Molecular Regulation of SATB1 in Regulatory T-cells

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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Taiz, Yemen

Bonn, 2012

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

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Tag der Promotion: 20.02.2013
Erscheinungsjahr: 2013

DECLARATION

This thesis is a presentation of my original research work. Wherever contributions of others are involved every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions. The work was done under the guidance of Professor Joachim Schultze at the LIMES Institute, Bonn.

Bonn, December the 3rd, 2012 …..……

Yasser A.Thabet

Acknowledgements

I would like to express my heartfelt thanks and gratitude to my supervisor Prof. Joachim Schultze for giving me the opportunity to do my thesis in his lab and on top of that for his unlimited support, patience, and guidance. It would have been a hard hard time for me without the outstanding ideas and supervision of Dr. Marc Beyer, who did not save any effort to support and guide me.

My deepest thanks go to the members of the Schultze lab, past and present, Eva Schönfeld, Svenja Debey-Pascher, Andrea Hoffmann, Andrea Nino-Castro, Wolfgang Krebs, Daniel Sommer, Timm Weber, Tristan Wirtz, and others for the great time and the excellent working atmosphere. I would like to give Andrea Nino-Castro again with a special thank for her valuable discussions and suggesstions while I was writing my thesis.

I deeply appreciate the collaborative work with many groups have been mentioned in this thesis. In particular, Prof. M. Famulok and Prof. G. Meyer for providing me the whole support to perform filter binding assays in their lab.

Finally, I would like to acknowledge my mother and my family who have been a constant beacon of support, encouragement and love.

Summary

In this study we have identified SATB1, a nuclear protein that recruits chromatin-remodeling factors and regulates numerous genes, as a novel effector molecule in T_{reg} cells. Our interest in SATB1 resulted from a genome wide expression profile of T_{reg} cells and conventional T-cells (T_{conv} cells). SATB1 was a prominent candidate gene that constantly repressed in T_{reg} cells and highly expressed in T_{conv} cells. The dominant repression of SATB1 expression in T_{reg} cells could be confirmed at mRNA, protein, and single cell level under resting and different stimulation conditions in humans and mice. In contrast, SATB1 is expressed at high levels in T_{conv} cells and is further enhanced following physiological stimulation.

The inverse expression pattern of FOXP3, the main transcription factor in shaping and maintaining T_{reg} cell identity, in relation to SATB1 led us to hypothesize its active involvement in regulation of SATB1. On the one hand, induction of FOXP3 was associated with inhibition of SATB1. This could be demonstrated by induction of FOXP3 in naïve $CD4^+$ T-cells converted to induced T_{reg} cells (iT_{reg}) or in $CD4^+$ T-cells ectopically overexpressing FOXP3 after lentiviral transduction. On the other hand, using different genetic approaches loss of FOXP3 expression in T_{reg} cells results in relieving the FOXP3-mediated repression and leads to an upregulation of SATB1. Furthermore, confocal microscopy on lymphocytes form *scurfy* and normal mice interestingly showed mutually excluding staining patterns. While the SATB1 signal is low in normal FOXP3-expressing thymocytes, it is high in thymocytes expressing a mutated non-functional FOXP3 from *scurfy* animals.

FOXP3 as a transcription factor has been linked to direct binding to DNA, thereby regulating gene expression. To investigate whether FOXP3 can directly bind to the SATB1 genomic locus FOXP3-ChIP tiling arrays were performed. The analysis of tiling array data provided us with several putative FOXP3 binding sites in the promoter and intronic regions of the SATB1 locus which were confirmed by ChIP qRT-PCR. Furthermore, we were able to demonstrate high specificity of the binding and determine the binding coefficients of FOXP3 to several motifs in the SATB1 locus by filter retention assays. To assess whether this binding has functional relevance, we performed reporter assays and showed that FOXP3 reduces lucifierase activity for several binding regions clearly supporting that FOXP3 regulates SATB1 transcription by direct binding to the genomic locus. Interstingly, we showed that FOXP3 also controls SATB1 gene expression indirectly at post-transcriptional level via miRNAs. Indeed we identified several FOXP3 dependent miRNA that have been linked to

post-transcriptional regulation of gene expression. FOXP3-ChIP tiling arrays showed FOXP3 peaks within these miRNAs loci. Furthermore, silencing of FOXP3 reversed this enrichment, whereas over-expression of FOXP3 induced their expression. Binding of FOXP3-dependent miRNAs to the 3´UTR of SATB1 in reporter assays confirms the suppressive effect of these miRNAs on SATB1 expression. An additional level of regulation of gene expression is exerted by epigenetic modifactions of the respective genomic locus. Epigenetic changes control the accessibility of a genomic locus by permissive or inhibitory histone modifications as well as methylation of CpG islands. Although, we did not observe differences in the methylation pattern of the CpG islands at the SATB1 locus between T_{reg} cells and T_{conv} cells, we observed more permissive and less repressive histone marks at the SATB1 genomic locus in T_{conv} cells and the opposite in T_{reg} cells which is in line with the expression data and aforementioned described regulatory mechanism of SATB1 expression in T_{reg} cells.

Besides the molecular mechanism regulating SATB1 expression in T_{reg} cells, we further delineated the functional consequences of induction of SATB1 in T_{reg} cells. Lentiviral over-expression of SATB1 in human and murine T_{reg} cells resulted in the edition of gene expression and function of T_{reg} cells. The striking observation was the abrogation of the capacity of T_{reg} cells to suppress the proliferation of responder cells *in vitro,* in addition to the production of proinflammatory cytokines like IL-4 and IFN-γ. These findings suggested that T_{reg} cells acquire an effector phenotype a finding which is further corroborated on a genome wide level. Gene expression profiles of SATB1 overexpressing T_{reg} cells showed that many proinflammatory genes have been switched on upon induction of SATB1 expression in T_{reg} cells which promotes skewing of regulatory towards effector programs. To further prove the antagonistic effect of SATB1 on the regulatory function of T_{reg} cells *in vivo,* we adoptively transferred T_{reg} cells overexpressing SATB1 with naïve $CD4^+$ cells into *RAG2*-/- mice. In this experimental setting T_{reg} cells failed to suppress inflammation *in vivo* and subsequently the mice developed colitis.

In conclusion, SATB1 is an important effector molecule whose expression is tightly regulated in T_{reg} cells. SATB1 upregulation in T_{reg} cells results in aquisition of proinflammatory properties and attenuated suppressive function *in vitro* and *in vivo*. Therefore, FOXP3-mediated repression of SATB1 expression in T_{reg} cells seems to be an important regulatory circuit crucial to maintain suppressive function of these cells.

Abbreviations

ab	Antibody
Ag	Antigen
AIRE	Autoimmune regulator
AP-1	Activator protein1
APC	Antigen presenting cell
APS	Ammoniumperoxodisulfate
BCA	Bicinchoninic acid assay
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanat

FOXP3	Forkhead Box Protein 3
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced Tumor Necrosis Factor Receptor
HLA	Human leukocyte antigen
ICOS	Inducible T-cell co-stimulator
ICS	Intracellular cytokine staining
IFN	Interferon
Ig	Immunoglobulin
IKK	Inhibitor of NF-κB kinase
IL	Interleukin
IL-2R	Interleukin-2 receptor
iTreg	Induced regulatory T-cells
IκB	Inhibitor of nuclear factor κ light chain gene enhancer in B cells
JAK	Janus kinase
mab	Monoclonal antibody
MACS	Magnetic activated cell sorting
mDC	Myeloid dendritic
MEKI	MEK kinase
MHC	Major Histocompatibility Complex
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor kappa-light-chain enhancer of activated T-cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline

pDC	Plasmacytoid dendritic *cells*
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein Complex
PI	Propidium iodide
PI3K	Phosphoinosite 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PIP_3	Phosphatidylinositol (3,4,5)-trisphosphate
PK	Protein kinase
PLC	Phospholipase C
RNA	Ribonucleic acid
SAP	Shrimp Alkaline Phosphatase
SATB1	Special AT-rich sequence-binding protein-1
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
siRNA	Small interfering RNA
STAT	Signal Transducer and activator of transcription
TBE	Tris/Borate/EDTA
T-bet	T-box expressed in T-cells
T_{conv}	Conventional T-cells
TCR	T cell receptor
TEC	Thymus epithelial cells
TGF-β	Transforming growth factor-β

Th1/2/3/9/17	T helper cell 1/2/3/9/17
TNFα	Tumor necrosis factor α
Tr1 cells	Type-I regulatory T-cells
T_{reg} cells	Regulatory T-cells
Tris	Tris-[hydroxymethyl]-aminomethan
WT	Wild-type

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1 Introduction

1.1 Tolerance

1.1.1 Introduction

The immune system faces a unique challenge. While it must deal with and control a broad spectrum of pathogens, it has to coexist peacefully at the same time with self-tissues. This challenge persists over the life time of an individuum as the immune system is chronically xposed to molecular components of various sources. Consistent contact of the immune system with newly presented intrinsic and extrinsic antigens by changing microbiotic colonization and fluctuating gene expression over its life span complicates its function toward keeping these two opposing challenges evenly balanced [1]. Maintaining the balance between a protective and a detrimental immune response is specifically controlled by mechanisms subsummized under the term tolerance. Both the innate and adaptive immune system cooperate to maintain tolerance towards certain antigens [2-5]. Although tolerance is mediated by many mechanisms, it has been grouped into two main categories according the site of induction: central tolerance in central lymphoid organs and peripheral tolerance in peripheral tissues [6]. Disturbance of tolerance has a dual sided effect. Diminished tolerance is associated with autoimmune diseases [7-12], while excessive tolerance may interfere with pathogen clearance and initiate a cancer promoting environment [13-15]. However, this also makes its manipulation highly desirable in diseased individuals as this might provide promising solutions for many urgent medical challenges including transplantation tolerance, autoimmunity and tumorogenesis [16].

1.1.2 Central tolerance

Central or recessive tolerance mainly deals with emerging autoreactive lymphocytes and prevents them from reaching the circulation [17]. This process includes two major check points, postitive and negative selection, in which the developing lymphocytes are screened for tissue restricted self peptide-MHC presented by medullary thymic epithelial cells (mTEC) and medullary dendritic cells (DC) [18-20]. The selection process has two unequivocal outcomes, either life or death [21-23]. Positive selection occurs when double positive thymocytes (DP) with a minimal threshold of reactivity to the particular self-MHC haplotypes survive; whereas thymocytes failing to express a T-cell receptor (TCR) without this basal self-recognition undergo apoptosis [24]. After receiving a selection signal and migrating to the thymic

medulla, thymocytes undergo the process of negative selection. Thymocytes bearing strongly self-reactive TCRs undergo apoptosis, thus preventing their maturation and subsequent ability to mount an autoimmune response in the periphery [25]. Negative selection of auto-reactive thymocytes is under control of autoimmune regulator (AIRE) which is a transcription factor that controls ectopic expression of tissue restricted antigens within mTEC. Nevertheless, there is an alternative fate in which these cells are rendered functionally idle through receptor editing in which the autoreactive T-cell receptors are replaced with nonreactive ones [26],[17, 24, 25].

1.1.3 Peripheral tolerance

Peripheral tolerance is the second line of immunoregulation exerted by the immune system to preserve immune homeostasis [1, 6, 17, 27]. It has a proof reading function for the output of central tolerance. The elimination of autoreactive cells by central tolerance is not without error, therefore a complementary machinery is required to track and eliminate or inactivate autoreactive cells which have escaped negative selection or receptor editing and prevent them from activation and expansion [28]. The major mechanisms of peripheral tolerance are anergy induction, clonal deletion by activation induced cell death (AICD), and cell mediated immune suppression in which T_{reg} cells play the main role [7, 29-32]. Anergy as a mechanism of tolerance is induced in response to partial or suboptimal stimulation and results in functional inactivation of T-cells, which become incapable of clonally expanding or producing cytokines upon re-encounter with antigen [24]. AICD deletion of autoreactive cells is mediated via induction of apoptosis in a Fas/FasL dependent manner [33]. The cellular control of peripheral tolerance comprises different myeloid and lymphoid subpopulations including T_{reg} cells which play a central role in maintaining self-tolerance [34, 35].

1.1.4 Cellular componentsof peripheral tolerance

1.1.4.1 Antigen presenting cells

Dendritic cells (DCs) have attracted a great deal of attention as a highly specialized population of well equipped antigen-presenting cells. Recently the focus has shifted towards their role in peripheral tolerance. Both myeloid DC (mDC) and plasmacytoid DC (pDC) have been implicated in tolerance induction. The tolerizing effect of DCs is mediated at least partly through their interaction with regulatory T-cells [7, 36]. In non-inflammatory settings cross presentation by DCs induces tolerance in $CD8^+$ T-cells rather than activation [15]. DCs with

lower levels of MHC class II (MHC-II) and co-stimulatory molecules (CD40, CD80, CD86) as immature DCs (imDC) can induce antigen-specific tolerance [28, 37]. On the opposite, high expression of the aforementioned molecules on DCs induces T cell immunity. In addition, mature DCs cells can induce tolerance under some *in vitro* circumstances such as antigen cross-presentation to $CD8^+$ T-cells in the absence of $CD4^+$ T-cells. Furthermore, maturation of DCs with TNFα and prostaglandin E2 induces tolerogenic T-cells [15].

1.1.4.2 B-cells

B-cells, in addition to their contribution to humoral immune responses, are important for $CD4^+$ T-cell activation, proliferation and differentiation [38-40]. B-cells are effective antigen-presenting cells when antigen is limited and they are able to induce tolerance under various settings [41, 42]. B-cells may also modulate regulatory T-cell development, proliferation and survival. In support of this possibility, a recent study showed that culturing $CD19^+$ human B-cells with $CD4^+CD25^+$ alloreactive T-cells plus IL-2 and CD28-specific antibody induced a 40-fold expansion of regulatory T-cells [43].

Additionally, regulatory B-cells or IL-10 secreting B-cells (B10 cells) are a subset of B-cells that have been recently identified in mice and their role in inducing immune tolerance in an IL-10 dependent manner has been clearly established. The existence and the role of B10-cells in humans is less defined as few reports have described and characterized this rare subpopulation [44, 45], [46].

1.1.4.3 Regulatory T-cells

The first evidence for the existence of cell mediated tolerance was the breakdown of tolerance in mice which had been thymectomized within the first 3 days after birth and that these mice could be rescued by adoptive transfer of cells from normal mice [47, 48]. This concept was further fortified when Sakaguchi described for the first time a subpopulation of $CD4^+$ T-cells constitutively expressing IL-2Rα (CD25) that have an effective suppressive function in a variety of autoimmune models [49]. Characterization of this subpopulation relying solely on the expression of CD25 was difficult as T-cells upregulate CD25 upon activation [47, 50]. Later on, the discovery of the transcription factor FOXP3 as a master regulator for regulatory T-cells improved the identification and the functional characterization of T_{reg} cells [51, 52]. Altough $CD4^+CD25^+FOXP3^+$ T_{reg} cells were reported to have the major role in exerting suppression in different autoimmune disease models, several subpopulations of regulatory T-

cells have been reported to exert regulatory function. In addition to $CD4^+$ $FOXP3^+$ T_{reg} cells, Th3 transforming growth factor beta (TGF-β)-producing cells, and type 1 regulatoryT-cells (Tr1 cells) that secrete IL-10 and lack FOXP3 expression have been described [53-56]. Furthermore, $CD4^+CD25^+FOXP3^+$ T_{reg} cells can be further subdivided according to their site of generation. Naturally occurring T_{reg} cells (nT_{reg}) are generated in the thymus and are mainly reactive against self-antigens, whereas induced T_{reg} cells (iT_{reg}) are generated in the periphery and show specificity for neo-antigens [57, 58].

1.2 Development of regulatory T-cells

1.2.1 Thymic development of nT_{reg}

T-cell development takes place in the thymus. The developmental stages of lymphocytes were first classified according to the expression of CD4 and CD8 co-receptors to double negative (DN), double positive (DP), and CD4 or CD8 single positive (SP) cells [18]. Positive selection and negative selection are two vital checkpoints occuring during the developmental progression towards mature T cells [59-61]. These two events ensure the deletion of autoreactive T-cells in the thymus before they reach the periphery. Positive selection and negative selection occur very close together or even simultaneously [59]. Positive selection ensures the ablity of TCR with CD4 and CD8 co-receptor to recognize self-peptide-MHC-I or II complex with low affinity/avidity. The ligation of the TCR with cognate self-peptide-MHC induces signaling events that provide a survival signal through the TCR; whereas the failure to recognize these complexes induces apoptosis by neglect [5, 6, 17, 27, 59, 62]. On the other side, negative selection occurs when the recognition of self-peptide with high affinity/avidity by the TCR causes deletion of these thymocytes via apoptosis from the repertoire [18, 63] .

Natural T_{reg} cells are not an exception; they undergo the same selection pressure and developmental checkpoints as conventional T-cells. Although the recognition of self-antigen on MHC-I & II that are presented by mTEC and intrathymic dendritic cells (tDCs) with high affinity is a hallmark of autoreactive T-cells and these cells have to be eliminated, this high affinity interaction is a key step in the positive selection of nT_{reg} cells [64, 65].

The importance of the thymus in the development and generation of nT_{reg} cells was shown as mice aquire severe autoimmune lesions when they are subjected to thymectomy within the first 3 days after birth [8, 66]. The development pathway through which nT_{reg} cells arise is still ambiguous. When nT_{reg} cells diverge from other thymocytes towards lineage commitment

remains unclear. Nonetheless, FOXP3 upregulation occurs rather late during thymic development. FOXP3-GFP reporter mice have shown that the majority of $FOXP3^+$ cells are detected at the $CD4^+CD8^-$ single positive stage [67]. However, the expression of FOXP3 follows the upregulation of CD25 on DP which is associated also with expression of nT_{reg} characteristics markers like CTLA-4 and GITR [68, 69].

The anatomic site of FOXP3 expression induction in thymus is also still a source of debate. The majority of data are supporting thymic medulla as the enhancing compartment, where FOXP3 expression is up-regulated [64, 70-72]. Several lines of evidence support this conclusion, since the majority of the $FOXP3^+$ population was identified in the medulla. Furthermore, a study showed that the expression of AIRE in mTEC in the medulla is required for nT_{reg} cells development, whereas the presence of MHC-II negative mTEC in the medullary region led to significantly lower frequency $FOXP3^+$ T_{reg} cells [11, 64, 73]. Besides AIRE, thymic stromal lymphopoietin (TSLP) is produced in the medulla and is critical for nT_{reg} cells [74, 75]. These notions showed the importance of the medulla in the development of nT_{reg} cells but did not exclude a supporting role of the cortex. Accumulation of $CD4^+FOXP3^+$ thymocytes within the cortex upon blocking of thymic migration from the cortex to the medulla in CCR7 deficient mice supported the involvement of the cortex in the induction of FOXP3 [71]. However, evidence suggest that FOXP3 induction is not limited to a single anatomical location as multiple thymic components of the medulla as well as the cortex support the generation of FOXP3 thymocytes [76].

1.2.2 Induced regulatory T-cells (iT_{reg})

For a long period of time the generation of T_{reg} cells in the periphery remained controversial. The first clue for the presence of functional extra-thymic T_{reg} cells was the ability of $CD4^+CD25^-$ T-cells transferred into *RAG2*-/- mice to convert and expand to $FOXP3^+$ $CD4^+$ T-cells [58, 77-79] and the capacity of these cells to maintain suppressive function against effector cells *in vitro* and *in vivo.* These cells were later called induced T_{reg} cells [58, 78]. The major known difference between nTreg and iT_{reg} cells is their origin as iT_{reg} cells differentiate in the periphery from naïve $CD4^+$ T-cells in various tissues like lymph nodes, lamina propria of the gut and in response to several conditions as chronical inflamation, tumors [80] and in response to foreign antigens such as food and microbiota [78].

The generation of iT_{reg} cells is favored under two conditions, suboptimal stimulation and costimulation provided via CTLA-4 rather than CD28. In agreement with these data, mice

deficient in CTLA-4 were devoid of iT_{reg} cells [70, 81]. Moreover, strong ligation of CD28 molecules results in the inhibition of TGF-β induced iT_{reg} cell induction [82].

Besides TCR engagement and weaker co-stimulation signals through B7, TGF-β and IL-2 are needed for the acquisition of an iTreg-cell phenotype [72, 83]. *In vivo* neutralization of TGF-β impaired oral tolerance and inhibited T_{reg}-cell induction [37]. The reported mechanism of FOXP3 induction in TGF-β-induced T_{reg} cells is mediated by the cooperative binding of TGF-β signaling molecule SMAD3 and NFAT to the FOXP3 gene enhancer [84]. Furthermore, TGF-β also cooperates with CTLA-4 to attenuate the strong TCR signal which is required for effector T cell differentiation to a suboptimal signal that favors T_{reg} cell induction [57]. The importance of IL-2 in iT_{reg} cell generation is mediated through attenuating the TGF-β mediated proliferation inhibition and enhancing IL-2-STAT5 mediated FOXP3 induction [83].

Another prominent difference between both populations is the stability of FOXP3 mRNA expression. It is less stable in iT_{reg} cells and its expression fades rapidly on withdrawal of IL-2 and TGF-β [85]. The preferential stability of FOXP3 in nT_{reg} cells might be attributed to continuous stimulation of nT_{reg} cells by self-antigens and the methylation status of FOXP3 specific CpG islands at the genomic FOXP3 locus which is completely demethylated in nT_{reg} cells and only partially demethylated in iT_{reg} cells [86, 87].

Induction of iT_{reg} cells normally does not occur under promoting conditions like in nT_{reg} cells. The generaration of iTreg takes place in proiflammatory environments like in the gut that antagonizes their generation [88]. Therefore, in such conversion conditions other mediators like retinoic acid are favoring and augmenting iT_{reg} conversion. In addition, DCs under specific settings are important players in this situation. Gut and mesenteric lymph node $CD103^+$ DCs effectively induce T_{reg} cells via TGF-β and production of retinoic acid through metabolizing vitamin A [89, 90]. $CD8^+DEC205^+$ splenic DCs are another TGF-β provider for conversion of naïve $CD4^+$ to iT_{reg} cells [91].

In summary, although nT_{reg} and iT_{reg} cells express FOXP3 as a master transcription factor, they are different in many aspects regarding the site and the conditions of generation, the stability, and might even differ at a functional level as nT_{reg} cells mainly deal with self-antigen and iT_{reg} cells control the immune response to newly generated antigens.

1.3 Mechanism of T_{reg} cell suppression

Regulatory T-cells control immune homeostasis through the suppression of activation and proliferation of immunocompetent cells. So far several mechanisms of T_{reg} mediated suppression have been proposed. According to their main effector mechanisms they have been grouped into two categories namely cell-dependent and cell-independent. However, it is not yet clear whether the aforementioned mechanisms act synergistically or independently in response to a specific situation [6, 92, 93]. T_{reg} cells have to react in response to a variety of immunological contexts; hence it is reasonable to assume that no single mechanism could be applied to all situations. Therefore, the mechanism of suppression of T_{reg} cells might not be pre-determined but it is rather shaped by the local immune context. The targets of T_{reg}-cell mediated suppression are widely spread. T_{reg} cells can suppress both $CD4^+$ and $CD8^+$ activation, proliferation, and cytokine production [94-96]. Alterations in B-cell immunoglobulin production and class switching have been described as major effects exerted by T_{reg} cells [97, 98]. T_{reg} cells further inhibit cytolytic function of NK [99] and NKT-cells [100] and interfere with the activation and maturation of DCs [101]. The suppression exerted by T_{reg} cells requires T_{reg} cell activation via TCR ligation but the suppressive activity itself does not depend on antigen specificity [92].

1.3.1 Cell-to-cell contact dependent suppression

Early studies could show that physical contact between T_{reg} cells and effector cells is required for suppression at least *in vitro*. This suppression was abolished when the two cell populations were separated by a semi-permeable membrane [102]. Over the last years, many molecules have been associated with the contact dependent mechanism of T_{reg}-cell mediated suppression. For instance, CTLA-4 (CD152) has been shown to be critical for suppression. Blocking of CTLA-4 in normal mice led to multiorgan autoimmune disease through loss of T_{reg} cell suppressive activity [103, 104]. *In vivo*, T_{reg} cell specific CTLA-4 deficiency directly affected the suppressive activity of T_{reg} cells in the periphery while it had no effect on their thymic development [12]. CTLA-4 downmodulates DC stimulatory function via reduction of CD80 and CD86 expression. Furthermore, CTLA-4 induces indolamine-2,3-deoxygenase (IDO) expression which catalyzes the conversion of tryptophan to kynurenine and other metabolites. This metabolic change acts as a potent suppressive effect on the neighboring T-cells mediated by depletion of the essential amino-acid tryptophan and the accumulation of the immunomodulatory kynurenines [105-107]. Cytolysis is another potential mechanism for T_{reg}-cell mediated suppression. T_{reg} cells can express perforin and Granzyme B, therefore, the

intimate cell contact between T_{reg} cells and effector cells cannot only hamper the activation and proliferation of the effector cells but it might also result in cell death. Granzyme B/perforin mediated killing by T_{reg} cells was reported for $CD4^+$ and $CD8^+$ T-cells as well as NK cells [98, 108]. T_{reg} cells from Granzyme B deficient mice were less efficient than normal T_{reg} cells in their suppressive activity *in vitro* [108]. Metabolic disruption of target cells through two main mechanisms has also been proposed: release of cAMP and pericellular accumulation of adenosines. On the one hand, T_{reg} cells produce high levels of the second messenger cyclic adenosine monophosphate (cAMP). It inhibits the proliferation and IL-2 synthesis by effector T-cells. It has been reported that T_{reg} cells inject cAMP via a gap junction into responder cells [109]. On the other hand, adenosines are released into the extracellular space following cellular distress or injury and sensing of these molecules by immune cells can be either anti- or proinflammatory depending on the local microenvironment [110]. It has been shown that a subset of regulatory T-cells constitutively expresses CD39, the ectonuclase converting enzyme which hydrolyzes ATP. T_{reg} cells from CD39 deficient mice show an altered phenotype with a capability to proliferate under TCR stimulation without exogenous IL2 and impaired suppressive activity [111, 112]. The immunomodulatory effect of CD39 can be augmented in the presence of CD73 (ecto-5´-nucleotidase) which dephosphorylates AMP generating adenosine. The production of adenosine results in an inhibitory signal delivered to effector cells via activation of the A2A receptor upon binding of adenosine. DCs in particular might be the main target by this mechanism as pre-exposure of T_{reg} cells to ATP containing medium reduced ATP-driven DC maturation [112] .

Furthermore, the lymphocyte activation gene 3 (LAG-3) or (CD223) is a CD4-associated adhesion molecule that binds MHC-II. It is expressed on the surface of murine T_{reg} cells upon activation. LAG-3 neutralizing antibodies resulted in abrogation of T_{reg}-cell function *in vitro* and *in vivo* without any manifestation of overt autoimmunity. The absence of hyperimmune manifestations suggests that other modes of suppression might compensate for this deficiency [113]. Furthermore, T_{reg} cells isolated from LAG3 knock-out mice showed impaired suppressive activity *in vitro*. Ectopic expression of LAG-3 in naïve $CD4^+$ T-cells reduced their proliferation and rendered them suppressive. The binding of LAG-3 to MHC-II on immature DCs induces ITAM-mediated inhibitory signals that interfered with the maturating and co-stimulatory function of these cells [114].

1.3.2 Cell-to-cell contact independent suppression

Although the initial papers described cell-to-cell contact to be the dominant mechanism of suppression, the active involvement of many soluble mediators has gained substantial leverage over the last years. Several studies in the 1990s reported that both TGF-β and IL-10 are rarely found in the supernatant of T_{reg} cells in *in vitro* suppression assays and neutralization of these cytokines failed to abrogate the suppressive activity of T_{reg} cells [27, 115]. However, several studies have shown that both factors play an important role *in vivo*. Adoptive colitis in *RAG*$^{-/-}$ deficient mice could not be prevented by adoptive transfer of $CD4^+CD25^+CD45RB^{low}$ T_{reg} cells from IL-10 knockout mice [116]. Similarly, another study using a T_{reg}-cell-specific IL-10 knockout could show that the production of IL-10 by T_{reg} cells is essential for limiting active immune response at environmental interfaces such as skin and colon but it was not required for the control of systemic autoimmune responses [117]. In contrast to IL-10 deficient T_{reg} cells, T_{reg} cells isolated from TGF-β$^{-/-}$ mice showed normal suppressive activity and rescued *RAG2*$^{-/-}$ mice from developing adoptive colitis [118, 119]. These studies showed that autocrine production of TGF-β by T_{reg} cells might not be critical for their function. However, administration of an anti-TGF-β antibody to recipient mice resulted in abrogation of T_{reg}-cell mediated suppressive activity, suggesting that TGF-β is required for protection from inflammatory bowel disease (IBD) [120]. Over the last years it has become more and more clear, that TGF-β might mediate suppression through cell-to-cell contact via membrane bound complexes rather than acting as a humoral mediator [99, 121]. TGF-β is synthesized as a precursor molecule. Through proteolytic processing, active TGF-β is produced in association with latency associated peptide (LAP). LAP binds TGF-β forming a latent complex. Active TGF-β is produced by dissociation from this complex [122]. Indeed, a subset of T_{reg} cells expressing LAP has been described [123]. Membrane bound TGF-β might inhibit activation of effector cells via interaction and activation of the Notch/HES-1 axis [124]. However, neither active nor LAP-bound TGF-β can be detected on the surface of non-activated T_{reg} cells in mice and man [57]. However, a high percentage of activated T_{reg} cells could be stained with anti-LAP antibodies suggesting that LAP and possible also TGF-β could have a role in the suppressive function of activated T_{reg} cells [57]. In addition to IL-10 and TGF-β, T_{reg} cells produce the inhibitory cytokine IL-35 which is a member of the IL-12 family of cytokines and consist of a heterodimer of EBI3 and IL-12α/p35. Recent studies have shown that IL-35 is an active soluble mediator of T_{reg} cells with suppressive function in mice

and man [125, 126]. T_{reg} cells from $EBI3^{-/-}$ and $IL12A^{-/-}$ mice showed significantly reduced suppressive capacity *in vitro* and failed to control experimental colitis *in vivo* [126].

Consuming of growth factors during cell contact is another potential mechanism that mediates cell suppression or even induction of apoptosis. IL-2 is required for peripheral survival, activation, and function of T_{reg} cells and the main sources for IL-2 cells seem to be activated T-cells [127]. In addition to the inability of T_{reg} cells to produce IL-2, they express high levels of the high-affinity α-chain of the IL2 receptor (CD25) which might enable T_{reg} cells to consume local IL-2 and therefore induce cytokine-deprivation-mediated apoptosis of effector T-cells [128].

1.4 MicroRNA and T_{reg} cells

The importance of microRNAs for the biology of T_{reg} cells has been shown by deletion of DICER specifically in the T_{reg}-cell lineage [129, 130]. DICER is an RNaseIII-microRNA processing enzyme which is critical for miRNAs biogenesis. It is responsible for cleaving of dsRNA and pre-microRNA into about 20-25 nucleotides short ds-RNA [131]. Depletion of DICER in T_{reg} cells resulted in fatal early onset of lymphoprolferative autoimmune diesease, indistinguishable from *scurfy* mice [132, 133]. Although neither the development, proliferation, nor survival of thymic T_{reg} cells deficient in miRNAs were grossly perturbed, Dicer-deficient T_{reg} cells showed impaired homeostasis and lack of suppressive activity in the periphery [132]. The specific role of individual miRNAs in the function of T_{reg} cells has recently been addressed. T_{reg} cells have a distinct profile of miRNAs and many of these miRNAs are FOXP3-associated as the ectopic expression of FOXP3 in T_{conv} cells confered a partial T_{reg}-cell miRNAs profile [134]. The study of genome-wide FOXP3 target genes revealed that FOXP3 binds to intergenic regions, demonstrating that FOXP3 regulates the expression of non-coding RNA [135]. MiRNA-155 is a well known example of FOXP3-driven miRNAs, highly enriched in T_{reg} cells. It promotes cell homeostasis in competitive lymphopenic conditions as inflammation. Mechanistically, miRNA-155 increases the sensitivity of T_{reg} cells to IL-2 through targeting the SOCS1 gene in the presence of limiting amounts of IL-2 [136].

1.5 Forkhead Box P3 (FOXP3)

1.5.1 Molecular structure of FOXP3

FOXP3 is a member of the Forkhead/winged-helix family of transcription factors. It is the master regulator for the development and function of T_{reg} cells [51, 137]. It is highly conserved amongs humans, dogs, cats, mice, macaques, and cattle. The similarity of human and mouse FOXP3 protein is around 91% and the identity is 86% in their amino acids [138].

FOXP3 has three functional domains: a single C2H2 zinc-finger-like motif (amino acid 200-223) with unknown function, a leucine-zipper-like motif (amino acid 240-261) which is critical for homodimer formation, and carboxy-terminal forkhead domain (amino acid 338-421) which is critical for both DNA binding and nuclear localization [27, 139]. The N-terminal domain is rich in proline, acts as transcriptional repressor domain and interacts with histone deacetylase (HDAC) (Fig.1). In contrast to mice where only one functional isoform exists, two main functional isoforms of FOXP3 are expressed in humans. The main isoform is the full-length isoform, while the second isoform lacks the proline-rich exon 2, which encodes the Leu-X-X-Leu-Leu motif. This region interacts with and represses the retinoic acid-related-orphan receptor-α (ROR-α) and ROR-γt [140].

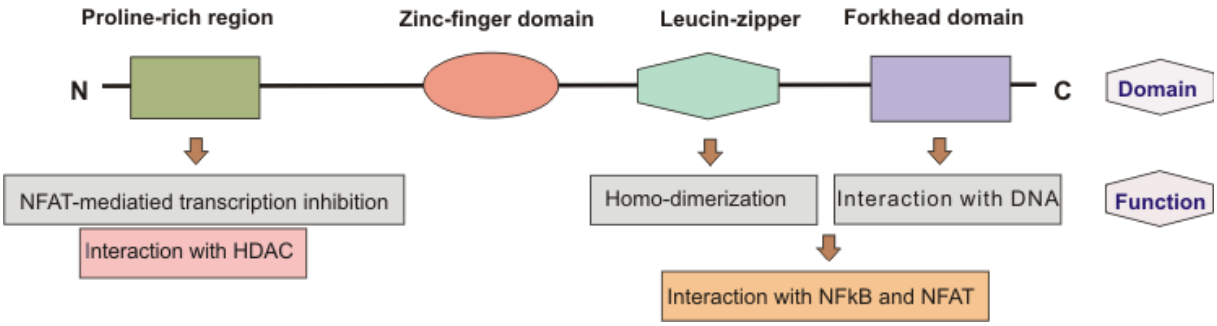

Figure 1. Schematic representation of the functional domains of FOXP3.

NFAT: Nuclear factor of activated T-cells, **HDAC**: Histone deacetylases, **NF-kB**: nuclear factor kappa-light-chain-enhancer of activated B cells. This figure is adapted from Sakaguchi *et al*., 2010 [27].

The identification of FOXP3 as a specific marker for T_{reg} cells followed the characterization of fatal autoimmune manifestations in male *scurfy* mice. Scurfy mice have an insertion in the FOXP3 gene that results in the induction of a premature stop codon and therefore a production of a truncated non-functional protein [141]. The importance of FOXP3 in controlling immune homeostasis has been shown experimentally in FOXP3-deficient mice.

Germ-line deletion of FOXP3 in mice resulted in a disease phenotype similar to scurfy [142-144]. The requirement of FOXP3 for T_{reg}-cell fate has attracted a lot of discussion until recently, when it was shown that the function of FOXP3 is not absolutely required for the commitment of developing thymocytes to the T_{reg}-cell lineage but it rather stabilizes and amplifies T_{reg}-cell function once T_{reg}-cell fate is determined [145, 146]. In humans, the deficiency of FOXP3 and thereby a loss of T_{reg} cells either in numbers or function underlies the lympho-proliferation and multi-organ autoimmunity of scurfy mutant mice and is linked with immunodysregulation, polyendocrinopathy, and the X-linked syndrome (IPEX) [141, 147, 148].

