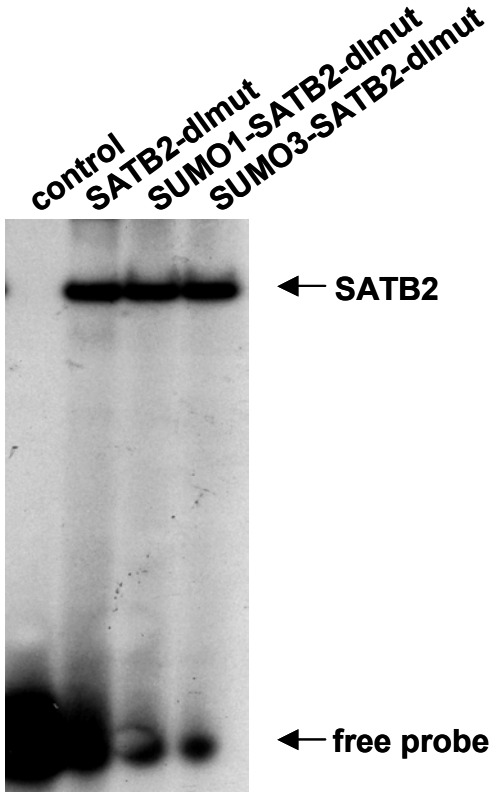


Figure 31. Covalent fusion of SUMO to SATB2 does not affect its DNA binding *in vitro*. 100 ng of recombinant His-SATB2-dlmut, His-SUMO1-SATB2-dlmut and His-SUMO3-SATB2-dlmut were tested for their ability to bind to a ^{32}P-labeled wild-type MAR consensus sequence by electrophoretic mobility shift assays. The binding reactions were performed as described in Materials and Methods and separated on a 6% polyacrylamide gel.

electromobility shift assay. The results shown on Fig. 31 revealed no significant difference in the DNA binding of the SUMO fusions in comparison with SATB2.

3.6. SUMO modification does not inhibit the dimerization of SATB2

Previous studies have shown that SATB1 associates with chromatin as a dimer or multimer and that the PDZ-like domain is responsible for the dimerization of the protein. Furthermore, it was shown that during the negative selection of T-cells in the thymus SATB1 is proteolytically cleaved by caspase 6, which results in SATB1 monomers that dissociate from chromatin *in vivo*. It thus became clear that the PDZ-like dimerization domain, together with the MAR-binding domain, is absolutely necessary for the chromatin association of SATB1. Since the PDZ-like domain is conserved between SATB1 and SATB2, we decided to test whether SUMO conjugation might inhibit SATB2 dimerization and thus downregulate the transcriptional activity of the protein by decreasing its DNA binding *in vivo*. Toward this end, 293T cells were transfected with myc- and FLAG-epitope tagged SATB2, SATB2 double mutant, SUMO1-SATB2-dlmut and SUMO3-SATB2-dlmut, in different combinations, as indicated (Fig. 32). Co-immunoprecipitation was performed using an anti-FLAG antibody and subsequently the co-immunoprecipitated proteins were detected by an anti-myc Western blot.

As seen on Fig. 32, the covalent conjugation of SUMO1 or SUMO3 to SATB2 had no inhibitory effect on the dimerization of the protein (lanes 8, 10). Therefore, the inhibition of the SATB2-mediated transcriptional activation by the covalent fusion of SUMO does not appear to be dependent on the suppression of dimerization. Thus, the SUMO conjugation uses a different mechanism to antagonize SATB2 mediated transactivation, which is not related to the dimerization of the protein.

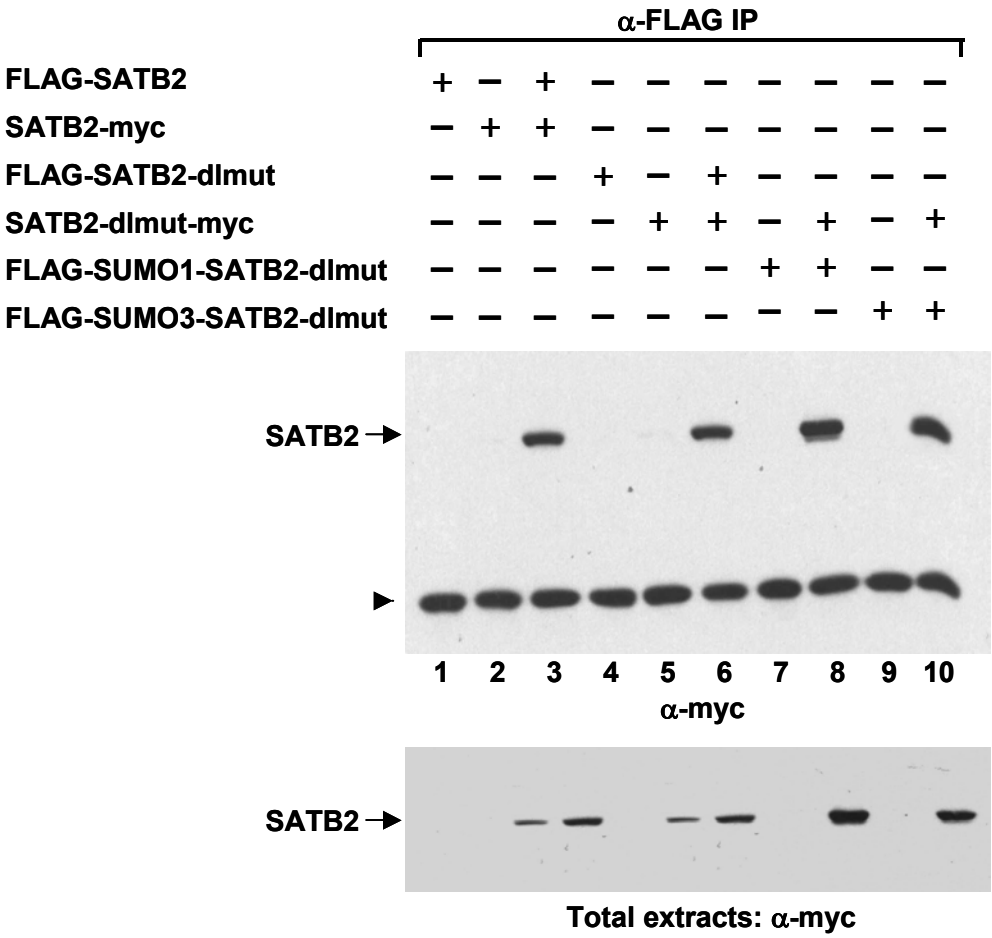


Figure 32. Covalent fusion of SUMO1 and SUMO3 to SATB2 does not affect the dimerization of the protein. Myc epitope-tagged SATB2 or SATB2 double mutant (dlmut) were co-expressed alone or together with FLAG epitope-tagged SATB2, SATB2-dlmut, SUMO1-SATB2-dlmut or SUMO3-SATB2-dlmut in 293T cells. Equal amounts of each extract were used for anti-FLAG immunoprecipitation and the co-immunoprecipitated proteins were detected by an anti-myc Western blot. The arrowhead (*top panel*) indicates the antibody heavy chain.

3.7. SUMO-modification alters the subnuclear localization of SATB2

We determined that SUMO conjugation of SATB2 reduces the binding to chromatin *in vivo*, but this is not due to a loss of the ability to form dimers. Another possible mechanism to influence the chromatin association is by changing the subnuclear localization of the protein. Indeed, conjugation of SUMO to multiple target proteins has been shown to influence their subcellular localization (Sachdev et al., 2001; Kirsh et al., 2002; Ross et al., 2002). We therefore wanted to examine the effects of sumoylation on the

subnuclear localization of SATB2 in stable cell lines of J558L cells, expressing similar amounts of FLAG-SATB2, FLAG-SATB2 double mutant, FLAG-SUMO1-SATB2-dlmut or FLAG-SUMO3-SATB2-dlmut. The stable cell lines were established to avoid the side effects of overexpression, which can influence the subcellular distribution of the protein.

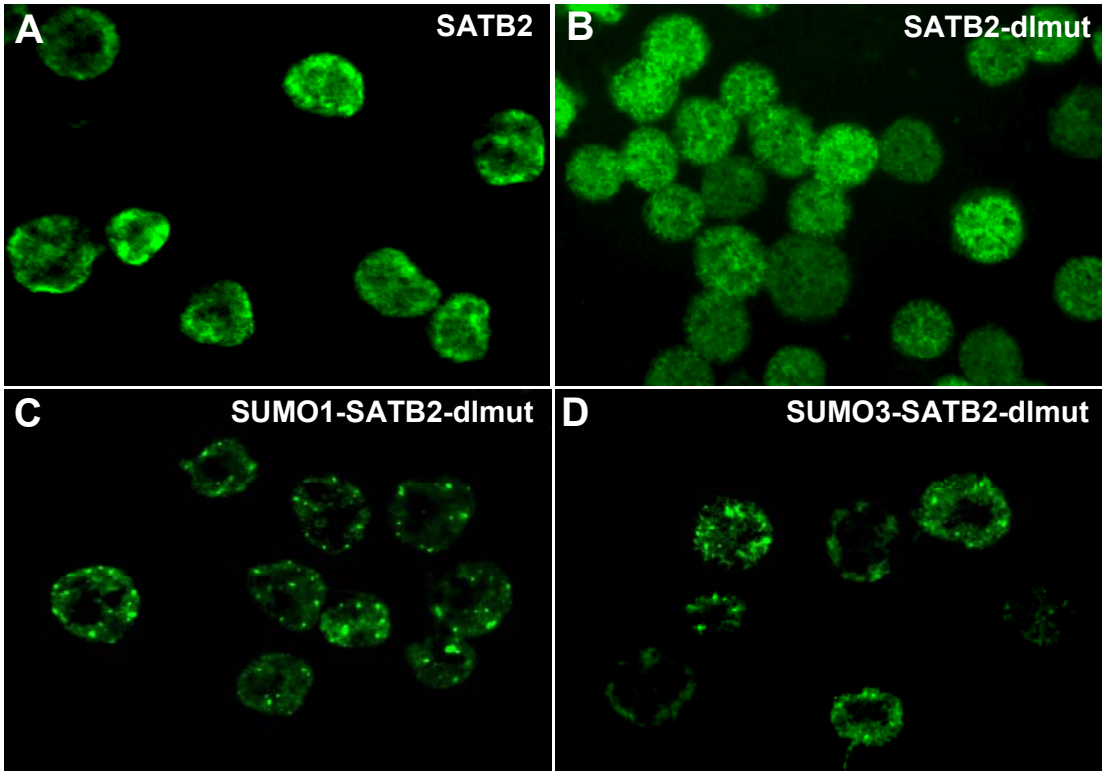


Figure 33. SUMO-modification alters the subnuclear localization of SATB2. Stable cell lines in J558L cells, expressing equal amounts of FLAG-SATB2, FLAG-SATB2 double mutant or SUMO1-SATB2-dlmut and SUMO3-SATB2-dlmut were analyzed by indirect immunofluorescence to study the intracellular distribution of the indicated proteins. The detection was performed with an anti-FLAG monoclonal antibody. SATB2 localizes to the nuclear periphery ***(A)***, while the SATB2 double mutant is diffusely distributed throughout the nucleus ***(B)***. Covalent attachment of SUMO1 relocalizes SATB2 into nuclear bodies (speckles) ***(C)***. Covalent attachment of SUMO3 relocalizes SATB2 into nuclear bodies, distributed mainly in the nuclear periphery ***(D)***. The experiment was done with the assistance of Julia Dambacher.