Together, these studies showed that FOXP3 is absolutely required for suppressive activity, proliferation, lineage stability, and metabolic fitness of T_{reg} cells [137, 141, 147, 149].

The complexity of the FOXP3-mediated control of the T_{reg} cell program has been studied over the last years. Genome wide analysis of the transcriptional program induced by FOXP3 has shown that it can act as an activator or repressor of gene expression [135, 139]. An elegant study used chromatin immunoprecipitation (ChIP) combined with tiling array or promoter array analysis revealed that 10% of FOXP3-dependent genes are directly regulated by FOXP3 [135]. It was also shown that FOXP3 might impart epigenetic marks on its target genes as the FOXP3 binding sites correlate with significant enrichment of permissive trimethyled histone3 lysine 4 (H3K4me3) and suppressive trimethyled histone3 lysine 27 (H3K27me3) markers [150]. Furthermore, a genome wide comparative analysis of FOXP3 occupancy in $FOXP3^{+}$ and $FOXP3^{-}$ cells supported the importance of FOXP3-mediated gene regulation in T_{reg} cells. This study showed that FOXP3 binds to around 700 genes and intergenically encoded miRNAs. In agreement with the previous study, gene expression analysis revealed that FOXP3 can activate or repress the transcription of target genes [135].

1.5.2 Regulation of FOXP3 expression

Rather unexpected, the promoter region of FOXP3 showed only weak promoter activity in reporter assays using a murine T-cell line [84, 151]. This interesting observation has suggested the involvement of other proximal regulatory elements in the induction of FOXP3 in T_{reg} cells. The regulatory regions of FOXP3 expression consist of a promoter, two enhancers or conserved non-coding sequence regions (CNS1 and CNS2) and a third conserved non-coding sequence region (CNS3) [152, 153]. Recent studies showed that FOXP3 induction is under synergetic control of signaling molecules downstream of several

pathways including TCR, co-stimulatory molecules and cytokines receptors [27, 84, 140, 151, 154-162]. These molecules mediate their action through binding to the regulatory elements within the FOXP3 genomic locus [163]. The FOXP3 promoter is activated in response to TCR signaling through binding of NFAT and AP-1 [164]. CNS1 contains binding sites for NFAT and Smad3 which are important for the induction of FOXP3 in peripheral naïve $CD4^+$ T-cells [84]. CNS2 and 3 are intronic regulatory regions which are not only sites for transcriptional regulation but CNS2 also harbors several CpG rich islands (Fig. 2) [86, 151]. AP-1, STAT5, and FOXO1 and FOXO2 act as positive transcriptional regulators through binding to CNS2, whereas STAT3 binding to CNS2 antagonizes FOXP3 expression [86, 140]. Similarly, c-Rel, an essential NF-kB family transcription factor, directly controls FOXP3 gene expression through biniding to CNS3 [153, 157, 165]. Demethylation of the CpG islands in CNS2 is a prequisite for the binding of interaction partners [153]. Taken together, several signaling molecules are actively involved in regulation of FOXP3 expression and the net effect of these interactions is either positive or negative depending on the interaction partners (Fig.2).

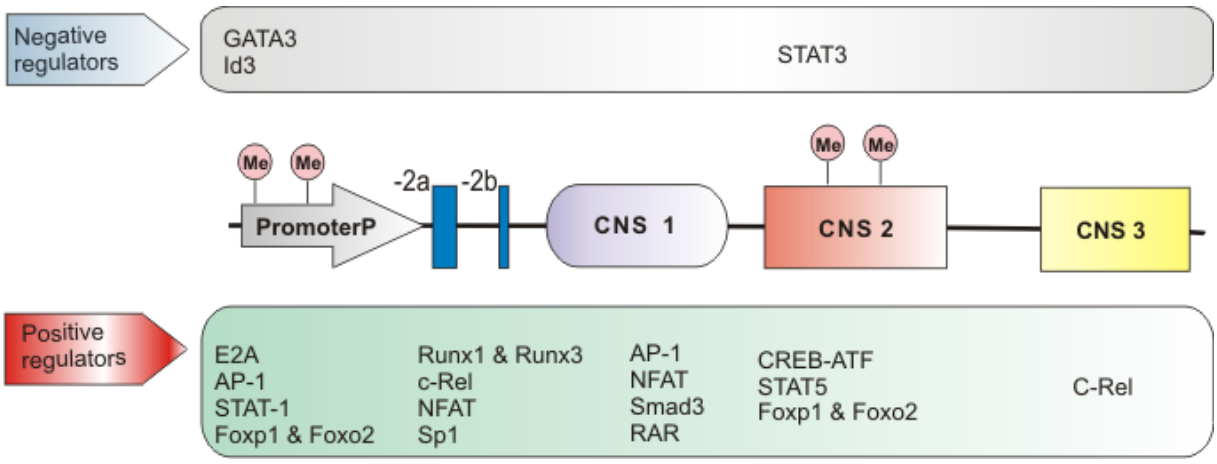

Figure 2. **Transcriptional regulation of FOXP3 expression.**

Regulatory elements within the genomic FOXP3 locus. The FOXP3 locus contains several moieties governing its expression: the FOXP3 promoter region, exons −2a, −2b, two enhancers (CNS1 and 2), and a conserved non-coding sequence 3 (CNS3). Depicted above and below the regulatory factors that either positively or negatively control FOXP3 expression .The diagram is adapted from Mashide Tone and Mark I. Greene, 2011 [155].

Additionally, epigenetic modifications of the FOXP3 genomic locus are an additional level of regulation. Both methylation of CpG residues and covalent post-transcriptional modification

of histone proteins are involved in this process [166]. The DNA methylation status of the proximal promoter and CNS2 correlates with the expression of FOXP3. The CNS2 region is called T_{reg}-cell specific demethylated region (TSDR) and was found to be fully demethylated in T_{reg} cells and methylated in T_{conv} cells [87, 167]. This difference in methylation of the TSDR has been regarded as an explanation for the preferential stability of FOXP3 expression in nT_{reg} cells rather than in iT_{reg} cells where the TSDR is only partially demethylated [86, 168]. Besides its methylation status, di- and trimethylation of histone 3 lysine 4 residue (H3K4) at the FOXP3 locus was reported to enhance FOXP3 induction upon TCR activation of naïve $CD4^+$ T-cells [134]. In addition to its methylation status, histone acetylation enhances gene accessibility and therefore gene expression [140]. Recruitment of histone deacetylases (HDACs) like HDAC7 or HDAC9 to the genomic FOXP3 locus inhibits it expression. Consistent with these findings, treatment of T_{reg} cells with the HDAC inhibitor like trichostatin A increases FOXP3 gene acetylation and results in enhanced T_{reg} cells function [169]. Taken together, accumulating data supports the involvement of methylation and histone modifications in the regulation of FOXP3 expression [170, 171].

In summary, the FOXP3 locus contains several regulatory elements that serve as a platform for the interaction of different transcription factors and epigenetic modifications. Consequently, FOXP3 expression is under tight control of various signal crosstalks that determine the fate of FOXP3 expression in terms of induction or repression.

1.6 Specific AT rich binding protein 1 (SATB1)

SATB1 was the first matrix attachment region (MAR) binding protein to be identified from a thymic cDNA expression library screened with a concatamer containing the nucleation site for unwinding of the 3`MAR flanking the IgH enhancer [172, 173]. MARs often contain base-unpairing regions (BURs) which are typically 100-150 bp regions within MARs, possess an intrinsic propensity to unwind under negative superhelical strain, and are considered to be hallmarks of MARs. MAR binding proteins bind via MARs to the DNA allowing binding to the nuclear matrix as a structural component inside the nucleus and thereby forming the looped chromatin structure.

SATB1 itself is highly conserved between vertebrates [174] and was among the first cell-type-restricted MAR binders [175]. It is expressed predominantly but not exclusively in thymocytes with additional expression in brain and testis as well as various cell lines [174]. SATB1 binds selectively and uniquely to AT-rich DNA reffered to as BUR dsDNA sequences

in the minor groove with little contact between the bases. It recognizes a specific DNA sequence context rather than a consensus DNA sequence. In general, SATB1 binds to promoters, intronic regions and gene loci [176].

1.6.1 Functional structure of the SATB1 protein

SATB1 is a 763 amino acid protein containing six functional domains: a nuclear localization signal (NLS), a PDZ-like domain, a Base Unpairing Region (BUR)-binding domain, two Cut repeats (CUT1 and CUT2), and a typical homeodomain (HD) in the distal protein region [95, 177-179]. PDZ is an acronym combining the first letters of three proteins: post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1) which first discovered to share the domain. These multiple domains are all necessary for SATB1 to excert its function. The NLS mediates SATB1 localization to the nucleus. The N-terminal PDZ-like domain is a protein-protein interaction domain found mostly in signaling proteins, necessary for dimerization of proteins [95, 177]. Hence, the PDZ-like domain provides the dimerization interface of SATB1. In addition, it allows SATB1 to interact with multiple protein partners including co-repressors and co-activators. Over the last years, it could be established that SATB1 has to dimerize in order to bind to DNA and that this homodimerization is mediated by the PDZ-like domain [95]. The CUT domains contain a DNA-binding motif. In addition, CUT1 and part of the CUT2 domain are part of the BUR-binding domain. The BUR domain is the module responsible for specific recognition of the BURs as opposed to any AT-rich sequence motif [96, 174]. Homeodomains are DNA-binding motifs typically found in transcription factors. [179]. The homeodomain of SATB1 together with the CUT repeat containing domains confer specific binding with high affinity to the core unwinding elements of BURs [180].

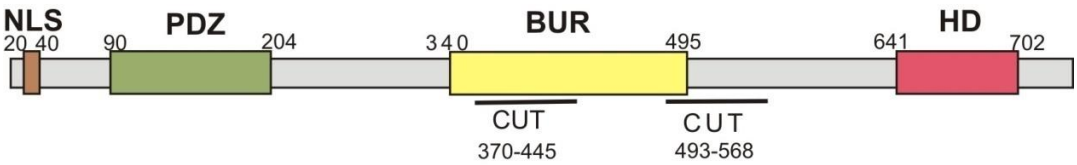

Figure 3. Illustration of the functional structure of SATB1.

NLS: nuclear localization signal, **PDZ**: *PDZ* is an acronym for a protein binding-domain combining the first letters of three proteins PSD95, Dlg1, and zo-1 which were first discovered to share the domain, **BUR**: Base Unpairing Region, **HD**: homeodomain. This figure is adapted from http://atlasgeneticsoncology.org/Genes/SATB1ID44225ch3p24.html.

1.6.2 SATB1 as a matrix binding protein

The organization of high-order chromatin is started at a basal level with packaging of genomic DNA into nucleosomes in 10 nm beads-on-a-string fibers. This is followed by the next level of organization by folding of nucleoprotein octameres (nucleosomes) into 30 nm fibers [181]. Chromatin compaction is further refined by loop formation which is independent of basal level packaging. The main players in formation and organization of loops are the nuclear matrix and matrix or scaffold attachment regions [181, 182].

The nuclear matrix is a non-chromatin structure which is composed of an insoluble fibrous network in the nucleus that provides an architectural support for high-order chromatin packaging and for the central processes of nucleic acid metabolism [183]. Furthermore, it is resistant to salt extraction and the majority of nuclear matrix composites are acidic and non-histone proteins [184].

MARs are sequences of DNA that exhibit high affinity for the nuclear matrix *in vitro* [181]. Organizing the genomic DNA into topologically distinct loops is the outcome of anchoring of the chromatin through MARs onto the nuclear matrix [185-187]. Co-localization or enrichment of MARs adjacent to regulatory elements is an indication of the importance of these sequences in regulation of gene replication and transcription [188, 189]. A set of characteristics are proposed for MAR sequences including their enrichment in inverted sequence repeats, AT tracts, DNA unwinding elements, DNAse I hypersensitivity sites, replication initiation sites, nucleosome free stretches, poly purine stretches, and motifs with potential for left handed and triplex structure [190, 191]. Another sub-specificity of MARs is small regions inside their sequences that reveal higher affinity to the isolated nuclear network *in vitro*, called BURs. BURs have a unique DNA content as they contain well mixed ATC sequences devoid of Gs. The importance of BUR sequences has been evaluated by mutation of their sequences which results in weak association with the nuclear matrix [172, 175].

The DNA binding proteins which bind directly to matrix associated region of DNA are called MAR binding proteins (MARBP). Several MARPBs have been characterized like SATB1, SATB2, BRIGHT, Cux/CDP, Lamin A/B/C, HMG, and SMAR1 [192-195]. It has been found that MARPBs are dynamic and their distribution is cell and cell cycle specific. This dynamic expression and distribution of proteins may dictate the functional state of the cell. The co-localization of MARPBs with many trans-activators and co-repressor has raised the question whether they directly or indirectly participate in gene regulation in addition to their role as

chromatin organizers. Various studies of different MARPBs have highlighted their influential role not only on gene expression but also on other important aspects of DNA biology such as translation and repair [188, 196]. In addition, MARPBs may directly modulate target gene expression or indirectly influence their transcription through recruitment and interaction with chromatin remodeling complexes. This modulation can result in either activation or repression of gene expression [192, 197, 198]. Another interesting aspect in the biology of these proteins is their ability to modulate gene expression over long distances which might range from a kilobase to hundreds of kilobases through forming chromatin loop domains that are important for organization of chromatin into units of genomic function [199, 200].

SATB1 is a prototypical example of one of these MARBPs. It functions as a global gene regulator and acts as a cis- or trans-acting factor over long distances of thousands of kilobases by reorganizing and modifying the chromatin into topologically distinct loop domains which are critical for bringing together different transcription complexes and coordinating their actions [95, 96, 201]. The expression of genes bracketed within SATB1 loops is either repressed or activated directly by influencing the promoter activity of these genes and indirectly by recruitment and interaction with chromatin remodeling complexes [190, 193, 202-206].

This interaction of SATB1 with different modifiers further controls the gene expression by inducing histone modifications, thus deciding its fate to act as repressor or an activator. It is reported that SATB1 overexpression in K562 cells increases ε-globin and decreases γ-globin gene expression accompanied by histone hyperacetylation and hypomethylation at the ε-globin promoter and hypersensitive site 2 HS2, and histone hypoacetylation and hypermethylation at the γ-globin promoter [198].

The importance of SATB1 for gene regulation has been shown in several publications [207-215]. SATB1 ablation by gene targeting led to a global gene dysregulation affecting hundreds of genes (around 2% of all T-cell genes). A genome wide expression approach using 19k cDNA microarrays identified 10% of the hybridized genes are either positively or negatively regulated by SATB1 [216].

How SATB1 switches between acting as a repressor or activator is an interesting phenomenon. It has been reported that SATB1 itself is subjected to post-translational modifications which act as molecular switches. Thereby SATB1 can acquire either activating or repressing functions through interacting and recruiting several chromatin remodeling

proteins. The choice of interaction partner is dependent on the phosphorylation and the acetylation status of the PDZ-like interaction domain in SATB1 [217-219]. Phosphorylation is correlated with gene repression, whereas acetylation is associated with release of repression [212, 215, 216, 220-222]. While phosphorylation of SATB1 regulates its mutual exclusive interaction with histone deacetylase 1 (HDAC1), acetylation directly affects SATB1 binding affinity to DNA. In the absence of activation, SATB1 is phosphorylated by PKC at S186 and shows higher binding affinity to DNA and association with HDAC1. Upon activation SATB1 is dephosphorylated, alternatively acetylated at K136 and the binding partners are replaced by the histone acetyltransferase PCAF [217]. This modification is negatively affecting the binding affinity to DNA and results in the release of HDAC1 from the complex [217].

1.6.3 SATB1 in T-cell biology

Generation of SATB1 null mice was the first evidence of the biological function of MARBP *in vivo* specifically in T-cells [223]. These mice exhibited neurological defects, reduced size of thymi and lymph nodes and succumbed to death after about three weeks later. Multiple defects at early stages of T cell development have been reported. Loss of SATB1 results in reduced number of immature $CD3^{-}CD4^{-}CD8^{-}$ triple negative (TN) thymocytes, and arrest of thymocyte development at the double positive (DP) stage, inappropriate migration of DP cells, increased apoptosis rates and impaired proliferation after activation. Ablation of SATB1 in thymocytes leads to temporal and spatial misexpression of numerous genes related to chemokines, cytokines, developmental surface markers, apoptosis, and tumor genes as assessed by gene expression profiling [223]. Overall more than 2% of T-cell genes were dysregulated with around 10% of genes either positively or negatively regulated by SATB1 [223].

SATB1 is one of the genes that show up-regulation after ligation of the TCR complex [224]. The mechanism of oscillation between activation and repression in order to explain how SATB1 represses gene expression has been shown for the expression of IL-2 in T-cells. In resting T-cells, IL-2 and IL2RA are repressed through occupancy of a SATB1 binding site (SBS) in both the IL-2 and IL-2R-α locus. However, activation of T-cells leads to changes in the loop structure and a loss of the SATB1 occupancy within the IL-2 and IL2RA locus which culminates in the derepression of IL-2 and IL-2R-α expression [217]. Similarly, SATB1 has been linked to both repression and derepression of gene expression during Wnt signaling in T-cells [215]. Many genes are repressed by SATB1 when it is bound to DNA in close proximity

with c-terminal binding protein (CtPB) and this repression is dependent on the interaction of CtPB with HDAC1 [221]. Acetylation of SATB1 and binding of PCAF reverses gene repression by disruption of the SATB1-CtPB interaction and recruitment of coactivators [212, 215].

The role of SATB1 in regulation of T-cell specific genes in differentiated T helper cells has been described in a Th2 clone [211]. The Th2 specific cytokine genes are arranged in a cluster of around 200 kb and this cluster consists of the genomic loci of IL-4, IL-5, IL-13, Rad50 and Kif3a [225]. Several important regulatory elements have been identified including specific hypersensitivity sites and conserved non-coding sequences (e.g. CNS1 and CNS2) which are critical for the expression of these genes [226]. Various cis-acting factors including GATA3 and STAT6 have been shown to control the expression of these genes through directly influencing their expression or recruitment of different chromatin remodellers and histone modifiers [227]. The importance of a locus control region (LCR) in coordinating expression of Th2-cell specific interleukins but not Rad50 through conformational changes in the formation of higher order chromatin structure was reported [228]. The involvement of the LCR in coregulation of these genes however does not explain the coregulation of the IL-5 locus as the LCR interacts with the IL-4 and IL-13 but not the IL-5 locus [229]. Furthermore, it was shown that SATB1 in Th1 cells is important for packaging of the Th2 locus and regulation of cytokine gene expression [211].

SATB1 interaction at the Th2 locus has resulted in a model that explains how a set of genes are organized in a poised chromatin conformation to which all necessary transcription factors and regulatory element are recruited. *In vitro*, nine SATB1 binding sites (SBS) were identified in a BAC clone encompassing the Th2 cell cytokine locus. SATB1 folds the chromatin across the Th2 cytokine locus by anchoring BURs within the cluster. After resting, these Th2 cells revealed 3D loops that involved only the two distal SBSs, CNS1, the IL-5 promoter, and the 3´region of the LCR. Whereas after activation, large numbers of smaller sized loops are formed and additional matrix-associated elements are involved in the formation of the loop structure such as an additional SBS, the IL-13 promoter, CNS2, and the IL-4 promoter. Moreover, H3K4/14 acetylation was observed at most sites and colocalization of GATA3 with SATB1 upon activation could be detected. These modifications at the Th2 locus after activation result in the coordinated expression of IL-4, IL-5, and IL-13 [211].

Furthermore, SATB1 is an important factor in the differentiation of Th2 cells. SATB1 mediates this role by regulating GATA3 expression and thereby regulating the expression of the Th2 cytokine signature in a Wnt/β-catenin dependent manner. SATB1 enhances the expression of GATA3 through cooperative interaction of SATB1 and β-catenin directly at the GATA3 promoter. Blocking of Wnt signaling or silencing of β-catenin drastically reduced the expression of GATA3 and subsequently the production of Th2 cell specific cytokines. It has been shown that SATB1 competes with T cell factor (TCF) for β-catenin and recruits β-catenin to its genomic locus. Therefore, genes formerly repressed by SATB1 are upregulated by Wnt signaling. SATB1 deacetylation occurring upon Wnt/β-catinin signaling is associated with increased occupancy of SATB1 on genomic targets and altered histone actylation of H3K9 on its targets. This increased binding of SATB1 to genes is mirrored by increased levels of β-catenin on the same gene as SATB1 recruits β-catenin to DNA. Once this complex of SATB1 and β-catenin is formed, β-catenin can recruit additional partners to potentiate gene expression and thereby switch SATB1 into a transcriptional activator [215]. Taken together, SATB1 regulates GATA3 expression in Th2 lineage differentiation by regulating Wnt/β-catenin signaling.

The specific role of SATB1 in CD8 development was studied in mice engineered to express reduced SATB1 in T-cells under the control of a CD2 cassette. This allowed to study the influence of SATB1 in single positive cells which was not possible in SATB1 null mice as thymocyte development is arrested at the DP stage [230]. These mice showed a reduced $CD8^+$ SP T-cell population with enhanced expression of CD3. L2a is a cis-acting DNA element with properties of a MAR acting as a potential silencer for CD8α expression. It could be shown that SATB1 and CDP/Cux are interacting with L2a. The outcome of this interaction on gene expression is dependent on the interaction partner. Binding of SATB1 to the L sub-motif of L2a results in displacement of CDP/Cux and correlates with enhanced CD8α expression and development of $CD8^+$ T-cells [230].

Interestingly, a link between SATB1 and the phenomenon of co-receptor reversal in SP $CD8^+$ T-cell lineage development had been proposed. The co-receptor reversal model proposes that DP thymocytes that have selectively terminated CD8 transcription can be signaled by IL-7 to differentiate into $CD8^+$ T-cells by silencing CD4 transcription and reinitiating CD8 transcription. It was shown that thymocytes from SATB1 null mice that have selectively terminated CD8 expression failed to reinitiate CD8 transcription in the presence of IL7 whereas cells from wild type mice re-expressed CD8 co-receptor again. How SATB1 is

getting involved in this process is not clear at the molecular level. It is reported that a ~1.5 kb sub-region of the E8III enhancer necessary for CD8 expression was sufficient to promote co-receptor reversal during positive selection of DP thymocytes. The authors hypothesized that SATB1 recruits a chromatin modeling complex to the p12 site within the E8III enhancer [231].

Finally, a recent study has linked SATB1 to T_{reg} cells where gene expression data clearly showed that SATB1 is differentially repressed in T_{reg} cells and suggested a functional consequence for this repression in comparison to T_{conv} cells [232].

In summary, T-cell thymic development and differentiation is a highly coordinated phenomenon. The transition from a developmental phase to another is mediated by interaction between several effector molecules. SATB1 as a global genome organizer is an active regulatory molecule in T-cell biology. SATB1 is not only required at the early stages of T-cell development in the thymus but its significance extends to the peripheral T-cell homeostasis as well. The importance of SATB1 in this context has been shown in differentiation and cytokine production of Th2 T-cells where SATB1 coordinates genes expression through organization of regulatory chromatin loopscapes.

2 Objectives

Regulatory T-cells (T_{reg} cells) are the stronghold of peripheral tolerance through modulation and control of activation, proliferation, and function of effector cells under both homeostatic and pathogenic conditions. The function and phenotype of T_{reg} cells depends to great extent on their unique transcriptional profile and its master regulator, the transcription factor FOXP3. One of the major functions of FOXP3 is to modify the transcriptional landscape of T_{reg} cells antagonizing effector programs while preserving their T-cell identity. Despite their key importance for T_{reg} cell function and identity the molecular mechanisms that allow FOXP3 to suppress effector programs in T_{reg} cells are only partially understood.

The main aim of this study was to gain a better understanding of the molecular mechanisms that mediate the inhibitory functions of FOXP3 over proinflammatory transcriptional programs. In initial experiments, we identified the chromatin modifier and transcription factor SATB1 as an interesting candidate that showed a low expression in T_{reg} cells in comparison to T_{eff} cells. We hypothesized that SATB 1 is an effector molecule whose expression is repressed by FOXP3. In order to prove this hypothesis we tested whether the SATB1 locus is under the control of FOXP3, therefore we undertook an in silico approach to find FOXP3 binding motifs at the SATB1 locus followed by experimental validation via FOXP3-ChIP tilling arrays, qRT-PCR, functional assays,and filter binding assays. We also evaluated post-transcriptional control of SATB1 mediated by FOXP3 dependent miRNAs in addition to epigenetic modifications. As a next step, we evaluated the effects of overexpression of SATB1 on the transcriptional program of T_{reg} cells. Finally, we evaluated the functional consequences of SATB1 overexpression for T_{reg} cells in vivo and in vitro.

3 Materials

3.1 Chemicals and reagents

Agar	Applichem, Darmstadt, GER
Agarose	Applichem, Darmstadt, GER
Ammonium persulfate (APS)	Sigma-Aldrich, St Louis, USA, USA
Ampicillin	Applichem, Darmstadt, GER, GER
BCA protein assay kit	Pierce, Rockford, US
Bovine serum albumin (BSA)	Sigma-Aldrich, St. Louis, USA
Brefeldin A	Sigma-Aldrich, St. Louis, USA
Bromophenole blue	Roth, Karlsruhe, GER
β-Mercaptoethanol	Sigma-Aldrich, München, GER
CFSE	Sigma-Aldrich, München, GER
Chloroform	AppliChem, Darmstadt, GER
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, München, GER
Dithiothreitol (DTT)	Fermentas GmbH, GER
Ethanol	Roth, Karlsruhe, GER
Ethidium bromide	AppliChem, Darmstadt, GER
Ethylendiamintetraacetat (EDTA)	Sigma, St Louis, US
ExVivo 15	Lonza, Basel, CH
Fetal calf serum (FCS)	Invitrogen Life Technologies, Karlsruhe, GER
Ficoll-Paque PLUS	Amersham, Piscataway, US
Formaldehyde	Sigma-Aldrich, München, GER
GeneRuler1 kb Plus DNA Ladder	Fermentas GmbH, St. Leon-Rot, GER
Glutamax	Invitrogen Life Technologies, Karlsruhe, GER
H_2O (sterile)	Fresenius Kabi AG, Bad Homburg, GER
Human Pancoll	PAN BiotechGmbH, Aidenbach, GER
Hydrochlorid acid (37 %)	Merck, Darmstadt, GER
Ionomycin	Sigma-Aldrich, St. Louis, USA
Isopropanol	Roth, Karlsruhe, GER
Laemmli buffer for SDS-PAGE (10 x)	Serva Electrophoresis GmbH, Heidelberg, GER
Methanol	Roth, Karlsruhe, GER
Milk Powder (Blotting Grade)	Roth, Karlsruhe, GER
Monensin	Sigma-Aldrich, St. Louis, USA

N,N,N',N'-Tetramethylethylenediamine	AppliChem, Darmstadt, GER
Odyssey 4X Protein Loading Buffer	Licor Biosciences, Bad Homburg, GER
Odyssey Blocking Buffer	Licor Biosciences, Bad Homburg, GER
Odyssey Two-Colour MW Marker	Licor Biosciences, Bad Homburg, GER
Paraformaldehyde (PFA)	Sigma-Aldrich, München, GER
PBS	PAA Laboratories GmbH, Pasching, AT
Penicillin/Streptomycin	PAA Labotories GmbH, Pasching, AT
Phorbol Myristate Acetate (PMA)	Sigma-Aldrich, St. Louis, USA
Re-Blot plus mild solution	Millipore, Temecula, USA
RNeasy MinElute Cleanup Kit	Qiagen, Hilden, GER
RPMI 1640	Invitrogen Life Technologies, Karlsruhe, GER
Running buffer 20x	Invitrogen Life Technologies, Karlsruhe, GER
Sodium chloride(NaCl)	Roth, Karlsruhe, GER
Sodium hydroxide (NaOH)	Merck, Darmstadt, GER
Sodiumdodecylsulfate (SDS)	AppliChem, Darmstadt, GER
TRIS (hydroxymethyl)-aminomethane	Carl Roth GmbH Co KG, Karlsruhe, GER
Tris	Applichem, Darmstadt, GER
Tris-HCl	Applichem, Darmstadt, GER
Triton X-100	Promega Corporation, Madison, US
TRIzol Reagent	Invitrogen Life Technologies,Karlsruhe, GER
Trypan blue (0.4%)	Merck, Darmstadt, GER
Tryptone	Roth, Karlsruhe, GER
Yeast extracts	Applichem, Darmstadt, GER

3.2 Cytokines

Interleukin 2 (IL-2)	Immunotools, Friesoythe, GER
TGF-β1	PeproTech EC Ltd., London, UK

3.3 Equipments

AutoMACS Pro Separator	Miltenyi Biotech, Berg. Gladbach, GER
BD FACSAria III flow cytometer	BD Biosciences, Heidelberg, GER
BD LSRII flow cytometer	BD Biosciences, Heidelberg, GER
BioDoc Analyze	Biometra, Jena, GER
Centrifuge 5415R	Eppendorf GmbH, Hamburg, GER
Centrifuge 5810R	Eppendorf GmbH, Hamburg GER
Centrifuge Mikro-200R	Hettich, Tuttlingen, GER
Centrifuge Rotina 420R	Hettich, Tuttlingen, GER
Dynal MPC 15 Magnet	Dynal Biotech, Hamburg, GER
Electroporator	Amaxa, Köln, GER
Eppendorf Concentrator Plus	Eppendorf GmbH, Hamburg, GER
Magnet MACS Multi Stand	Miltenyi Biotech, Berg. Gladbach, GER
Mini Trans-Blot system	BioRad Laboratories, München, GER
Mini-Protean Electrophoresis System	BioRad Laboratories, München, GER
NanoDrop 2000	Thermo Scientific, Rockford, USA
Odyssey Infrared Imaging System	Licor Biosciences, Bad Homburg, GER
PowerPac HC Power Supply	BioRad Laboratories, München, GER
Real-time PCR system LightCycler 480	Roche Diagnostics, Basel, Switzerland
Spectrophotometer NanoDrop 2000	Thermo Scientific, Waltham, USA
SRT9D Roller Mixer Stuart	Bibby Scientific Ltd, Staffordshire, UK
Synergy HT Multi-Mode Micro-plate Reader	Bio-Tek Instruments GmbH, GER
Thermomixer comfort	Eppendorf GmbH, Hamburg , GER
T-Professional Basic Gradient PCR Cycler	Biometra, Jena, GER
Trans-Blot Semi-Dry Transfer Cell	Bio-Rad Laboratories, München , GER
Vortexer	Velp Scientifica, Usmate, Italy
Water bath	Memmert, Schwabach , GER

3.4 Antibodies

3.4.1 Antibodies for Western blotting

Antigen	Species	Distributor
Anti-*β*-actin (primary antibody)	mouse	Chemicon, Temecula (US)
SATB1	mouse	BD Biosciences, USA
Anti-mouse IgG IRDye 680 (secondary antibody)	goat	Licor Biosciences, Bad Homburg (USA)
Anti-mouse IgG, IRDye 800CW (secondary antibody)	goat	Licor Biosciences, Bad Homburg (USA)

3.4.2 Antibodies for flow cytometry

Antigen	Clone	Isotype	Fluorophore	Manufacturer
Anti-human-CD127	hIL-7R-M21	Mouse $IgG_{1,}\kappa$	Alexa Fluor 647	BD Biosciences, USA
Anti-human-FOXP3	206D	Mouse $IgG_{1,}\kappa$	PE	BioLegend, USA
Anti-human SATB1	Alexa Fluor 647	Mouse $IgG_{1,}\kappa$	Alexa Fluor 647	BD Biosciences, USA
Anti-human-CD25	M-A251	Mouse $IgG_{1,}\kappa$	APC	BD Biosciences, USA
Anti-human-CD25	M-A251	Mouse $IgG_{1,}\kappa$	FITC	BioLegend, USA
Anti-human-CD25	M-A251	Mouse $IgG_{1,}\kappa$	PE	BD Biosciences, USA
Anti-human-CD25	2A3	Mouse $IgG_{1,}\kappa$	PE-Cy7	BD Biosciences, USA
Anti-human-CD3	SK7	Mouse $IgG_{1,}\kappa$	APC-Cy7	BD Biosciences, USA
Anti-human-CD3	SK7	Mouse $IgG_{1,}\kappa$	FITC	BD Biosciences, USA
Anti-human-CD3	UCHT1	Mouse $IgG_{1,}\kappa$	PE	BD Biosciences, USA
Anti-human-CD4	SK3	Mouse $IgG_{1,}\kappa$	FITC	BD Biosciences, USA
Anti-human-CD4	SK3	Mouse $IgG_{1,}\kappa$	PE	BD Biosciences, USA
Anti-human-CD4	SK3	Mouse $IgG_{1,}\kappa$	PE-Cy7	BD Biosciences, USA
Anti-human-CD4	L200	Mouse $IgG_{1,}\kappa$	PerCp-Cy5.5	BD Biosciences, USA
Anti-human-CD45RA	L48	Mouse $IgG_{1,}\kappa$	FITC	BD Biosciences, USA

Anti-human-CD45RA	HI100	Mouse $IgG_{2b}\kappa$	PE-Cy5	BioLegend, USA
Anti-human-CD45RO	UCHL1	Mouse $IgG_{2b}\kappa$	APC	BD Biosciences, USA
Anti-human-CD45RO	UCHL1	Mouse $IgG_{2b}\kappa$	PE	BD Biosciences, USA
Anti-human-CD56	MY31	Mouse $IgG_{1,}\kappa$	PE	BD Biosciences, USA
Anti-human-CD8	SK1	Mouse $IgG_{1,}\kappa$	APC	BD Biosciences, USA
Anti-human-CD8	SK1	Mouse $IgG_{1,}\kappa$	PerCP	BD Biosciences, USA
Anti-human-CD8	SK1	Mouse $IgG_{1,}\kappa$	PE	BD Biosciences, USA
Anti-mouse-CD4	GK1.5	Rat IgG_{2b},k	PerCp-Cy5.5	BioLegend, USA
Anti-mouse-CD25	PC-61	Rat IgG_1, λ	Alexa Fluor 647	BioLegend, USA
Anti-mouse-CD3	500A2	Syrian hamster IgG_2,k	V500	BD Biosciences, USA
Anti-mouse-CD62L	MEL-14	Rat IgG2a,k	PE-Cy7	BioLegend, USA
Anti-mouse-CD8	53-6.7	Rat IgG_{2a},k	Pacific Blue	BioLegend, USA
Anti-mouse-CD90.1	OX-7	Mouse IgG1, κ	V450	BD Biosciences, USA
Anti-mouse-Foxp3	MF-14	Rat IgG_{2b},k	Alexa Flour 488	BioLegend, USA
Isotype Control	MOPC-21	Mouse $IgG_{1,}\kappa$	Alexa Fluor 647	BD Biosciences, USA
Isotype Control	MOPC-21	Mouse $IgG_{1,}\kappa$	PE	BD Biosciences, USA
Isotype Control	MOPC-21	Mouse $IgG_{1,}\kappa$	Alexa Fluor 488	BD Biosciences, USA
Isotype Control	MOPC-21	Mouse $IgG_{1,}\kappa$	PerCp-Cy5.5	BioLegend, USA
Isotype Control	MOPC-21	Mouse $IgG_{1,}\kappa$	Pacific Blue	BioLegend, USA
Isotype Control	MOPC-21	Mouse $IgG_{1,}\kappa$	PECy7	BioLegend, USA

3.5 Buffers and media

50 x TAE buffer

242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH=8, ad 1 l H_2O

ACK lysis buffer:

0.15 M NH4Cl, 10.0 mM KHCO3, 0.1 mM Na2EDTA,

pH=7.2-7.4 adjusted with 1 N HCl, solution was sterile filtered

10 x TBS

12.14 g/l Tris-HCl, 87.66 g/l NaCl

5 x Laemmli loading buffer

0.3 M Tris-HCl pH6.8, 50% glycerol, 25 % β-Mercaptoethanol, 2 % SDS,

0.01 % bromophenol blue

Lysis buffer

20 mM Tris-HCl pH=8, 10 % Triton X-100, 100 mM NaCl,

1 mM EDTA, 1 M DTT, a Complete Mini Protease inhibitor tablet

MACS buffer

1 x PBS supplemented with 0.5 % BSA, 2 mM EDTA, pH=7.2 sterile-filtered

4.5 % stacking gel

1.83 ml H_2O_{dest}, 0.83 ml 1M Tris-HCl (pH6,8), 25 µl 10 x SDS,

0.42 ml 30 % Acrylamide, 17 µl 10 % APS, 3.3 µl TEMED

10 % separating gel

2.01 ml H_2O_{dest}, 1.25 ml 1.5 M Tris-HCl (pH8,8), 50 µl 10x SDS,

1.67 ml 30 % polyacrylamide, 16.65 µl 10 % APS, 7 µl TEMED

4 % native polyacrylamide gel (for 20 ml)

2.5 ml 40% polyacrylamide (Polyacrylamide-BIS ratio =29:1),

1 ml 1M Tris (pH=7,5), 3.8 ml 1 M Glycine, 80 µl 0.5M EDTA,

13 ml H_2O, 100 µl, 10 % APS, 15 µl TEMED

Western blot transfer buffer (for semi dry blotting)

25 mM Tris, 192 mM glycine

Western blot transfer buffer (for wet blotting)

4.8 mM Tris-Base, 3.9 mM glycine, 20 % methanol

SOB-medium

5 g yeast extract, 20 g trypton, 0.6 g NaCl, 0.2 g KCl ad 1 l H_2O, Medium was autoclaved and further supplemented with 10 ml of a 1 M $MgCl_2$ solution and 10 ml of a 1 M $MgSO_4$ solution before use.

SOC-medium

SOB-medium was supplemented with 20mM glucose solution

LB growth medium

10 g/l Bacto-tryptone, 5 g/l Bacto yeast extract, 10 g/l NaCl

3.6 Disposables

6-well tissue culture plates	Sarstedt, Nürnbrecht , GER
12-well tissue culture plates	Sarstedt, Nürnbrecht , GER
24-well tissue culture plates	Sarstedt, Nürnbrecht , GER
48-well tissue culture plates	Sarstedt, Nürnbrecht , GER
96-well tissue culture plates	Sarstedt, Nürnbrecht , GER
10 cm plates	Sarstedt,Nürnbrecht , GER
LightCycler 480 Multiwell Plate	Roche Diagnostics, Basel, Switzerland
0.5-2.0 ml reaction tubes	Eppendorf GmbH, Hamburg , GER
0.2 ml PCR reaction tubes	Eppendorf GmbH, Hamburg , GER
15 ml Falcon tubes	Sarstedt, Nürnbrecht , GER
50 ml Falcon tubes	Sarstedt, Nürnbrecht , GER
MACS LS columns	Miltenyi Biotech, Bergisch Gladbach , GER
MACS LD columns	Miltenyi Biotech, Bergisch Gladbach , GER
MACS MS columns	Miltenyi Biotech, Bergisch Gladbach , GER
Nitrocellulose-membrane Hybond-C Extra	GE healthcare, Piscataway, USA
Parafilm	Pechiney, Chicago, USA
2, 5, 10 & 25 ml Pipettes	Sarstedt, Nürnbrecht , GER
10, 200 & 1.000 µl Pipette tips	Sarstedt, Nürnbrecht , GER
Pre-Separation Filters	Miltenyi Biotech, Bergisch Gladbach , GER
10, 20, 100, 200 & 1.000 µl Filter Tips	Starlab, Ahrensburg , GER
0.2 µm Sterile filter	Sartorius, Hannover , GER
2, 5, 10, &50 ml Syringe	Braun, Melsungen , GER

3.7 Software

BD CBA	BD Biosciences, Heidelberg, GER
CorelDRAW X4	Corel Corporation, USA
Endnote X5	Thomson Reuters, Carlsbad, USA
FACSDiva	BD Biosciences, Heidelberg, GER
FlowJo 7.5 and later	Tree Star, USA

LightCycler 480 Software	Roche Diagnostics GmbH, Mannheim, GER
Mircosoft Office 2007	Microsoft Deutschland GmbH, GER
Odyssey V3.0 software	Licor Biosciences, GER
SigmaPlot 10.0	Systat Software GmbH, GER
Vector NTI	Invitrogen Life Technologies, Karlsruhe, GER

3.8 Kits and reagents

Amaxa Human T Cell NucleofectorKit	Lonza Cologne AG, Köln, GER
BCA protein assay kit	Thermo Scientific, Rockford, USA
BLOCK-iT™ miR RNAi Select	Invitrogen Life Technologies
$CD4^+$ T cell isolation Kit II	Miltenyi Biotech, Bergisch Gladbach, GER
cDNA Synthesis Kit	
Conventional and FastDigest-restriction enzymes	Thermo Scientific, GER
DNA loading dye 6X	Thermo Scientific, GER
Dual-Luciferase® Assay System	Promega, Mannheim
Dynalbeads®CD3/CD28 T Cell Expander	Dynal Biotech, Oslo, NO
FOXP3 Fix/Perm Buffer Set	BioLegend, San Diego, USA
Gateway® LR Clonase™ Enzyme Mix	Invitrogen Life Technologies
GeneJET™ Plasmid Miniprep Kit	Fermentas GmbH, St. Leon-Rot, GER
LightCycler 480 Probe Master Kit	Roche Diagnostics, Basel, Switzerland
MicroBeads $CD25^+$	Miltenyi Biotech, Bergisch Gladbach, GER
MicroBeads $CD45RA^+$	Miltenyi Biotech, Bergisch Gladbach, GER
Midi Plasmid HiSpeed prep kit	Qiagen, Hilden, GER
QIAquick Gel Extraction Kit	Qiagen, Hilden, GER
QIAquick PCR Purification Kit	Qiagen, Hilden, GER
QuickChange Lightning Multi-Site-Directed Mutagenesis kit	Agilent Technologies, USA
RosetteSep (CD4+ T cell enrichment kit)	Stem Cell Technologies, Grenoble, France
T4 DNA Ligase	Thermo Scientific, GER
T4 Polynucleotide Kinase	New England Biolabs, UK
TaqMan MicroRNA assay	Applied Biosystems, USA
TaqMan® Lightcycler Kit	Roche Diagnostics GmbH, Mannheim, GER
Transcriptor First Strand	Roche Diagnostics GmbH, Mannheim, GER

Turbofect *in vitro* Transfection Reagent	Thermo Scientific, GER

3.9 Bacterial strains

BL-20	Agilent Technologies, USA
DH5 α	Invitrogen Life Technologies
One Shot Stbl3	Invitrogen Life Technologies
One Shot TOP10	Invitrogen Life Technologies
XL-1 blue	Invitrogen Life Technologies
XL-10 gold	Agilent Technologies, USA

3.10 Plasmids

pGL4.24	Promega Corp., Madison, USA
pGL4.72	Promega Corp., Madison, USA
pcDNA6.2-GW/EmGFP-miR	Invitrogen Life Technologies
pDONR221	Invitrogen Life Technologies
pLenti6.3/V5-DEST	Invitrogen Life Technologies
pLVTHM	Addgene, USA
pMD2-G	Addgene, USA
pCMVRΔ8.74	Addgene, USA
psiCHECK-II	Promega Corp., Madison, USA
pIRES	Clontech Laboratories, Inc

4 Methods

4.1 Mouse strains

C57BL/6 mice were purchased from Jackson Laboratory. *RAG2*$^{-/-}$, *DEREG*, *scurfy* and *DEREG x scurfy* mice were previously described [233-236]. Mice were housed under specific pathogen-free conditions at TWINCORE, Hannover, the Centre for Experimental and Clinical Infection Research or at the LIMES Institute, University of Bonn and used in accordance with the German legislation governing animal studies following the principles of laboratory animal care (NIH publication No. 85-23, revised in 1996).FOXP3-GFP-hCre BAC Dicer$^{lox/lox}$ mice and FOXP3-GFP-hCre BAC Dicer$^{lox/lox}$ ROSA26R-loxP-Stop-loxP-YFP mice have been previously described [237]. Mice were housed and bred under specific pathogen-free conditions at the UCSF Animal Barrier Facility of the University of California, San Francisco. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco

4.2 Cell culture

4.2.1 Human Peripheral Blood Mononuclear Cell (PBMC) isolation

All human cell populations in this study were purified from whole blood of healthy human donors in compliance with institutional review board (IRB) protocols. PBMCs were isolated from human buffy coats. Buffy coats were diluted 1:2 with PBS, then 35 ml was over-layered on 13 ml of Pancoll (PAA Laboratories GmbH, Austria), and centrifuged for 30 min at room temperature at 1000x g without break. PBMCs were then collected and washed twice with PBS and used for further experiments.

4.2.2 Human CD4$^+$ T-cell isolation (Rossettesep)

For the isolation of human CD4$^+$ T-cells each ml of buffy coat was mixed with 40 µl CD4$^+$ antibodies (STEMCELL technologies Inc, Canada) and incubated at room temperature for 20 min. Then the cells were diluted 1:2 with PBS. Every 35 ml of diluted cells was over-layered on 13 ml of Pancoll, centrifuged for 30 min at room temperature at 1000x g without break. CD4$^+$ T-cells were collected from the interface and washed two times with PBS. Afterwards, purity of the cells was assessed by flow cytometry after staining of CD3, CD4, and CD8 with fluorescently conjugated antibodies.

4.2.3 Human CD25$^+$ T-cell isolation

To isolate human T_{reg} cells, 10^7 $CD4^+$ Tcells were incubated with 10 µl of $CD25^+$ microbeads II for 20 min (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were then washed with MACS buffer. $CD4^+$ $CD25^+$ T_{reg} cells were enriched by magnetic separation using either a MIDI-Macs equipped with LS columns or the AutoMACS pro with the "possls" program. Cell purity was routinely determined by flow cytometry (staining cells with CD3, CD4, CD25, CD127 antibodies) and was always higher than 90%.

4.2.4 Human naïve CD4$^+$CD25$^-$CD45RA$^+$ T-cell isolation

PBMCs isolated from human buffy coats were isolated as described above. To islate human naïve T-cells the naïve T-cell isolation kit was used (Milteny Biotec). Therefore 40 µl of Biotin-antibody cocktail II were mixed with 10^7 PBMCs and incubated for 25 minutes at 4°C. After incubation the cells were washed two times and re-suspended in MACS buffer 80 µl per 10^7 cells. The magnetic labeling was done by adding 20 µl of anti-biotin micro-beads per 10^7 cells. Incubation was performed for 20 minutes at 4^oC followed by washing for two times. Each 10^8 cells were resuspeded in 500 µl MACS buffer then enriched by depletion using either a MIDI-Macs equipped with LS columns or the AutoMACS pro with the "depletes" program. The enrichment of naïve T-cells was assessed after stainig of CD3, CD4, CD45RA, and CD8 using flow cytometry.

4.2.5 In vitro generation of human iT$_{reg}$ cells

Human naïve $CD4^+CD25^-CD45RA^+$ T-cells were purified from buffy coats of healthy human donors as described above. Naïve $CD4^+$ T-cells (5 x 10^4 cells per well) were stimulated in serum-free Aim-V/X-Cell (50%/50% V/V) medium with 5 x 10^4 magnetic beads per well coated with 5% CD3 (OKT3, Ortho Biotech), 12% CD28 (9.3), and 83% anti-MHC-I (W6/32) monoclonal antibody and TGF-β(R&D systems, 5 ng/ml) for a period of 5 days in the presence of IL-2 (200 IU/ml). The described composition of beads was optimized for the induction of iT_{reg} cells. The FOXP3 induction was examined by flow cytometry after intracellular staining of FOXP3 as described below relative to its induction in the absence of TGF-β.

4.2.6 Human T$_{reg}$-cell expansion

Human T_{reg} and T_{conv} cells were isolated by negative selection using CD4-RosetteSep (Stem Cell), followed by positive-selection using CD25-specific MACS beads (Miltenyi Biotech).

Isolated cells were stained with combinations of fluorochrome-labeled monoclonal antibodies to CD3, CD4, CD25, and CD127 at 4°C for 30 min. The cells were then sorted on a FACSAria III cell sorter (Becton Dickinson) by gating on the $CD3^+CD4^+CD25^{high}CD127^{low}$ population. The purity of the isolated cell was assessed after sorting and was always above 98%. The sorted cells were then stimulated with Human T_{reg} Expander DynalBeads (Invitrogen, USA) at bead-to-cell ratio of 4:1 in the presence of 1000 IU IL-2/ml. The expanding time was between 2–3 weeks with addition of IL-2 every 3 days and restimulation of the expanding cells with beads every 8-10 days. After expansion, FOXP3 expression of the T_{reg}-cell expansion cultures was assessed by flow cytometry and usually was above 90%.

4.2.7 siRNA-mediated gene silencing

siRNA were obtained either form Dharmacon, Thermo Scientific or synthesized by biomol GmbH, Germany. Upon delivery siRNA was reconstituted in siRNA buffer (Dharmacon, Thermo Scientific, Belgium) at a final concentration of 100 µM. Then each pair of siRNA was annealed at 90ºC for 5 min and allowed to cool down to room temperature over 45 min. Annealed siRNAs for target genes were then pooled and stored at -80°C.

For gene silencing, freshly isolated conventional $CD4^+$ T-cells or T_{reg} cells were transfected using the human T-cells nucleofector kit (Lonza AG, Germany) according to the manufacturer's instructions. Around 5-10 x 10^6 freshly isolated T-cells were mixed with 1000 pmol of gene specific or control siRNA, mixed and 100 µl of the nucleofector solution provided with the kit was added. Next, the mixture was transferred into an Amaxa certified cuvette. The transfection was performed with the U-14 program. To enhance the cell survival 500 µl of pre-warmed medium was added and the transfected cells were transferred into a 12-well plate. The cells were rested overnight in 5% CO_2 at 37°C and restimulated with CD3/CD28-coated beads the next day for 2-3 days. The efficiency of gene silencing was assessed on RNA and protein level after 48-72 hrs by qRT-PCR, immunoblotting, and flow cytometry.

4.2.8 Isolation of murine $CD4^+$ T-cells

Murine $CD4^+$ T-cells were isolated from mouse spleen and lymph nodes. First, single cell-suspensions were prepared by smashing tissues in a cell strainer inside a petri dish using a syringe plunger then sieving the cells through a 70 µm cell strainer to obtain single cells. The pelleted cells were resuspended in 1-2 ml of gentle hypotonic solution (ACK) for 1-2 min to

lyse erythrocytes. The lysis buffer was removed by washing the cells with 30-40 ml of PBS. Trypan blue staining was used to determine the total number and viability of isolated cells.

Next, the single cell suspension from spleen and lymph nodes was used to isolate $CD4^+$ lymphocytes by depletion of non-$CD4^+$ cells using the mouse $CD4^+$ T cell isolation kit (Miltenyi Biotec). CD4 negative cells were labeled with a cocktail of biotin-conjugated monoclonal antibodies. Then these labeled cells were further stained by coupling them to anti-biotin labeled MicroBeads. $CD4^+$ T-cells were collected in the negative elution fraction. In brief, according to the manufacturer's protocol 10^7 mouse cells were resuspended in 40 µl of MACS buffer then incubated with 10 µl of antibodies cocktail for 10 min followed by addition of an extra volume of 30 µl of buffer in addition to 20 µl of MicroBeads, mixed and incubated for 20 min. All incubations were performed at 4°C. The cell suspension was washed and resuspended in 500 µl of buffer per 10^8 cells. The separation was performed with a MIDI-Macs equiped with LS columns or the AutoMACS pro with the "Deplete" program.

4.2.9 Isolation of murine $CD4^+$ $CD45RB^{hi}$ T cells

Single cell suspensions from mice spleen and lymph nodes were prepared as described above. Afterwards, $CD4^+$ cells were enriched using the mouse-$CD4^+$ T cell isolation kit (Miltenyi Biotech). Enriched $CD4^+$ T-cells were stained with a combination of monoclonal antibodies against CD4, CD25, and CD45RB. Naive $CD4^+CD45RB^{hi}$ T-cells were sorted with a FACSAria III (Beckton & Dickinson).

4.2.10 Mouse T_{reg} cell expansion

Mouse DEREG $CD4^+$ T-cells were isolated as described above then cells were stained for CD3, CD4, and CD25 with fluorescently labeled antibodies. The stained cells were sorted on the $CD3^+CD4^+CD25^{high}GFP^+$ population using a FACSAria III. After sorting, the cells were expanded with Mouse T-Activator CD3/CD28 DynalBeads (Invitrogen, USA). Briefly, four beads were used per cell in addition to IL-2 (1000 IU/ml). IL-2 was added evey 3 days. The beads were changed on day 8-11 and novel beads added at a 1:1 bead per cell ratio. The cells were expanded for 2-3 weeks. The expression of FOXP3 was monitored by intracellular staining and by assessing expression of GFP.

4.2.11 Cell viability and counting

The number of cells was determined using trypan blue based exclusion method and expressed as number of cells per ml. Therefore, cells were diluted with 0.4% tryban blue and loaded into a haemocytometer (Neubaur, Assistent, Germany).

Cell number was calculated using the following equation:

Cell number = number of living cells x dilution factor x volume x 10^4

4.2.12 Intracellular staining

Intracellular staining was used to stain either nuclear or cytoplasmic proteins. The FOXP3 staining kit (Biolegend Inc, USA) was used according to the manufacturer's instructions. Briefly, the cells were first stained for extracellular markers then washed. Fixation was performed for 20 min at room temperature then the fixative was removed by washing. Next, the cells were permeabilized for 15 min with a subsequent washing step. The cells were resuspended in permeabilization buffer and incubated with blocking antibodies (murine CD16/CD32 or human irrelevant IgG) for 10 min. The staining was performed with the required amount of antibodies for 30 min in the dark at room temperature. Finally, cells were washed and resuspended in 200 µl of PBS. Samples were analyzed by flow cytometry within 18 hrs and data were analyzed using FlowJo software.

4.2.13 Generation of high titer lentiviral stocks

Lentivirus-containing supernatants were generated by co-transfection of plasmids encoding vector components into the highly transfectable HEK-293T cell line. Next ultracentrifugation was used to generate stock solutions of highly concentrated virus.

One day before transfection, 3-4 x 10^6 HEK293T-cells were plated on 10 cm petri dishes. On the next day, lentiviral vectors were co-transfected with helper constructs using lipofection with TurboFect (Fermentas, Thermo Scientific, Germany) according to the manufacturer's instructions. The medium was exchanged 4-6 hrs after transfection. The plates were incubated for 72 hrs for virus production. On the third day, the supernatant was collected into 50 ml falcon tubes and centrifuged for 10 min. The virus-containing supernatant was passed through a 0.45 µm filter and transferred into autoclaved ultracentrifugation tubes (Beckman Coulter, Optima, LE-80K). The tubes containing viral supernatants were centrifuged at 82,000xg at

16°C for 140 min. The supernatants were decanted and the pellets were resuspended with the remaining medium and an additional 200 µl of PBS. The concentrated virus stock was stored at 4^{o} C for a short storage period or alternatively at -80°C for long-term storage.

Virus titers were determined by transduction of HEK293T using polybrene with serial dilutions of viral stock. The efficiency of transduction was determined by flow cytometry either directly for GFP encoding virus or after staining for reporter genes like Thy1.1. The dilution resulting in less than 10% transduced cells was used for the calculation of the multiplicity of infection (MOI) - the number of viral particles per cell - according to the following formulas:

$$\text{Number of transducing units} = \frac{\text{Number of plated cells x Percentage of transduced cells}}{\text{Volume of added supernatant}}$$

$$\text{Multiplicity of Infection (MOI)} = \frac{\text{Number of transducing units (TU) deposited in a well}}{\text{Number of target cells present in that well}}$$

4.2.14 Transduction of lymphocytes

Human or mouse $CD4^{+}$ T cells were stimulated overnight with CD3/CD28-coated beads before transduction. The cell concentration was kept at 10^{6} per ml of medium with stimulating beads and 6 µg/ml polybrene at the day of transduction. Spinoculation was used to infect the cells with lentiviral constructs (MOI 20-50). The cells were centrifuged for 90 min at 30°C then left in 5% CO_2 at 37°C for 3-5 hrs. Then transduction media was removed and the cells were washed twice. The transduced cells were resuspended in fresh complete media and incubated for three days with CD3/CD28-coated beads and 100 IU/ml IL-2. Transduction efficacies were estimated using the expression of reporter genes which were also used for purification of transduced cells on a FACS Aria III. The level of gene expression was determined at protein and mRNA levels relative to its expression in control vector transduced cells and untransduced cells.

4.2.14.1 miRNA-mediated gene silencing in human T_{reg} cells

Human T_{reg} cells were expanded and transduced as as described above. The transduced cells were sorted on GFP expression and used for further analysis as summarized in Figure 4.

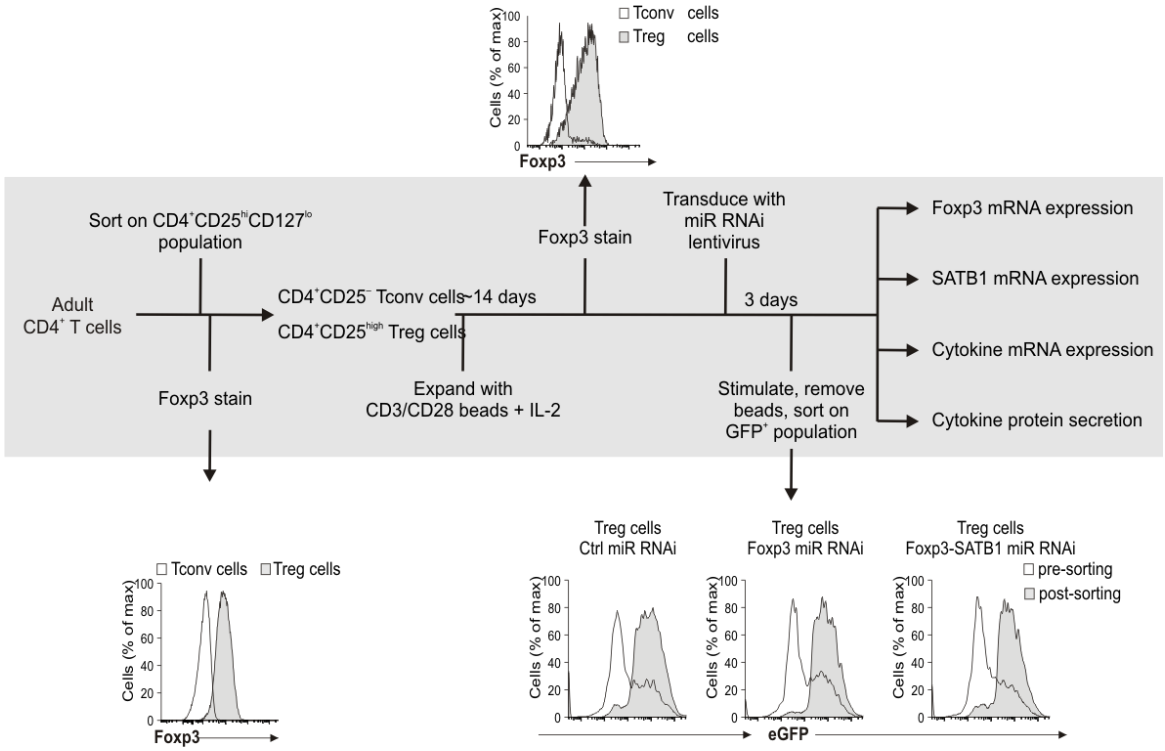

Figure 4. Experimental protocol for combined silencing of SATB1 and FOXP3 in expanded human T_{reg} cells with artificial miRNA.

FACS-sorted human $CD4^+CD25^{high}CD127^{low}CD45RA^+$ T_{reg} cells were expanded for 14 days with CD3 and CD28-coated Treg-cell expanding beads in the presence of 1000 IU/ml IL-2. After this initial expansion, T_{reg} cells were lentivirally transduced in the presence of 6 µg/ml polybrene with miR-RNAi against FOXP3, FOXP3 and SATB1, or control plasmids containing EmGFP and expanded for 3 additional days in the presence of CD3 and CD28-coated beads and IL-2. Cells were sorted on EmGFP-positivity using a FACS Aria III sorter (with cells showing highly similar EmGFP expression used for further analysis.

4.2.14.2 Transduction of human T_{reg} cells with SATB1

To assess the influence of SATB1 on T_{reg} cell phenotype and function, lentiviral overexpression of SATB1 in human T_{reg} cells was done (all experiements were performed in the lab of James Riley, Perelman School of Medicine, University of Pennsylvania). Therefore, expanded human T_{reg} cells were transduced with pELNS lentivirus encoding for full-length SATB1 and DsRED as reporter. Co-expression of full length SATB1 and DsRED at a 1:1 ratio was achieved using the 2A peptide (Fig. 5). After sorting of cells on DsRED-positive cells, these were used for further experiments.

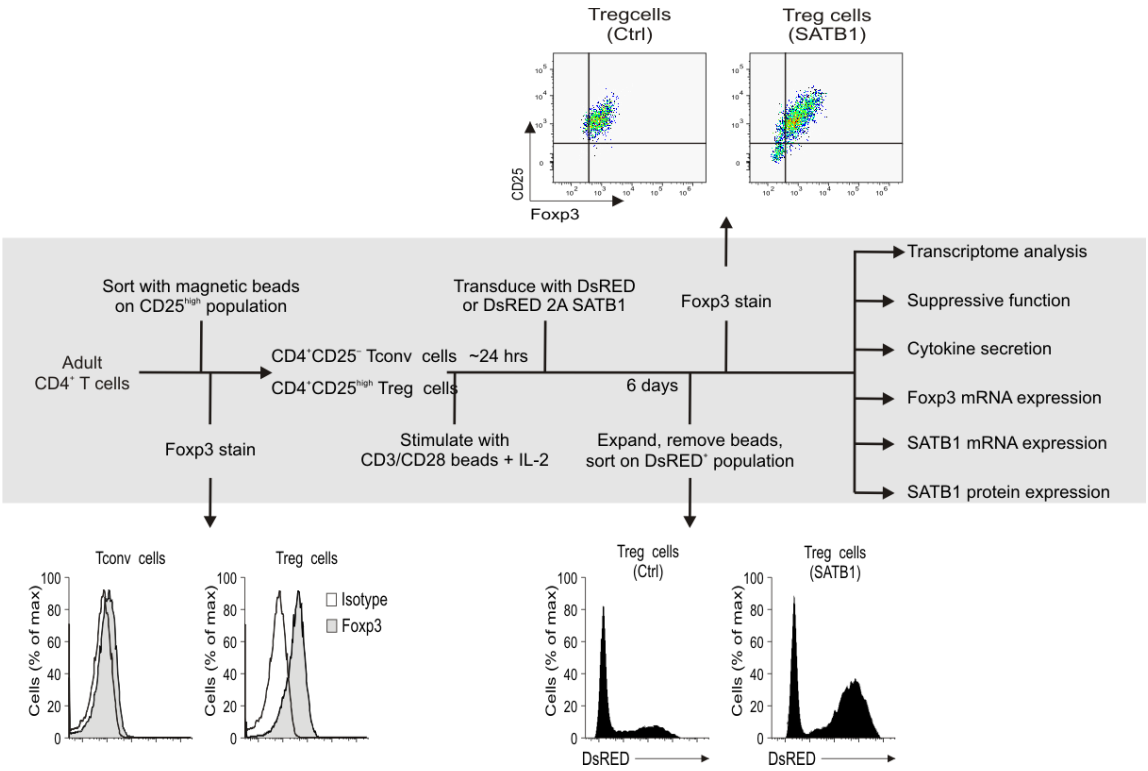

Figure 5. Experimental protocol for overexpression of SATB1 in primary human T_{reg} cells.

MACS-isolated human $CD4^{+}CD25^{high}$ T_{reg} or $CD4^{+}CD25^{-}$ T_{conv} cells were stimulated overnight with CD3 and CD28-coated beads in the presence of IL-2. After this initial stimulation, T_{reg} cells were lentivirally transduced with pELNS DsRED 2A SATB1 or control plasmids, , expanded for 6 days in the presence of CD3 and CD28-coated beads and IL-2. Cells were sorted for DsRED-positivity using a MoFlo sorter (DakoCytomation) with cells showing highly similar DsRED expression used for further analysis.

4.2.14.3 Transduction of murine T_{reg} cells with SATB1

Expanded murine T_{reg} cells from DEREG mice were transduced with either pLVTM lentiviral vectors encoding for SATB1 and Thy1.1 or Thy1.1 alone. Co-expression of full length SATB1 and Thy 1.1 as a reporter protein was achieved using an internal ribosomal entry site (IRES). The transduction was performed with 20-50 MOI in the presence of CD3 and CD28 coated beads, 1000 IU/ml IL-2, and 6 µg/ml polybrene. In order to enhance the infection process, the cells were centrifuged for at 30°C for 90 min followed by incubation for 4 hrs. After incubation, the cells were washed twice and then resuspened in complete medium containing IL-2 and incubated for 3-4 days. Only T_{reg} cells overexpressing SATB1 were used for downstream experiments by sorting on co-expression of GFP as reporter for FOXP3 and Thy1.1. The level of overexpression was assessed at protein level by intracellular staining and on mRNA level using qRT-PCR. The experimental setup is further outlined in Figure 6.

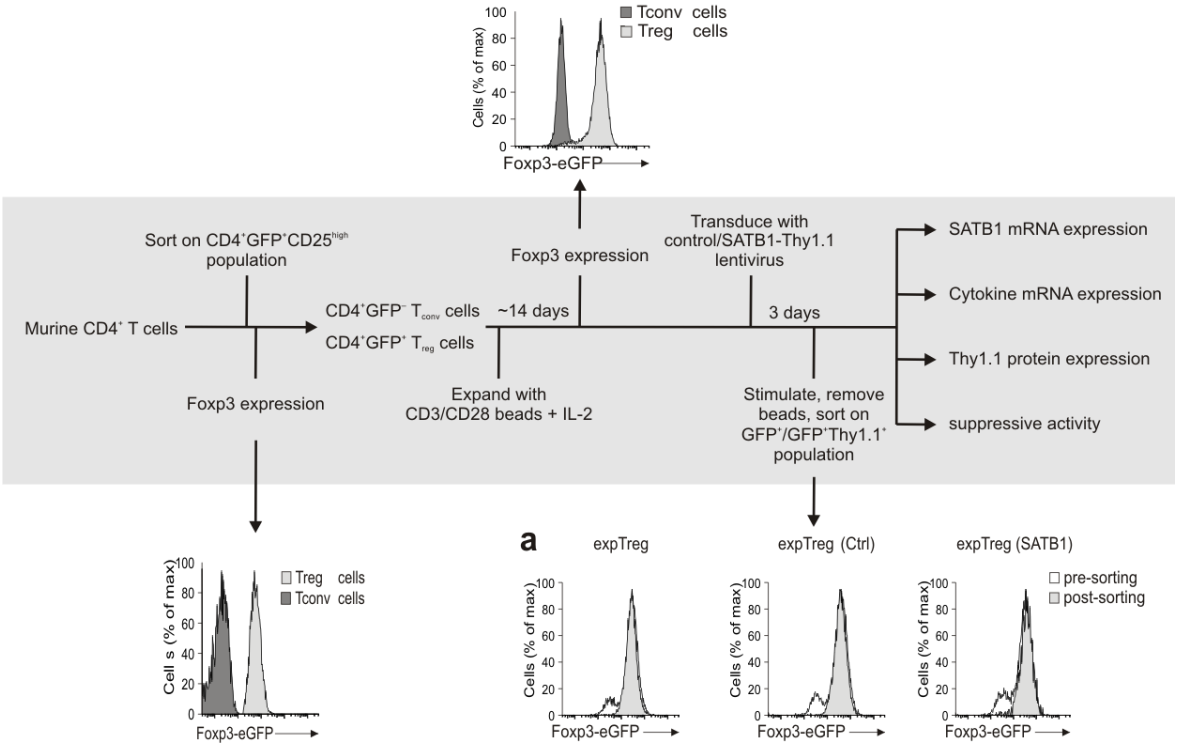

Figure 6. Experimental protocol for overexpression of SATB1 in murine T_{reg} cells.

FACS sorted murine $CD4^+GFP^+$ T_{reg} cells from DEREG mice were expanded for 10-14 days with CD3/CD28-coated beads in the presence of 1000 IU/ml IL-2. After this expansion period, T_{reg} cells were transduced with pLVTHM-SATB1-IRES-Thy1.1 or control lentivirus and cultivated in the presence of CD3/CD28-coated beads and IL-2 for 3-4 days. SATB1-Thy1.1-transduced, control-transduced or non-transduced expanded T_{reg} cells were sorted on GFP and Thy1.1 expression or GFP expression alone on a FACS Aria III (BD).

4.2.15 Proliferation assay

Amine interacting dyes were used to monitor cell division. CFSE (Carboxyfluorescein diacetate succinimidyl ester, Molecular probes, Invitrogen, USA) was used in most experiments. Labeling was done by mixing 10^7 cells in 1 ml of PBS with CFSE dye for 8 min at room temperature in the dark (final concentration 0.5-1 µM). The reaction was then quenched with 10 ml of FCS and washed twice with complete medium. Proliferation of cells was measured by flow cytometry.

Cell proliferation dye eFlour 670 (eBioscience, Inc, USA) was used to monitor the proliferation of GFP-expressing cells where usage of CFSE was not applicable. Shortly, 5 µM of the dye was used to stain 10^7 cells at 37°C for 10 min in the dark. The labeling was stopped by adding ice cold FCS followed by washing two times with complete medium.

In both cases, the labeled cells were stimulated after staining with CD3/CD28 coated beads for 72 hrs. Labeled but unstimulated cells were also included as reference population. FlowJo software (Tree Star, USA) was used to analyse the data.

4.2.16 Suppression assay

Responder cells (conventional $CD4^+$ T-cells or $CD8^+$ T-cells) were labeled either with CFSE or Cell Proliferation Dye eFluor 670 as described above. In a 96 well-plate, fixed numbers of labeled responder cells (5-10 x 10^4) were co-cultured with different ratios of shortly stimulated and unlabeled T_{reg} cells (1:1 through 8:1). The cells were then stimulated with anti-CD3/CD28 beads for 72 hrs at a ratio of 1:1 responder cells. The proliferation of responder cells was measured by flow cytometry. Analysis was performed with FlowJo software using unstimulated labeled responder cells as a negative control and the stimulated responder cells without T_{reg} cells as 100% proliferation. Suppression was expressed as a reduction in the percentage of proliferation of the labeled responder cells.

4.2.17 Detection of cytokines using cytometric bead arrays (CBA)

Cytokines were quantified in supernatants with multiplex cytokine detection systems which allows for the simultaneous detection of multiple cytokines (human cytometric bead array Th1/Th2 cytokine kit, BD Biosciences, or human or mouse Th1/Th2 11-plex FlowCytomix multiplex kits, eBiosience, USA) according to the manufacturer's instructions. Beads with two different sizes and with distinct fluorescence intensities have been coated with capture antibodies specific for the respective cytokines. PE conjugated detection antibodies are used to quantify the interaction between antibody-bead complexes and respective cytokines. The intensity of fluorescence of each sandwich complex is proportional to the concentration of the corresponding cytokine. In brief, the supernatants of cells are thawed at room temprature. Next, the bead populations in addition to the PE detection reagent were mixed together and incubated with serially diluted standards or cell culture supernatants for 3 hours at room temperature protected from light. The beads were washed and resuspended in 300 µl of PBS. The data were analysed either with FCAP array software (Soft Flow Inc., Pecs, Hungary) or with FlowCytomix Pro Software (eBioscience).