Indirect immunofluorescence analysis with an anti-FLAG antibody indicated that wild-type SATB2 is localized predominantly in the nuclear periphery (Fig. 33A), whereas the SATB2 double mutant was localized more diffusely

throughout the nucleus (Fig. 33B). Notably, the covalent fusion of SUMO1 resulted in an accumulation of SATB2 in distinct nuclear speckles (Fig. 33C), in contrast to the covalent fusion with SUMO3, which resembled the staining of wild-type SATB2 at the nuclear periphery (Fig. 33D).

3.8. SUMO conjugation is stimulated by stress conditions

Having demonstrated that SUMO conjugation of SATB2 antagonizes SATB2-mediated transcriptional activation we were further interested to see how this process might be regulated. It was previously shown that SUMO conjugation of proteins is cell cycle-dependent with a peak of sumoylated species in S-phase (Chakrabarti et al., 2000). This is most likely due to changes in the level of Aos1 and Ubc9 that was reported to increase as cells progress trough S-phase and to substantially decrease in G2 phase (Azuma et al., 2001).

Experiments involving a double thymidine or aphidicolin block of cell-cycle progression, as well as sorting of cells in S-phase using the elutriation method, revealed only a modest increase in the sumoylation of SATB2 during S-phase (up to two fold; data not shown). Since the subcellular distribution of SATB2 resembles the localization of the SUMO3 covalent fusion and it is known that SUMO conjugation can be promoted by stress-inducing stimuli, such as acute temperature shift and high osmolarity (Saitoh and Hinchey, 2000), we decided to induce stress by culturing the cells for 3 hours at 42 °C, increasing the salt concentration in the cell culture medium with NaCl at a final concentration of 0.5 M, or dehydrating the cells via treatment with 7% ethanol. We observed a significant augmentation of SUMO conjugation to SATB2 when the cells were shifted to 42 °C or cultured in medium with high osmolarity. We further examined whether this process was reversible, by shifting the heat shocked cells back to 37 °C for 5 hours. We found that SATB2 sumoylation was indeed dynamic, since the SUMO-conjugated species decreased after reversal of the heat shock (Fig. 34).

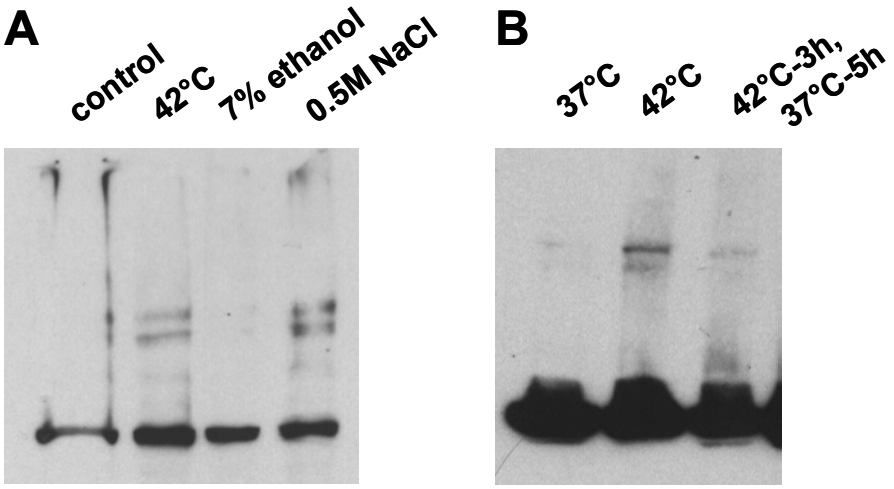


Figure 34. SUMO modification of SATB2 is augmented by stress-inducing stimuli. ***(A)*** 293T cells were transiently transfected with SATB2-myc. 36 h. post transfection the cells were transferred to a 42 °C incubator or subjected to treatment with 0.5 M NaCl or 7% ethanol. ***(B)*** Cells that had been heat shocked by incubation at 42 °C were transferred back to 37 °C and 5 hours later harvested in CoIP buffer. Using equal amounts of protein extract from each condition in ***A*** and ***B***, SATB2 and the SUMO-conjugated SATB2 species were detected by an anti-myc Western blot.

III. Discussion

The nuclear matrix participates in diverse processes such as DNA replication, transcription, recombination, RNA processing and transport, as well as signal transduction and apoptotic events. One fundamental issue concerning the function of the nuclear matrix is the precise mechanism through which it regulates the transcriptional activity of the associated chromatin. This thesis was aimed at investigating the mechanism by which matrix attachments regions (MARs) and in particular the MARs in the intronic enhancer of the immunoglobulin μ heavy chain modulate transcription.

1. Synthetic multimerized MAR-binding sites mimic the natural MARs in augmenting transcription of the IgH gene

It was already shown in transgenic mice that the expression of a rearranged μ gene is dependent on the presence of both the core μ enhancer and the flanking MARs (Forrester et al., 1994). The natural immunoglobulin MARs, however, are fairly long sequences and contain binding sites for a number of general transcription factors. Thus, it was unclear whether a specific portion of the natural MARs is essential for μ gene transcription. By substituting these sites with short, multimerized MAR consensus sequences (Dickinson et al., 1992) we could prove that indeed the AT-rich sequences in the 5' and 3' MAR regions are the ones collaborating with the core μ enhancer in augmenting the transcription of the IgH gene (Fig. 8). These experiments raised the question how the MARs modulate transcription. One basic hypothesis would be that this is accomplished through the action of transcription factors, which are component of the nuclear matrix and specifically interact with the IgH MAR sequences. However, no MAR-binding proteins that are expressed in pre-B cells (where the action of MARs is involved in immunoglobulin expression)

had been previously discovered. Therefore, our initial efforts were focused on the identification of MAR-binding proteins expressed in pre-B cells.

2. SATB2 is a novel MAR-binding protein

We identified SATB2 as a novel, cell-type specific MAR-binding protein that is expressed abundantly in pre-B and B cells, as well as in kidney and brain (Fig. 11). Furthermore, SATB2 binds to MAR consensus sequences (Dickinson et al., 1992) *in vitro* and is a component of the nuclear matrix (Fig. 12 and Fig. 13). Moreover, in pre-B cells, stably transfected with a tagged SATB2 gene, the SATB2 protein was shown to bind MAR sequences flanking the enhancer of the endogenous immunoglobulin gene (Fig. 14).

In contrast to SATB2, the other lymphoid specific MAR-binding proteins, SATB1 and Bright, which can also interact with multiple sites in the μ MARs *in vitro*, are not expressed in early B cells, where the μ enhancer and MARs collaborate to augment the Vh promoter activity. SATB1 is expressed predominantly in T lymphocytes and Bright – in activated B cells and late stages of the B cell lineage (Dickinson et al., 1992; Herrscher et al., 1995).

Using the minimal AT-rich MAR consensus sequence, which is sufficient for nuclear matrix association (Dickinson et al., 1992), we demonstrated that SATB2, like SATB1 and Bright, binds *in vitro* to these MAR sites. Furthermore, we could also show by chromatin immunoprecipitation that SATB2 is bound the immunoglobulin μ enhancer *in vivo*, which has not been shown for either SATB1 or Bright.

3. SATB2 augments immunoglobulin gene expression

The binding of SATB2 to μ MAR sequences *in vivo* was found to correlate with an increase in the expression of a transfected, rearranged wild-type μ gene, but not of a μΔMAR gene, lacking the MAR sequences (Fig. 8 and Fig. 17). In addition, the expression of SATB2 in stably transfected plasmocytoma cells results in a modest but reproducible increase in the expression of the

endogenous immunoglobulin gene. Finally, SATB2 was found to augment the expression of a reporter gene construct containing multimerized SATB2 binding sites, indicating that SATB2 acts as a *bona fide* transcriptional activator (Fig. 15).

3.1. Possible mechanisms of SATB2-mediated transcriptional activation – comparison with other MAR-binding proteins

In contrast to the stimulation of the transcription of immunoglobulin gene constructs by SATB2, the closely related thymocyte-specific MAR-binding protein SATB1 has been shown to repress target genes, such as IL-2R. In SATB1-knockout thymocytes, which arrest at the $CD4^+CD8^+$ stage of differentiation, IL-2Rα expression is upregulated and this effect correlates with changes in the acetylation pattern in histone H3 over a large chromatin domain (Yasui et al., 2002). Consistent with the repressive effect of SATB1, this protein has been found to associate with the histone deacetylase (HDAC) of the NURD complex and to recruit the chromatin remodeling complexes ACF and ISWI *in vitro* (Yasui et al., 2002). Another histone deacetylase, HDAC1, could also be cross-linked to a MAR region in the first intron of the IL-2R gene in wild-type, but not in SATB1-deficient thymocytes. Recently, SATB1 has also been implicated in the positive regulation of genes, based on the finding that the c-myc locus, which contains a SATB1-binding region upstream of the transcription start site, is not properly up-regulated in PMA-stimulated thymocytes from SATB1-deficient mice (Cai et al., 2003). Furthermore, Cai et al., (2003) identified multiple SATB1-associated genes that are actively transcribed in the presence of SATB1 and are downregulated after ablation of the protein, supporting the notion that SATB1 can in some cases act as an activator. The fact that SATB1 could act both as a transcriptional activator and as a repressor raises the question as to how it chooses its function with respect to any given gene. One possibility might be that at different sites SATB1 could interact specifically with other factors (activators or repressors) binding to neighbouring sequences. Another

explanation may be that at different sites SATB1 is differently modified which leads to the recruitment of distinct transcriptional regulators.

Another MAR-binding protein, Bright, which is expressed predominantly in activated B cells and antibody-secreting plasma cells, has also been shown to augment the expression of the immunoglobulin μ gene. Bright, which shares no sequence similarity with SATB1 and SATB2, binds to MAR sequences through a distinct region, termed the ARID domain (Kortschak et al., 2000; Webb, 2001). In addition, the tetramerization of Bright is mediated through a REKLES domain (Schandala et al., 2002). Some insights into the potential mechanism by which Bright stimulates μ gene expression came from a search for interacting partners of Bright, which identified a B-cell specific isoform of the speckle protein 100 (LYSp100B) as a co-activator of Bright.