4.2.18 In vivo assessment of SATB1 overexpression in murine T_{reg} cells

4.2.18.1 Induction of colitis by adoptive transfer of naïve $CD4^+$ T-cells

After isolation of naïve $CD4^+CD45RB^{hi}$ T-cells from wild-type mice by magnetic isolation of $CD4^+$ T-cells and sorting of CD45RB highly expressing $CD4^+$ T-cells on a FACS Aria III, *RAG2*$^{-/-}$ mice were injected by tail vene injection with 6 x 10 cells. While the first group of animals only received naïve T-cells, the second group of animals was transferred additionally with 2 x 10^5 freshly isolated T_{reg} cells. The third group of animals additionally received 2 x 10^5 expanded and sorted T_{reg} cells expressing the control plasmid. The fourth group additionally received 2 x 10^5 $Thy1.1^+$ T_{reg} cells overexpressing SATB1.
The recipient mice were weighed prior T cell transfer and subsequently three times per week. They were observed for clinical signs of illness, including hunched over appearance, piloerection of the coat, and diarrhea. Diseased animals were sacrificed 9 weeks after T cell transfer.

4.2.18.2 Microscopic and macroscopic characterization of colitis model

In order to assess the distribution of transplanted cells, spleens, peripheral and mesenteric lymph nodes were isolated from reconstituted *RAG2*$^{-/-}$ mice from each mouse in each group. Single cell suspensions were obtained as described above. The isolated cells were stained, characterized and quantified using BD counting beads by flow cytometery.

To examine the characteristic histological changes of colitis, the animals were sacrificed and the colons were excised and fixed in 10% neutral buffered formalin. The samples were routinely processed, sectioned at 5 µm thickness, and stained with hematoxylin and eosin (H&E) for light microscopic examination. The histological sections and analysis were performed by Dr. Claudia Wickenhauser (Leipzig University Hospital, Leipzig).

4.3 Biochemistry and molecular biology

4.3.1 Cell lysis and immunoblotting

Total cell lysates were prepared by lysing cells in lysis buffer containing protease inhibitors (Roche Applied Science, Switzerland). Cell lysates were incubated on ice for 30 min, centrifuged at 12000 g, and the supernatant transferred to a new tube. The total concentration of protein contents was determined using the BCA assay kit (Thermo Scientific, USA) according to the manufacturer's recommendations. Immunoblots were performed with 20-50

µg of total protein. The protein was denatured with Laemmli buffer at 90°C for 5 min. The mixture was loaded on SDS-PAGE gels (Bio-Rad Laboratories, France) and run at 80 V for 20 min then at 160 V for 1 hr. Proteins were transferred either by wet or semi-dry blotting onto a nitrocellulose membrane (Amersham Bioscience, UK). After blotting, the membrane was blocked with Odyssey blocking buffer (LI-COR Bioscience, USA) for 1 hr. Next, the membrane was incubated with primary monoclonal antibodies diluted in Odyssey blocking buffer at 4°C overnight. The blot was washed with PBS plus 0.2% Tween-20 three times. Next, the membrane was incubated with IRDye secondary antibodies diluted in Odyssey blocking buffer for 1 hr followed by washing as described above. The membrane was scanned using the Odyssey imaging system (LI-COR Biosciences, USA). Expression of β-actin was used as an internal control after stripping of the membrane according to the manufacturer's protocol (Amersham, UK).

4.3.2 Immunofluorescence microscopy

Thymocytes from *DEREG* mice or $CD4^+$ GFP^+ T_{reg} cells from female heterozygous *DEREG x scurfy* mice isolated by sorting using a FACS Diva cell sorter were provided by Katharina Lahl and Tim Sparwasser (Institute of Infection Immunology, Hannover Medical School). These slides were stained and analysed by Eva Schönfeld and Thomas Quast. Briefly, after centrifugation the cells fixed in 4% cold paraformaldehyde for 10 min, washed with PBS, permeabilized with Triton-X and pre-blocked in PBS containing 10% normal goat serum and 1% gelatine from cold water fish skin for 30 min. Slides were then incubated in combinations of primary antibodies (rabbit anti-GFP, mouse anti-FOXP3, mouse anti-SATB1-AF647) for 60 min, washed twice, and incubated with secondary antibodies (anti-rabbit-AF488, anti-mouse-AF555) for 60 min, stained with DAPI and immunofluorescence was examined using an Olympus FluoView FV1000 or Zeiss LSM 5 LIVE confocal microscope.

4.3.3 Chromatin Immunoprecipitation

4.3.3.1 Genome-wide analysis of FOXP3 binding

Genome-wide FOXP3 binding sites were identified by Simon Barry and Timothy Sadlon (Molecular Immunology Laboratory, University of North Adelaide, Australia). The detailed method and analysis was described previously [238]. In brief, cord blood $CD4^+CD25^+$ T_{reg} and $CD4^+CD25^-$ T_{conv} cells were isolated from purified mononuclear cells using the regulatory $CD4^+CD25^+$ T Cell Kit (Invitrogen, USA). The *ex vivo* expansion was performed as described

above at a bead-to-cell ratio of 3:1. Expanded cord blood T_{reg} cells were restimulated with 1 mM ionomycin (Sigma-Aldrich, St. Louis, MO) for 2 hrs. Isolated T-cells were cross-linked for 10 min in 1% formaldehyde solution. Formaldehyde was quenched by the addition of glycine. Anti-FOXP3 or control rabbit IgG was used to precipitate cross-linked protein-DNA complexes from nuclear lysates. The cross-linking of the immunoprecipitated material was removed and the material was proteinase K treated, and the DNA was purified and amplified. The resultant material was labeled and hybridized to Affymetrix Human Tiling 2.0R arrays. Data from two independent ChIP-on-chip experiments were analyzed as replicates using model-based analysis of tiling-arrays (MAT, Model-based Analysis of Tiling-array) [239] to identify the locations of binding sites for FOXP3. Significantly enriched regions in FOXP3 binding relative to input chromatin were identified with a false discovery rate (FDR) of 0.5%. Gene accession numbers were assigned to an individual ChIP region if the peak of the enriched region was within 20 kb upstream of a transcriptionstart site (TSS) or 20 kb downstream of the transcription end site. Annotation was performed using gene accession numbers both from the University of California, Santa Cruz and the National Center for Biotechnology Information.

4.3.3.2 Analysis of histone modifications at the SATB1 locus

To study differences in histone modifications at the SATB1 locus in T_{reg} and T_{conv} cells chromatin immunoprecipitations of histone proteins was performed by Wolfgang Krebs. In brief, expanded T_{reg} and T_{conv} cells were harvested on day 14, treated with MNase to generate approximately 80% mononucleosomes and 20% dinucleosomes. Chromatin from 2.5×10^6 cells was used for each ChIP experiment, which yielded approximately 500 pg of DNA. Antibodies against histone H4Ac, H3K4me3 and H3K27me3 (Millipore) were used. Analysis of histone binding to the genomic SATB1 locus was carried out by ChIP-qRT-PCR. Reactions were performed using SYBR green qRT-PCR master mix (Fermentas, Thermo Scientific, Germany). The relative enrichment of target regions in histone immunoprecipitated material relative to input chromatin analysis was carried out using the $2^{-\Delta\Delta CT}$ method. Immunoprecipitations using control IgG were used to normalize for non-specific background.

4.3.4 Bisulphite sequencing

To assess and compare the methylation status of the SATB1 locus in T_{reg} and T_{conv} cells. human T_{reg} and T_{conv} cells were purified by negative selection using RosetteSep (Stem Cell) followed by sorting on a FACSDiVa cell sorter (Becton & Dickinson) at the Institutes of

Molecular Medicine and Experimental Immunology (IMMEI), University Hospital Bonn, after incubating cells with a combination of fluorochrome-labeled monoclonal antibodies to CD4, CD25, and CD127. Genomic DNA was isolated using phenol/chloroform extraction. Sodium bisulphate treatment of genomic DNA results in the deamination of unmethylated cytosines to uracil, whereas methylated cytosines remain unchanged. After amplification, PCR products were purified and sequenced in both directions. Bisulphate sequencing and analysis of the CpG islands was perfomed by Dr. Andreas Waha, University Hospital Bonn.

4.3.5 Gene expression

4.3.5.1 RNA isolation

For RNA isolation, cells were washed, pelleted and lysed with 1ml of TRizol reagent for 10 min at room temperature and stored at -80°C or processed directly. Shortly, 200 µl of chloroform were added per 1 ml of TRizol then centrifuged at 12000x g for 10 min. The upper phase, containing the RNA, was collected and mixed with 500 µl of isopropanol and incubated for 10 min at room temperature then centrifuges at 12000x g for 30 min at 4°C. After removal of isopropanol, the RNA was washed with at least 1 ml of 75% ethanol and centrifuged at 12000x g at 4°C. After removal of the ethanol, the pellet was dried for 10-20 min and dissolved with nuclease free water. The concentration and quality of the RNA was assessed using a spectrophotometer (Nanodrop, Thermo Scientific, USA).

4.3.5.2 cDNA synthesis

mRNA was converted to cDNA using the Transcripter First Strand cDNA Synthesis Kit (Roche Applied Science, Switzerland). The method is summarized in the table below.

Total RNA	50-1000 ng
Anchored-oligo(dT)18 Primer 50 pmol/ µl	1 µl
H2O	Upto 13 µl
The template was denatured at 65°C for 10 min then kept on the ice for 5 min	
Reaction Buffer, 5× conc	4 µl
Protector RNase Inhibitor 40 U/ µl	0.5 µl
Deoxynucleotide Mix, 10 mM each	2 µl
Transcriptor Reverse Transcriptase 20 U/ µl	0.5 µl
The reaction mix was incubated at 50°C for 60 min then inactivated for 5 min at 85°C	

4.3.5.3 Real time PCR

cDNAs were synthesized as described above and diluted with nuclease free ddH_2O up to 100 µl before usage. For qRT-PCR, each sample was run in triplicates in a 96-well-plate. The reaction was performed with 4 µl of cDNA in a final volume of 10 µl. Threfore, the universal library probe and the primers were mixed with 5 µL of TaqMan Universal PCR Mastermix containing PCR buffer, dNTPs, and Taq polymerase. The master mix was then plated in triplicates in 96-well optical reaction plates. After addition of the respective DNAs the plate was centrifuged shortly and run on a LightCycler 480 II (Roche Applied Science, Switzerland). The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Housekeeping genes like β2-microglobulin for human samples and β-actin for murine samples were used for normalization.

Universal ProbeLibrary probe	0.1 µl
Forward primer, 10 µM	0.2 µl
Reverse primer, 10 µM	0.2 µl
ddH_2O	0.5 µl
TaqMan Universal PCR Mastermix	5 µl
cDNA	4 µl

The Roche Light Cycler 480 II was used with the following amplification program:

		Temp.°C	Hold	No. of cycles	Acquisition mode	Analysis mode
pre-incubation	Initial-denaturation	95	10 min	1	None	None
Amplification	Denaturation	95	10 sec	45-55 cycles	None	Quantitative
	Annealing	60	30 sec		Single	
	Extension	72	5 sec		None	
Cooling		40	10 sec		None	None

4.3.5.4 Whole-genome gene expression in human cells

All RNA was extracted using TRIzol and purified in our laboratory using standard methods. Sample amplification, labeling and hybridization on Illumina WG6 Sentrix BeadChips V1 were performed for all arrays shown in Figure 7 according to the manufacturer's instructions (Illumina) using an Illumina BeadStation. All data analyses were performed by using

Bioconductor for the statistical software R (http://www.r-project.org). Expression values were normalized and summarized by using the IlluminaGUI package [240]. From the resulting data sets we extracted a list of genes with a significant different expression in T_{reg} compared to T_{conv} cells. Data generation and analysis was conducted by Dr. Marc Beyer and former group members Dr. Sabine Claßen and Dr. Daniela Eggle. Details concerning sample processing, data collection, data assessment and statistical analysis can be obtained from the Ph.D. thesis of Dr. Sabine Claßen and Dr. Daniela Eggle.

For SATB1-transduced T_{reg} cells as well as FOXP3-transduced T_{conv} cells Illumina WG6 Sentrix BeadChips V3 were used according to the manufacturer's instructions (Illumina) using an Illumina BeadStation. All data analyses were performed as described above. From the resulting data sets we extracted a list of genes with a significant different expression in SATB1-transduced T_{reg} cells compared to control-transduced T_{reg} cells. These were analyzed in comparison to a dataset consisting of $CD4^+CD25^+$ T_{reg} cells, $CD4^+CD25^-$ T-cells, naive $CD4^+CD25^-CD45RA^+$ T-cells activated for 5 days with CD3/CD28-coated beads, as well as unstimulated naive T-cells to define T_{conv}-cell dependent genes (differentially expressed between T_{conv} and T_{reg} cells), T-cell activation dependent genes (differentially expressed between activated and unstimulated naive T-cells), and common T-cell genes (changed in the same direction in both comparisons). For enrichment analysis of Th-cell gene sets, the expression dataset was filtered for expressed probes and significance of enrichment was calculated using χ^2 statistic implemented in R.

4.3.6 Generation of plasmids containing the ORF or genomic sequences of SATB1 and FOXP3

4.3.6.1 PCR amplification

PCR reactions were carried out in reaction volumes of 50 µl as described in the table below.

10x Buffer with Mg^{2+}	5 µl
Forward primer,20 µM	1.5 µl
Reverse primer, 20 µM	1.5 µl
dNTPs, 2mM	5 µl
DNA Polymerase	Varying
Template DNA	10 pg-1 ug
Nuclease free water	To 50 µl

General set-up for amplification

	Temp(°C)	Hold	No. of cycles
Initial denaturation	95	4 min	1
Denaturation	95	30 sec	30 cycles
Annealing	varying	30 sec	
Extension	72	1min/kb of plasmid	
Final extension	72	10 min	1
Hold	8	∞	

After amplification, the size of the PCR products was assessed using agarose gel electrophoresis. 1-1.5% agarose gels were used. For further processing, the amplified product was excised and isolated using the QIAquick gel extraction kit according to manufacturer's instructions (Qiagen GmbH, Hilden, Germany).

4.3.6.2 Restriction digestion and gel extraction

All restriction endonucleases were used according to the manufacturer's instructions. In general, restriction digests were set up in a total volume of 20 µl. The mixture contained 1 µg of purified template/vector dissolved in ddH_2O, together with 2 µl of the appropriate 10x enzyme buffer and 0.5 U of restriction enzyme. Reaction mixtures were incubated for a minimum of 1 hour at 37ºC. Agarose gel electrophoresis was used to separate the digested fragments. The desired band was excised and extracted with the QIAquick gel extraction kit as described above.

4.3.6.3 Ligation of DNA insert (sequence of interest)

Ligation of DNA was performed in a final volume of 20 µl. The plasmid DNA and 4-5 fold molar excess of PCR-amplified insert were mixed with 10x reaction buffer and 5 units of T4 DNA ligase (Fermentas, Thermo Sceintific, Germany) and incubated between 4 and 16 hr at 16-22^{o}C. Afterwards, the reaction was inactivated at 85°C for 5 min.

4.3.6.4 Bacterial transformation and plasmid purification

Chemically competent *E.coli* bacteria were used. 2-5 µl of ligation reaction were shortly mixed with 100 µl of competent cells and incubated for 30 min on ice. To induce DNA uptake by the bacteria, a heat-pulse for 45 sec in a water bath preheated to 42°C was performed.

Next, the bacteria were placed on ice for 2 min and 500 µl of prewarmed SOC medium was added. The transformed bacteria were incubated at 37°C for 1 hr. 50-200 µl of the transformed bacteria were plated on LB agar containing the proper antibiotic. The agar plates were incubated overnight at 37°C. On the next day, the plates were examined for bacterial growth and single colonies were screened for the insertion of the amplified product either by PCR or restriction digestions after bacterial amplification and plasmid extraction. In brief, several colonies were picked and inoculated in 2 ml LB medium containing the proper antibiotic. Next, the cultures were incubated overnight at37°C. Plasmid extraction was performed using the GeneJET Plasmid Miniprep Kit (fermentas, Thermo Scientific) according to manufacturer's instructions and the plasmid's concentration and purity was assessed on a spectrophotometer.

To generate high amounts of plasmid DNA, 1 ml of bacterial cultures were diluted 1:200 in LB medium and incubated overnight. Plasmid DNA was extracted with the High Speed Plamid MidiKit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. The plasmid's concentration and quality were assessed on a spectrophotometer.

4.3.7 Reporter assays to assess functional binding to the genomic SATB1 locus

All luciferase assays in this thesis were performed together with Roman Müller and Stefanie Keller in the lab of Bernhard Schermer/Thomas Benzing in University Hospital of Cologne. The dual Luciferase Reporter (DLR) assay system (Promega Corporation, USA) was used. In brief, activities of firefly (*Photinus pyralis*) and renilla (*Renilla reniformis*) luciferase are measured sequentially from a single sample. The firefly luciferase reporter is measured first, afterwards this reaction is quenched, while the renilla luciferase reaction is simultaneously initiated and quantified.

To assess the inhibitory effect of binding of FOXP3 and FOXP3-driven miRNA to the SATB1 locus the pGL4 and the psiCHECK II luciferase reporter systems were used. FOXP3 binding sites that had been identified in the genomic SATB1 locus by ChIP were cloned into the pGL4.24 (*luc2P*/minP) plasmid containing the luciferase reporter gene luc2P (*Photinus pyralis*) as described above. The pGL4.74 (hRluc/TK) vector encoding the luciferase reporter gene hRluc (*Renilla reniformis*) was used for normalization. Similarily, the putative binding sites of FOXP3-dependent miRNA to the 3´UTR region of SATB1 were cloned into psiCHECK II. The DLR was performed according to the supplier's instructions. In brief, HEK293T-cells were co-transfected with the pGL4.24 and pGL4.74 constructs in addition to

a FOXP3 expressing construct in a 96-well-plate. After 24 hrs, cells were lysed with passive lysis buffer for 15 min at room temperature. Next, 100 µl of LAR II Luciferase Assay Buffer was injected into each well containing 20 µl of cell lysate followed by reading the firefly luminescence on a Mitras plate reader (Berthold Technologies GmbH, Austria). Directly, 100 µl of Stop & Glo was injected into each well to quench the firefly luciferase and measure renilla luciferase. Triplicates of each transfection were measured. The luminescence signal of the constitutively expressed renilla luciferase is used as an internal control for normalization. For analysis, the ratio of firefly and renilla signals was calculated for each sample of three independent experiments.

4.3.8 Site directed mutagenesis of reporter constructs

For mutation of FOXP3 and miRNA binding motifs the QuickChange Lightning Multi Site-Directed mutagenesis kit (Stratagene, Agilent Technologies, USA) was used. In brief, supercoiled dsDNA vectors isolated from a Dam^+ bacteria strain and two synthetic oligonucleotide primers containing the desired mutations (listed in Appendix) are mixed. The oligonucleotide mutagenic primers, each complementary to opposite strands of the vector, are extended without primer displacement during temperature cycling by *pfu* DNA polymerase, generating dsDNA molecules with one strand bearing multiple mutations and containing nicks. Next, the nicks are sealed by components in the enzyme set. To remove the parental, methylated and non-mutated DNA strand, the thermal cycling reaction was treated for 5 min at 37°C with DpnI endonuclease which targets methylated and hemimethylated DNA. Next, the reaction mixture is used to transform XL10-Gold ultra-competent cells. Specific sequencing primers were used for selection of positive clones.

10x Buffer with Mg^{2+}	5 µl	5 µl
Forward primer, 20 µM	100 ng	Varying
Reverse primer, 20 µM	100 ng	Varying
dNTPs, 10 mM	0.2 mM	1 µl
pfu DNA Polymerase		1 µl
Template DNA	50-100 ng	Varying
Nuclease free water		To 50 µl

Cycling parameters:

	Temp(C)	Hold	No. of cycles
Initial-denaturation	95	3 min	1
Denaturation	95	20 sec	18 cycles
Annealing	Varying	30 sec	
Extension	68	30 sec/kb of plasmid	
Final extension	68	5 min	1
Hold	8	∞	

4.3.9 Protein expression and purification

For *in vitro* binding assays, FOXP3 was recombinantly expressed in *E. coli* BL21. Therefore, the FOXP3 open reading frame was amplified by PCR and cloned into the pASK-IBA43plus vector under control of the *tet* promoter/repressor which allows for protein expression only after addition of anhydro-tetracycline. This vector contains both a N-terminal 6x histidine-tag and a C-terminal strep-tag II that can be used for protein purification and detection.

Not in all bacterial colonies integrating the construct protein expression can be induced. After, screening for inducible expression in a small scale experiment, glycerol stocks of positive colonies were generated and preserved. To express the protein at a high-scale, the colony that showed the highest protein induction was used for protein expression. 500 µl of inducible bacteria culture were grown overnight in 50 ml of LB medium with the appropriate antibiotic. On the next day, 1 liter of LB medium containing the proper antibiotic was added to the culture and incubated on a shaking platform at room temperature and the growth was monitored spectrophotometrically every 1 hr. When the OD600 reached between 6.0-8.0 one ml of the culture was removed, centrifuged and kept as uninduced control, anhydro-tetracycline (200 µg/ml medium) was added and the culture incubated for 3-5 hr. At the end of the incubation period, the culture was centrifuged and washed once with PBS and bacterial pellets stored at -80 °C.

Protein was purified with Ni-NTA (Nickel-Nitrilotriacetic acid) affinity chromatography (Qiagen GmbH, Hilden, Germany) which binds histidine tags with high affinity to the beads. This tight binding can be reversed with high concentrations of imidazole which is used for elution. All experimental steps were carried out on ice. Bacterial cells were lysed in sonification buffer with lysozyme and protease inhibitors for 30 min followed by mechanical

lysis by sonification. The cell lysate was centrifuged and incubated with 2 ml of pre-equilibrated Ni-NTA slurry for 1 hr with gentle rotation. Next, the cell lysate resin mixture was loaded onto the column and the column was washed twice with washing buffers I and II. The protein was eluted with imidazole (500 mM). The eluted protein was further cleaned using a PD-10 desalting column (GE Healthcare, UK). The protein concentration was determined by BCA assay kit (Thermo Scientific, USA). Afterwards, the purity and the size of the expressed protein were assessed by SDS-PAGE gel.

4.3.10 Filter retention assays

Wild type and mutated FOXP3 binding sites in the SATB1 promoter were used for this assay to determine the binding coefficient of FOXP3 to the SATB1 locus. Synthetic ssDNA oligonucleotides were purchased form Ella Biotec GmbH, Germany. Synthetic ssDNA was annealed in TE buffer at 94°C for 5 min and cooled down to room temperature. [$^{\gamma\text{-}32}$P]-labeled dsDNA was generated using the T4 polynucleotide kinase (New England Biolabs, UK) and [$^{\gamma\text{-}32}$P]-ATP. [$^{\gamma\text{-}32}$P]-end labeled DNA (10 nM) was incubated with increasing concentrations of FOXP3 protein in binding buffer for 30 min at 37°C. After incubation, the reactions were passed through a 0.45 µm nitrocellulose membrane and washed four times with 200 ml binding buffer. Bound protein-DNA was quantified by phosphorimager (FLA 5000, Fuji photo film Co. Ltd, Japan). All assays were performed in quadruplicates.

4.3.11 Lentiviral expression system

In preliminary experiments, several lentiviral vectors with promoters like CMV, EF-1α and hPGK were tested for efficient gene transfer into primary cells and a high and relatively stable level of protein expression or gene silencing in case of shRNA.

For SATB1 overexpression in murine T_{reg} cells, we subsequently used the pLVTHM lentiviral backbone and cloned the murine SATB1 ORF in front of an IRES cassette with a subsequent Thy1.1 as reporter gene. As control an IRES-Thy1.1 construct was generated as well.

For silencing of FOXP3 and SATB1 in human expanded T_{reg} cells, we generated pLenti6.3-V5-GW-EmGFP lentiviral backbone containing either a miR RNAi against FOXP3, SATB1, both, or an empty control by chaining the miR RNAi using the Gateway system from Invitrogen. miRNAs mediated gene silencing were designed using the Invitrogen website (***https://rnaidesigner.invitrogen.com/rnaiexpress/***) and for this purpose BLOCK-iT Pol II miR RNAi Expression Vector Kits (Invitrogen, UAS) were used. The advantages of this

system over conventional shRNA systems are the higher silencing success rate, expression tracking through co-expression of GFP, polycistronic expression which allows multiple targeting through chaining, and it's pol II expression system which enables co-cistronic expression of multiple miRNAs. miRNAs were synthesized by Sigma Aldrich, Germany. Annealing of each complementary oligonucleotide was performed as described above. miR RNAi-ds oligos were cloned into linearized pcDNA6.2-GW/miR using T4 DNA ligases. The ligation reaction was used to transfect One Shot TOP10 competent *E.coli* and plated into Spectinomycin containing LB agar. The correct oligo sequence and the orientation are confirmed by sequencing the plasmid prepared from MiniPrep. The pri-miRNAi expression cassette was transferred to a lentivirally based destination vector using Gateway Technology. pLenti 6.3/V5 Dest as a Gateway adapted destination vector was used to host the respective pre-miRNA. The recombination was carried out as stated in the vendor manual. The transfer process of the cassette includes two recombination reactions. Rapid BP/LR recombination was performed. The first recombination reaction was done between linearized attB expression clone and donor vector in the presence of PB clonase for 6 hrs at room temperature. PB clonase catalyzes the recombination of attB substrate with an attP substrate to create an attL-containing entry vector. LR clonase promotes in vitro recombination between an entry clone (attL-flanked sequence) and any number of attR-containing destination vectors to generate attB-containing expression clones The LR reaction was the second step and performed directly without an intermediate bacterial transformation step. The destination vector was mixed with PB reaction in the presence of LR clonase II overnight at room temperature. In this reaction an attL (entry clone) is recombined with attR (destination vector) to create an attB-containing expression clone. Subsequently the LR reaction was used to transfect One Shot TOP10 competent *E.coli* which were then plated on ampicillin LB agar.

miR RNAi encoding lentivirus containing supernatants were generated by transfecting HEK293T-cells with pLenti 3.6 letivirus vector with the following packaging plasmids: pMD2.G, pMDLg/p, pRSV-Rev.

To generate murine SATB1 encoding supernatants HEK293T-cells were transfected with the pLVTHM plasmid together with the pMD2.G and pCMVΔR8.74 packaging plasmids.

4.3.12 MicroRNA quantification

MicroRNAs were quantified using the TaqMan MicroRNA assays as recommended by the manufacturer (Applied Biosystems, USA). In brief, total RNA was isolated with the standard

TRizol method. Afterwards, miRNA is reversely transcribed form total RNA using specific primers with the TaqMan Universal PCR master mix. Next, looped-primer RT-PCR amplification was performed by addition of miRNA specific primers in triplicates using the LightCycler 480 II. The relative miRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method. Constitutively expressed small RNA or miRNA like U6 small nuclear RNA or miR-26b were used for normalization.

4.4 Statistical analysis.

Student's t-tests and ANOVA with least significant difference (LSD) were performed with SPSS 19.0 software.

5 Results

While the main function of T_{eff} cells is to respond against potential threats, T_{reg} cells have the task to limit and shape the immune response to prevent a destructive overreaction against healthy tissues and harmless antigens. Although it is clear that T_{reg} and T_{eff} cells develop from a common progenitor, the mechanisms that regulate the acquisition of these opposing programs is not yet completely understood. To understand the molecular mechanisms and find new targets that control the functional specificity of T_{reg} and T_{eff} cells is of high importance as this might allow us to restore homeostasis in pathological conditions where the balance between tolerance and inflammatory responses has been altered.

SATB1 is required for terminal thymocyte differentiation in the thymus. Furthermore, SATB1 is involved in the acquisition of effector function of T-cells particularly in the production of Th2 related cytokines. However, the role of SATB1 for the acquisition of effector and regulatory programs in T-cells has not yet been described in detail. Preliminary data from a former PhD student in the laboratory, Sabine Claßen, revealed that SATB1 is highly expressed in conventional T-cells, whereas its expression is lower in T_{reg} cells indicating that SATB1 might initiate and maintain T-cell effector programs [232]. This thesis was intended to elucidate how SATB1 expression is regulated between effector and regulatory T-cells and how its expression contributes to shape the antagonistic responses observed between these cells. To gain insight into the regulation of SATB1, its expression patterns were analyzed in effector and regulatory T-cells in mice and human. Enforced SATB1 expression in T_{reg} cells was performed to address the functional consequences of its expression *in vitro*. Furthermore, the regulation of SATB1 expression in T_{reg} cells was investigated and the active role of FOXP3 in this process explored. Finally the functional effect of gaining high SATB1 expression in T_{reg} cells in an *in vivo* model was examined.

5.1 Low SATB1 expression in human natural T_{reg} cells

Previous data showed that SATB1 is expressed in a tissue specific pattern, mainly in thymocytes [173, 174, 241]. Its expression is low in peripheral T-cells but is induced upon stimulation which suggests the involvement of SATB1 in effector mediating functions [224]. To our knowledge no previous study has specifcally addressed the expression of SATB1 in T_{reg} cells. Our first question was whether SATB1 is differentially expressed between T_{reg} and T_{conv} cells. To explore the expression pattern of SATB1 amongst T_{conv} and T_{reg}, $CD4^+CD25^+$ T_{reg} and $CD4^+CD25^-$ T_{conv} cells were isolated from human blood. The cells were either left

untreated or stimulated with anti CD3/CD28-coated beads alone or in combination with TGF-β. Cells from the different experimental conditions where lysed in TRIzol and the RNA was extracted to perform whole transcriptome profiling. The experimental procedures and data analysis were performed by former members of the Schultze laboratory. Transcriptome analysis showed lower expression of SATB1 in thymic derived natural T_{reg} cells, whereas it is highly expressed in conventional $CD4^+$ T-cells. Next, SATB1 expression was assessed on mRNA and protein levels. The inverse expression of SATB1 between T_{reg} and T_{conv} cells was corroborated at mRNA level using qRT-PCR (Fig. 7, b). Moreover, this difference in SATB1 expression was confirmed by immunoblotting (Fig. 7, c) and flow cytometric analysis by intracellular staining of $CD4^+$ cells using a directly labeled SATB1 antibody (Fig. 7, d). In summary, our data showed that SATB1 expression is lower in T_{reg} cells relative to T_{conv} cells.

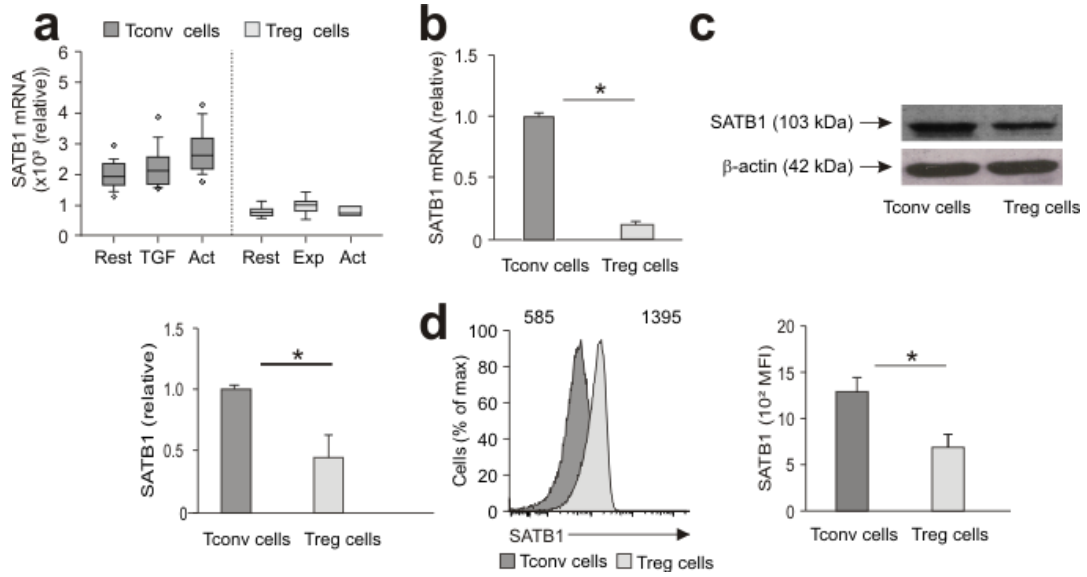

Figure 7. Low SATB1 expression in human naturally occurring T_{reg} cells.

Human $CD4^+CD25^-$ T-cells (T_{conv}), and regulatory $CD4^+CD25^+$ T-cells (T_{reg}) were isolated from peripheral blood of healthy donors. The isolated cells were then either rested or stimulated with CD3/CD28 beads alone or with TGF-β. Expanded T_{reg} cells were stimulated with CD3/CD28 expander beads. (**a**) Microarray analysis of SATB1 mRNA expression in T_{conv} and T_{reg} cells (rest=resting, act=activated, TGF=TGFβ treated, exp=expanded). (**b**) Relative SATB1 mRNA expression compared to B2M in freshly isolated T_{reg} and T_{conv} cells assessed by qRT-PCR (mean±s.d., n=5; * $p<0.05$). (**c**) Immunoblotting of SATB1 in freshly isolated T_{reg} cells and T_{conv} cells, relative expression of SATB1 was measured densitometrically in comparison to β-actin (representative donor (left), relative expression (right; n=6, mean±s.d.; * $p<0.05$)). (**d**) Flow cytometric analysis of SATB1 expression in freshly isolated T_{reg} and T_{conv} cells. Cells were stained intracellularly with SATB1 monoclonal antibodies after fixation and permeabilization with Biolegend's FOXP3-staining kit. Data presented as mean fluorescence intensity (MFI) (left: representative donor; right: mean±s.d., n=11; * $p<0.05$).

5.2 SATB1 is expressed at low levels in murine T_{reg} cells

The protein sequence of SATB1 is highly conserved between mice and men which implies functional similarity across the species. The study of human T_{reg} cells is challenging due to the lack of reliable surrogate markers for isolation and characterization. FOXP3 is commonly used as a specific marker for T_{reg} cells; however, the transient expression of FOXP3 in activated human T_{conv} cells is further complicating the analysis and understanding of human T_{reg}-cell biology. Such challenges are overcome at least in part in mouse models where FOXP3 is co-expressed with a reporter gene such as GFP providing a reliable marker for isolation and characterization of T_{reg} cells. To precisely address some aspects of SATB1 biology in T_{reg} cells, FOXP3-GFP reporter mice were used. Therefore, the DEREG (DEpletion of REGulatory T-cells) mouse model was used in this study in collaboration with the group of T. Sparwasser (Hannover Medical School, Hannover) [242]. This model is a BAC (bacterial artificial chromosome) transgenic mouse line carrying a DTR-eGFP fusion transgene under the control of the endogeneous FOXP3 promoter, thereby allowing specific depletion of T_{reg} cells by application of diphtheria toxin and at the same time allowing for isolation of highly pure GFP-marked T_{reg} cells for downstream analyses [242]. To asses the expression of SATB1 in murine $CD4^+$ T-cells, T_{reg} and T_{conv} cells were freshly isolated and sorted from spleens of DEREG mice. SATB1 protein expression was assessed by flow cytometry and immunoblott. The experiments clearly established lower expression of SATB1 in murine T_{reg} cells. For flow cytometry the isolated cells were fixed, permeabilized and stained intracellularly for SATB1. Immunoblotting was performed in Jeffrey Bluestone's laboratory (UCSF, San Francisco) on cells which were isolated from FOXP3-GFP mice [243]. Both methods revealed that the pattern of expression of SATB1 is similar to the pattern observed in humans with lower SATB1 expression in T_{reg} compared to T_{conv} cells (Fig. 8, a and b). Altogether, our data suggest that the expression pattern of SATB1 is conserved between murine and human T_{reg} cells, supporting a conserved function and regulation of SATB1 across different species.

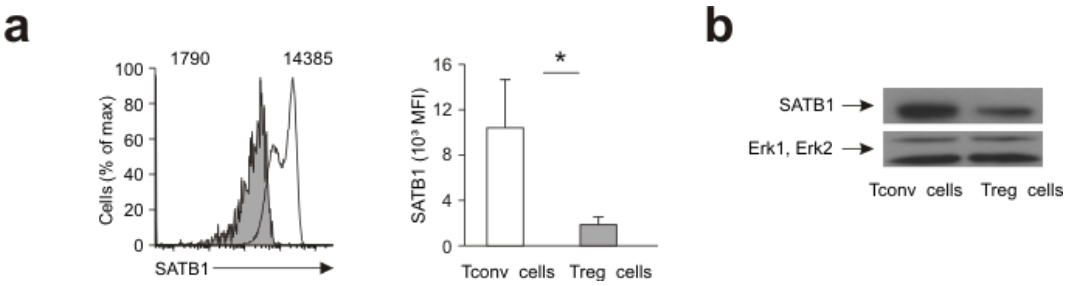

Figure 8. Low SATB1 expression in murine regulatory T-cells.

(**a**) Flow cytometric analysis of SATB1 in freshly isolated T_{reg} cells and T_{conv} from spleens of male DEREG mice (left: representative mouse; right: mean±s.d. n=3; * $p<0.05$). MFI values are presented for Treg and T_{conv} cells respectively. (**b**) Western blot analysis of SATB1 protein expression in murine Treg and T_{conv} cells. Erk1 and Erk2 were used as loading control.

5.3 Reduced SATB1 expression levels in human induced T_{reg} cells

T_{reg} cells can be subdivided according to their sites of induction into thymus-derived natural T_{reg} cells and peripherally induced iT_{reg} cells. *In vitro*, TGF-β in concert with high concentration of IL-2 and the presence of a TCR and a co-stimulatory signal mediates the induction of FOXP3 in naïve $CD4^+$ T-cells [80, 244, 245]. Briefly, iT_{reg} cells were generated from naive $CD4^+CD45RA^+$ T-cells stimulated with CD3/CD28-coated beads in combination with TGF-β and IL-2. FOXP3 and SATB1 expression was analysed by flow cytometry and qRT-PCR. To assess the influence of FOXP3 induction on SATB1 expression and consequently on the differentiation of effector T-cell cytokines were measured in the supernatants. It could be observed that FOXP3 induction in naïve $CD4^+$T-cells was correlated with repression of SATB1 expression in iT_{reg} cells relative to stimulated $CD4^+$ Tcells (Fig. 9, a). Furthermore, FOXP3 induction and SATB1 suppression were associated with a decrease in the production of proinflammatory cytokines such as IFN-γ and IL-4 as typical Th1/Th2 cytokines as potential targets of SATB1-dependent gene regulation (Fig. 9, b).

In summary, independently of the origin of T_{reg} cells, SATB1 expression is lower in T_{reg} cells in comparison to T_{conv} cells. Moreover, in iT_{reg} cells FOXP3 upregulation was concomitant with SATB1 repression, suggesting a possible regulatory loop between these two molecules.

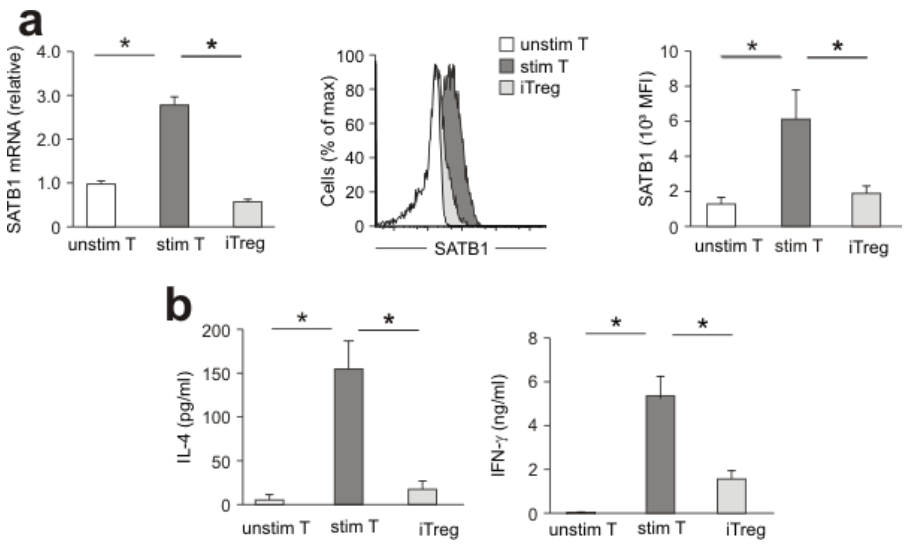

Figure 9. SATB1 expression is reduced in human iT_{reg} cells and correlates with the induction of FOXP3.

Human naïve CD4+CD45RA+ T-cells were isolated and stimulated for 5 days either with CD3/CD28 beads alone or in combination with TGF-β and IL-2 to generate iT_{reg} cells. (**a**) Left, qRT-PCR, right, flow cytometry of SATB1 in unstimulated T-cells (unstim T), stimulated T-cells (stim T), and iT_{reg} cells (iTreg) on day 5 (left: representative experiment; right: n=3, mean±s.d.; * $p<0.05$)). (**b**) Cytokine bead array for IL-4 and IFN-γ secretion in the supernatants of unstimulated T-cells, stimulated T-cells, and iT_{reg} cells; n=3 (mean±s.d.; * $p<0.05$).

5.4 SATB1 expression is significantly upregulated in T_{conv} cells upon stimulation in comparison to T_{reg} cells

T-cell survival, differentiation, and function are dependent on the integration of diverse signals provided during the interaction of lymphocytes with antigen-presenting cells. TCR, costimulatory/inhibitory molecules, and cytokines signals can positively or negatively shape the immune response via modulating the activation status of the major downstream signaling molecules. The ability to transduce extrinsic signals is mediated through several proximal and distal effector molecules and signal transduction cascades such as Lck, Zap70, Jaks, MAPK, PKC and others. The strength and duration of these signaling events subsequently resulting in differential and combinatorial activation of several transcription factors such as AP-1, NFAT, NF-kB, ERK and STATs that shape the fate of the immune response [246, 247]. We observed that following TCR ligation in $CD4^+$ T-cells, SATB1 expression was rapidly upregulated. To examine the difference in the expression kinetics of SATB1 in T_{reg} cells in comparison to T_{conv} cells under activation conditions, T_{reg} and T_{conv} cells were freshly isolated and activated with CD3 alone in the presence of IL-2 or CD3/CD28-coated beads. In contrast to T_{reg} cells where SATB1 showed only minimal induction upon TCR activation in combination with

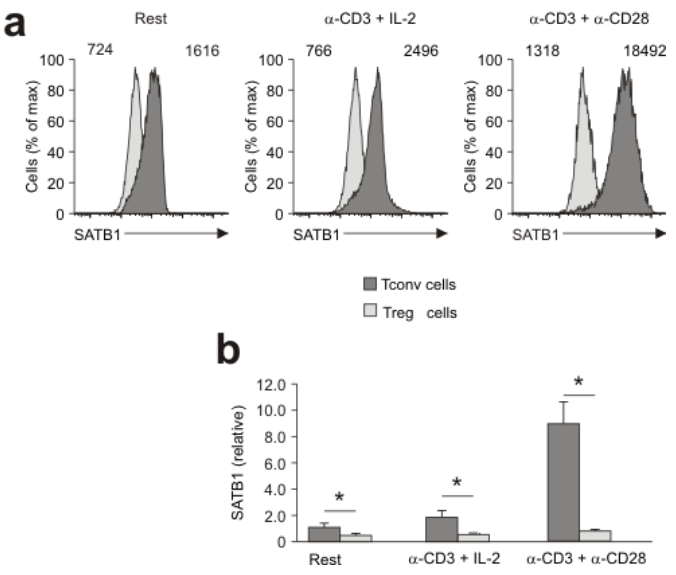

Figure 10. SATB1 expression in CD4+ T-cells upon stimulation.

T_{conv} and T_{reg} cells were isolated and either rested or stimulated with CD3/CD28-coated beads or CD3 and IL-2 for 48 hrs. (**a**) Flow cytometry of SATB1 expression after stimulation of T_{reg} cells and T_{conv} for 2 days. (**b)** Relative protein expression of SATB1, normalized to resting T_{conv} cells (mean±s.d. n=5; * p<0.05)

costimulation, T_{conv} cells showed around 7-fold increase in SATB1 expression under sub-optimal stimulation (Fig.10, a and b). The induction of SATB1 expression was lower in the absence of co-stimulation which might indicate a probable role of the costimulation signaling pathway in SATB1 activation (Fig.10, b).

Overall, upon TCR activation, SATB1 expression is only slightly increased in T_{reg} cells, whereas it is highly induced in T_{conv} cells. This evidence suggests the presence of a dominant inhibitory mechanism on SATB1 expression in T_{reg} cells.

5.5 SATB1 gene expression is under control of FOXP3

FOXP3 is the master regulator of T_{reg} cell function and many studies have dissected the role of FOXP3 in maintaining T_{reg} cell functional identity and stability [135, 145, 146, 150, 248-251] which clearly suggests that FOXP3 largely defines the transcriptional program of T_{reg} cells. This evidence was further supported by ectopic expression of FOXP3 in non-T_{reg} cells ($CD4^+CD25^-$) which resulted in endowing T_{conv} cells with a T_{reg}-cell-like phenotype [52].

Genome wide expression studies performed by our group and others have shown that several genes are repressed in T_{reg} cells. The repressive status of many of these genes is functionally relevant, as their upregulation antagonizes T_{reg} cell identity and function, e.g. RORγt, Akt, and proinflammatory cytokines genes. Our data showed that SATB1 was repressed in T_{reg} cells. Moreover, FOXP3 induction was negatively correlated with the expression of SATB1. This led us to hypothesize that FOXP3 imposes a potential regulatory circuit on SATB1

expression in T_{reg} cells. To test this hypothesis, we combined gain and loss of function genetic approaches to study the proposed effect of FOXP3 on SATB1 expression. Furthermore, we examined the staining pattern of FOXP3 and SATB1 in FOXP3-competent and FOXP3-mutated thymocytes.

5.5.1 SATB1 shows an inverse staining pattern to FOXP3 in murine thymocytes

To dissect SATB1 localization and abundance in T_{reg} and T_{conv} cells cells, were isolated from DEREG reporter mice and confocal microscopy was performed. To investigate how the staining of SATB1 in thymocytes correlated with FOXP3 staining, thymocytes were purified from male DEREG mice, fixed with paraformaldehyde, permeabilized with Triton-X, and stained with primary antibodies against SATB1 followed by staining with secondary antibodies and DAPI to stain chromatin (all slides and images were prepared by Eva Schönfeld and Marc Beyer). Consistent with the expression data, the nuclear staining pattern of SATB1 (shown in red) is more intense in FOXP3 negative thymocytes which reflects higher expression of SATB1 in non-T_{reg} cells. In contrast, SATB1 staining was dim in FOXP3-expressing T_{reg} cells (GFP-positive cells) (Fig.11).

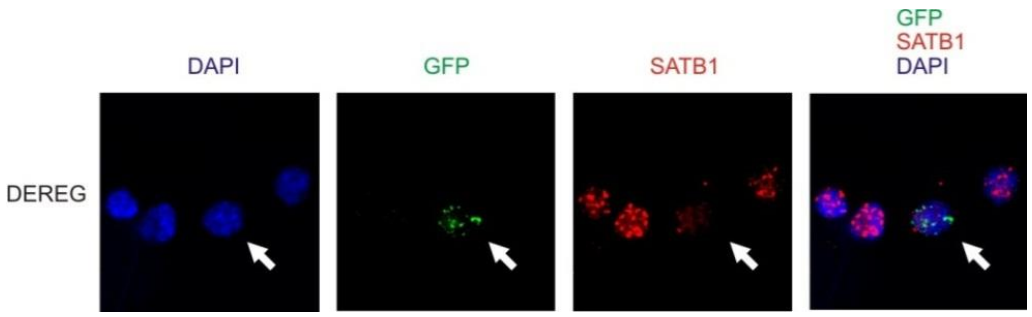

Figure 11. SATB1 is expressed at high levels in FOXP3-negative thymocytes.

Confocal immunofluorescence for SATB1 (red) and FOXP3 (green) in freshly sorted thymocytes, Z-projection of immunofluorescence for SATB1 GFP protein expression in thymocytes from male DEREG mice counterstained with DAPI (blue). White arrow depicts a T_{reg}-cell.

5.5.2 Ectopic expression of FOXP3 in $CD4^+$ T-cells represses SATB1 expression

The data obtained in iT_{reg} cells clearly demonstrated that the induction of FOXP3 is inversely correlated with SATB1 expression supporting the hypothesis of a putative suppressive mechanism exerted by FOXP3 on SATB1 gene expression. To test whether FOXP3 regulates SATB1 expression, the full-length cDNA of FOXP3 variant 1 was cloned into a lentiviral vector in the laboratory of James Riley (University of Pennsylvania, Philadelphia). Afterwads,

lentivirus was produced in HEK293T-cells and concentrated by ultracentrifugation. Human naïve $CD4^+CD25^-CD45RA^+$ T_{conv} cells were isolated by magnetic separation and transduced with high concentrations of FOXP3 encoding virus or control virus, expanded for several days, and sorted after a resting period on reporter gene expression in the laboratory of James Riley (University of Pennsylvania, Philadelphia). After RNA isolation and cDNA generation, FOXP3 and SATB1 expression were assessed by qRT-PCR. Under these conditions, SATB1 was highly expressed in control virus transduced T_{conv} cells but ectopic expression of FOXP3 resulted in a drastic inhibition in the expression of SATB1 in T_{conv} cells (Fig. 12, a). The FOXP3 associated reduction of SATB1 expression in T_{conv} cells was also coupled with a significant decrease in the production of Th1/Th2 cytokine genes e.g. IL-5 and IFN-γ (Fig. 12 b). Taken together, this evidence further supported the idea of FOXP3-mediated repression of SATB1 gene expression.

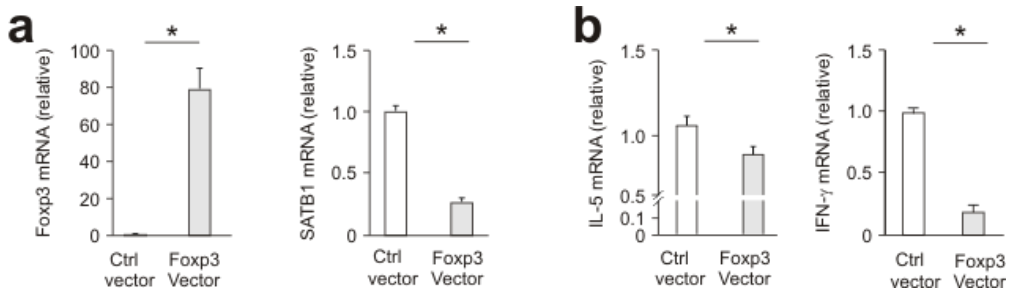

Figure 12. FOXP3 represses SATB1 transcription.

Human naïve CD4+CD25-CD45RA+ T_{conv} cells were isolated by magnetic separation, activated for 48 hrs and lentivirally transduced with FOXP3 and control virus and expanded for several days. After a resting period of 3 days, reporter gene expressing cells were isolated by flow cytometry and analyzed. **a**) qRT-PCR analysis of FOXP3 and SATB1 expression. (**b**) qRT-PCR analysis of IL-5 and IFN-γ expression. mean±s.d. n=5, * $p<0.05$.

5.5.3 Loss of function of FOXP3 results in the upregulation of SATB1 in T_{reg} cells

Since we observed that ectopic expression of FOXP3 in T_{conv} cells antagonized SATB1 induction, we wanted to examine whether a reduction in FOXP3 expression would result in an increase in SATB1 expression in FOXP3-expressing cells. We studied this effect in T_{reg} cells where FOXP3 was either intrinsically defective as in *scurfy* mice where FOXP3 is inactive because of a point mutation at the FOXP3 locus or in artificially FOXP3-silenced human T_{reg} cells. For this we either silenced FOXP3 expression in human T_{reg} cells by sequence specific siRNAs or lentivirally transduced human Treg cell with artificial miRNA (RNAi) targeting FOXP3.

5.5.3.1 Intrinsic loss of FOXP3 results in increased SATB1 expression and expression of Th1/Th2 cytokine genes in T_{reg} cells

Scurfy mice harbor a mutated allele for FOXP3 which results in rapid protein degradation. To characterize "would-be" T_{reg} cells from *scurfy* mice, *scurfy* mice were crossed with DEREG mice in the laborytory of Timm Sparwasser (Hannover Medical School, Hannover) providing us with a tool to visualize and isolate T_{reg} cells with non-functional FOXP3. Thymocytes were isolated from female DEREG mice heterozygous for the *scurfy* mutation. As FOXP3 is encoded on the X chromosome and because of the random inactivation of the second X chromosome in cells from female animals, these mice have both normal T_{reg} cells ($CD4^+GFP^+FOXP3^+$) as well as T_{reg} cells expressing the mutated FOXP3 allele ($CD4^+GFP^+FOXP3^-$). Confocal microscopy of SATB1 in FOXP3-sufficient and FOXP3-deficient T_{reg} cells (performed by Eva Schönfeld and Marc Beyer) showed that SATB1 signal intensity was inversely correlated with FOXP3 expression. While the SATB1 signal was higher in *scurfy* T_{reg} cells where FOXP3 expression is defective (corresponding to a weak GFP signal), SATB1 signal was lower in wild type T_{reg} cells (corresponding to a strong GFP signal, Fig. 13 a).

Next, we asked whether the loss of FOXP3 expression also results in increased levels of SATB1 mRNA expression. Therefore, T_{reg} cells from male DEREG mice harbouring the *scurfy* mutation as well as T_{reg} cells from normal DEREG mice were isolated by flow cytometry and mRNA expression of SATB1 was quantified by qRT-PCR. Our data demonstrate that non-functional FOXP3 correlated with induction of SATB1 expression at even higher levels than the levels observed in T_{conv} cells from DEREG or DEREG mice with non-functional FOXP3 (Fig. 13, b).

Using flow cytometry of thymocytes from female mice heterozygous for the *scurfy* mutation, we could confirm that *scurfy* T_{reg} cells showed higher SATB1 protein expression relative to wild type counterparts (Fig. 13, c).

Functional wild type T_{reg} cells are unable to produce proinflammatory cytokines. In contrast, T_{reg} cells from *scurfy* mice have been reported to produce Th2 cytokines, suggesting that they mediate inflammatory rather than regulatory responses. Moreover, SATB1 has been reported to be involved in the production of Th2 cytokines [190, 249, 252, 253]. To investigate whether enhanced expression of SATB1 in T_{reg} cells is associated with the production of proinflammatory cytokines we assessed mRNA expression of proinflammatory cytokines by

qRT-PCR. Indeed, the derepression of SATB1 expression in T_{reg} cells from *scurfy mice* was associated with a significant production of Th1/Th2 cytokines (IL-6 and IFN-γ) in comparison to wild type T_{reg} cells from DEREG mice (Fig. 13, d).

In summary, our data indicate that the loss of functional FOXP3 increases SATB1 expression and results in the production of Th1/Th2 cytokine gene expresison in *scurfy* T_{reg} cells. This evidence further supported the association that FOXP3 might actively repress SATB1 expression.

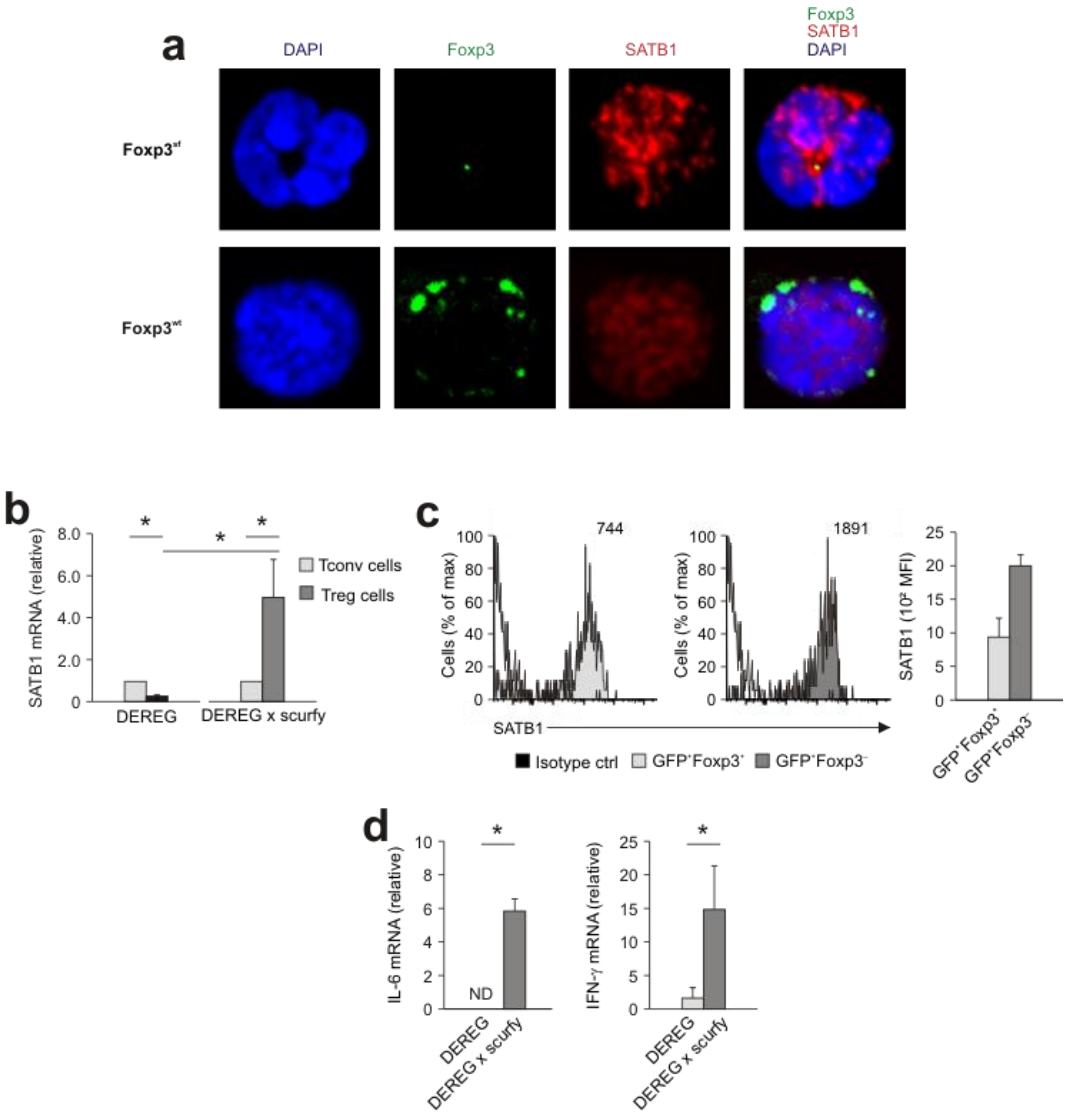

Figure 13. Loss of FOXP3 function results in SATB1 induction.

(**a**) Confocal microscopy for SATB1 (red) and FOXP3 (green) in freshly sorted thymic T_{reg} cells from female DEREG mice heterozygous for the *scurfy* mutation counterstained with DAPI (blue). FOXP3 competent $CD4^+GFP^+FOXP3^+$ ($FOXP3^{wt}$) T_{reg} cells or FOXP3 incompetent $CD4^+GFP^+FOXP3^-$ T_{reg} cells ($FOXP3^{sf}$) T_{reg} cells. (**b**) qRT-PCR for SATB1 in freshly sorted $CD4^+GFP^+$ T_{reg} cells and $CD4^+GFP^-$ T_{conv} cells (♂ DEREG mice; ♂ FOXP3-deficient DEREG x *scurfy* mice). Data are representative of two experiments (mean ± s.d. of three replicates; * $p<0.05$). (**c**) qRT-PCR for IL-6 and IFN-γ mRNA production by T_{reg} cells derived from male DEREG or DEREG x *scurfy* mice. mean±s.d; * $p<0.05$; n.d. = not detectable. (**d**) Flow cytometry for SATB1 in freshly isolated thymic single positive $FOXP3^+$ (left, light grey) and $FOXP3^-$ (right, dark grey) GFP^+ T_{reg} cells from female DEREG mice heterozygous for the *scurfy* mutation; one representative experiment (left); mean±s.d. (right, n=2). Isotype control shown as solid line.

5.5.3.2 siRNA-mediated silencing of FOXP3 abrogates T_{reg}-cell function and upregulates SATB1 expression

To further characterize the role of FOXP3 in SATB1 gene repression, we used a siRNA-mediated silencing approach of FOXP3 in nT_{reg} cells (performed together with Anne Flach). Therefore, human nT_{reg} cells were freshly isolated and electroporated with siRNA targeting FOXP3. After electroporation, cells were rested and then incubated for 24 hrs in the presence of CD3 and IL-2 and used for further experiments. Specific silencing of FOXP3 resulted in low levels of FOXP3 mRNA and protein in FOXP3-silenced T_{reg} cells (Fig. 14, a). Next, the suppressive function of FOXP3-silenced T_{reg} cells was analyzed by co-cultivating the siRNA-treated T_{reg} cells with T_{conv} cells stimulated with beads as artificial antigen presenting cells (aAPC). These experiments clearly revealed that loss of FOXP3 resulted in an impairment of the suppressive activity of T_{reg} cells (Fig. 14, b). In addition, we assessed the expression of several well-known marker genes for T_{reg} cells in FOXP3-deficient cells by qRT-PCR and observed a reduction in the expression of several important T_{reg} cells markers genes (Fig. 14, c).

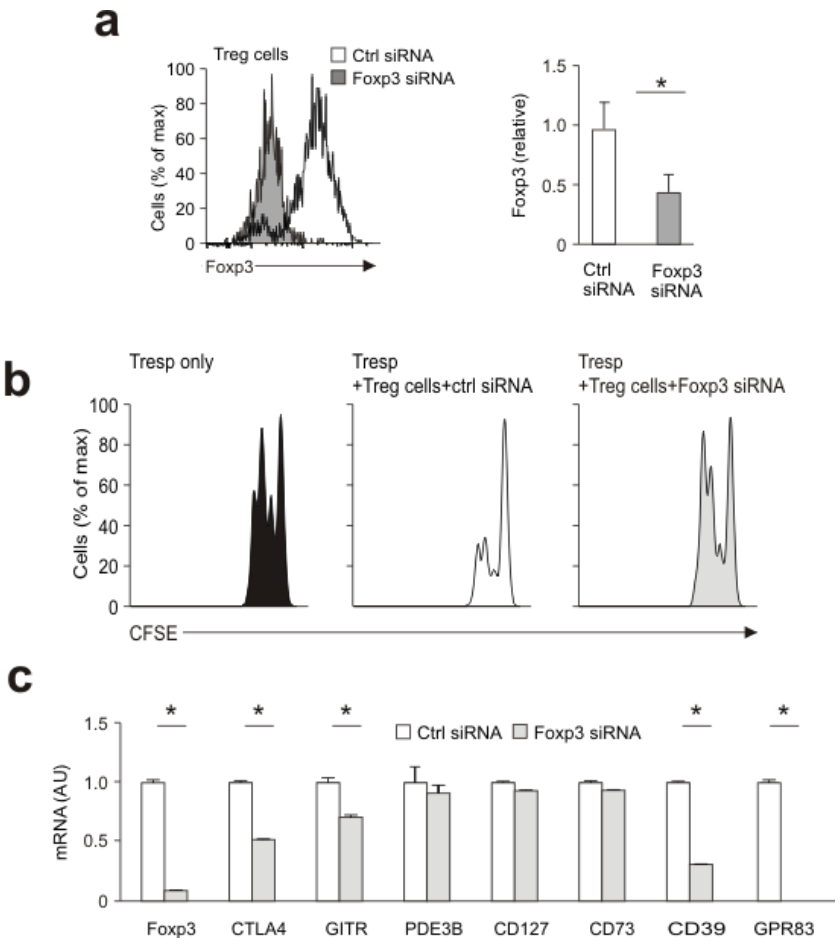

Figure 14. FOXP3 silencing impairs suppressive function and T_{reg}-cell associated genes expression.

To assess whether silencing of FOXP3 reverses the phenotype and function of T_{reg} cells, we isolated human T_{reg} cells and performed siRNA-mediated knockdown of FOXP3 and analyzed suppressive function after 24 hrs of incubation with CD3 and IL-2 as well as mRNA expression of typical Treg cell genes after stimulation for 48 hours with CD3 and CD28 (mean±s.d., n=3; * $p<0.05$). (**a**) Flow cytometric analysis of intracellular FOXP3 expression in T_{reg} cells post FOXP3 knockdown compared to control siRNA transfected T_{reg} cells (left: representative donor; right: mean±s.d., n=6; * $p<0.05$). (**b**) *In vitro* suppressive function of control or FOXP3 siRNA treated T_{reg} cells cultured for 4 d together with CFSE-labeled allogeneic $CD4^+$ T-cells at a 1:1 ratio; after stimulation of cultures, CFSE dilution was assessed by flow cytometry. (**c**) Treg-cell associated gene expression in FOXP3-silenced human T_{reg} cells (mean±s.d., n=3; * $p<0.05$).

Next, SATB1 expression levels were assessed in FOXP3-silenced and control siRNA treated T_{reg} cells. It could be shown that FOXP3-silenced T_{reg} cells expressed significantly higher levels of SATB1 in comparison to control-siRNA treated T_{reg} cells. Furthermore, SATB1 expression was further enhanced in FOXP3-silenced T_{reg} cells after stimulation with CD3 together with IL-2 or in combination with CD28 (Fig. 15, a). This increase in SATB1

expression upon FOXP3 silencing in T_{reg} cells was also associated with the expression of Th1/Th2 cytokine genes (IL-5 and IFN-γ, Fig. 15, b) and the production of Th1/Th2 cytokines (IL-4, and IFN-γ, Fig. 15 c) suggesting that SATB1 is actively involved in the production of these cytokines.

In conclusion, siRNA mediated knock down of FOXP3 in nT_{reg} cells reverses their suppressive function and results in a T_{eff} cell-like phenotype, manifested by an increase in the expression of SATB1 and the production of Th1/Th2 cytokines.

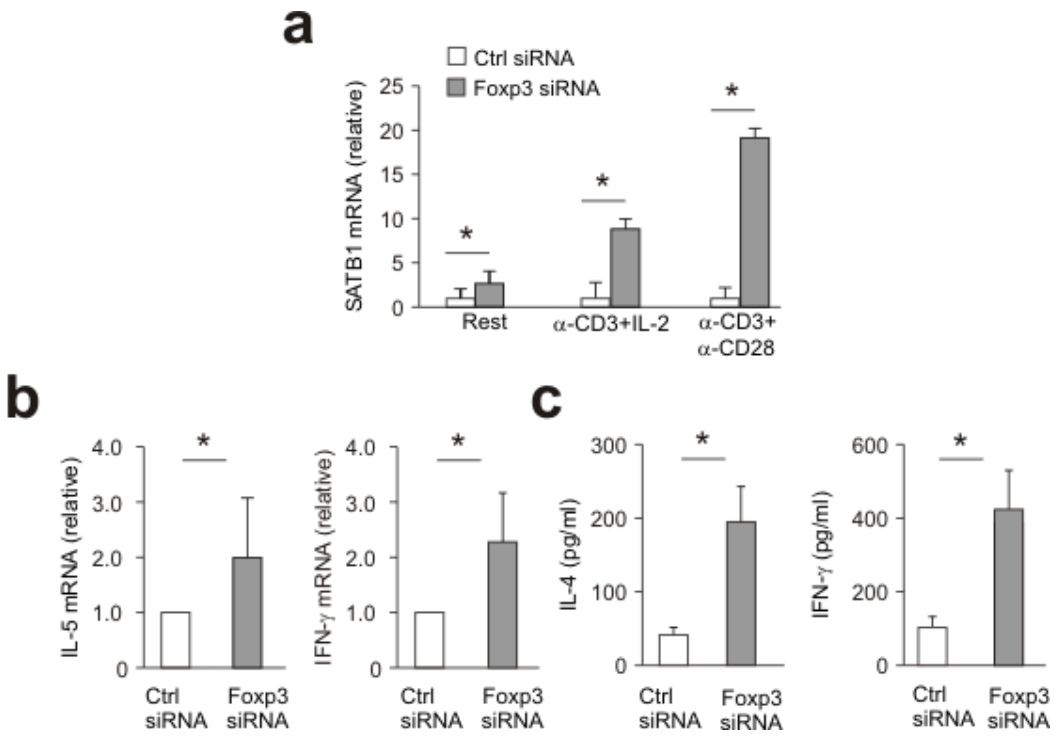

Figure 15. Rescue of SATB1 expression after silencing of FOXP3 in human T_{reg} cells.

Human T_{reg} cells were either transfected with control siRNA or FOXP3-specific siRNA. (**a**) qRT-PCR for SATB1 (mean±s.d., n=6; * $p<0.05$) in human T_{reg} cells after silencing of FOXP3. T_{reg} cells were transfected and cultivated for 48 hours without stimulation or in the presence of CD3 and IL-2 or CD3 and CD28. (**b**) qRT-PCR for IL-5 and IFN-γ (n=4, mean±s.d.; * $p<0.05$) in siRNA-treated T_{reg} cells stimulated for 48 hours in the presence CD3 and IL-2. (**c**) Cytometric bead array for IL-4 and IFN-γ (mean±s.d.; * $p<0.05$) in the supernatant of siRNA-treated T_{reg} cells stimulated for 48 hours in the presence of CD3 and IL-2.

5.5.3.3 SATB1 mediates proinflammatory cytokine production in FOXP3 deficient T_{reg} cells

A direct role of SATB1 in the transcription of proinflammatory cytokines has been reported [254]. However, the aforementioned experiments could only indirectly suggest that SATB1 is

required for the induction of the Th1/Th2 cytokine genes. To investigate whether the induction of SATB1 upon the loss of FOXP3 in T_{reg} cells drives the production of Th1/Th2 cytokines, we silenced FOXP3 alone or in combination with SATB1 in T_{reg} cells using siRNA and a miRNA (RNAi) based approach and assessed the production of cytokines by qRT-PCR.

Freshly isolated human T_{reg} cells were transfected either with FOXP3-siRNA alone or in combination with SATB1. Scrambled-siRNA treated T_{reg} cells were used as a control. Nucleofected cells were rested overnight, and then stimulated with CD3/CD28 antibodies for 48 hrs. Cells were harvested and the expression of FOXP3, SATB1 and cytokine genes was analysed. On the one hand, we observed that upon silencing of FOXP3 (Fig. 16, a) the expression of SATB1 as well as IL-5 and IFN-γ was increased in comparison with control siRNA treated cells (Fig. 16, a and b, gray bars). On the other hand, double knock down of FOXP3 and SATB1 significantly reduced both the expression of SATB1 and FOXP3 as well as the expression of cytokine genes (Fig. 16, a and b, black bars).

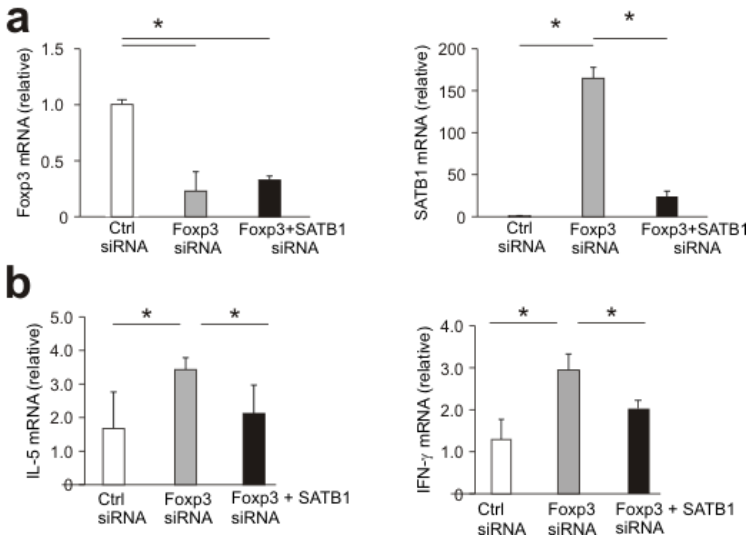

Figure 16. SATB1 induces the expression of cytokine genes in FOXP3-silenced T_{reg} cells.