The main focus of interest of this work was the molecular mechanism by which MARs augment the transcription of the immunoglobulin gene. Our experiments demonstrated that SATB2 binds the μ enhancer MAR regions *in vivo* and stimulates μ gene transcription. We further established that SATB2 stimulates transcription in plasma B cells but not in non-lymphoid cells. One explanation of that fact could be that in B cells the transcriptional repressor Cux/CDP, which also binds the IgH MAR regions, is not expressed (Wang et al., 1999), allowing SATB2 to bind and stimulate the transcription of the immunoglobulin gene. Another possibility is that SATB2, like Bright, may interact with a B cell-specific transcriptional co-activator. In this context it will be important to search for interaction partners of SATB2 and such experiments are currently under way. Future studies should address the question whether SATB2, in analogy to SATB1, can also act as a transcriptional repressor at specific sites.

Overall, the multiple roles of MAR-binding proteins in gene regulation suggest that their function depends on the context of their cis-acting DNA elements. Although no direct interactions of MAR-binding proteins with transcription factors have been identified to date, two lines of evidence suggest that MAR-binding proteins collaborate functionally with the μ enhancer-binding proteins. First, MAR elements that are combined with the μ enhancer generate DNase I

hypersensitive sites and alter chromatin accessibility, whereas MAR elements alone fail to modify the chromatin structure (Jenuwein et al., 1993, 1997). Second, the potentiation of the μ enhancer by the MAR-binding protein Bright has been shown to depend on the presence of a binding site for the Oct transcription factor in the μ enhancer (Webb et al., 1999). In addition, Bright transactivates only from a subset of binding sites and acts by competing with the repressor Cux/CDP, which recognizes the same nucleotide sequence (Kaplan et al., 2001). Thus, it seems likely that the context of the regulatory sequences may determine whether MAR-binding proteins act as activators or repressors of transcription.

4. Regulation of SATB2 function

After determining that SATB2 act as transcriptional activator of the immunoglobulin gene, our interest shifted towards the mechanisms that regulate its function. To date, the regulation of the activity of MAR-binding proteins is still fairly obscure. One common mechanism appears to be proteolytic cleavage by caspases that leads to functional inactivation of the respective protein and its dissociation from chromatin – as shown for SATB1 (Galande et al., 2001) and SAF-A (SAF-A - Gohring et al., 1997). However, in the case of SATB2 such regulation does not appear to be applicable, since SATB2, in contrast to SATB1, does not contain a caspase cleavage site and is not proteolysed *in vitro* (Fig. 22 and Fig. 23). Therefore we concentrated on other posttranslational modifications, as a possible means for the regulation of SATB2 activity.

4.1. SATB2 is SUMO-modified

In recent years the covalent conjugation of SUMO has emerged as an important mechanism for the posttranslational regulation of diverse proteins, including numerous transcription factors (see Introduction – 4.5). Our results demonstrated that SATB2 is modified by SUMO conjugation at two SUMO consensus sites around lysine 233 and lysine 350 (K233 and K350). It is

interesting to note that the consensus SUMO site, IK^{233}VE, is located at almost the same position where a caspase cleavage site is found in SATB1 (Fig. 22). The second SUMO consensus site, VK^{350}PE, is positioned immediately upstream of the first CUT repeat of the DNA-binding domain; this may explain the inhibitory effects of SATB2 sumoylation on its binding to MAR regions and on its transcriptional activation ability. The SUMO conjugation sites of SATB2 are not conserved in SATB1 and our experiments revealed that *in vivo* SATB1 is not SUMO-modified under conditions that produce a significant sumoylation of SATB2. Therefore, this dynamic modification is a distinctive feature of SATB2, compared to SATB1, whose activity is known to be tightly regulated by irreversible inactivation through proteolytic cleavage in apoptotic T cells during negative selection. The caspase 6 cleavage separates the DNA-binding domains from the PDZ-like dimerization domain, results in a monomeric SATB1 which lacks BUR-binding activity, despite containing both its DNA-binding domains, and rapidly dissociates from chromatin *in vivo* (Galande et al., 2001). In comparison, our results indicated that the sumoylation of SATB2 does not affect its dimerization, although it also reduces its association with chromatin. This further underlines the dissimilarities in the regulation of the closely homologous SATB1 and SATB2.

We demonstrated that the SUMO modification of SATB2 is augmented under conditions of cellular stress. In this respect, it is relevant to note that stress factors, such us temperature shifts, high osmolarity, or DNA damage, are reversible conditions that in most cases do not lead to cell death. A reversible inactivation of transcription factors during these periods (e.g. by sumoylation, in the case of SATB2) could be one mechanism for adjustment of the stress response. It will also be important to examine whether the SUMO modification of SATB2 is altered during normal physiological processes, e.g. the selection of B cells.

The dissimilarities between SATB1 and SATB2 are not unique. Similar differences in the sumoylation of highly related proteins have been shown for the Sp1 family of transcription factors. Sp1, Sp3 and Sp4 have similar transactivation domains and recognize the same binding sites, but only Sp3 can also act as a repressor. The ability of Sp3 to repress transcription is due

to SUMO modification at lysine 539 in its inhibitory domain, which is not present in Sp1 and Sp4 (Ross et al., 2002). Thus, SUMO modifications of specific members of protein families can differentially modulate the transcriptional activity of the conjugated protein.

4.2. PIAS1 is the E3 ligase for SATB2

The final step of the SUMO conjugation pathway is catalyzed by E3 ligases, which are specific for the modified substrate (see Introduction – 4.3). Thus, it was intriguing to determine which is the E3 ligase mediating SATB2 sumoylation. Our experiments identified PIAS1 as the SUMO E3 ligase for SATB2. Co-expression of SATB2 with PIAS1, but not with other members of the PIAS family, led to significant augmentation of the sumoylation of SATB2 *in vivo*. We could further demonstrate that PIAS1 is directly involved in this enzymatic process, using a reconstituted *in vitro* system. It is interesting to note that in contrast to the other SUMO targets that associate with and are sumoylated by several PIAS proteins, SATB2 shows high specificity for PIAS1. For example, all PIAS family members stimulate sumoylation of LEF1 (Rick Sachdev, personal communication), while both PIAS1 and PIASxβ can modify p53 (Schmidt and Müller, 2002). In contrast, only PIAS1 showed any detectable binding to SATB2 and led to its sumoylation.

Recently, additional E3 ligases for SUMO conjugation have been identified. RanBP2, which has no homology to PIAS proteins and is localized at the cytoplasmic side of the nuclear pore, acts as an E3 ligase for RanGAP, p53 and HDAC4 (Pichler et al., 2002; Kirsh et al., 2002). Sumoylation by this E3 ligase has been implicated in the regulation of nucleocytoplasmic shuttling of proteins. In addition, polycomb group protein-2 (Pc2), a component of Pc nuclear bodies, which are involved in the stable repression of genes, has been shown to stimulate SUMO modification of the transcriptional co-repressor CtBP (Kagey et al., 2003). These SUMO E3 ligases have an overlapping substrate specificity but differ in their subcellular localization. PIAS proteins contain a conserved domain, termed the SAP domain, which mediates their association with the nuclear matrix and localization to PML

nuclear bodies (Sachdev et al., 2001). Polycomb group protein-2 on the other hand, localizes to Pc nuclear bodies that are distinct from PML bodies (Kagey et al., 2003). Thus, different E3 ligases may impart a spatial or temporal regulation on SUMO modification. Therefore, further experiments will need to investigate whether under specific conditions or at specific locations SATB2 can be SUMO-modified by some of the other E3 ligases mentioned above.

4.3. SUMO modification antagonizes SATB2-mediated transcriptional activation

Even though sumoylation is now an established posttranslational modification for a large number of proteins, the functional consequences of SUMO conjugation in most cases remain unclear. It was therefore important to attempt characterizing the effect of SUMO modification on the function of SATB2.

A functional role for the sumoylation of SATB2 was inferred from the analysis of a mutant protein (K233R/K350R) that cannot be modified with SUMO. This sumoylation-deficient SATB2 protein stimulated expression of a co-transfected reporter plasmid, carrying multimerized SATB2-binding sites, more efficiently than the wild-type SATB2. Although lysines can also be modified by ubiquitination, acetylation or methylation, an amino-terminal fusion of SUMO to the K233R/K350R double mutant of SATB2 resulted in a decrease of the transcriptional activation potential, suggesting that the effects of the lysine mutations of SATB2 are most likely due to deficiency in sumoylation. Therefore, SUMO modification appears to contribute to the regulation of the transcriptional activity of SATB2, as in the case of Sp3 (Ross et al., 2002). In this regard, SATB2 differs from LEF1, p53 and STAT1, which are also SUMO-modified by PIAS proteins. For these proteins, mutations of the sumoylation sites do not alter the transcriptional activity. However, the sumoylation activity of PIASy was found to be important for the PIASy-mediated transcriptional repression and subnuclear targeting of LEF1 to PML nuclear bodies (Sachdev et al., 2001). These observations were interpreted to suggest that sequestration of LEF1 and p53 to sumoylation centers, such as

PML bodies, may be the primary event in the regulation of LEF1 by PIAS family members, whereas sumoylation of SATB2 and Sp3 by PIAS proteins may alter the activity of these proteins irrespective of the location where they function. Thus, SUMO modification may generate different pools of proteins that are functionally distinct and/or localized to different subnuclear compartments.

4.4. SUMO modification of SATB2 reduces its chromatin association

In addition to its increased transactivation capacity, the sumoylation-deficient SATB2 protein was found to associate more efficiently with the MAR sequences of the endogenous immunoglobulin μ locus than the wild-type SATB2 protein, suggesting that sumoylation may regulate DNA binding. Similar observations have been reported for other transcription factors e.g. the heat shock factors HSF1 and 2, although in that case the SUMO modification leads to increased association with DNA (Goodson et al., 2001). However, using the covalent SUMO–SATB2 fusion proteins we could show that the difference in the transactivation potential and chromatin association is unlikely to be due to differences in the direct protein-DNA interactions (Fig. 31). A decrease in DNA binding has been reported for STAT proteins, which are also targets for sumoylation (Liu et al., 1998). In this case sumoylation does not appear to affect the DNA binding *per se*, but rather serves to stabilize the interaction between STAT and PIAS proteins, that is responsible for the decreased association with DNA (Liu et al., 1998). In fact, some of our experiments suggest that an analogous interaction between SATB2 and PIAS1 may have the same effect and inhibit the DNA binding of SATB2 (data not shown).

The dimerization domain of SATB1 was shown to be absolutely required for SATB1 association to chromatin *in vivo* (Galande et al., 2001). The experiments shown on Fig. 32 used N-terminal SUMO1 and SUMO3 fusions to SATB2 and showed no decrease in the dimerization capacity of the protein. This most likely indicates that the negative effect of sumoylation on the

transactivation potential and chromatin association of SATB2 is exerted by a mechanism that is independent of SATB2 dimerization.