Freshly isolated human T_{reg} cells were transfected with either FOXP3 and SATB1 siRNA, or control siRNA. The cells were rested overnight and afterwards stimulated for 48 hrs with CD3/CD28-coated beads. (**a**) Relative expression of FOXP3 and SATB1 after siRNA transfectionby qRT-PCR. (**b**) qRT-PCR for IL-5 (left) and IFN-γ (right) in T_{reg} cells (n=4, mean±s.d. one-way ANOVA with LSD; * $p<0.05$).

5.5.3.4 miRNA-mediated silencing of FOXP3 rescues SATB1 and cytokine gene expression in T_{reg} cells

Although the use of siRNAs has been used successfully for gene targeting, miRNA (RNAi) have many advantages over siRNAs. For instance, the short RNAs used for knock down are endogenously produced by the cellular machinery, multiple gene targeting is applicable, and higher success rates by stable expression can be achieved. Targeting oligo-sequences for FOXP3 and SATB1 were designed using the Block-it RNAi designer. Next, oligos were annealed and cloned into an intermediate vector, chained and recombined into a lentiviral expression vector. Finally, the lentivirus for different constructs were produced and used to transduce expanded T_{reg} cells. Subsequently, the transduced T_{reg} cells were sorted on GFP expression on the third day after transduction and stimulated for an additional 3 days. The supernatant and mRNA were used to analyse the expression level of cytokines in T_{reg} cells under different silencing conditions relative to control miRNA (RNAi) transduced cells. In accordance with the data obtained using siRNAs, successful silencing of FOXP3 (Fig. 17, a, gray bar) in T_{reg} cells was associated with the induction of SATB1 (Fig. 17, b, gray bar) and proinflammatory cytokines (IL-5 and IFN-γ, Fig. 17, c and d, gray bars). Moreover, co-silencing of FOXP3 and SATB1, significanly decreased the production of proinflammatory cytokines at mRNA and protein levels (Fig. 17, c and d, black bars).

Altogether the results obtained by loss of function approaches for FOXP3 support the hypothesis that FOXP3 represses SATB1 expression. Furthermore, FOXP3 and SATB1 double knockdown dampened the expression of Th1/Th2 cytokines in T_{reg} cells which suggests that SATB1 is directly involved in the expression of these genes.

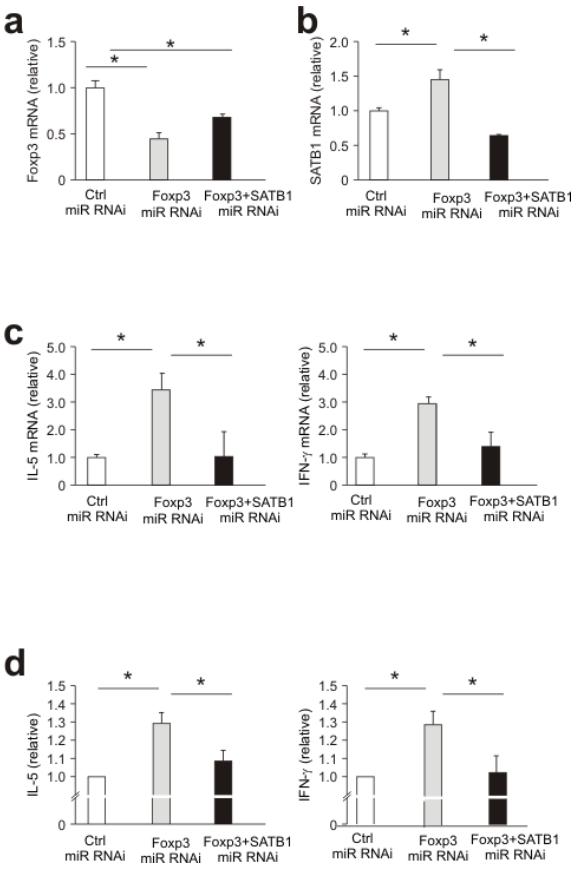

Figure 17. SATB1 expression is induced upon silencing of FOXP3 in expanded human T_{reg} cells with artificial miRNAs.

(**a**) Relative FOXP3, (**b**) SATB1, (**c**) IL-5 (left), and IFN-γ (right) mRNA expression in GFP^+ sorted expanded human T_{reg} cells transduced with nontargeting miR RNAi (Ctrl miR RNAi), FOXP3-targeting miR RNAi (FOXP3 miR RNAi) or FOXP3- and SATB1-targeting miR RNAi (FOXP3 + SATB1 miR RNAi). *P < 0.05 (Student's *t*-test). Data are representative of three experiments (mean and s.d.) with cells derived from different donors. (**d**) Relative IL-5 and IFN-γ secretion in the supernatants T_{reg} cells sorted as described above. *P < 0.05 (Student's *t*-test). Data is representative of one experiment representative of two (mean ± s.d. of triplicate wells) with cells derived from different donors.

5.6 FOXP3 directly controls SATB1 transcription

5.7 Identification of FOXP3 binding sites at the genomic SATB1 locus

Both loss and gain of function experiments pointed towards the existence of a putative FOXP3-SATB1 regulatory axis. To define the potential targets of FOXP3 in T_{reg} cells, FOXP3-ChIP tiling arrays using chromatin isolated from expanded cord blood natural T_{reg} cells were performed by the group of Simon Barry (University of Adelaide, North Adelaide, Australia). For this purpose, isolated $CD4^+CD25^+$ T_{reg} cells were expanded ex vivo using CD3/CD28 expander beads. The expanded T_{reg} cells were characterized phenotypically by the expression of FOXP3 and functionally by *in vitro* suppression assays and retained T_{reg} cell proterties over the expansion period. Chromatin preparations from expanded T_{reg} cells for ChIP were performed as follows: expanded T_{reg} cells were stimulated with ionomycin for 2 hrs, cross-linked with formaldehyde solution followed by quenching the formaldehyde with glycine and washing. Afterwards, anti-FOXP3 or control rabbit IgG were used to precipitate cross-linked protein–DNA complexes from nuclear lysates. The cross-linking of the

immunoprecipitated material was removed and the samples were proteinase treated. The obtained purified DNA was amplified and hybridized to probes covering only the known promoter regions of known genes in the whole human genome using Affymetrix GeneChip Human Tiling Array Set.

The data were analysed by Dr. Marc Beyer using model based analysis of tiling arrays (MAT) with a false discovery rate of (FDR) of 5%. 13 potential FOXP3 binding regions at the SATB1 gene locus were identified with significant enrichment in FOXP3 binding. The FOXP3 binding sites are distributed in the promoter and within the genomic region of SATB1 (Fig. 18, a and b). Combining these data with bioinformatic *in silico* prediction, we could identify 16 regions for qRT-PCR validation. Remarkably, FOXP3 binding was preferentially enriched in all identified binding regions (Fig. 18, c). To corroborate the specificity of FOXP3 enrichment at the SATB1 locus, analysis of known T_{reg}-cell genes was performed, FOXP3 binding to the promoter regions of CTLA-4, IL-7R, and two regions within the PDE3B locus as positive controls as well as AFM and intron 10 at the PDE3B locus as negative controls were assessed by promoter arrays, whole genome tiling arrays, and ChIP-qRT-PCR. While no enrichment was observed at the AFM locus or at intron 10 at the PDE3B locus, FOXP3 binding was significantly enriched at the promoters of CTLA-4, IL-7R, and at two regions within the PDE3B locus as well as at the SATB1 locus (Fig. 19).

Taken together, results from both tiling array and qRT-PCR strongly indicated that FOXP3 binds directly at the SATB1 locus. This observation, in concert with the decreased levels of SATB1 mRNA and protein in the presence of FOXP3 suggests that FOXP3 directly represses SATB1 expression.

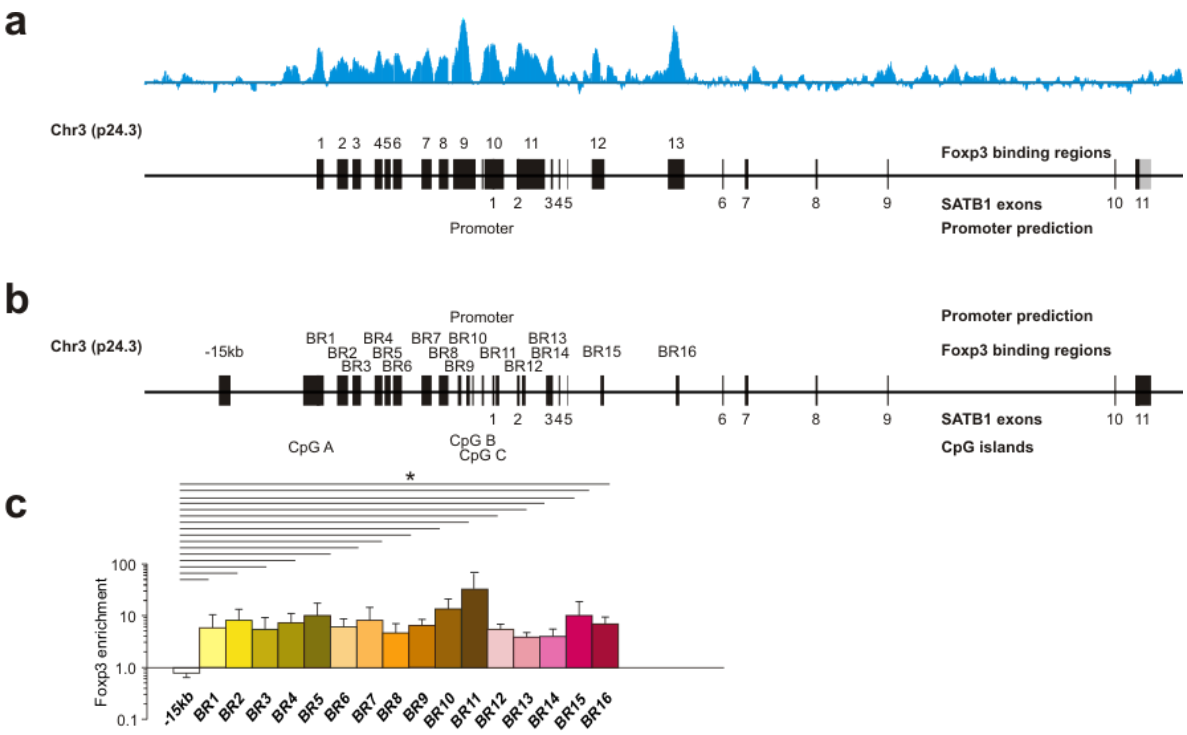

Figure 18. FOXP3 binds to the genomics SATB1 locus.

(**a**) FOXP3 ChIP tiling array data from human expanded cord-blood T_{reg} cells assessed with a FOXP3 antibody and hybridized to Affymetrix tiling arrays. Data were analyzed with MAT and overlayed to the SATB1 locus to identify binding regions (1-13, $p<10^{-5}$ and FDR<0.5%). (**b**) Schematic representation of FOXP3 binding regions (BR) at the human genomic SATB1 locus identified by *in silico* prediction within the indentified regions. (**c**) ChIP-qRT-PCR for FOXP3 binding at the SATB1 locus in human expanded cord-blood T_{reg} cells. Input DNA and precipitated DNA were quantified by qRT-PCR; the same chromatin was used for control ChIP experiments with immunoglobulin G–coupled Dynabeads. Enrichment over input DNA was normalized to control IgG (mean±s.d.; * $p<0.05$; n=3).

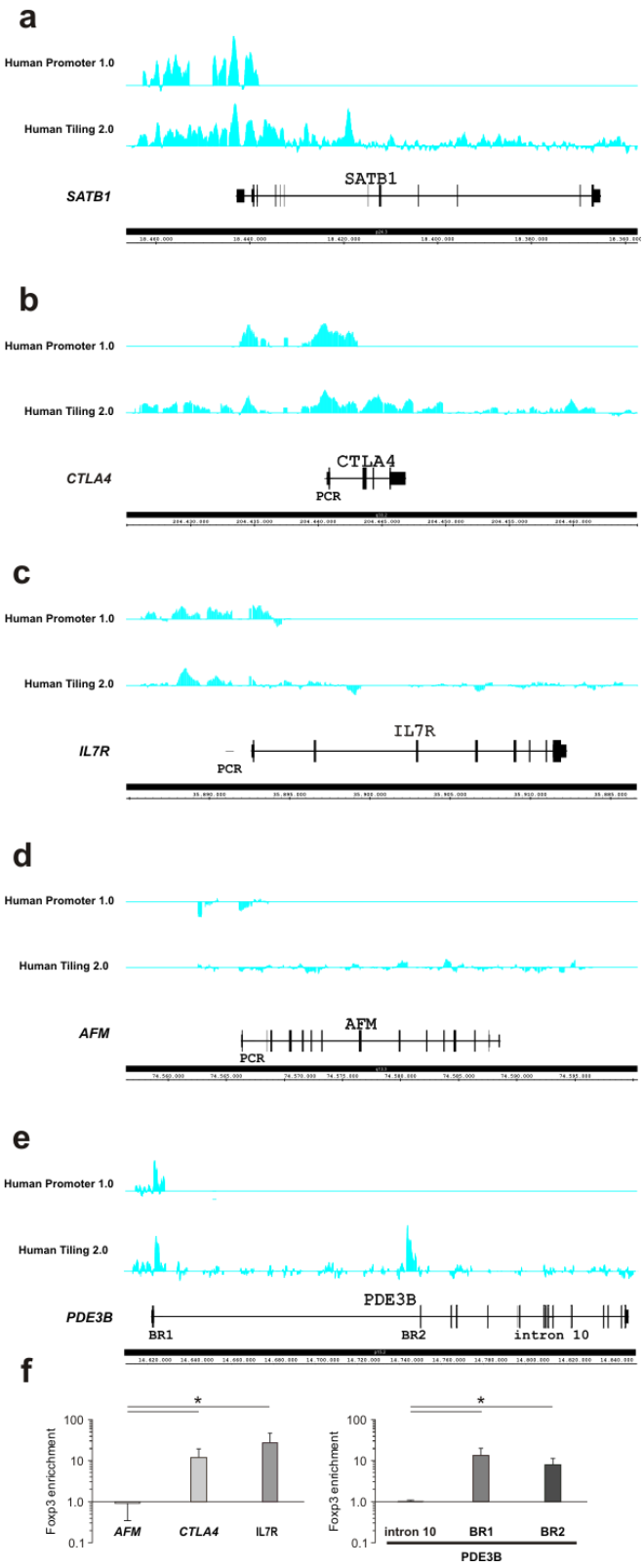

Figure 19. Array and ChIP-qRT-PCR results for FOXP3 binding known FOXP3 target genes.

FOXP3 ChIP-on-chip experiments and data analysis using MAT were previously described [248]. Each bar represents the signal intensity of an individual oligonucleotide probe on Affymetrix Human Promoter 1.0 (upper panel) or Human Tiling 2.0R Arrays (lower panel). ((**a**) FOXP3 binds to the human SATB1 locus. (**b**) FOXP3 binds to the promoter region of the human CTLA4 locus. (**c**) FOXP3 binds to the human IL7R locus. (**d**) No FOXP3 binding to the human AFM locus. (**e**) FOXP3 binds to the human PDE3B locus. (**f**) Confirmation of FOXP3 binding by ChIP-qRT-PCR. Quantitative PCR was performed using primer sets corresponding to the marked regions and FOXP3 antibody or control IgG precipitated chromatin isolated from T_{reg} cells. Relative enrichment of FOXP3 Chip over input normalized to IgG was calculated. The AFM locus or intron 10 of PDE3B were used as negative controls.

5.7.1 FOXP3 binds with high affinity to motifs within the human SATB1 locus

Next, to demonstrate the specificity of the binding of FOXP3 to the genomic SATB1 locus and assess the affinity of this binding we characterized FOXP3 binding at the SATB1 locus in a cell-free assay. Filter retention assays are a simple method used to study the binding affinity between two molecules such as nucleic acids and protein. Briefly, dsDNA oligos containing a FOXP3 binding motif of the specific binding regions 9 and 10 at the SATB1 locus were synthesized and labeled with [$^{\gamma\text{-}32}$P]. The labeled oligos were incubated with different concentrations of FOXP3 recombinant protein. Then, the complexes were passed through a nitrocellulose membrane and bound protein-DNA complexes quantified by phosphor-imaging. Filter binding assays revealed that FOXP3 binds with a K_D of 516.2 nM and 579.8 nM to a FOXP3 binding motif within BR9 and BR10, respectively. Furthermore, the high affinity of FOXP3 binding to the SATB1 locus was abrogated upon mutation of the specific motifs in the dsDNA oligos (Fig. 20).

Taken together, these experiments confirmed the specific binding of FOXP3 to the genomic SATB1 locus and could establish for the first time the binding affinity of FOXP3 to specific DNA sequences.

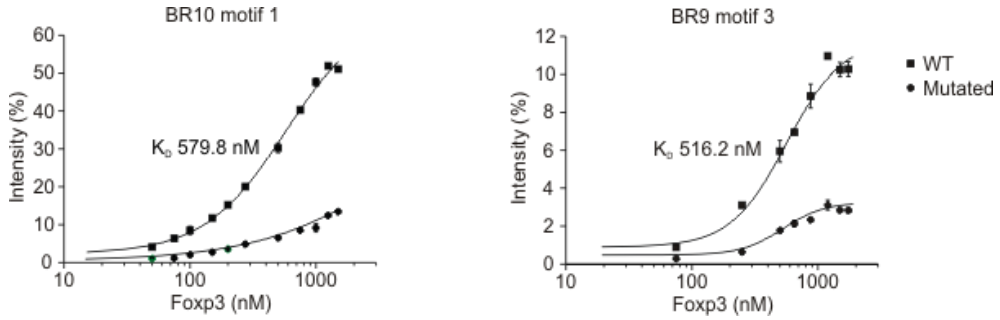

Figure 20. FOXP3 binds with high affinity to the SATB1 locus.

Filter binding assays to determine the kinetic of binding of FOXP3 at the SATB1 locus expressed as K_D-values of FOXP3 binding to FOXP3 binding motifs in BR9 and BR10 at the SATB1 locus. His-tagged-FOXP3 protein was expressed in E.coli and purified using Ni-NTA resins. dsOligos were labeled with [$^{\gamma\text{-}32}$P] using T4 polynucleotide kinase. The labeled dsOligos were incubated with increasing concentration of FOXP3 proteins, filtered through a nitrocellulose membrane, washed tree times with binding buffer and dried. DNA retained on the membranes was quantified with a phosphorimager (mean±s.d.; * $p<0.05$; n=3).

5.7.2 Binding of FOXP3 to the SATB1 locus suppresses SATB1 transcription

The identification of putative FOXP3 binding sites at the genomic SATB1 locus requires further functional verification to demonstrate regulation of transcriptional activity. To study the functional significance of this binding, reporter assays using conventional luciferase plasmids were performed. The six binding regions with the highest probability score were cloned into the pGL4.24 firefly reporter construct. These constructs were co-transfected into HEK293 cells with an expression vector encoding for full length FOXP3 gene and the pGL4.74 plasmid, a renilla luciferase expressing vector under control of a constitutive promoter as an internal control. The activity of luciferases was measured using dual luciferase kit. (The cell culture, transfections, and luciferase assays were performed in the laboratory of Bernhard Schermer and Thomas Benzing (University Hospital Cologne). Measurements of luciferase activity showed a significant reduction for 4 of the 6 regions analyzed in the presence of FOXP3. Interestingly, all sites that showed reduced activity had more than one FOXP3 binding motif suggesting that cooperative or multiple binding of FOXP3 might be necessary to functionally alter gene expression. Next, the specificity of the binding was assessed by mutating FOXP3 binding sites. The luciferase assays were repeated under the same conditions that had been described above. The luciferase activity was rescued upon mutation of these sites which confirmed the specificity of FOXP3 binding to the SATB1 locus (Fig. 21).

Taken together, these reporter assays could establish a functional link between FOXP3 expression and transcriptional control of the SATB1 locus, supporting the repressive activity of FOXP3 on the SATB1 locus at the functional level.

In summary, the data generated by loss and gain of function experiments, ChIP tiling arrays, ChIP qRT-PCR, filter binding, and luciferase assays provided strong evidence of the presence of a FOXP3-SATB1 regulatory axis, in which FOXP3 suppresses SATB1 gene expression.

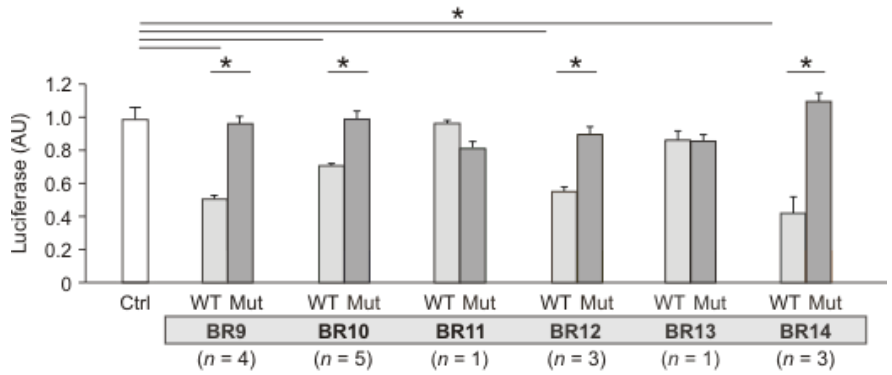

Figure 21. Direct suppression of SATB1 mRNA transcription by FOXP3.

Luciferase reporter assays comparing wildtype to mutated FOXP3 binding regions (mean±s.d.; * p<0.05; n=3). FOXP3 binding was assessed by transfecting a reporter construct containing the potential FOXP3 binding region in cells expressing FOXP3 in comparison to an empty control. Constructs with mutated motifs were used to demonstrate specificity.

5.8 Posttranscriptional regulation of SATB1 by FOXP3 dependent microRNAs

5.8.1 Identification of T_{reg} cell distinctive miRNAs

miRNAs regulate gene expression post-transcriptionally by forming imperfect base pairing with sequences within the 3` untranslated region (3`UTR) of genes to prevent protein accumulation by repressing translation or by inducing mRNA degradation [129, 255]. miRNAs have been reported as important players shaping the T_{reg}-cell phenotype and function [130, 243, 256-258]. In order to explore miRNA-dependent regulation of SATB1 expression in T_{reg} cells, miRNA profiling was performed in T_{reg} and T_{conv} cells by Dr. Marc Beyer using whole miRNA transcriptome arrays. miRNA expression analysis revealed that T_{reg} and T_{conv} cells harbor a distinctive miRNA signature (Fig.22). Next, we wanted to identify T_{reg}-cell specific miRNAs targeting the 3´UTR of SATB1. Using the miRNA expression data as well as bioinformatic *in silico* analysis, we identified miR-155, miR-21, miR-18a, miR-7, and miR-34 as potential cadidates binding to the SATB1 3`UTR (Fig. 22, a). To validate the preferential expression of these miRNAs in T_{reg} cells, we assessed their expression in T_{reg} and T_{conv} cells using miRNA-specific qRT-PCR. In fact, the predicted miRNAs showed an increase of 4 to 10 fold in T_{reg} compared with T_{conv} cells (Fig. 23, b).

The increased expression of miR-155, miR-21, miR-18a, miR-7, and miR-34 in T_{reg} cells in addition to their predicted binding to the 3´UTR of SATB1 region suggests that the high

prevalence of these miRNAs in T_{reg} cells might contribute to the regulation of SATB1 expression.

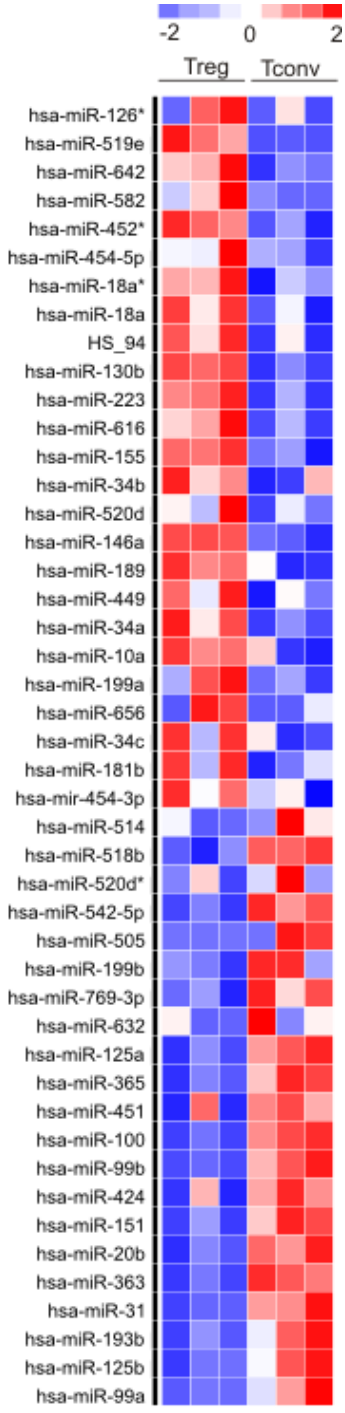

Figure 22. T_{reg} cells show a distinct miRNA profile.

All RNAs were extracted and purified from freshly isolated human T_{reg} and T_{conv} cells.
Heatmap displaying expression of 46 miRNAs differentially expressed in T_{reg} cells and T_{conv} cells. Average expression signals were standardized using Z score transformation.

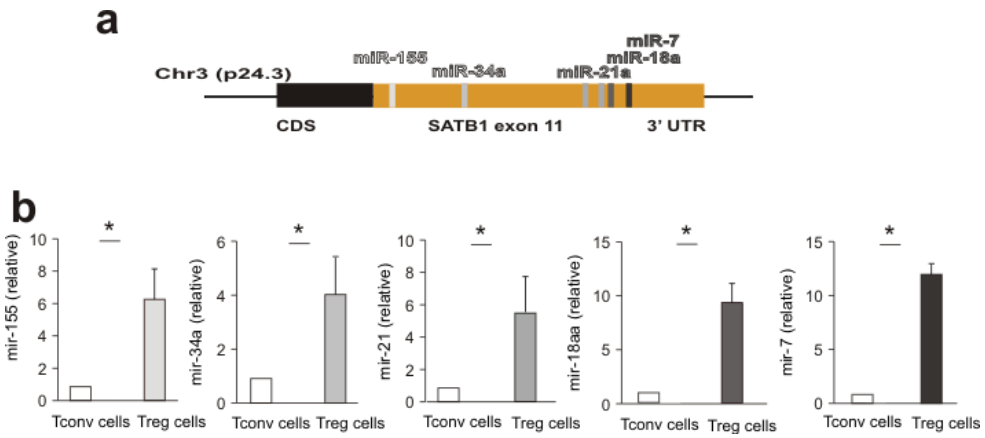

Figure 23. Identification of miRNAs potentially targeting SATB1 in T_{reg} cells.

(**a**) Representation of the human genomic SATB1 3′ UTR and the conserved miRNA binding sites. (**b**) Quantification of differentially expressed miRNAs in T_{conv} and T_{reg} cells by qRT-PCR for miR-155, miR-21, miR-7, miR-34a, and miR-18a in human T_{reg} and T_{conv} cells (mean±s.d. n=5; * $p<0.05$).

5.8.2 FOXP3 binds to the genomic loci of the identified miRNAs

Previous studies have shown that FOXP3 drives several miRNAs expression such as miR-155, miR-146a and miR-21 [259, 260]. Genome wide FOXP3 ChIP tiling array data provided by the group of Simon Barry was analyzed by Dr. Marc Beyer in order to identify FOXP3 target miRNAs. FOXP3 binding peaks were indentified in close proximity to the sequences encoding miR-155, miR-21, and miR-7 suggesting that FOXP3 might regulate expression of these miRNA. Afterwards, binding of FOXP3 was validated by ChIP-qRT-PCR for all three loci (Fig. 24, a and b). These data supported that FOXP3 induces miRNA that can potentially target the 3´UTR of SATB1 and thereby regulate SATB1 expression.

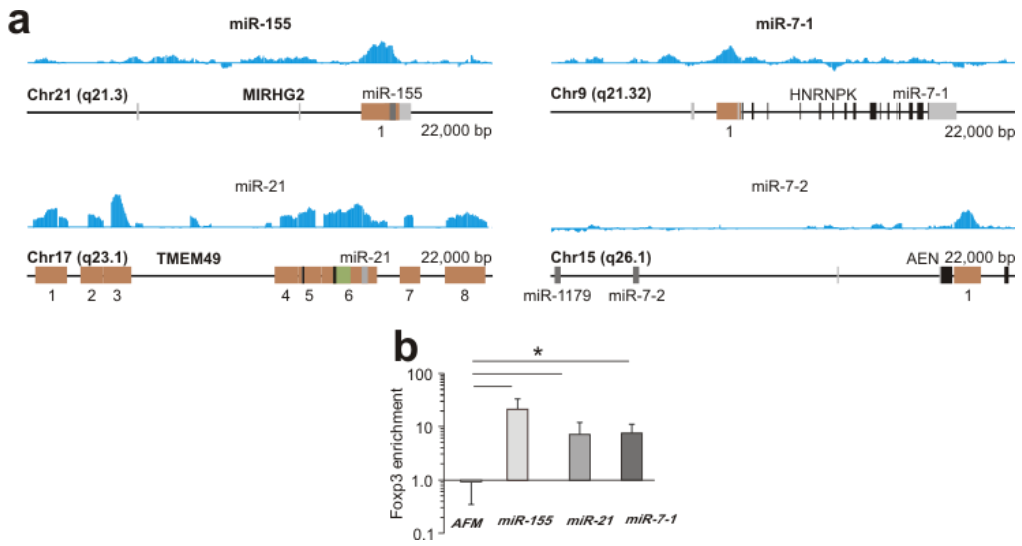

Figure 24. FOXP3 binds to genomic loci encoding for miRNAs.

(**a**) FOXP3 ChIP tiling array data for miR-155, miR-21, and miR-7. (**b**) Confirmation of FOXP3 binding to the miR-155, miR-21, and miR-7 loci by ChIP-qRT-PCR. Quantitative RT-PCR was performed using primer sets corresponding to the marked regions. Relative enrichment of FOXP3 Chip over input normalized to IgG was calculated. The AFM locus was used as a negative control.

5.8.3 FOXP3 overexpression in CD4$^+$ T-cells induces T_{reg}-cell associated miRNAs

To investigate whether expression of the aforementioned miRNAs is FOXP3 dependent, FOXP3 was overexpressed in T_{reg}-cell depleted naïve CD4$^+$T-cells as described above (in the lab of James Riley, Perelman School of Medicine, University of Pennsylvania). The expression of individual miRNA (miR-155, miR-21, miR-18a, miR-7, and miR-34) was quantified using qRT-PCR. This analysis revealed that enforced FOXP3 expression in naïve CD4$^+$T-cells resulted in an enrichment of these T_{reg}-cell associated miRNAs (Fig. 25, a).

Next, to further corroborate the feed-forward loop between FOXP3 expression and the expression of these miRNA, we performed siRNA mediated knockdown of FOXP3 in human T_{reg} cells and assessed miRNA expression in these cells. Remarkably, the loss of FOXP3 function resulted in the downregulation of the expression of these miRNAs in human T_{reg} cells in comparison to control siRNA treated T_{reg} cells (Fig. 25, b).

In conclusion, FOXP3 gain and loss of function experiments cleary showed that FOXP3 induces T_{reg}-cell associated miRNAs as an important part of its regulatory program.

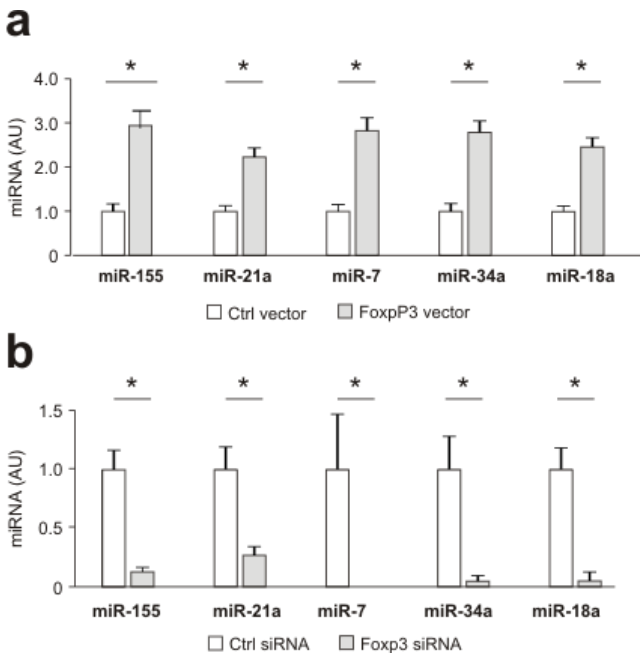

Figure 25. Expression of miRNAs is dependent on FOXP3.

(**a,b**) To assess whether FOXP3 is responsible for the increased expression of miR-155, miR-21a, miR-7, miR-34a, and miR-18a, expression of these miRNAs was assessed by qRT-PCR. (**a**) T_{conv} cells were transduced with lentivirus containing the full length human FOXP3 cDNA and assessed adter sorting on FOXP3-expressing cells. (**b**) SiRNA-mediated knockdown of FOXP3 was performed in isolated human T_{reg} cells and miRNA expression was analyzed after stimulation for 48 hours with CD3 and IL-2. Data are representative of 3 independent experiments (mean and s.d.), with cells derived from different donors.

5.8.4 T_{reg} cell-associated miRNAs regulate SATB1 expression in T_{reg} cells

As shown above, SATB1 is a direct target of FOXP3. Moreover, several FOXP3-driven miRNAs potentially target the 3´UTR of SATB1. To address whether expression of FOXP3-induced miRNA can functionally regulate transcription of SATB1, luciferase reporter assays were performed. The 3`UTR sequence of SATB1 was cloned into a reporter plasmid downstream of the renilla luciferase. Transfection of synthetic miRNAs (miR-155, miR-21, miR-18a, miR-7, and miR-34) together with the reporter plasmid harbouring the 3`UTR sequence of SATB1 resulted in a significant inhibition of luciferase activity (Figure 25, a). (All transfections and luciferase assays were performed in the laboratory of Bernhard Schermer and Thomas Benzing, University Hospital Cologne). This inhibition of transcription suggested that these miRNAs are potential inhibitors of SATB1 expression in T_{reg} cells. To further support the specific impact of these miRNA, the binding sites for these miRNAs within the 3´UTR of SATB1 were mutated and luciferase activities were assessed under the

same conditions. Mutation of the seed sequences of the binding sites restored luciferase activity (Fig. 26, a) suggesting the involvement of FOXP3-dependent miRNAs in the repression of SATB1 expression.

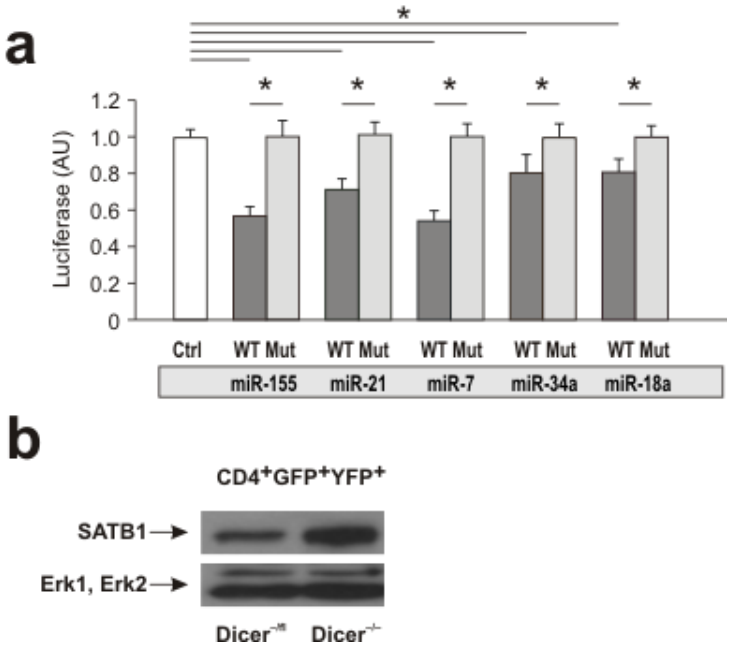

Figure 26. SATB1 is repressed by miRNAs in T_{reg} cells.