Nevertheless, the possibility exists that SUMO modification thorough an isopeptide bond at the SUMO consensus sites (K233 and K350) could affect the dimerization, as well as the DNA association of SATB2, in a manner different from that shown for the N-terminal SUMO fusion proteins, since this conjugation would lead to branching of the polypeptide chain in close proximity to the DNA-binding domains and might influence the protein conformation. Further experiments involving electromobility shift analysis with 100% SUMO-modified SATB2 would answer that question.

4.5. Sumoylation alters the subnuclear localization of SATB2

In a number of instances, SUMO conjugation leads to changes in the subcellular localization of the modified proteins. In the case of SATB2, we also found a striking correlation between sumoylation of the protein and its subnuclear localization. For these experiments, we used stably transfected plasmocytoma cells that express SATB2 at moderate levels, to avoid artificial targeting of excess proteins to sites of protein storage or degradation. Both SATB2 and the SUMO3-SATB2-dlmut fusion protein are localized at the nuclear periphery, whereas the sumoylation-deficient SATB2-dlmut protein has a more diffuse nuclear localization. Importantly, the SUMO1-SATB2-dlmut fusion protein accumulated in prominent nuclear speckles (bodies). As SATB2-dlmut and SUMO1-SATB2-dlmut have different localization patterns, it seems likely that the subnuclear distribution of SATB2 is influenced by the added SUMO peptide. This can probably be explained by associations with other proteins that may lead to targeting of SATB2 to specific sites. In connection with this, it has to be mentioned that association of the MAR-binding protein, Bright, to the speckle protein LYSp100 targets Bright to distinct subnuclear sites, termed LANDs and leads to augmentation of immunoglobulin transcription, whereas the related Sp100 protein, which represses Bright-mediated activation by antagonizing tetramerization, targets Bright to PML nuclear bodies (Zong et al., 2000).

It would obviously be intriguing to see whether the SATB2-dlmut or the SUMO1 and SUMO3 covalent fusions have different interaction partners, that may account for the different subnuclear localization and such immunoprecipitation experiments are currently under way.

The differences in the subnuclear localization of wild-type SATB2 and the SUMO-deficient SATB2 double mutant raise the question of whether the sumoylation of this MAR-binding protein may be involved in the changes of the localization of genes during transcriptional activation and/or repression. The immunoglobulin μ gene has been shown to localize at the nuclear periphery in primary T cells that do not express the gene, whereas the μ gene is localized away from the nuclear periphery in pre-B cells in which the gene is activated (Kosak et al., 2002). In addition, the nuclear localization of genes that are silenced by the transcription factor Ikaros is altered in comparison to cells in which Ikaros is not present (Cobb et al., 2000). In this case, the silenced genes co-localize with heterochromatic chromatin in foci that are located near the nuclear periphery. Our experiments indicate that the sumoylation of SATB2 downregulates its association with MAR sequences and the transcriptional activation of MAR-containing target genes. In addition, sumoylation targets SATB2 to the nuclear periphery. However, we consider it unlikely that these processes are linked and that SUMO modification of SATB2 is involved in localizing silent immunoglobulin genes to the nuclear periphery. Instead, we favor the view that other mechanisms, including epigenetic modifications of chromatin and/or binding to other proteins, may account for the localization of the silent immunoglobulin loci to the nuclear periphery. If SATB2 had a similar function as the proposed nuclear scaffold role of SATB1 (Cai et al., 2003), SATB2 could be involved in localizing the immunoglobulin loci to sites of active transcription. According to this scheme, sumoylation of SATB2 could be a mechanism to regulate chromatin tethering during the cell cycle, cellular stress or upon receiving extracellular signals that alter the balance between sumoylation and desumoylation.

In conclusion, the work presented in this thesis identified SATB2 as a novel MAR-binding protein with high homology to the previously described SATB1,

but with a number of distinct properties. SATB2 is expressed in early B cells and specifically stimulates immunoglobulin transcription upon association with the MAR sequences of the μ gene. Furthermore, SATB2, unlike SATB1, is SUMO-modified by PIAS1 at two consesus sites, and this decreases the transactivation potential of SATB2. In addition to suppressing the activity of the protein, sumoylation also leads to the sequestration of SATB2 in distinct nuclear structures.

5. Perspectives

The exact mechanisms, through which SATB2 activates transcription upon binding to MAR sites, remain unclear. Therefore it is important to identify interacting partners for this protein, which may provide essential clues about the transcriptional activation, and possibly other cellular functions, mediated by SATB2. In addition, selected candidates can be assayed for their ability to act as co-activators for SATB2. It would be interesting to examine whether the SATB2 double mutant or the SUMO1 and SUMO3 covalent fusions associate with different proteins, which may account for their different subnuclear localization and chromatin association. To this end, both the purified SATB2 protein and the stable cell lines expressing TAPtag-SATB2 or FLAG-tagged SATB2 and its derivatives, that we have established, will represent valuable tools.

Further work is needed to elucidate the physiological stimuli that lead to SATB2 sumoylation, as well as the spatio-temporal regulation of this process. In the same line of work, it will be interesting to analyze in more detail the speckled structures, where SUMO-modified SATB2 is targeted, and to determine whether it colocalizes there with other nuclear proteins.

IV. Materials and methods

1. Reagents

1.1. Chemicals

Except where otherwise stated, reagents were from Sigma (Deisenhofen, Germany), Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany).

1.2. Enzymes

Enzyme	Company
Klenow fragment	Roche
Pfu DNA polymerase	Stratagene
Restriction enzymes	New England Biolabs
RNase A	Roche
Superscript II reverse transcriptase	Invitrogen
T4 DNA ligase	New England Biolabs
T4 DNA polymerase	Roche
T4 polynucleotide kinase	New England Biolabs
Taq polymerase	Roche
Expand High Fidelity PCR system	Roche

2. General buffers

BBS (BES-buffered saline), 2×: 50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES; Calbiochem), 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95. Filter sterilize through a 0.45-μm nitrocellulose filter (Millipore). Store in aliquots at –20 °C.

Denhardt solution, 100×: 10 g Ficoll 400, 10 g polyvinylpyrrolidone, 10 g bovine serum albumin in 500 ml dH_2O. Filter sterilize and store at –20 °C in 25 ml aliquots.

HeBS (HEPES-buffered saline) solution, 2×: 16.4 g NaCl, 11.9 g HEPES, 0.21 g Na_2HPO_4 in 1l dH_2O. Titrate to pH 7.05 with 5 N NaOH.

Filter sterilize through a 0.45-μm nitrocellulose filter (Millipore). Store in aliquots at –20 °C.

MOPS buffer: 0.2 M MOPS [3-(*N*-morpholino)-propanesulfonic acid], pH 7.0, 0.5 M sodium acetate, 0.01 M EDTA. Store in the dark.

10× PBS (phosphate-buffered saline): 1,37 M NaCl, 27 mM KCl, 43 mM $Na_2HPO_4{\cdot}7H_2O$, 14 mM KH_2PO_4, pH 7.4. Dilute with dH_2O to a 1× working solution.

SDS electrophoresis (Tris/glycine) buffer, 5×: 15.1 g Tris base, 72.0 g glycine, 5.0 g SDS in 1 liter H_2O.

SSC (sodium chloride/sodium citrate), 20×: 3 M NaCl (175 g/liter), 0.3 M tri-sodium citrate. Adjust pH to 7.0 with 1M HCl.

TBE (Tris/borate/EDTA) electrophoresis buffer (10×): 890 mM Tris base

890 mM boric acid, 20 mM EDTA.

1× protease inhibitor mix (PIM): 1.5 mM $MgCl_2$, 10 mM KCl, 5 μg/ml Soybean Trypsin/Chymotrypsin inhibitor, 5 μg/ml Antipain, 5 μg/ml Aprotinin; 5 μg/ml Leupeptin, 0.5 μg/ml Pepstatin A, 5 μg/ml Bestain.

Z buffer: 60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.7H_2O$, 10 mM KCl, 10 mM $MgSO_4.7H_2O$.

3. Cloning and related techniques

3.1. Cloning of SATB2 constructs

Full-length SATB2 was cloned in pBluescript SK(+/-) by RT-PCR from RNA isolated from Abelson transformed pre-B cell line (19-1-4) using the following primers: SATB1h-Uf (5' ATGGAGCGGCGGAGCGAGAG 3') and SATB1h-Lf (5' TTATCTCTGGTCAATTTCGGCAGGTGC 3').

pcDNA3.1-SATB2-myc-His was constructed by two fragment ligation of an *EcoR*I*, Kpn*I SATB2 fragment derived from pBluescript (pBs) SK-SATB1h and a *Kpn*I*, Xba*I digested PCR fragment amplified using the following primers: 5' AAGCCATCCACACACTCTCC 3' and 5' GCTCTAGACTCGAGTTGTCTCTG GTCAATTTCGGCAGGTGC 3').

pET21a-SATB2 was constructed by two fragment ligation of a *BamH*I*, Msc*I digested PCR fragment, amplified using the primers: 5' GCGGATCCATGGAG CGGCGGAGCGAGAG 3' and 5' GTGCTCTTCTCGGTTGTCGT 3', and a *Msc*I*, Xho*I fragment derived from pcDNA3.1-SATB2-myc-His.

pEF-FLAG-SATB2 was cloned by two fragment ligation a *BamH*I*, Msc*I digested PCR fragment, amplified using the primers: 5' GCGGATCCATGGAG CGGCGGAGCGAGAG 3' and 5' GTGCTCTTCTCGGTTGTCGT 3', and a *Msc*I*, Xba*I fragment derived from pcDNA3.1-SATB2-myc-His.

pcDNA3.1-SATB2-TAPtag was cloned by ligation of a SATB2 *BamH*I fragment, derived from pET21a-SATB2, into pcDNA3.1-TAPtag.

All of the above PCRs were performed with Expand High Fidelity PCR System (Roche Molecular Biochemicals, Mannheim, Germany) or with PfuTurbo DNA polymerase (Stratagene, La Jolla, CA), using deoxyribonucleosidetri-phosphate mix (final concentration 250 mM) and reaction buffers, supplied by the manufacturers.

3.2. Cloning of the luciferase reporter constructs

pfosluc-μ enhancer and pfosluc-μ enhancerΔMAR were cloned by introducing the 1 kb or respectively 450 bp *Xba*I fragment of the μ enhancer derived from pμ or pμΔMAR plasmids, respectively (Grosschedl et al., 1984), into the *Xba*I site of pfosluc.

pfosluc-wt$(MAR)_7$ was cloned by ligation of a *Sac*I*, Xho*I fragment, containing the multimerized wild-type MAR consensus sequences - wt$(MAR)_7$, derived from pBs-wt$(MAR)_7$ into the *Sac*I*, Xho*I sites of pfosluc.

pfosluc-mut$(MAR)_7$ was cloned by ligation of of a *Sac*I*, Xho*I fragment, containing the multimerized mutated MAR consensus sequences - mut$(MAR)_8$, derived from pBs-mut$(MAR)_8$, into the *Sac*I*, Xho*I sites of pfosluc.