(**a**) Luciferase reporter assays comparing the wildtype 3' UTR of SATB1 to a 3' UTR containing mutated miRNA-binding sites (mean±s.d.; * $p<0.05$, n=3). Functional miRNA binding was assessed by transfecting either a reporter construct containing the WT or the mutated SATB1 3′ UTR in cells transfected with miRNA mimics. (**b**) Western blot of SATB1 in sorted T_{reg} cells from mice with a T_{reg} cell-specific complete loss of Dicer (*Dicer*$^{fl/fl}$) in comparison to heterozygous DICER$^{wt/fl}$ T_{reg} cells.

We could observe that inhibition of a single miRNA in human T_{reg} cells did not exert a major inhibitory effect on SATB1 expression. This finding suggested that several miRNAs might act synergistically at the locus of SATB1 and that expression of several miRNA might be required to suppress SATB1 gene expression. To study the cooperative effect of multiple miRNAs on the expression of SATB1, T_{reg} cells from conditional *Dicer* knock-out mice (devoid of functional miRNAs in T_{reg} cells) or heterozygous *Dicer* $^{wt/fl}$ mice were isolated and assessed by immunobloting for SATB1 in the laboratory of Jeffrey Bluestone (University of California, San Francisco, California, USA). T_{reg} cells deficient in Dicer expression showed elevated levels of SATB1 protein compared to Dicer expressing T_{reg} cells. This result

supported that miRNAs are important for the regulation of SATB1 expression in T_{reg} cells (Fig. 26, b).

Taken together, these experiments showed that the expression of SATB1 is regulated at the post-transcriptional level by FOXP3 via FOXP3-induced miRNAs.

5.9 Epigenetic control of SATB1

Besides gene regulation by direct binding and post-transcriptional mechanisms, epigenetic regulation constitutes an additional layer of how gene expression can be modulated. Both methylation of CpG islands within the upstream promoter or enhancer elements as well as permissive or repressive histone modifications have been described to modulate gene accessibility enhancing or repressing gene transcription.

5.9.1 Methylation status of CpG islands at the SATB1 locus in T_{reg} cells is similar to T_{conv} cells

DNA methylation is involved in the regulation of many cellular processes including X-chromosome inactivation, genomic imprinting, and transcription. DNA methylation involves the covalent modification of the fifth carbon in cytosine residues of CG dinucleotides. CpG islands are stretches of DNA rich in CG dinucleotides that occur near the transcription start sites of approximately 50% of all mammalian genes [140, 261].

CpG islands near the SATB1 promoter have been indentified via analysis of the distribution of 5-methylcytosine at the SATB1 locus by Dr. Marc Beyer. This approach resulted in the prediction of three CpG-rich sites upstream of exon 1 at the SATB1 locus in $CD4^+$ T-cells (Fig. 27, a). To explore the contribution of CpG methylation in the control of SATB1 expression, bisulphate sequencing of human T_{reg} and T_{conv} cells was performed by the group of Andreas Waha (Neuropathology Institute, University Hospital Bonn). In brief, human $CD4^+$ Tcells were isolated, and sorted into T_{reg} and T_{conv} cells. Genomic DNA was isolated from both cell populations and subjected to bisulphate sequencing. This experimental approach relies on the conversion of only unmethylated cytosine to uracil mediated by bisulfite treatment. Uracil residues are subsequently converted into thymidines upon PCR amplification.

Bisulphate sequencing analysis showed no significant differences in methylation between T_{reg} and conventional $CD4^+$ T-cells as CpG islands are almost completely demethylated in both

cells populations as indicated by the yellow color code (Fig. 27, a). Conversely, as a positive control a well described CpG island within the promoter of the human FOXP3 locus showed differential methylation between T_{reg} and T_{conv} cells with heavy methylation in T conv cells; while T_{reg} cells showed a largely demethylated CpG island (Fig. 27, b).

Taken together these data indicate that CpG methylation is similar in T_{reg} and T_{conv} cells supporting a model where SATB1 expression in T_{reg} cells is repressed by reversible mechanisms allowing for a possible induction of SATB1 expression which would be prohibited by methylation of DNA bases.

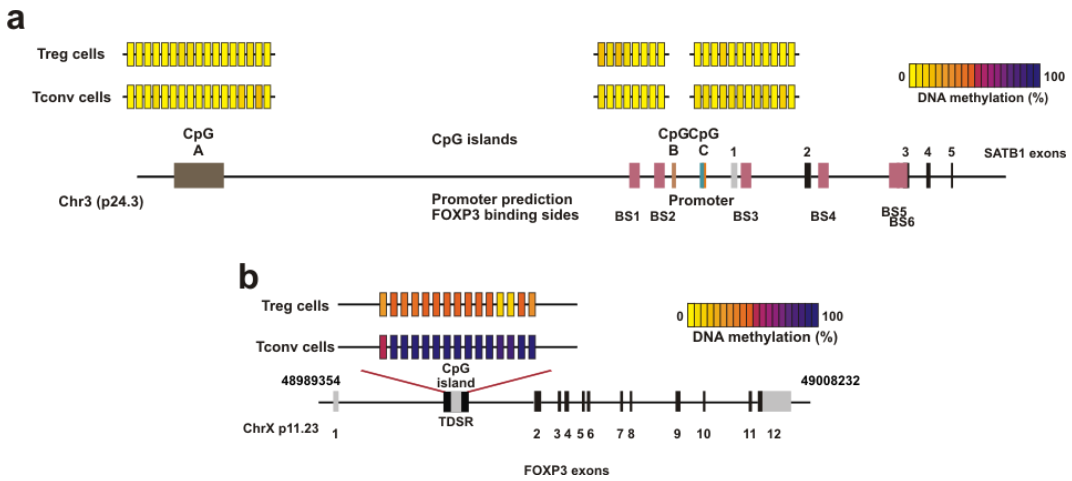

Figure 27. No difference in DNA methylation of the CpG islands at the human SATB1 in freshly isolated T_{reg} cells and T_{conv} cells.

(**a**) DNA methylation of the three predicted CpG-islands in the genomic region of *SATB1* in T_{reg} cells and T_{conv} cells.T_{reg} cells Each box represents an individual CpG motif after normalization and quantification of methylation signals from sequencing data by calculating ratios of T and C signals at CpG sites. The methylation status of individual CpG motifs is color coded according to the degree of methylation at that site. The color code ranges from yellow (0% methylation) to blue (100% methylation) according to the color scale on the right. Data are representative of three independent experiments (mean) with cells derived from different donors. (**b**) As a positive control DNA methylation of a described CpG-island within the human *FOXP3* promoter was assessed in T_{reg} cells and T_{conv} cells. T_{reg} cells Data are representative of three independent experiments (mean) with cells derived from different donors.

5.9.2 Enrichment of non-permissive histone modifications at the SATB1 locus in human T_{reg} cells

The covalent modification of nucleosomal histones has emerged as an important determinant of chromatin structure and gene activity. The nature, position and degree of modification correlates with the level of transcription and modifications are largely restricted to transcribed regions, suggesting that their regulation is tightly linked to polymerase activity [262, 263].

To investigate histone modifications at the genomic SATB1 locus and whether these histone marks correlate with its differential expression in T_{reg} and T_{conv} cells, ChIP for several histone modifications with subsequent qRT-PCR was performed. Briefly, expanded human T_{reg} and T_{conv} cells were lysed and treated with micrococcal nuclease (MNase) to generate mononucleosomes and dinucleosomes. Afterwards, ChIP was performed by Wolfgang Krebs by using antibodies against trimethylation of histone H3 at Lysine residue 4 (H3K4me3), acetylation of histone H4 (H4Ac) and trimethylation of histone H3 at Lysine ridue 27 (H3K27me3) as well as the isotype control antibodies. After ChIP, DNA was isolated and qRT-PCR performed with primers every 1-2 kb within the upstream and promoter region of SATB1.

Consistent with the SATB1 expression data, we observed an enrichment of the permissive histone modifications H3K4me3 and H4Ac in T_{conv} cells (Fig. 28, a and b); whereas the repressive mark H3K4me27 was enriched in T_{reg} cells (Fig. 28, c).

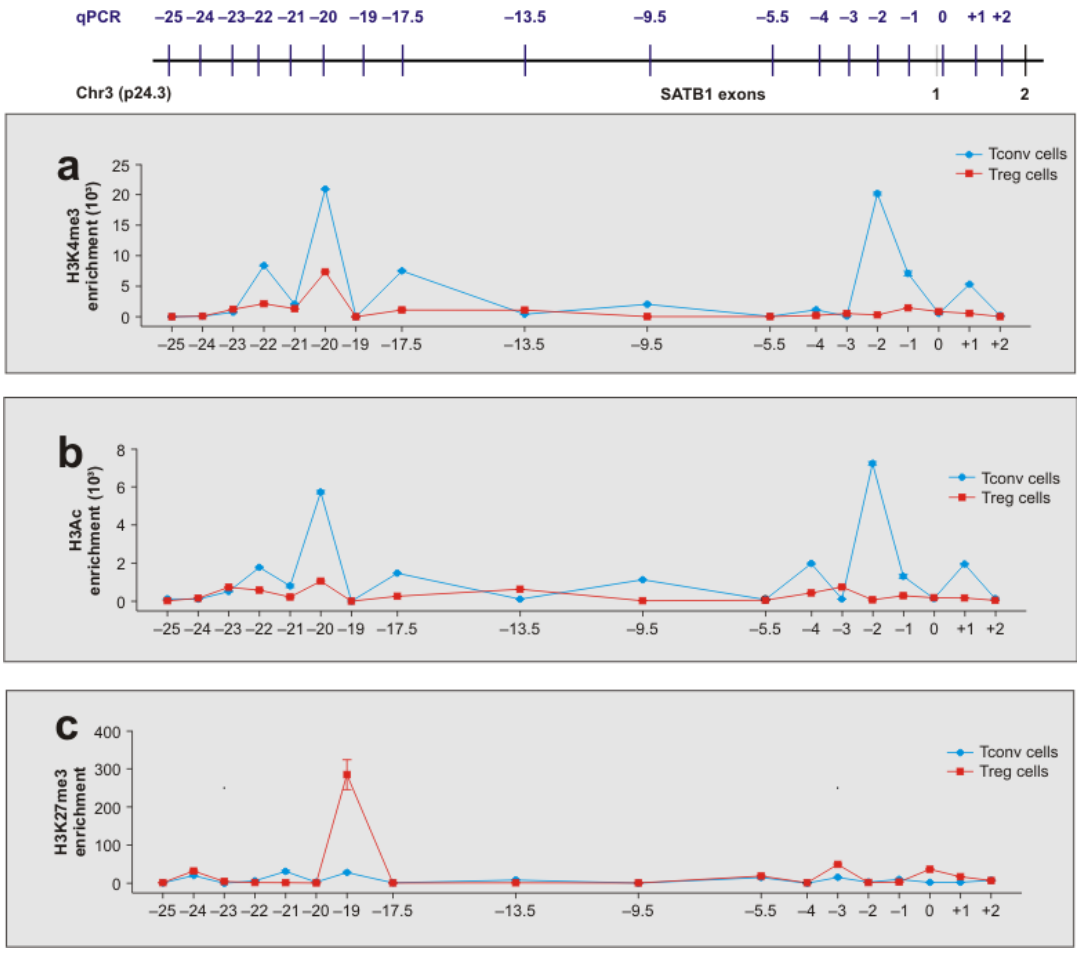

Figure 28. Differences in epigenetic status contribute to the differential expression of SATB1 in human T_{reg} cells.

Freshly isolated human CD4+ T-cells were stained with antibodies against CD4, CD25, and CD127 and sorted into T_{reg} cells$CD4^+CD25^+CD127^{lo}$ Treg and $CD4^+CD25^-CD127^{hi}$ T_{conv} cells. The sorted cells were expanded with Treg expander beads for 2-3 weeks. 10^7 cells were used for ChIP experiment. (**a-c**) ChIP analysis of human expanded T_{reg} cellsand T_{conv} cells with antibodies specific for the permissive histone modifications (**a**) H3K4me3 and (**b**) H4Ac and (**c**) the repressive histone modification H3K27me3 and PCR primers specific for the genomic *SATB1* locus. Relative enrichment of histone ChIP over input normalized to IgG was calculated. *P < 0.05 (Student's *t*-test). Data are representative of three independent experiments (mean and s.d.) with cells derived from different donors.

Recently an elegant study has explored the genome-wide distribution of several histone modifications of different murine $CD4^+$ T cell-lineages and correlated these histone landscapes with the corresponding transcriptional profiles [264]. These data were reanalyzed by Dr. Marc Beyer to explore histone methylation at the SATB1 locus in different murine $CD4^+$ T-helper cell lineages. Analysis of histone methylations showed that the repressive methylation mark H3K27me3 was enriched in T_{reg} cells at the genomic SATB1 locus which

correlates with the expression pattern of SATB1 in this cell lineage. On the contrary, the SATB1 locus was enriched for permissive H3K4me3 mainly in the helper $CD4^+$ T cell lineages (Th1, Th2, Th17) (Fig. 29).

Taken together, the human as well as the murine data suggest that histone modifications can modulate the expression of SATB1 in T_{reg} and T_{conv} cells.

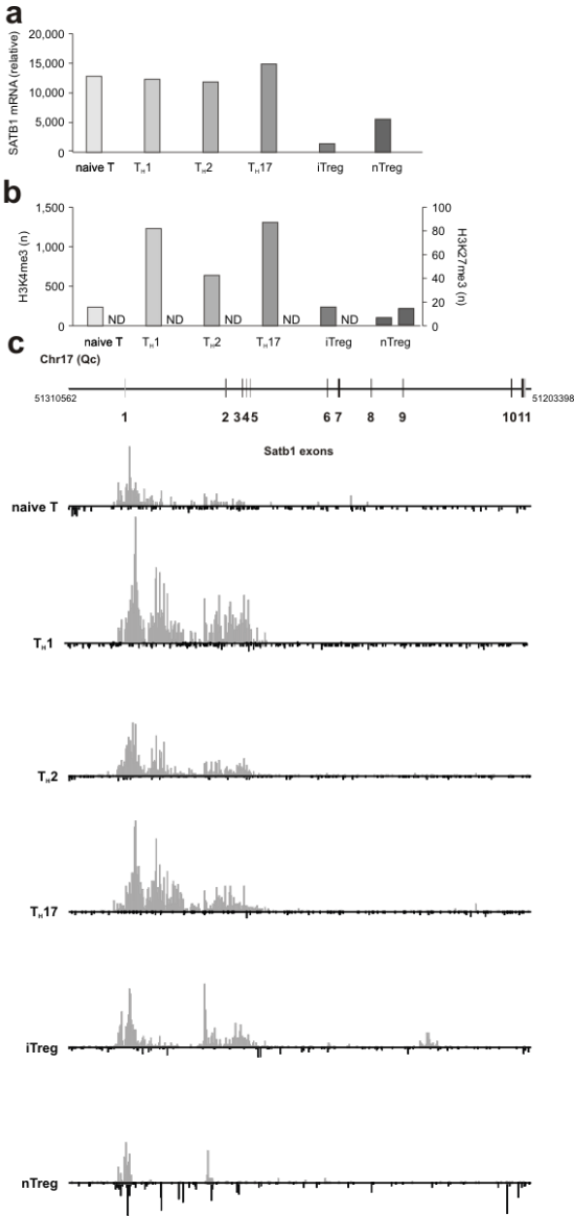

Figure 29 . Histone methylation at the murine SATB1 gene locus correlates with its expression in different $CD4^+$T-cell subpopulations.

Recently published data on genome-wide histone methylation were reanalyzed for SATB1 expression and histone methylation in murine naive T-cells, T_{eff} (Th1, Th2, resp. Th17), iT_{reg} and nT_{reg} cells [265]. (**a**) Expression of SATB1 as assessed by microarray analysis. (**b**) and (**c**) ChIP-sequencing data were re-analyzed for the SATB1 locus. **,** Cumulative data for trimethylation of H3K4 (left) and di- and trimethylation of H3K27 (right) in T_{naive}, Th1, Th2, Th17. iT_{reg}, and nT_{reg} cells (ND= not detectable)**,** Analysis of trimethylation islands (gray: H3K4, black: H3K27) mapped to the genomic SATB1 locus.

5.10 SATB1 overexpression in T_{reg} cells induces transcriptional profiles of T_{eff} cells while maintaining the T_{reg}-cell program

As outlined above, several lines of evidence have linked SATB1 to effector function in T-cells e.g. the expression of Th1/Th2 cytokine genes. Moreover, we could show that SATB1 is tightly controled at several levels in T_{reg} cells. To evaluate the impact of high SATB1 expression on the transcriptional profile of T_{reg} cells, SATB1 was overexpressed in human T_{reg} cells by lentiviral gene transfer in the laboratory of James Riley. In brief, the coding sequence of SATB1 was cloned into a lentiviral expression plasmid downstream of a DsRED reporter gene and an adjacent 2A petide sequence allowing for the joint expression of both genes. Following virus production, human T_{reg} cells were transduced with either the control or the SATB1 encoding virus. DsRED-expressing cells were sorted in the laboratory of James Riley and used for subsequent analysis of the transcriptome of transduced cells. For this analysis, RNA was isolated from lysed cells and cleaned. Next, the purified RNAs were used for the synthesis of cRNA. Then cRNAs were labeled and hybridized to IlluminaV3 bead chips. After scanning and data acquisition, analysis was performed by Dr. Marc Beyer. Remarkably, analysis of the transcriptome of T_{reg} cells overexpressing SATB1 showed a distinct expression profile. ANOVA analysis (FC >2, p-value <0.05) provided a list of around 120 differentially expressed genes in T_{reg} cells overexpressing SATB1. The expression of 100 genes was increased in T_{reg} cells overexpressing SATB1 in comparison to control T_{reg} cells, whereas only 20 genes showed a decrease in expression in SATB1-overexpressing T_{reg} cells. Expanding the analysis of these differentially expressed genes to data sets previously established in the laboratory revealed that 20% of the genes were associated with elevated expression in T_{conv} cells (in comparison to T_{reg} cells), 29% of the differentially expressed genes were primarily linked to T-cell activation, and 16% were classified as common T-cell genes. The remaining genes (35%) showed no association with T-cell function or lineage and were classified as SATB1-induced (Fig. 30, a and b). Next, using precompiled lists of genes previously associated with T_{eff} cell differentiation, we could establish that SATB1 overexpression in T_{reg} cells induces transcriptional programs of T_{eff} cells (Fig. 30, c).

This observation raised a question whether overexpression of SATB1 resulted in reprogramming of T_{reg} cells into T_{eff} cells or this newly acquired program is superimposed onto the transcriptional T_{reg} cell program. We could demonstrate, that the majority of the T_{reg} cell related genes were unchanged in T_{reg} cells upon overexpression of SATB1 (Fig. 30, d).

Since polyclonal human T_{reg} cells were used for this analysis, it is not surprising that the three major T-cell differentiation programs were simultaneously represented.

In summary, overexpression of SATB1 in T_{reg} cells resulted in a considerable reprograming of the T_{reg} cell transcriptome towards a T_{eff} cell associated signature. Therefore, SATB1 repression in T_{reg} cells might be necessary to inhibit the acquisition of a T_{eff} cell programs.

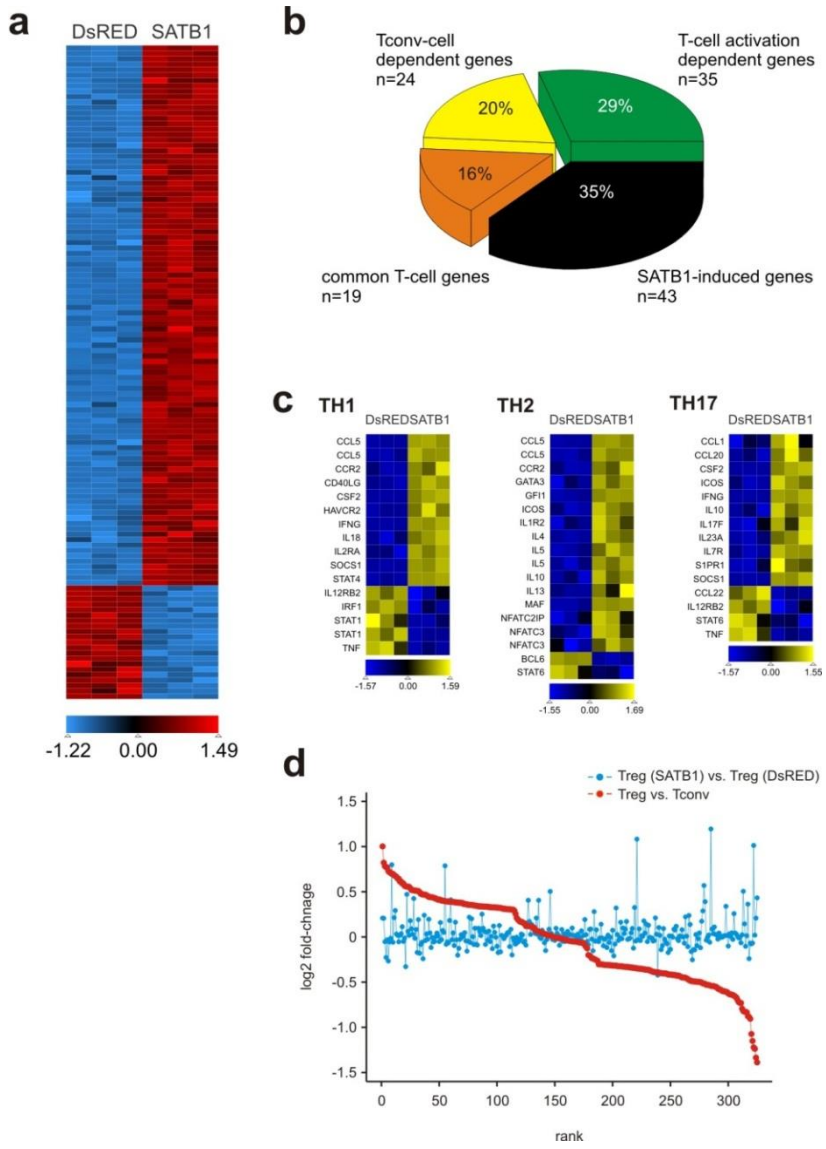

Figure 30. Transcriptional T_{eff} cell programs are induced in SATB1 overexpressing T_{reg} cells.

(**a**) Up- and down-regulated genes in T_{reg} cells lentivirally transduced with SATB1 or control vector after 16 hours stimulation with CD3/CD28-coated beads. Data were z-score normalized. (**b**) Cross annotation analysis using 4 classes: Genes associated with T_{conv} but not T_{reg} cells (yellow), with T cell activation (green), common T-cell genes (orange), and SATB1-induced genes (black). (**c**) Visualization of gene expression levels of genes previously associated with Th1, Th2, or Th17 differentiation; data were z-score normalized. p-values for T-helper associated genes determined by x^2 test in comparison to the complete data set (= Th specific gene enrichment) were p = 3.236e-06 (Th1), p = 9.017e-15 (Th2), resp. p = 1.157e-06 (Th17). (**d**) Changes in genes associated with the human T_{reg} cells signature in T_{reg} cells lentivirally transduced with SATB1 in comparison to control vector transduced T_{reg} cells. The mean log2 fold-changes of the comparison between T_{reg} and T_{conv} cells (red dots) and SATB1 to control-transduced T_{reg} cells (blue dots) were plotted and both comparisons were ranked by fold change in the T_{reg} vs. T_{conv} cell comparison.

5.11 SATB1 repression does not lead to FOXP3 induction in peripheral naïve CD4$^+$ T-cells

After establishing that FOXP3 regulates SATB1 expression on multiple levels, we asked whether SATB1 in turn would also regulate FOXP3 expression. To address this question, SATB1 was silenced by siRNA mediated knockdown in naïve CD4$^+$ T-cells. These cells were activated with aAPC for 48 hours in the presence and absence of TGF-β and IL-2. While aAPC and particularly iT$_{reg}$-cell inducing conditions resulted in an induction of FOXP3 protein expression, there were no differences in the induction of FOXP3 between control and SATB1 deficient cells (Fig. 31, a and b).

As a next step the expression of T$_{reg}$ cell associated genes was assessed, CD4$^+$ T$_{conv}$ cells were isolated, electroporated with SATB1 or control siRNA, and stimulated with CD3/CD28-coated beads for 4 days. mRNA expression of several T$_{reg}$-cell associated genes like FOXP3, GITR, CTLA, CD25, CD39, CD73, and PDE3B was assessed in these cells by qRT-PCR. In agreement with FOXP3 protein expression data, no significant differences in the expression of T$_{reg}$-cell associated genes between SATB1 silenced or control T$_{conv}$ cells were observed (Fig.31, c). In conclusion these observations do not support the direct involvement of SATB1 in FOXP3 induction in naïve CD4$^+$ T-cells.

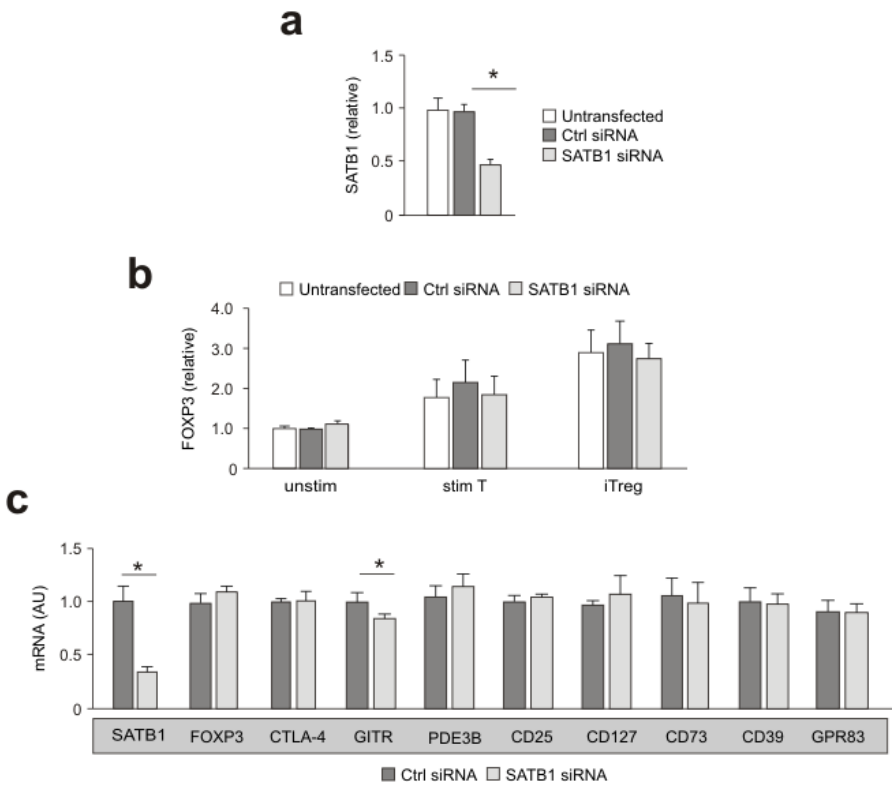

Figure 31. SATB1 silencing does not enhance the induction of Treg-cell T_{reg} cells associated genes in human $CD4^+$ T_{conv} cells.

(**a,b**) To assess whether silencing of SATB1 induces FOXP3, we isolated naïve T_{conv} cells, performed siRNA-mediated knockdown of SATB1, rested them (resting), stimulated them with CD3/CD28 coated beads (stimulated) or cultivated them in the presence of CD3/CD28 coated beads, TGF-α and IL-2 to induce iT_{reg} cells (iT_{reg} cells) and analyzed FOXP3 expression by flow cytometry (mean±s.d., n=4; * $p<0.05$). (**c**) To assess whether silencing of SATB1 induces other genes associated with the T_{reg}-cell phenotype, we isolated T_{conv} cells, performed siRNA-mediated knockdown of SATB1, stimulated them with CD3/CD28 coated beads and analyzed mRNA expression for a set of known T_{reg}-cell marker genes (mean±s.d., n=3; * $p<0.05$)

5.12 Functional analysis of the consequences of sustained high SATB1 expression in T_{reg} cells

5.12.1 SATB1 overexpression in human nT_{reg} cells results in a loss of suppressive activity and a gain in the production of cytokines

We have shown the inverse correlation between FOXP3 and SATB1 expression and the global impact of SATB1 overexpression on the transcriptional profile of T_{reg} cells. To address the functional significance of SATB1 overexpression on the suppressive activity of human T_{reg} cells, the suppressive activity of control T_{reg} cells and T_{reg} cells overexpressing SATB1

cells was evaluated *in vitro* in the laboratory of James Riley. Briefly, a DsRED-2A-SATB1 expression cassette was cloned into the pELNS lentiviral vector. Human $CD4^+CD25^+$ T_{reg} cells were isolated and stimulated with CD3/CD28-coated beads. The stimulated human T_{reg} cells were transduced with either the SATB1 encoding lentivirus or control lentivirus. T_{reg} cells with high SATB1 expression level were sorted according to the expression of the DsRED reporter. Transduced T_{reg} cells retained high expression of FOXP3 and showed a significant induction of SATB1 expression compared to control-transduced T_{reg} cells (Fig. 32, a-c).

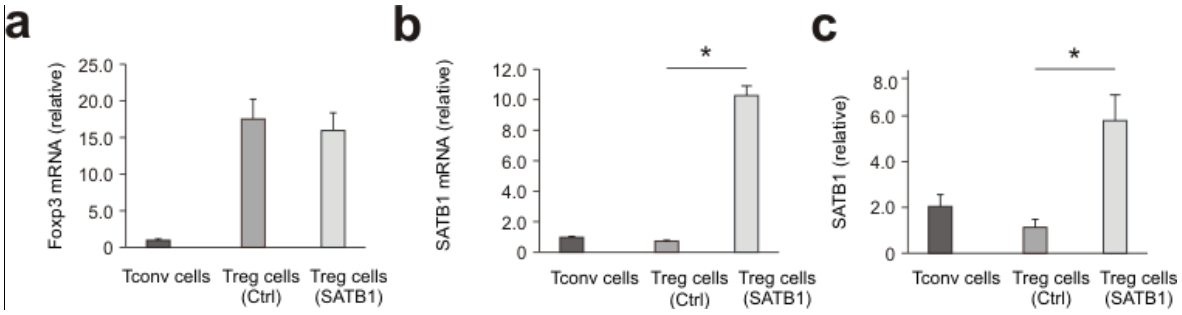

Figure 32. Overexpression of SATB1 in primary human T_{reg} cells.

(**a,b**) Relative FOXP3 (**a**) and SATB1 (**b**) mRNA expression in DsRED-transduced T_{reg} cells (T_{reg} cells (Ctrl)) resp. SATB1-transduced T_{reg} cells (T_{reg} cells (SATB1)) was assessed by qRT-PCR and compared with freshly isolated T_{conv}. *P < 0.05 (Student's *t*-test). Data are representative of three independent experiments (mean and s.d.) with cells derived from different donors. (**c**) Relative SATB1 expression was determined by flow cytometry, assessed and presented as in **a**. * p < 0.05 (Student's *t*-test). Data are representative of three independent experiments (mean and s.d.) with cells derived from different donors

The suppressive activity of T_{reg} cells was assessed by coculturing allogeneic CFSE-labeled-PBMC T_{reg} cells transduced with SATB1 or control vector at different dilutions in the laboratory of Jimes Riley. The cultured cells were stimulated and incubated for 4 days. The efficiency of T_{reg} cells to restrain effector cell expansion was monitored by analysis of CFSE dilution in the dividing $CD8^+$ T-cells. Flow cytometric analysis revealed that T_{reg} cells overexpressing SATB1 had a significantly reduced capability to suppress responder cells *in vitro* in comparison to control T_{reg} cells which showed a potent suppressive activity (Fig.33, a). The role of SATB1 in the coordinating expression of proinflammatory cytokines has been reported at least in Th2 [211]. We have shown also that silencing of SATB1 was associated with low levels of cytokine production. To corroborate the role of SATB1 in acquiring *de novo* expression of proinflammatory cytokines in T_{reg} cells, we quantified the cytokines in T_{reg}

cells after overexpression of SATB1. We could observe that the impairment in the supressive function of T_{reg} cells overexpressing SATB1 was accompanied with the production of proinflammatory cytokines (IL-4, IL-17 and INF-γ) (Fig.33, b-e).

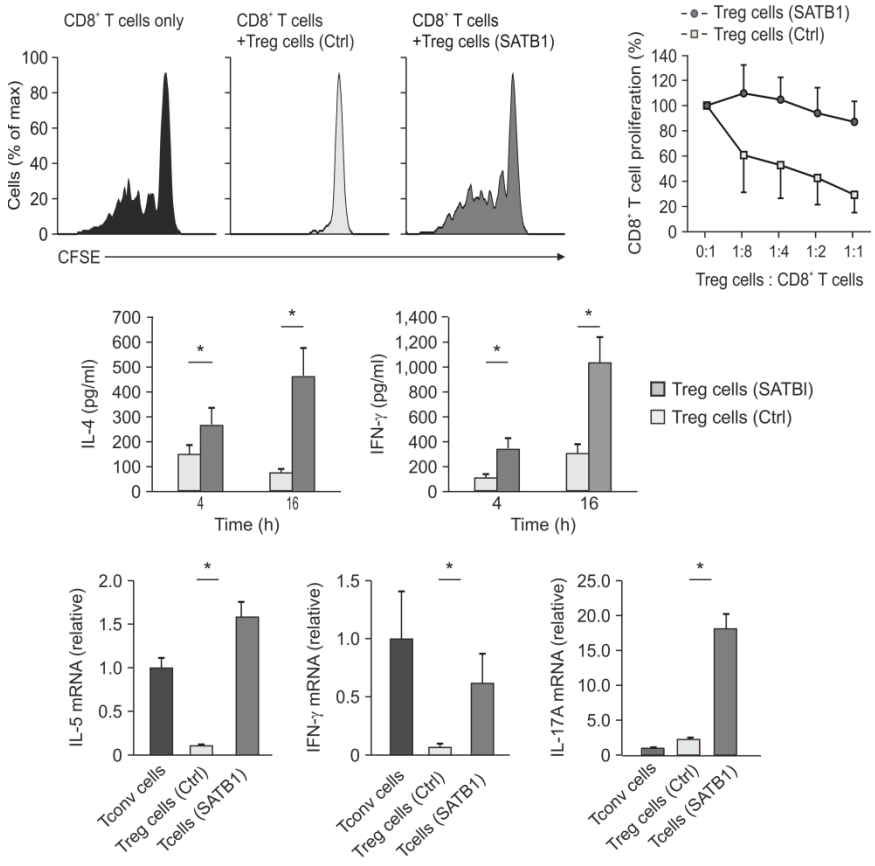

Figure 33. SATB1 expression in T_{reg} cells reprograms T_{reg} cells into T_{eff} cells.