3.3. Preparation of competent *E. coli*

50 ml LB medium were inoculated with 0.5 ml overnight culture of *E. coli* DH5α or BL21(DE3) and grown to A_{600} of 0.5. The bacteria were then centrifuged in a 50 ml conical tube (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) at 3000 rpm, 5 min., 4 °C, resuspended in 25 ml cold sterile 0.1 M $CaCl_2$ and stored on ice for 30 min. After a second spin as above, the bacteria were resuspended in 1 ml cold 0.1 M $CaCl_2$ and frozen in 15% sterile glycerol at –80 °C, in aliquots.

3.4. Transformation and growth of transformed bacteria

100 μl competent bacterial cells were mixed with 0.1–100 ng of plasmid DNA and incubated on ice for 30 min. The cells were then heat shocked by placing in a 42 °C water bath for 90 s. 1 ml LB medium was added to the transformation mix and the tubes were placed in a 37 °C shaker for 1h. Afterwards the cells were spread onto LB agar plates containing the respective antibiotic: 100 μg/ml ampicillin, 50 μg/ml kanamycin, or 34 μg/ml

chloramphenicol and grown overnight at 37 °C. Single colonies were used to inoculate 2 ml LB cultures. These cultures were expanded to 400 ml and grown overnight at 37 °C, shaking at 200 rpm.

3.5."Mini" and "maxi" plasmid preparation

Maxi preps were done using kits from Qiagen (Chatsworth, CA) according to the manufacturer's instructions. Mini preps were done according to Clewell et al., 1970.

3.6. Restriction digests

Restriction digests were typically done for 2 h. at 37 °C with 2 units restriction endonuclease per μg DNA in the appropriate buffer as recommended by the manufacturer (New England Biolabs, Beverly, MA).

3.7. Ligation of DNA

Ligation was done with T4 DNA ligase in buffer supplied by the manufacturer (New England Biolabs, Beverly, MA) in a final volume of 10 μl for 1-4 h. at RT.

3.8. Agarose gel electrophoresis

Agarose (electrophoresis grade, GibcoBRL, Grand Island, NY) was dissolved in 0.5× TBE buffer to the desired concentration (1 to 2.5% depending on the size of DNA fragments) by boiling in a microwave oven, and after cooling down, ethidium bromide was added to a final concentration of 0.5 μg/ml and the agarose solution poured in a gel chamber (Peqlab Biotechnologie GmbH, Erlangen). The DNA sample was mixed 1:10 with 10× DNA sample buffer (30% glycerol, 0.25% Bromphenol Blue, 0.25% Xylene Cyanole in TBE buffer). The gels were run in 0.5× TBE buffer and visualized on a UV transilluminator.

3.9. Point mutant generation

The point mutants in the SUMO consensus sites of SATB2 were introduced following the instructions of the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) using the pEF-FLAG-SATB2 clone (previously verified by sequencing) as a template and PfuTurbo DNA polymerase (Stratagene) with the following primers:

233 K/R: 5' AAGTATAAGAAGATAAGAGTGGAAAGAGTGGAGCGAGAG 3'

350 K/R: 5' ATTCCCAGAGCAGTTAGGCCAGAGCCAACAAAC 3'

4. Tissues culture and related techniques

4.1. Cell lines

38B9 (Engler and Storb, 1987)	Murine fetal liver-derived Abelson-transformed pre-B lymphocytes.
PD36 (Travis et al., 1991)	Murine adult bone marrow-derived pre-B lymphocytes.
70Z/3 (Maki et al., 1980)	Murine late pre-B lymphocytes derived from adult bone marrow.
BJAB (Menezes et al., 1975)	Human Epstein-Barr virus negative B lymphoblastoid cell line established from an African Burkitt's lymphoma.
M12 (Laskov et al., 1981)	Murine mature B cell line.
Sp2 (Hurwitz et al., 1980)	Murine myeloid cell line.
J558L (Gehring et al., 1972)	Murine plasmocytoma cell line.
WEHI231 (Boyd et al., 1981)	Murine B cell lymphoblasts. Secrete IgM under lipopolysaccharide stimuli.
WEHI3 (Kersten et al., 1980)	Murine myeloid cell line.
EL4 (Johnson, 1972)	Murine T cell lymphoma.

BW5147 (Ralph et al., 1973)	Murine T cell lymphoma.
Jurkat (Gillis et al., 1980)	Human T lymphoblastoid cell line. Heterogeneous in ploidy..
293T (Xie et al., 1996)	Adenovirus 5-transformed human embryonic kidney cell line.
HeLa (Boshart et al., 1984)	Aneuploid, human epithelial cell line originating from a cervical carcinoma.
NIH 3T3 (Jainchill et al., 1969)	Fibroblastic cell line from mouse embryo.
MEL (Orkin et al., 1975)	Murine erythroleukemia cell line.

4.2. Culture conditions

The cell lines, used in this study were grown in the following media:

1) HeLa - Eagle minimal essential medium (MEM) supplemented with 10% heat-inactivated foetal bovine (calf) serum (FBS=FCS, Invitrogen Life Technologies) and 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate and 2 mM L-glutamine (Invitrogen Life Technologies).

2) 293T and NIH3T3 - Dulbecco's modified Eagle medium (DMEM), containing 1 g/l glucose and supplemented with 10% FBS, 4 mM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate (Invitrogen Life Technologies).

3) 38B9, PD36, 70Z/3, BJAB, M12, Sp2, J558L, WEHI231, WEHI3, Jurkat, EL4, BW5147, MEL - RPMI 1640 medium, supplemented with 10% heat-inactivated foetal calf serum (FCS) and 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 4 mM L-glutamine.

The adherent cell lines, used in this study were usually grown in 10-cm or 15-cm tissue culture dishes (Falcon, Becton Dickinson Labware) in a tissue culture incubator with 5% CO_2 atmosphere at 37 °C. Subculturing of the

adherent cells was done by trypsinization with 0.25% trypsin, 0.2% EDTA (Invitrogen Life Technologies).

Suspension cell lines were grown in 25, 75 or 175 cm^2 tissue culture flasks. Subculturing of suspension cells was done by diluting with fresh medium.

The freezing of cells was done in 90% FCS and 10% (v/v) dimethyl-sulfoxide (DMSO) in a freezing box at -80°C. The frozen aliquots were stored in a liquid nitrogen tank or at -80°C.

Thawing of the cells was done by submerging the frozen vial in a 37 °C water bath. The cells were then washed with medium by centrifugation at 1200 rpm for 3 min. and resuspended in pre-warmed medium before transferring them to culture plates or flasks.

4.3. Calcium phosphate transfection

The day before transfection, 293T or HeLa cells were seeded in 10 cm tissue culture dishes or in 6-well plates (for reporter assays) at 30% confluency. 20 μg or 4 μg total DNA (the total DNA concentration in each transfection experiment was kept constant by adding vector plasmid DNA) was used and the reactions were filled to 450 μl or 90 μl with H_2O for 10-cm dishes or 6-well plates, respectively. 50 μl or 10 μl of 2.5 M $CaCl_2$ were added, the tubes were vortexed, and left at room temperature for 20 min. 500 μl of 2× BES or 2× HBS (depending on the cell type) were added dropwise while gently vortexing, and the solution was incubated another 20 min. at room temperature. The calcium phosphate-DNA solution was added dropwise onto the cell culture plate while swirling. The plates were incubated overnight in a 3% CO_2-humidified incubator at 37 °C to allow for a calcium phosphate-DNA complex to gradually form. The cells were then washed three times in PBS, and incubated in complete medium in a 5% CO_2 humidified incubator at 37 °C.

4.4. Transfection by electroporation

5×10^6 cells per transfection were spun down and resuspended in 500 μl pre-warmed RPMI medium. 35 μg total plasmid DNA (the total DNA concentration in each transfection experiment was kept constant by adding vector plasmid DNA) was added to the cell suspension and mixed carefully. The sample was then transferred to a 4 mm Gene Pulser® cuvette (Bio-Rad Laboratories, Hercules, CA) and a pulse of 250 mV and 975 μF was applied. The cell suspension was then transferred to a 10 cm tissue culture dish, containing 10 ml pre-warmed RPMI medium and the cells were incubated for the desired period of time (at least 36 h).

This protocol was applied successfully for the transfecion of Jurkat, EL4, J558L, Sp2, BJAB and 38B9 cells.

4.5. Stable cell line establishment

For stable cell line establishment, J558L or 38B9 cells were electroporated with 30 μg linearized DNA, carring neomycin resistance or together with 3 μg pSVneo linerized with *Not*I, for plasmids that did not contain a neomycin resistance gene. 24 h. post transfection 100 mg/ml (active concentration) G-418 (Invitrogen Life Technologies) in 100 mM HEPES (pH 7.4) was added to a final concentration of 2 mg/ml. Cell clones were established by diluting to densities of 10^4 cells/ml and seeding of 200 μl aliquots into 96-well plates. 14 days after plating, G-418-resistant clones were expanded in RPMI medium containing 200 μg/ml G-418.

4.6. Nuclear matrix preparation

Transfected or untransfected cells, were plated on glass coverslips (11 mm, Menzel-Gläser) at least 8 h. prior to fixation. The cells were washed with 1× PBS and then treated with 0.5% Triton X-100 in CSK buffer (10mM PIPES pH 7.1, 1 mM EGTA, 3 mM $MgCl_2$, 20 % sucrose) for 5 min. at 4 °C to remove the membrane and soluble proteins. Subsequently the slides were treated

with extraction buffer (42.5 mM Tris.HCl pH 8.3, 8.5 mM NaCl, 2.6 mM $MgCl_2$, 1.2 mM phenyl methylsulfonyl flouride (PMSF), 1% Tween 40 and 0.5 % deoxycholic acid) for 5 min. at 4 °C to remove the cytoskeletal proteins. After extraction the cells were transferred to a humid chamber and treated with 100 μl digestion buffer (50 mM NaCl, 300 mM sucrose, 10 mM PIPES pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM phenylmethyl sulfonyl flouride, 100 μg/ml DNaseI). The slides were incubated at 37 °C for 1 h. and then extracted with 0.25 M ammonium sulfate in digestion buffer at 4 °C for 5 min. to remove the chromatin fraction from the nuclei. The slides were washed once with 1× PBS, fixed consecutively in methanol and acetone at –20 °C for 3 min. each, and allowed to air dry.

4.7. Immunofluorescence

Transfected or untransfected cells, were plated on glass coverslips (11 mm, Menzel-Gläser) at least 8 h. prior to fixation. The cells were washed 1× with PBS and fixed (see below). After washing 3× with PBS, blocking was done with 10% foetal bovine serum in PBS by incubating 30 min. on a 50 μl drop. The coverslips were then transferred to a 50 μl drop primary antibody diluted in PBS and incubated 30-60 min. Following a 3× washing with PBS, the cells were incubated 30 min. with the secondary antibody, diluted in PBS. After a final wash (3× with PBS followed by dH_2O) the coverslips were mounted on a precleaned microscope slide (Menzel-Gläser) in MOWIOL 4-88 medium (Calbiochem, La Jolla, CA) containing 1 mg/ml *p*-phenylenediamine (Sigma) as an anti-fade reagent.

For immunofluorescence of suspension cells, the cells were spun down and then resuspended in PBS to a final density of 10^6 cells/ml. 1 cm^2 squares were made with a hydrophobic pen (Super PAP pen; Electron Microscopy Sciences, Fort Washington, PA) on a poly-lysine coated slide (Poly-Prep™ slides, Sigma Diagnostics), 100 μl of the cell suspension was added in the square and the cells were allowed to attach for 30 min. at 37 °C. The cells

were washed once with 1× PBS and the immunofuorescence was performed essentially as described above.

The fixation procedures were as follows:

“PFA”: Fix with 3% paraformaldehyde (PFA) for 10-30 min., wash 1× with PBS and permeabilize with 0.5% Triton X-100 in PBS for 5 min. at RT.

“P-T”: Fix with 3% paraformaldehyde, 0.1% Triton X-100 in PBS for 10 min. at RT.

“MeOH” - Fix with cold (–20 °C) methanol for 15 min. at –20 °C.

"MeOH-Acetone" - Fix with cold (–20 °C) methanol for 5 min. followed by incubation with –20 °C acetone for 5 min.

“Acetone” – Fix with cold (–20 °C) acetone at RT for 5 min.

For the “P-M”, “MeOH”, and “MeOH-Acetone” fixations it is important to aspirate all the PBS before adding methanol.

4.8. Microscopy

Microscopy was done with a fluorescent microscope (Zeiss, Jena, Germany) equipped with standard DAPI, FITC (or GFP) and Texas Red (or Cy3) filters. Images were acquired with a 63× objective, using a high-performance charged-coupled digital (CCD) camera with MetaMorph software (Universal Imaging Corporation).

4.9. Reporter assays: luciferase and β-galactosidase assay

Cells were collected, washed once with 1× PBS and resuspended in 200 μl 1× Reporter Lysis Buffer (Promega, Madison, WI). The cell suspension was frozen in a dry ice\ethanol bath and thawed at room temperature. The cell debris were spun down at 13000 rpm for 5 min. 20 μl of the protein extract were used for measurement of the luciferase activity using the Luciferase assay system (Promega, Madison, WI), following the manufacturer’s

instructions. The measurement was performed with the luminometer LUMAT LB9507 (EG&G® BERTHOLD, Wellesley, MA).

β-galactosidase assays were performed as follows: 20 μl of the protein extract were transferred to a 96-well plate and 180 μl β-galactosidase assay mix (10 ml Z buffer, 10 μl 50 mM CPRG (chlorophenol-red-β-galactopyranoside monosodium salt – Roche, Mannheim, Germany), 10 μl β-mercaptoethanol) were added to each reaction. 20 μl 1× reporter lysis buffer were used as a blank. The measurement of the enzymatic activity was performed using the SoftMax program on the Spectra MAX250 ELISA machine (Molecular Devices, Sunnyvale, CA) **.**

5. RNA isolation, purification and analysis

5.1. Isolation of total and poly-A^+ RNA

5.1.1. Preparation of total RNA

Cellular or tissue total RNA was prepared either with Trizol reagent (Gibco BRL) according to the manufacturer's instructions or following the single-step method for isolation (Acid Guanidine-Phenol Extraction) developed by Chomczynski and Sacchi (1987). The cells were washed with PBS, lysed in 4 ml of GITC solution (4 M Guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β-mercaptoethanol). 0.4 ml of 2 M sodium acetate pH 4.0, 4 ml of water-saturated phenol and 0.8 ml of chloroform-isoamylalcohol mixture (49:1) were added, mixed and incubated on ice for 15 min. The samples were centrifuged at 6000 rpm for 30 min. at 4 °C, the aqueous phase was removed and mixed with the same volume of 2-propanol. RNA was precipitated at –20 °C for 1 h. and pelleted by centrifugation at 6000 rpm for 20 min. at 4 °C. The RNA pellet was dissolved in 0.5 ml of GITC solution, re-precipitated with 2-propanol, washed with 80% ethanol, air dried and dissolved in 100-500 μl DEPC-treated water. RNA concentration was determined on a spectrophotometer by measuring the A_{260} and samples were stored at –80 °C.

The quality of isolated RNA was determined by electrophoresis in 1% MOPS agarose gel.

5.1.2. Preparation of poly-A$^+$ RNA

1 g of oligo(dT) cellulose powder (BMB) was added to 2 ml of 0.1 M NaOH and poly(A) loading buffer (0.5 M LiCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 0.1% (w/v) SDS) and the volume was filled to 10 ml. 1 ml of the slurry was added into BioRad plastic columns. The column was rinsed in 10 ml of DEPC-H_2O and equilibrated two times with 20 ml of poly(A) loading buffer until the pH of the flow-through reached neutral point. The RNA samples were heated to 70 °C for 10 min. and LiCl was added to 0.5 M final concentration. The RNA solution was loaded onto the oligo(dT) column and the column was washed with 1 ml of poly(A) loading buffer. The resulting flow-through containing mainly ribosomal and transfer RNA was used to test for RNA degradation. The column were rinsed with 2 ml of middle wash buffer (0.15 M LiCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 0.1% (w/v) SDS). The purified poly-A$^+$ RNA was eluted with 2 ml of 2 mM EDTA, pH 8.0, 0.1% (w/v) SDS and precipitated in 0.3 M NaAcetate, pH 7.0 and 100% ethanol at -20°C for 2 h. After centrifugation and two washes in 70% ethanol, the pellet was dried and resuspended in 50 μl of DEPC-H_2O. 5 μl of each sample was taken out to test for the quality of RNA. The concentration of RNA was determined by measurement of the absorbance at 260 nm (A_{260}).

5.2. Northern blot hybridization

5.2.1. Agarose gel electrophoresis

A volume corresponding to 10-20 μg of total RNA or 2-10 μg poly-A$^+$ RNA were adjusted to a volume of 30 μl with DEPC-H_2O and an equal volume of 2× Rotta loading dye (62.5% formamide, 10% formaldehyde, 1× MOPS) was added. The samples were subsequently heated to 65 °C for 10 min. (to denature secondary RNA structures) and placed on ice for 5 min. The RNA

was loaded on an agarose gel, containing 1% (w/v) agarose in 1× MOPS buffer and run at 5 V/cm in 1× MOPS buffer. The RNA was visualized and the gel photographed with a gel documentation system (IS-1000, Alpha Innotech Corporation). The locations of the 28S (corresponding to 4718 nt.) and 18S (corresponding to 1874 nt.) rRNA molecules were marked on the gel.

5.2.2. Transfer of RNA to nylon membrane

Two pieces of Whatmann 3MM paper, wetted in 20× SSC, were placed on two pieces of dry Whatmann 3MM paper and a stack of paper towels. Hybond-N+ nylon membrane was soaked first in dH_2O, then in 20× SSC and placed on the pre-wetted Whatmann 3MM papers. The gel was placed on the membrane and the whole sandwich was covered with Saran-Wrap. A heavy glass plate was placed on top of the stack for compression. The RNA was transferred to the nylon membrane overnight.

To immobilize the RNA, the membrane was UV-crosslinked in a transilluminator (Spectronics Corporation).

5.2.3. Preparation of probes

The probes were prepared with the Rediprime II random prime labeling kit (Amersham Biosciences). 20 ng of linearized DNA template in 45 μl of TE-buffer was denatured by boiling for 10 min. at 100 °C. The sample was placed immediately on ice for 5 min., collected by centrifugation, and added to the Rediprime reaction tube. 5 μl of α-^{32}P-dCTP (3000 Ci/mmol) were added to the reaction mix and incubated for 30 min. at 37 °C. The reaction was stopped by adding 5 μl of 0.2 M EDTA, pH 8.0. 50 μl of H_2O was added to increase the volume for more efficient elution. To remove unincorporated nucleotides, the probe was loaded on a Quick Spin Column G50 (Roche) and centrifuged at 1200×*g* for 3 min. 1 μl of the probe was used for counting the activity in a liquid scintillation counter.

PROBES:

Name of the probe	Source of the DNA fragment
full length SATB2	*XhoI/XbaI* fragment from pBluescript SK-SATB1h
GAPDH	*NotI/BamHI* fragment from pGAPDH
VDJ probe	PCR fragment, amplified from pμ using primers: 5' GGGATATCCACACCAAACATC 3' 5' AGAGGCCATTCTTACCTGAGG 3'
Cα	PCR fragment, amplified from J558L cDNA using primers: 5' TGCACAGTTACCCATCCTGA 3' 5' AGACGGTCGATGGTCTTCTG 3'

5.2.4. Hybridization

The nylon membrane was placed in a hybridization tube with the RNA-side facing the center of the tube. Depending on the probe, two different methods were used for hybridization:

1) 20 ml of hybridization mixture (50% formamide, 5× SSC, 50mM Na-phosphate pH 6.5, 5× Denhardt's reagent, 0.1% (w/v) SDS, 0.5 mg/ml yeast transfer RNA) were added and the membrane was pre-hybridized in a hybridization oven for 2 h. at 42 °C. The pre-hybridization solution was discarded and fresh 5-10 ml hybridization mix (depending of the hybridization tube), containing the respective radioactively labeled probe at a final concentration of $1\text{-}3\times10^6$ cpm/ml, was added. The hybridization was carried out at 42 °C overnight. After hybridization the membrane was washed once with 2× SSC, 0.1% (w/v) SDS at room temperature for 15 min. Two subsequent washes in 0.2× SSC, 0.1% (w/v) SDS for 15 min. at 68 °C were performed. The membrane was wrapped in Saran Wrap and exposed to autoradiography films AR (Kodak) in a cassette at –80°C.

2) 20 ml of hybridization mixture (5× SSC, 50 mM Na-phosphate pH 7.0, 1× Denhardt's reagent, 1% (w/v) SDS, 0.1 mg/ml yeast transfer RNA) were

added and the membrane was pre-hybridized in a hybridization oven for 2 h. at 68 °C. The pre-hybridization solution was discarded and fresh 5-10 ml hybridization mix (depending of the hybridization tube) were added, containing the respective radioactively labeled probe at a final concentration of 1-3×10^6 cpm/ml. The hybridization was carried out at 68 °C overnight. After hybridization the membrane was washed once with 5× SSC at 68 °C for 15 min. Two subsequent washes in 2× SSC, 0.5% (w/v) SDS for 15 min. at 68°C were performed. The membrane was wrapped in Saran Wrap and exposed to autoradiography films AR (Kodak) in a cassette at –80°C.

For reprobing, the membrane was stripped by 3 washes with 0.1× SSC, 0.1% (w/v) SDS on a rocking plate for 15 min. at 90 °C followed by washing at room temperature 0.1× SSC, 0.1% (w/v) SDS for 2 min.

5.3. RT-PCR (Reverse transcription polymerase chain reaction)

5 μg of cytoplasmic RNA or 1 μg of poly-A^+ tissue RNA in 15 μl of DEPC-H_2O were incubated at 65 °C for 5 min. to denature secondary RNA structures and subsequently placed on ice for 5 min.

The following components were mixed in DEPC-treated Eppendorf tubes:

Reagent	**Volume**
RNA	15μl
5× first strand buffer (Invitrogen)	5 μl
0.1 M DTT	2 μl
Oligo dT primer (50 pmol/μl)	1 μl
dNTPs (10 mM)	1 μl
Superscript II Reverse Transcriptase (Invitrogen, 200 U/μl)	1 μl
Final volume	25 μl

As a negative control, one reaction was mixed without Reverse Transcriptase. The reaction was carried out at 42 °C for 1 h. to allow the cDNA synthesis to take place. The samples were diluted 1:2 by addition of 50 µl of DEPC-H_2O and subsequently used for PCR analysis (see 6.3).

6. DNA isolation, purification and analysis

6.1. Isolation of genomic DNA

Genomic DNA was isolated according the method developed from Kirby, 1957. The cells were washed with PBS and resuspended in NET buffer (10 mM Tris-HCl pH 7.5, 25 mM EDTA, 100 mM NaCl). SDS to a final concentration of 1% and proteinase K to 50 µg/ml were added and the cell lysate was incubated for 4 h. at 56 °C or overnight at 37 °C. Two phenol-chloroform extractions were performed and the DNA was then precipitated with 2 to 3 volumes absolute ethanol. The precipitated DNA was harvested with a pasteur pipette, washed in 70% ethanol, air dried and subsequently resuspended in 100-500 µl dH_2O (depending on the starting material).

6.2. Southern blot hybridization

6.2.1. Restriction digestion and agarose gel electrophoresis

5-10 µg of genomic DNA was subjected to restriction digestion with 10 U per µg DNA of the respective restriction endonuclease in the appropriate buffer as recommended by the manufacturer (New England Biolabs, Beverly, MA) in a final volume of 60 µl. The restriction digest was typically carried out overnight at 37 °C. After the incubation, the DNA was mixed with 10× DNA loading dye, loaded on a 0.7% agarose gel and run at 2 V/cm in 1× TBE buffer. The DNA was visualized and the gel photographed with a gel documentation system (IS-1000, Alpha Innotech Corporation). The locations of marker bands relative to a ruler were marked.

6.2.2. Transfer of the digested DNA to nylon membrane

After running, the gel was incubated two times for 15 min. in denaturation solution (0.5 M NaOH, 1.5 M NaCl), then incubated two times for 15 min. in neutralization solution (1 M Tris pH 7.4, 0.5 M NaCl) and subsequently equilibrated in 20× SSC.

To assemble the transfer sandwich, a piece of Whatmann 3MM paper with both ends submerged in 20× SSC was placed on a solid support. The gel was placed topside facing down on the Whatmann 3MM paper and a piece of wetted Hybond-N nitrocellulose membrane (Amersham Biosciences) was placed on the gel carefully, avoiding any air bubbles. One piece of wetted and five pieces of dry Whatmann 3MM paper, lying on top of the membrane and a stack of paper towels, constituted a sponge. A heavy glass plate was placed on top of the stack for compression. The transfer was carried overnight. DNA transferred on the membrane was immobilized by UV-crosslinking.

6.2.3. Preparation of probes

The probes were prepared with the Rediprime II random prime labeling kit (Amersham Biosciences) essentially as described (5.2.3.).

PROBES:

Name of the probe	Source of the DNA fragment
VDJ probe	PCR fragment, amplified using primers: 5’ GGGATATCCACACCAAACATC 3’ 5’ AGAGGCCATTCTTACCTGAGG 3’

6.2.4. Hybridization

The hybridization was performed in hybridization tubes. 20 ml of hybridization mixture (50% formamide, 2.5× SSC, 10 mM Tris pH7.5, 1× Denhardt’s reagent, 1% (w/v) SDS, 0.1 mg/ml sonicated salmon sperm DNA) was added

and the membrane was pre-hybridized in a hybridization oven for 2 h. at 42 °C. The pre-hybridization solution was discarded and fresh 5-10 ml hybridization mix (depending of the hybridization tube), containing the respective radioactively labeled probe at a final concentration of 1-3×10^6 cpm/ml, were added. The hybridization was carried out at 42 °C overnight. After hybridization, the mixture was poured off and the membrane was washed twice with 2× SSC, 0.1% (w/v) SDS at room temperature for 15 min. Two subsequent washes in 0.2× SSC, 0.1% (w/v) SDS for 15 min. at 68 °C were performed. The membrane was wrapped in Saran Wrap and exposed to autoradiography films AR (Kodak) in a cassette at –80°C.

6.3. PCR (Polymerase chain reaction)

The standard PCR reaction is assembled by mixing the following components:

Reagent	**Amount**
DNA	2-200 ng
10× PCR buffer	5 μl
25 mM $MgCl_2$	5 μl
dNTPs (10 mM)	2 μl
Primers (forward and reverse)	20 pmol
FIREPol® polymerase (SOLIS BIODYNE)	0,5 μl
Final volume	50 μl

7. Protein purification and analysis

7.1. Expression and purification of recombinant proteins

For His-SATB2, His-SUMO1-SATB2 and His-SUMO3-SATB2 purification, *E. coli* (Rosetta) were transformed with pET21a-SATB2, pET21a-NtermSATB2,

pET21a-SUMO1-SATB2-dlmut and pET21a-SUMO3-SATB2-dlmut. A single colony was used to inoculate 10 ml LB, containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. 4 ml overnight culture was transferred to 800 ml LB with 50 μg/ml ampicillin and incubated at 37 °C until A_{600}=0.6. Protein expression was induced by 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and carried out at room temperature for 4 h. The bacteria were harvested by centrifugation, washed with 1× PBS and frozen at –80 °C. After defreezing the pellets were resuspended in 10 ml lysis buffer (300 mM NaCl, 5 mM $MgCl_2$, 20 mM sodium phosphate pH7.5, 40 mM imidazole, 10% glycerol, 0,1% Triton X-100, 1 mM DTT and protease inhibitors mix) and sonicated 3× 30 sec. The bacterial lysate was spun for 15 min. at 27 000 g (15 000 rpm in a SS-34 rotor (Sorvall, Newton, CT)). The soluble fraction was filtered (0.45 μm filter, Millipore, Bedford, MA) and loaded on Ni-NTA Agarose (Qiagen). After binding, the Ni-NTA Agarose was washed three times with 10 ml lysis buffer. The bound proteins were eluted with 300 mM imidazole in lysis buffer. Eluted proteins were dialysed against 20 mM Tris-HCl pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol.

For SUMO1 purification, bacteria lysed in 50 mM Tris-HCl pH 8.0 and 50 mM NaCl were precleared with Q Sepharose (Sigma) and purified by gel filtration.

For purification of catalytically active SUMO E1 enzyme, His-Aos1 and His-Uba2 were coexpressed in bacteria, lysed in 50 mM Na-phosphate buffer pH 8.0, 300 mM NaCl, 10 mM imidazole, and purified on ProBond Resin (Invitrogen), followed by molecular sieving (Superdex 200) and ion exchange chromatography (Mono Q, Pharmacia Biotech).

For purification of Ubc9, bacteria were lysed in 50 mM Na-phosphate buffer (pH 6.5), 50 mM NaCl, incubated with SP-Sepharose beads (Sigma), eluted with 50 mM Na-phosphate buffer (pH 6.5), 300 mM NaCl, and sieved through a Superdex 200 column. All of the proteins were dialyzed against transport buffer (20 mM HEPES pH 7.5, 110 mM K-acetate, 2 mM Mg-acetate, 0.5 mM EGTA) before their use in the *in vitro* sumoylation assays.

For GST-PIAS1 purification, GST-PIAS1 was expressed in Rosetta and lysed in GST-lysis buffer (10 mM Na-phosphate buffer pH 7.2, 150 mM NaCl, 10

mM EDTA, 1% Triton X-100, 1 mM DTT, 1 mM phenyl methylsulfonyl fluoride (PMSF)) and sonicated 3× 30 sec. The bacterial lysate was spun for 15 min. at 27 000 g (15 000 rpm in a SS-34 rotor (Sorvall, Newton, CT)). The soluble fraction was filtered (0.45 μm filter, Millipore, Bedford, MA) and loaded on Glutathione-Sepharose beads preequlibrated with GST-lysis buffer. After binding (typically for 1 hour), the Glutathione-Sepharose was washed three times with 10 ml GST-wash buffer (20 mM HEPES pH 7.9, 50 mM NaCl, 1 mM DTT, 10% glycerol). The bound proteins were eluted with 50 mM glutathione in GST-wash buffer. Eluted protein was dialyzed against 20 mM Tris-HCl pH 8.0, 100mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol.

7.2. Preparation of total protein extracts

7.2.1. Cell extracts

Plates of adherent cells were washed twice with Dulbecco's phosphate-buffered saline (PBS), scraped off with a rubber policeman and transferred to an Eppendorf tube. Suspension cells were collected by centrifugation, washed twice in Dulbecco's PBS without Ca^{2+} and Mg^{2+}, and transferred to Eppendorf tubes. All subsequent steps were performed on ice. The cells were resuspended in an appropriate volume (depending on the cell pellet) of RIPA buffer (10 mM Na-phosphate pH 7.2, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) Na-deoxycholate, 0.1% (w/v) SDS), supplemented with 1× protease inhibitors mix, 1 mM dithiotreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF), added just before harvesting, or CoIP buffer (50 mM Tris-HCl, pH 7.9, 15 mM EGTA pH 8.0, 100 mM NaCl, 0.1% (v/v) Triton X-100, 1× protease inhibitors mix, 1 mM DTT, 1 mM PMSF). The samples were sonicated 3× 30 s. and then centrifuged for 15 min. at 13000 rpm, 4 °C. The supernatant, containing the total protein extract, was transferred to a fresh Eppendorf tube and the protein concentration was determined by the Bradford assay. The total protein extract was stored at –20°C or –80°C.

7.2.2. Organ extracts

Mouse organ extracts for Western blot analysis were prepared by isolating the respective organs from anaesthetized mice, snap freezing them in liquid nitrogen and thawing by resuspension with a plastic homogenizer on ice in cold lysis buffer: 50 mM Tris-HCl 7.5, 1% Triton X-100, 120 mM NaCl, 20 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium vanadate, 10 μg/ml pepstatin, leupeptin, aprotinin, antipain and chymostatin, and 250 μg/ml Pefabloc SC (Roche).

7.3. Measurement of protein concentration

Protein concentration was determined using a Coomassie Brilliant Blue G-250-based protein assay reagent (Biorad; see also Krauspe and Scheer, 1976).

7.4. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was based on the discontinuous system described by Laemmli (1970). 8-15% separating gels (depending of the size of the proteins of interest) were cast using a 30% acrylamide/0.8% *N,N'*-methylene bisacrylamide solution (Roth). For electrophoresis, protein samples were mixed 1:1 with 2× Laemmli buffer, heat denatured for 10 min. at 95 °C and loaded onto the gel. Proteins were separated by applying a current of 40 mA until the dye front had reached the end of the gel. Pre-stained or unstained marker proteins (Bio-Rad) were run in parallel. Following electrophoresis, proteins were stained with Coomassie Brilliant Blue G250, silver staining or subjected to Western blotting (see below).

7.5. Coomassie staining of polyacrylamide gels

For Coomassie staining of polyacrylamide gels, the gels were incubated for 30 minutes on a slowly rocking platform with Staining solution (50% Methanol, 10% Glacial Acetic acid, 0,25 % Coomassie Brilliant Blue G250). To visualize the proteins the gels were incubated overnight in destaining solution (50% Methanol, 10% Glacial Acetic acid). For drying the gels were soaked in dH_20 containing 20% gycerol, placed between cellophane film and dried for 2 h. at 75 °C.

7.6. Silver staining of polyacrylamide gels

The gels were fixed in 2.5% acetic acid, 2.5% methanol, rinsed 2× with dH_2O and shaken in dH_2O for 2 h. to overnight. Sensitization was with 0.02% sodium thiosulphate for 2 min., followed by rinsing 2× 30 sec. with dH_2O. The gels were then incubated 30 min. in 0.1% $AgNO_3$, rinsed 2× 30 sec with dH_2O and developed with 0.01% formaldehyde, 2% sodium carbonate. When a sufficient degree of staining was reached the developing solution was poured out and the process was stopped with several changes of 1% acetic acid.

7.7. Western blotting (immunoblotting)

Proteins separated by SDS-PAGE were transferred to a nitrocellulose filter (membrane) using either wet or semi-dry blot transfer.

For semi-dry transfer ROTH „SEMI-DRY-BLOT“ apparatus was used applying constant current of 0.8 mA per cm^2 for 1 hour. For protein transfer, the gel was sandwiched between gel-sized Whatmann 3MM papers soaked in transfer buffer (20% methanol in 1× Tris/glycine buffer).

The wet blot was carefully set up under transfer buffer to avoid air pockets. The transfer was run in a cold room at 60 V for 2 hours or at 20 V overnight.

After transfer, nitrocellulose filters were incubated for 1 hour in a Blocking solution (PBS containing 5% dried milk and 0.1% Tween-20) in order to

reduce the unspecific background. The membrane was sealed in a plastic bag and incubated for 1 h. with in appropriate dilution of the primary antibodies, directed against the protein of interest.

Primary antibody	Dilution	Secondary antibody	Dilution
Mouse monoclonal α-myc (Roche)	1:1000	α-mouse-HRP	1:4000
Mouse monoclonal α-FLAG M2 (1 mg/ml, Sigma)	1:5000	α-mouse-HRP	1:5000
Mouse monoclonal α-T7 (Novagen)	1:5000	α-mouse-HRP	1:5000
Mouse monoclonal α-SUMO1 (Zymed)	1:1000	α-mouse-HRP	1:500

The membrane was washed 3 times for 10 min. in PBST (0.1% Tween-20 in PBS) and incubated for an additional hour with the appropriate secondary antibody conjugated to horseradish peroxidase. After 3 washes in PBST, antigen-antibody complexes were detected using the enhanced chemiluminescence detection system (NEN, Boston, MA or Amersham Biosciences), according to the manufacturer's instructions and exposed on Biomax-MR film (Eastman Kodak, Rochester, NY).

8. Analysis of protein-protein and protein-DNA interactions

8.1. Protein-protein interactions

8.1.1. Co-immunoprecipitation

Protein extracts (typically 500 μg) were diluted in Co-IP buffer (50 mM Tris-HCl, pH 7.9, 15 mM EGTA, pH 8.0, 100 mM NaCl, 0.1% (v/v) Triton X-100) to a final volume of 500 μl. To each sample, 1 μg monoclonal antibody (α-myc, α-FLAG, α-T7) was added. The samples were then incubated rotating at 4 °C

for 2 h. Meanwhile Protein G-Sepharose was washed three times and resuspended in Co-IP buffer to a 1:1 slurry. 50 μl of the slurry was added to each sample and incubated for another hour while rotating in a cold room. The Protein G-Sepharose beads were washed three times in washing buffers of different stringency: Co-IP buffer with 100 to 300 mM NaCl and/or 0.1% (w/v) SDS and subsequently resuspended in 30 μl of 2× Laemmli sample buffer and boiled at 100 °C for 10 min. The samples were immediately cooled on ice, centrifuged at 23000 rpm for 2 min. at 4 °C and analyzed by SDS PAGE and Western blotting.

8.1.2. TAPtag purification

The TAPtag purification was done essentially as described by Rigaut et al., 1999. In brief, cells were spun down and the packed cell volume was measured. An equal volume of Buffer A (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 1 mM leupeptin, 2 mM pepstatin A, 4 mM chymostatin, 2.6 mM aprotinin) was added to the pellet and the cells were broken by sonicating 5× 30 sec. The extract was then centrifuged at 20000×*g* for 1 h. 200 μl bead suspension of IgG-Sepharose was transferred into the Bio-Rad Poly-Prep columns (Bio-Rad). The beads were washed with 10 ml IPP150 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40). The extract was then transferred into the column containing the pre-washed beads and rotated for 2 h. at 4 °C. After the incubation, the beads were washed three times with 10 ml of IPP150 and once with 10 ml of TEV cleavage buffer (IPP150 adjusted to 0.5 mM EDTA and 1 mM DTT). Cleavage was done in the same column by adding 1 ml of TEV cleavage buffer and 100 units of TEV protease (Invitrogen). The beads were rotated for 2 h. at 16 °C and the eluate was recovered by gravity flow. 100 μl of calmodulin beads (Stratagene), corresponding to 200 μl of bead suspension, were transferred into a column and washed with 10 ml of calmodulin binding buffer (10 mM Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1 % NP-40). 3 ml of calmodulin binding buffer and 3

μl of 1 M $CaCl_2$ were added to the 1 ml of eluate recovered after TEV cleavage. The sample was then transferred to the column containing pre-washed calmodulin beads and rotated for 1 h. at 4 °C. After the beads were washed with 30 ml of calmodulin binding buffer, the bound proteins were eluted with 1 ml of calmodulin elution buffer (10 mM Tris-HCl pH 8.0, 10 mM 2-mercaptoethanol, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 0.1% NP-40, 2 mM EGTA). Five elution fractions of 200 μl each were collected.

8.2. Protein-DNA interactions

8.2.1. Electromobility shift assay (EMSA)

The wild-type MAR (5´-TCTTTAATTTCTAATATATTTAGAATTC-3´) or mutant MAR (5´-TCTTTAATTTCTACTGCTTTAGAATTC-3´) oligonucleotides were radiolabeled with ^{32}P and purified on a 20% native polyacrylamide gel. The MAR oligonucleotides have been previously described (Dickinson et al., 1992). DNA-binding reactions contained 20 mM HEPES (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 8% glycerol, 1 μg poly dI-dC and 10000 cpm radiolabeled probe. Electrophoretic mobility shift assays were performed for 20 min. at RT and subsequently run on a 6% native polyacrylamide gel in 0,33× TBE.

8.2.2. Chromatin immunoprecipitation (ChIP)

Proteins were crosslinked to DNA by adding formaldehyde directly in the culture medium to a final concentration of 1% and incubated for 10 min. at 37 °C. Cells were collected in a Falcon tube, washed once with 1× PBS, then with Triton buffer (10 mM Tris-HCl 8.0, 0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA) and finally with NaCl buffer (200 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0). After the washes the cells were resuspended in sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0). DNA was sheared by sonicating 15× 15 sec. Chromatin extracts were diluted 10-fold in

immunoprecipitation (IP) buffer (140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitors mix). Immunoprecipitation using anti-FLAG mAb and Protein G-Sepharose or two-step TAP-tag affinity chromatography purification (Rigaut G et al., 1999) was performed. The IPs were washed twice with 1 ml of IP buffer, once with 1 ml of IP buffer containing 500 mM NaCl, once with 0.5 ml wash buffer (10 mM Tris pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.25% sodium deoxycholate), and twice with 0.5 ml of TE buffer. The chromatin bound to the beads was eluted in 300 μl elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) by heating at 65 °C for 15 min. Bound and input chromatin samples were diluted to a final SDS concentration of 0.5%. All samples were incubated overnight at 65 °C to reverse formaldehyde crosslinking. RNA was digested for 30 min. at 37 °C with 3 μl of DNase-free RNase A (10 mg/ml), followed by proteinase K digestion. After phenol-chloroform extraction, the DNA was ethanol precipitated using glycogen (10 mg/ml; Sigma) as a carrier. Precipitated DNA was resuspended in 100 μl of TE. Semiquantitative PCRs using five-fold dilutions were performed, using primer pairs 5'MAR-CHIP-U (5' CCTGCAAAAGTCCAGCTTTC 3') and 5'MAR-CHIP-L (5' AGAGCCTCAC TCCCATTCCT 3') for the 5' MAR region in the immunoglobulin intronic enhancer, H7uA and H7uB for the β-globin locus (Litt et al., 2001).

9. Sequence analysis

Protein and DNA sequences were retrieved from the Entrez server (http://www.ncbi.nlm.nih.gov/Entrez/) or with the Sequence Retrieval System (http://srs.ebi.ac.uk/) and analysed for open reading frames (ORF) and restriction sites using the DNA Strider software (Douglas, 1995).

Sequence homology searches and alignments were done on the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul et al., 1997) or with the ClustalX programme (Thompson et al., 1997).

Domain searches and analyses were done using the SMART tool (http://smart.embl-heidelberg.de/; Schultz et al., 1998) or the NCBI CD-Search

engine (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer et al., 2002). EST sequences were searched on the EST database (http://www.ncbi.nlm.nih.gov/dbEST/; Boguski et al., 1993).

V. Publications

The work presented in this thesis will be published in the following article:

Gergana Dobreva, Julia Dambacher and Rudolf Grosschedl (2003). **"SUMO modification of a novel MAR-binding protein, SATB2, modulates immunoglobulin μ gene expression".** Genes and Development 2003 (*in press*)

Previous work has been published in:

Boyanovski B, Russeva M, **Dobreva G**, Ganev V, Mladenova A, Peicheva V, Nikolov K, and Baleva M.: **"Protein C Activity in Patients with Antiphospholipid Syndrome."** *Journal of Clinical Rheumatology* 2000 Oct;6:239-243.

Bodem J*, **Dobreva G*,** Hoffmann-Rohrer U, Iben S, Zentgraf H, Delius H, Vingron M, Grummt I.: **"TIF-IA, the factor mediating growth-dependent control of ribosomal RNA synthesis, is the mammalian homolog of yeast Rrn3p."** *EMBO Reports* 2000 Aug;1(2):171-5.

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