(**a**) Analysis of suppressive function of human T_{reg} cells lentivirally transduced with SATB1 or control vector (DsRED) cultured for 4 d together with CFSE-labeled allogeneic PBMC at a 1:1 ratio *in vitro*; after stimulation of cultures, CFSE dilution of human $CD8^+$ T-cells was assessed by flow cytometry. Left: representative experiment; right: mean±s.d., n=3; * p<0.05.(**b**) Cytometric bead array for IL-4 and IFN-γ secretion of human T_{reg} cells lentivirally transduced with SATB1 or control vector assessed 4 and 16 hours after stimulation with CD3/CD28-coated beads (mean±s.d.; * p<0.05). qRT-PCR analysis of (**c**) IL-5, (**d**) IFN-γ, and (**e**) IL-17A expression in T_{conv} cells activated for 16 hrs with CD3/CD28 coated beads as well as human T_{reg} cells lentivirally transduced with SATB1 or control vector (DsRED) (mean±s.d., n=3, * p<0.05).

Collectively, overexpression of SATB1 in T_{reg} cells was associated with an attenuation of suppressive function and acquisition of proinflammatory properties. This evidence suggests that SATB1 repression is critical to maintain the functional integrity of T_{reg} cells.

5.12.2 Murine SATB1- overexpressingT_{reg} cells exhibit impaired suppressive activity with inflammatory cytokine production in vitro

We have clearly shown, *in vitro*, that SATB1-overexpressing human T_{reg} cells had impaired suppressive function accompanied with the production of proinflammatory cytokines. To corroborate these findings in murine DEREG T_{reg} cells, the full length SATB1 coding sequence followed by an IRES-driven reporter gene (Thy1.1) was cloned into a lentiviral expression plasmid.

T_{reg} cells from DEREG mice were isolated based on FOXP3 promoter driven GFP expression. The sorted T_{reg} cells were expanded and lentivirally transduced with pLTVM-SATB1-IRES-Thy1.1 and the lentiviral control vector. After further expansion, Thy1.1-expressing GFP-positive T_{reg} cells were sorted and used for the indicated functional assays. Initially, SATB1 expression was analyzed at protein and RNA levels in Thy1.1$^+$ T_{reg} cells using intracellular staining and qRT-PCR. SATB1 expression in transduced T_{reg} cells showed an increase of 4-6 fold relative to control transduced T_{reg} cells without affecting the expression level of FOXP3 (Fig. 34, a and b).

The next question was, whether the overexpression of SATB1 in T_{reg} cells would dampen the suppressive function of these cells. To evaluate T_{reg}-cell function suppression assays with SATB1-overexpressing T_{reg} cells were performed. Briefly, CD4$^+$ T cells were isolated from mouse spleen and were further stimulated with CD3/CD28-coated beads and cocultured with T_{reg} cells at various dilutions. T_{reg} cells overexpressing SATB1 showed a severe impairment in their ability to control the proliferation of T_{eff} cells (Fig. 34, c). In line with the human data, overexpression of SATB1 in murine T_{reg} cells mediated the production of proinflammatory cytokines (IL-5 and IFN-γ) (Fig. 34, d).

In conclusion, overexpression of SATB1 in murine T_{reg} cells negatively interfered with their suppressive function and reprogramed their phenotype towards a T_{eff}-cell like program.

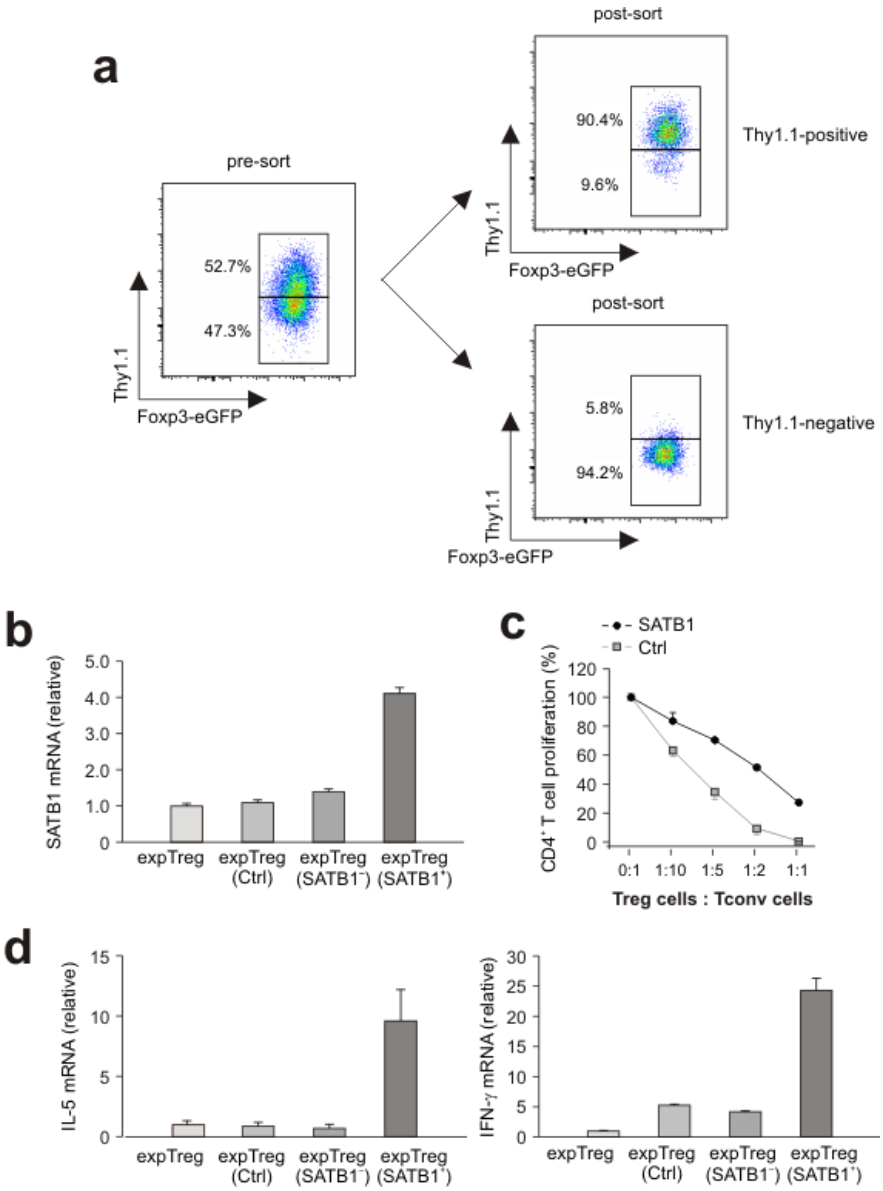

Figure 34. SATB1 overexpression in murine T_{reg} cells impairs their suppressive function and results in the production of proinflammatory cytokines.

(**a**) Transduced T_{reg} cells were sorted based on GFP as well as Thy1.1 expression as exemplified here for SATB1-Thy1.1 transduced T_{reg} cells from a representative experiment. (**b**) Relative expression of SATB1, (**c**) Analysis of the suppressive function of murine T_{reg} cells lentivirally transduced with SATB1 or control vector cultured for 4 d together with eFluor 670-labeled $CD4^+$ T-cells at the indicated ratio *in vitro*; after stimulation of cultures, eFluor 670 dilution of $CD4^+$ T-cells was assessed by flow cytometry. Mean±s.e.m. Data are representative of 2-3 independent experiments (mean and s.e.m. of triplicate cultures). (**d**) Relative expression of IL-5 (left), and IFN-γ (right). mRNA expression in SATB1-overexpressing SATB1-Thy1.1-transduced ($expT_{reg}$ cells (SATB1)), SATB1-negative SATB1-Thy1.1-transduced ($expT_{reg}$ cells (SATB1)), control-transduced ($expT_{reg}$ cells (Ctrl)), or non-transduced expanded T_{reg} cells ($expT_{reg}$ cells).

5.12.3 SATB1-overexpressing T_{reg} cells fail to prevent colitis in RAG2$^{-/-}$ mice in vivo

It has been shown that the adoptive transfer of $CD4^+CD45RB^{high}$ naïve T-cells into severe combined immunodeficiency (SCID) mice induced wasting disease and colitis which was ameliorated by the co-transfer of $CD4^+CD45RB^{low}$ T-cells [266, 267]. Similarly, transfer of T_{reg} cells into $RAG2^{-/-}$ mice challenged with naïve T-cells can rescue these animals from the development of colitis [268] .

During this thesis evidence of the deleterious effect of SATB1 overexpression on T_{reg}-cell phenotype and function has been demonstrated. To further investigate whether these findings can be translated to an *in vivo* situation, the adoptive transfer colitis model was used. In brief, $RAG2^{-/-}$ mice were reconstituted with $CD4^+CD45RB^{high}$ naïve T-cells either alone as a positive control, or in combination either with control vector transduced T_{reg} cells or SATB1 overexpressing T_{reg} cells. The onset of colitis was monitored by measuring the animals' weight.

$RAG2^{-/-}$ mice adoptively transferred with $CD4^+CD45RB^{high}$ naïve T-cells and T_{reg} cells overexpressing SATB1 showed symptoms of colitis like weight loss and bloody diarrhea around 8 weeks after transfer, whereas mice that coreceived control T_{reg} cells did show only mild or no signs of disease (Fig. 35, a).

To confirm and characterize colitis development the mice were sacrified and the colons were inspected macroscopically and microscopically. In mice reconstituted with $CD4^+CD45RB^{high}$ naïve T-cells alone or in combination with T_{reg} cells overexpressing SATB1, gross colon apprearance was consistent with colitis in terms of contracted coeca, opaque and swellen proximal colon. Furthermore, colon histological sections (performed in the laboratory of Claudia Wickenhauser (University Hospital Leipzig) from diseased mice showed leukocyte infiltration, depletion of goblet cells, epithelial cell hyperplasia and ulceration corresponding to an average colitis score of 8. On the opposite, mice transplanted with control-transduced T_{reg} cells showed normal colon structure and histology (Fig. 35, b and c).

This evidence suggested that SATB1 overexpresing T_{reg} cells are unable to control $CD4^+CD45RB^{high}$ naïve T-cells leading to colitis. However, it is possible to obtain similar results if the trafficking of T_{reg} cells to secondary lymphoid organs failed or survival of T_{reg} cells *in vivo* is impaired. To determine whether the differences in pathology between animals receiving T_{reg} cells overexpressing SATB1 and the control-transduced T_{reg} cells were due to

variations in T-cell reconstitution *in vivo*, the absolute $CD4^+$ T cell numbers in the spleen, mesenteric, and peripheral lymph nodes were determined. Flow cytometric analysis revealed that $CD4^+$ T-cells proliferated significantly at these sites in mice that had received only $CD4^+CD45RB^{high}$ naïve T-cells or in combination with T_{reg} cells overexpressing SATB1 (Fig. 35, d); whereas the control T_{reg} cells inhibited the inflammatory expansion of $CD4^+CD45RB^{high}$ naïve T-cells and prevented induction of colitits. (Fig. 35, d). To exclude that the decreased survival of T_{reg} cells was responsible for the induction of colitis, we also assessed T_{reg} cell numbers. No differences existed in numbers of T_{reg} cells between animals receiving SATB1-overexpressing or control-transduced T_{reg} cells was observed (Fig. 35; e).

Taken together, this evidence strongly indicated that FOXP3-mediated SATB1 repression is relevant to maintain T_{reg}-cell function *in vivo*. The concordant observations obtained in mice and humans suggest that FOXP3 repression of SATB1 expression by multiple layers of regulation is a conserved mechanism across species important to maintain T_{reg}-cell lineage functionality and identity.

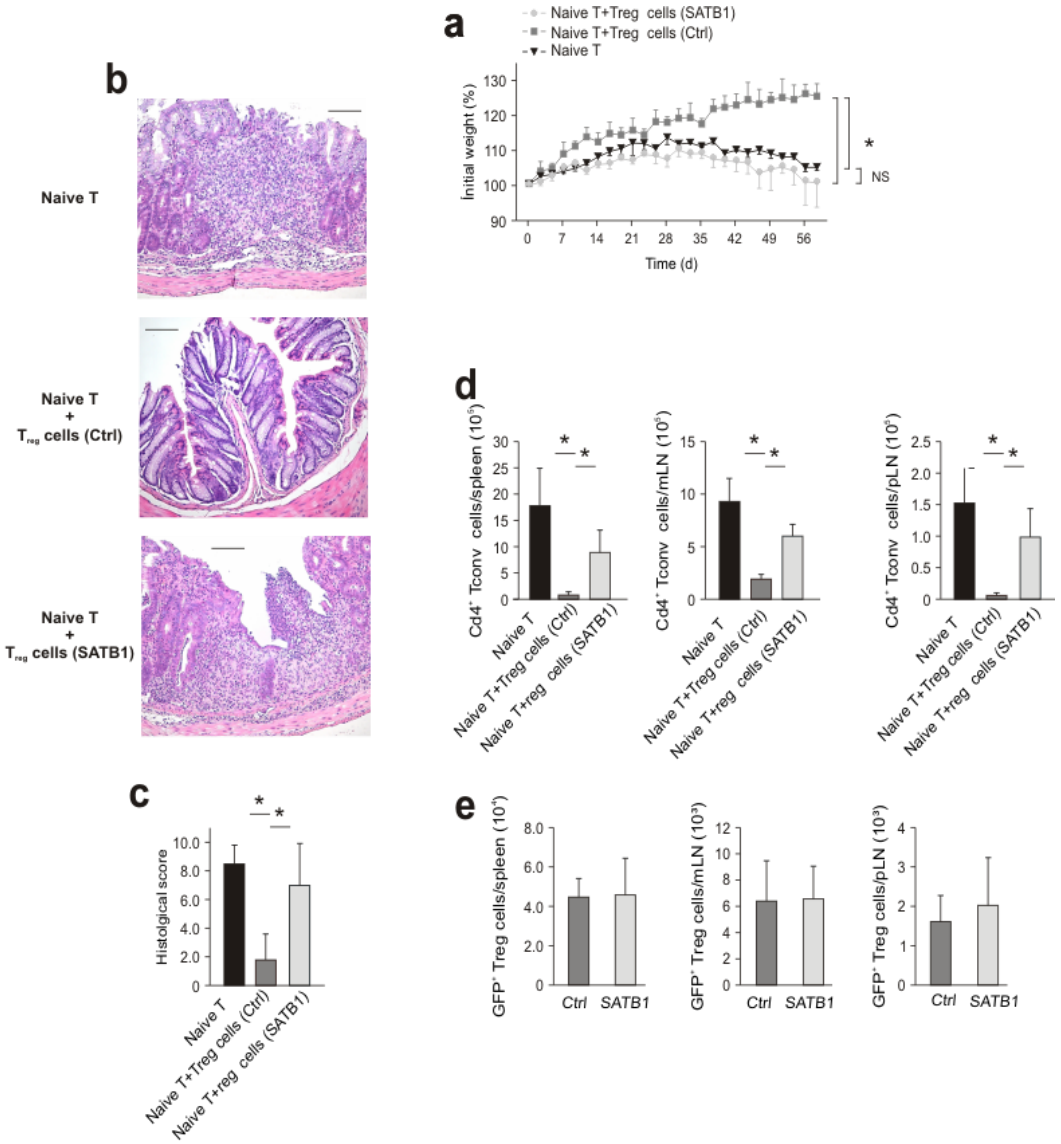

Figure 35. Ectopic SATB1 expression in T_{reg} cells induces colitis in RAG2$^{-/-}$ mice.

(**a**) Body weight of RAG2$^{-/-}$ recipients of naïve CD4^{+}CD45RB^{high} T-cells transferred alone or together with control- or SATB1-transduced T_{reg} cells, presented relative to initial body weight. Data are pooled from two independent experiments (average and s.d. of four to five recipient mice). (**b**) Hematoxylin and eosin staining of colon sections. Colons from mice that received naïve CD4^{+}CD45RB^{high} T-cells only or together with SATB1-overexpressing T_{reg} cells displayed infiltrates of inflammatory cells. In colons from mice that received naïve CD4^{+}CD45RB^{high} T-cells and control T_{reg} cells no inflammatory changes were evident. (**c**) Histology scores of sections of the colon at 8 weeks after cell transfer. (**d**) Recovery of T_{conv} cells from spleens, mesenteric, and peripheral lymph nodes. (**e**) Recovery of T_{reg} cells from spleens, mesenteric, and peripheral lymph nodes.

6 Discussion

Maintaining the proper function of the immune system requires complex and highly coordinated regulatory networks. This regulation begins in the thymus with the deletion of T-cells and B cells; a process termed recessive tolerance and extends to the periphery with the egress of immune cells maintaining a state of unresponsiveness which can be transferred by a subset of cells from a tolerant donor into an immunocompetent host (dominant tolerance) [33]. The comprehension of dominant tolerance has been expanded with the identification and characterization of new immuno-regulatory cell subsets, in particular a subset of $CD4^+$ T-cells constitutively expressing IL-2Rα (CD25) with a potent immunosuppressive function. This subpopulation is widely known as T_{reg} cells and its identity has been further delineated by the expression of the transcription factor FOXP3 which regulates their phenotypic stability and functional integrity. Therefore, FOXP3 is recognized as a master regulator and lineage-specific-modulator for T_{reg} cells [269-273] .

In this study, we have identified and characterized SATB1 (special AT-rich sequence-binding protein-1) as a novel effector molecule in the regulatory hub of T_{reg} cells. We demonstrated that SATB1 is dominantly repressed in T_{reg} cells and this low expression level is indispensable for the suppressor function of T_{reg} cells. Therefore, the expression of SATB1 is tightly regulated through several mechanisms. FOXP3 represses SATB1 expression at transcriptional level via direct binding or indirectly at post-transcriptional level via FOXP3-dependent miRNAs. In addition, post-translational repressive chromatin modifications constitute another layer of regulation. Functionally, we have demonstrated that sustained high SATB1 expression in T_{reg} cells was at the cost of their regulatory program. SATB1 overexpression reprograms the T_{reg}-cell transcriptome and promotes the expression of T_{eff} cell associated genes such as proinflammatory cytokines which consequently leads to impairment of suppresssive function in *vitro* and in *vivo.*

6.1 SATB1 is differentially expressed in $CD4^+$ T-cells

SATB1 is a chromatin organizer and transcription factor essential for controlling a large number of genes involved in T-cell development and activation [209, 211, 223, 230]. We observed that SATB1 is differentially expressed between $CD4^+CD25^-$ T_{conv} cells and $CD4^+CD25^+$ T_{reg} cells. Whereas it is significantly lower in human and murine T_{reg} cells, it is highly expressed in T_{conv} cells. In addition, its expression is further augmented upon TCR ligation. Interestingly, stimulation-dependent induction of SATB1 was greatly blunted in T_{reg}

cells. This is in agreement with a study that showed that SATB1 was among early genes were upregulated upon T cells stimulation [224]. Although T_{reg} cells also are dependent on TCR activation for survival and function, it has been well reported that the signaling outcomes considerably differ between T_{reg} and T_{conv} cells. This might explain reduced post-activation induction of SATB1 expression in T_{reg} cells. As a matter of fact, it is well described that T_{reg} cells show altered TCR signaling upon stimulation and this unique signaling is essential for their distinctive phenotype and function [274-279]. On biochemical level, several studies investigating TCR-mediated signaling in T_{reg} cells showed a global reduction in TCR-activated signaling pathways such as defects in calcium mobilization, Ras, Mitogen-activated protein kinase kinase (MEK1/2), as well as PI3K and Akt activation in comparison to T_{eff} cells [278, 280-282]. However, until now the circuit that controls T_{eff} cell program repression in T_{reg} cells has not yet been thoroughly elucidated. Therefore, low expression of SATB1 and refractory to respond to antigenic stimulation in T_{reg} cells makes SATB1 an attractive candidate to act as a molecular switch between T-cell effector and regulatory programs.

The key role of SATB1 as a transcriptional regulator has previously been described in a SATB1 knock-out mouse. Mice lacking SATB1 showed an abnormal thymocyte development, mostly T-cell development was arrested at the double positive (DP) stage. Moreover, at the molecular level SATB1 knock-out mice showed derepression of approximately 2% of genes at inappropriate stages of T-cell development amongst them IL-2Rα and IL-7Rα were ectopically transcribed in DP thymocytes [223]. In T_{eff} cells, TCR ligation induces SATB1 expression which suggests its active role in shaping the effector program. Indeed, SATB1 involvement in Th2 lineage differentiation has been reported [215]. Moreover, its role in Th2 cytokine production was investigated in a Th2 clone. In this context, SATB1 acts as a platform that creates a higher chromatin loop structure that brings together the Th2 gene cluster and recruits specific transcription factors such as GATA3, STAT6 and c-Maf [211]. This action enables the concerted transcription of Th2 cytokines. SATB1 was also shown to be induced in different T-helper cells upon activation and cytokine-mediated polarization [254, 283, 284].

Functionally, in contrast to T_{eff} cells, SATB1 is not upregulated in T_{reg} cells even upon TCR ligation. One explanation for this could be that SATB1 could counteract the suppressive programs necessary for the T_{reg}-cell phenotype, therefore a distinctive regulation in a T_{reg}-cell specific manner is needed through active repression of SATB1 expression in T_{reg} cells.

Alternatively, in T_{eff} cells SATB1 is actively involved in T-helper cell activation, proliferation, and differentiation; hence it must be potentially upregulated in T_{eff} cells. Furthermore, SATB1 expression could even include T helper-type specific regulation in which T-cell lineage specific transcription factors might modulate SATB1 expression induced by triggering events such as TCR activation and cytokines.

6.2 SATB1 expression in T_{reg} cells is under control of FOXP3

Generally, cellular identity and functionality is under control of a complex regulatory program. The key players in this regulatory circuit are transcription factors. Many of these factors fulfill the definition of master regulator where a specific transcription factor is necessary and sufficient to induce a specific cell type. The concept of "master transcription factors" is applicable to several T-helper cell subsets [285]. Accumulating evidence has assigned FOXP3 as the master regulator in T_{reg} cells. Not just non-functional FOXP3 is associated with the perturbation of immune homeostasis but also the level of its expression as expression of hypomorphic FOXP3 allele results in a marked impairment of T_{reg} cell function and their production of IL-4 [286, 287]. Disease-causing mutations in FOXP3 are linked to the lethal autoimmunity, immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome in humans and the scurfy phenotype in mice [141, 147, 288, 289]. In accordance with this concept, ectopic FOXP3 expression in $CD4^+CD25^-$ T-cells endowed them with a T_{reg} cell-like phenotype at least in murine T-cells [51, 52, 253]. Our study has shown for the first time that multiple layers of regulation are involved in the repression of SATB1 expression in T_{reg} cells. We have identified a key role for FOXP3 in keeping SATB1 expression at basal levels. However, this does not rule out the potential of active involvement of other regulatory pathways in this process.

Using gain- and loss-of-function we could establish that FOXP3 actively represses SATB1 expression. Initially, we observed a low expression of SATB1 in the presence of FOXP3. This was further corroborated with confocal microscopy in thymocytes. In fact, FOXP3-mediated repression has also been described for several genes promoting T_{eff}-cell function such as lineage specific cytokines like IFN-γ and transcription factors such as ROR-γt [290-292]. Strikingly, loss of function of FOXP3 in *vitro* via siRNA and miRNA against FOXP3 and in *vivo* in FOXP3 mutant *scurfy* mice rescued SATB1 expression even to higher levels than those observed in T_{conv} cells. In line with these findings, FOXP3 *de novo* expression in iT_{reg} cells as well as its ectopic expression in T_{conv} cells resulted in SATB1 repression, corroborating the central role of FOXP3 in this regulatory circuit. Our evidence is in

agreement with other studies showing that overexpression of FOXP3 in Jurkat cells and human $CD4^+$ T-cells favors induction of T_{reg}-cell associated genes, defective activation properties, and reduced production of proinflammatory cytokines with acquisition of suppressive activity [293, 294]. Functionally, FOXP3 induction in conventional T-cells was sufficient to confer these cells with suppressor function. Furthermore the transfer of these FOXP3 expressing cells together with effector T-cells in *RAG2*-/- mice prevented wasting disease and development of colitis in *RAG2*$^{-/-}$ mice [52].

These facts prompted us to further dissect the molecular mechanism of FOXP3-mediated SATB1 regulation. The direct involvement of FOXP3 in this regulation was demonstrated by the reduced expression of SATB1 in T_{conv} cells upon either enforced or *de novo* expression of FOXP3. Conversely, FOXP3 loss of function with either specific siRNA or miRNA in T_{reg} cells was associated with restored SATB1 expression levels and production of proinflammatory cytokines. Supporting this observation, murine T_{reg} cells from *scurfy* mice, where FOXP3 is non-functional, showed high expression levels of SATB1.

In our study, we have shown that FOXP3 restrains SATB1 transcription in several ways, suggesting that these diverse mechanisms might synergize to control SATB1 transcription in an efficient manner. One of the important steps in this regulation cascade occurs at the transcription stage. Coding and noncoding genes contain promoter and enhancer sequences that are bound by transcription activators and repressors [295]. We have identified several FOXP3 binding sites in the genomic sequence of SATB1. Our ChIP-on-ChIP and filter retention data showed that FOXP3 binds specifically with high affinity to the genomic SATB1 locus. Furthermore, we have shown in reporter assays that the occupancy of FOXP3 at the SATB1 locus is functional and substantially reduces the activity of the reporter gene.

FOXP3-mediated repression of genes involved in T-cell activation and cytokine production is a key to maintain T_{reg}-cell phenotype and function. Genome-wide analysis of FOXP3 binding genes have suggested that FOXP3 acts as a transcriptional repressor and activator and has identified a core set of around 700 genes that are targeted by FOXP3 via direct binding to their promoters [238, 296, 297]. Most of the downregulated genes upon FOXP3-induction are involved in T-cell activation and cytokine production and showed suppressed activation in stimulated $FOXP3^+$ hybridoma cells [150]. Despite the great importance of NFAT and NF-κB in the induction of FOXP3 in the thymus, their proinflammatory activities have to be repressed by FOXP3 in mature T_{reg} cells [156]. It is worth mentioning here that high cellular

level of NF-κB upon strong TCR activation antagonizes induction of FOXP3 in differentiating iT_{reg} cells [298]. In fact, lineage commitment during T-cell differentiation is usually accompanied by suppression of the alternative fates that the precursor could have assumed. Such suppression is mainly achieved by cross-regulation among transcription factors at both transcriptional and post-transcriptional levels [299]. A model of cross-regulation has been reported for the interaction between FOXP3 and RORγt which controls Th17-cell differentiation depending on the availability of TGF-β and particular metabolites and cytokines such as retinoic acid and IL-6 [300].

It is important to mention that the association of FOXP3 with other lineage-specific factors is not always antagonistic. It is well reported that T_{reg} cells can be co-expressed T-bet, STAT3, and GATA3 which confers ability to adaptat to different microenvironments in trems of boosted survival, expansion, and function to maintain tissue homeostasis [301-304]. FOXP3 may form complexes with these transcription factors in a manner that the capacity of these factors to induce effector cytokines is inhibited while the expression of other genes necessary to accommodate to new situations and adapt context dependent specialization, such as chemokine receptors, is preserved. To exemplify, the expression of Th1 transcription factor T-bet in T_{reg} cells promoted expression of CXCR3 on T_{reg} cells with enhanced suppressive activity. Therefore, it facilitates accumulation of T_{reg} cells to the sites of Th1 mediated inflammation [304]. Similarily, co-expression of GATA3 has been shown to endow T_{reg} cells with ability to accumulate and suppress Th2 immune responses at the inflammed tissues [305]. This mechanism of cross-regulation has been nicely demonstrated for the interaction of FOXP3 with NFAT-1 [306]. NFAT-1 interaction with AP-1 prtotein complex is important for effector immune response. It has been shown that FOXP3 disrupts NFAT1-AP-1 nuclear complex formation and instead it cooperates with NFAT-1 in T_{reg} cells. This cooperative association between FOXP3 and NFAT-1 divert the effector transcriptional program to regulatory program. Furthermore, NFAT-1-deficient T_{reg} cells are functionally defective because FOXP3-NFAT cooperation is required for the induction of important functional molecules such as CD25 and CTLA-4 as well as inhibition of IL-2 production [307]. These critical functions of NFAT-1 in T_{reg} cells might imply abundant expression of this gene under resting and stimulation conditions, however, NFAT expression was reported to remain constanty low in T_{reg} relative to T_{conv} cells [308]. In this scenario, we would assume that the low expression of SATB1 might have a functional role for T_{reg} cells by acting as a genome

organizer or exerting specific undescribed functions to maintain the balance between regulatory and inflammatory program which require further study.

We have shown that FOXP3 regulates SATB1 expression via direct binding to the genomic SATB1 locus. However, its is possible that FOXP3 can act in an indirect manner via association with intermediary factors [171]. Recently, Eos-zinc-finger transcription factor has been described as a missing link in the FOXP3-dependent gene repression. Eos was shown to directly interact with FOXP3 and this interaction is necessary to suppress the expression of IL-2 as well as other genes in T_{reg} cells. Eos recruits CtBP1 to the Eos–FOXP3 complex and this complex modulated the epigenetic status of the IL-2 promoter. siRNA-mediated knockdown of Eos resulted in a significant increase in permissive histone 3 lysine 4 trimethylation (H3K4me3) and H3 and H4 acetylation levels at the IL-2 promoter in T_{reg} cells. Furthermore, knockdown of either Eos or CtBP1 resulted in demethylation of genomic DNA at CpG dinucleotides in the IL-2 promoter. These epigenetic modifications observed in the absence of functional Eos resemble the epigenetic status of the IL-2 promoter in T_{eff} cells [252]. We have assessed the transcriptional profile of T-cells after Eos silencing. To our interest, SATB1 was one of the prominent genes in the cluster that was significantly repressed in T_{reg} cells and sharply upregulated upon Eos silencing. This observation supports our findings and suggests a possible FOXP3-Eos interaction in SATB1 repression.

6.3 SATB1 expression is post-transcriptionally regulated by FOXP3-dependent miRNAs

Post-transcriptional control of gene expression is an important layer of gene regulation excerted by microRNAs. MicroRNAs are a class of small non-coding RNAs implicated in the post-transcriptional regulation of gene expression, typically by base pairing to the 3´UTR of target mRNAs to mediate repression of that target mRNA either by transcript destabilization, translational inhibition, or both [309]. There are several hundred known miRNAs with each of them potentially targeting multiple transcripts [310]. Therefore, it is likely that a substantial proportion of protein-coding transcripts are subject to miRNA-dependent regulation. The importance of miRNAs for the immune system was demonstrated in a pioneer study by specific conditional deletion of the Dicer allele in T cells at different development stages. Dicer deletion early in T-cell development induced by a Cre transgene driven by the lck promoter resulted in a sharp reduction of miRNAs at the double-positive stage and a 10-fold drop in the number of TCR-αβ thymocytes [129, 311]. Deletion of Dicer later in T cell development induced by a Cre transgene driven by the CD4 promoter resulted in moderately

reduced T-cell numbers [129, 312]. In general, Dicer$^{-/-}$ T-cells exhibited decreased proliferative potential and increased propensity to apoptosis in response to activation [133]. The pivotal role of miRNA for the development and differentiation of T_{reg} cells was illustrated by disruption of Dicer-dependent miRNAs at the double positive stage that resulted in a 50-70% reduction in the frequency of FOXP3^{+} T_{reg} cells. Depletion of miRNA within the T_{reg}-cell lineage resulted in loss of their suppressor capacity *in vitro* and fatal autoimmunity indistinguishable from that observed in T_{reg}-cell–deficient mice [132, 243, 258]. Though The FOXP3-dependent induction of characteristic miRNAs has been described in several studies, the functional relevance of this finding remains largely elusive [129, 274]. The specific functions of individual miRNAs in the biology of T_{reg} cells have been addressed in few studies suggesting that single miRNAs are involved in specific functions and can sustain a certain properties in T_{reg} cells but unsurprisingly do not control the complete T_{reg}-cell transcriptional program [259, 260]. miRNAs are like transcription factors also show cell-lineage specific expression. This cell-type specific expression profile of miRNAs are largely goverened by conventional transcription factor-dependent transcriptional control mechanisms [313]. In our study we have identified a set of T_{reg}-cell-associated miRNAs which are FOXP3-dependent. Ectopic expression of FOXP3 in non-T_{reg} cells induced their expression and the opposite when FOXP3 was silenced; T_{reg} cells lost this distinct profile of miRNAs.

Our findings showed that several FOXP3-dependent miRNAs have an inhibitory effect on the expression of SATB1. In addition, we could demonstrate that silencing of individual miRNAs is not sufficient to significantly increase the expression of SATB1. However, T_{reg} cells that are completely devoid of miRNAs showed a significant upregulation of SATB1. The observation that silencing of a single mRNAs is not sufficient to rescue SATB1 expression could be attributed to functional redundancy among closely related miRNA family members. In agreement with this notion, it has been reported that multiple sites, either for the same or different miRNAs, are required for effective repression, and when the sites are close to each other, they tend to act cooperatively [314]. Furthermore, the phenotypic properties controlled by miRNAs are less defined and it seems that miRNA govern a more restrained program compared to transcription factors. In addition, miRNA usage may be more biased towards controlling specific aspects of terminal differentiation of individual cell types. In support of this notion, miRNAs tend to have highly cell type-specific expression profiles, both during normal development and in specific disease states, such as cancer [313]. miRNA-155 exemplifies this point as its deficiency in T_{reg} cells induced no overt dysfunctional phenotype

under physiological conditions. However, it was critical in competitive enviroments such as inflammatory and lymphopenic conditions through heightened T_{reg} cells responsiveness to IL-2 by targeting SOCS1, a nagive regulator of IL2R signaling [259]. miR-146a is another example of the fine tuning of T_{reg} cells function by miRNAs. It has been shown that it is essential for the ability of T_{reg} cells to restrain IFN-γ-mediated pathogenic Th1 responses and associated inflammation. Interestingly, miRNA-146 stabilizes regulatory program by targeting excessive activation of STAT1, therefore, preventing deviation of activated T_{reg} cells into IFN-γ-producing Th1-like cells [260].

6.4 The genomic SATB1 locus is a target of chromatin remodeling complexes

Epigenetic modifications have recently been recognized as a key step in the control of gene expression since this process determines in great extent the accessibility of regulatory elements within target loci [261, 315-317]. DNA methylation is usually involved in gene silencing by blocking access of transcription factors, and forming compact, inactive chromatin through recruitment of chromatin remodeling factors to methylated DNA binding proteins known as methyl-CpG-binding domain proteins (MBDs). DNA methylation is considered as a stable marker, and maintained in somatic cells by several methyltransferases [318-321]. In general, 40% of genes contain CpG-rich islands upstream from their transcriptional start sites and 80-90% of all CpG islands can be methylated in mammals. Our findings showed no differences in the methylation of CpG islands upstream of the genomic SATB1 locus in T_{conv} and T_{reg} cells. This led us to the assumption that DNA-methylation does not contribute to the downregulation of SATB1 in T_{reg} cells, but rather would allow for a rapid upregulation of SATB1 expression without the need for cell division first to remove methylation.

Histone can be modified by the enzyme-catalysed addition or removal of acetyl, methyl, phosphate, ubiquitin, sumoyl or ADP-ribose group. Histone modifications such us methylation, acetylation and phosphorylation are important defining events during gene regulation [227, 261, 316, 322, 323]. In general, acetylation histones 3 and 4 (H3Ac and H4 Ac), mono, di, and trimethylation of lysine 4 of H3 (H3K4) characterize active or recently transcribed genes. Conversely, di and trimethylation of H3K9 and H3K27 mark silenced gene [227]. To explore SATB1 regulation in T_{reg} cells in all its complexity, we examined permissive and repressive histone modifications at the SATB1 locus in T_{reg} cells. As histones can be ornamented with a set of modifications with dual impact on gene expression, we have used ChIP analysis of human expanded T_{reg} and T_{conv} cells with antibodies specific for the permissive histone modifications H3K4me3 and H4Ac and the repressive histone

modification H3K27me3. As a rule of thumb, histone modifications correlate well with gene expression as repressive marks are associated with gene silencing and permissive marks are in accordance with gene activation.

We identified differences in histone modifications between T_{reg} and T_{conv} cells which correlated well with the expression status of SATB1 in T_{reg} cells and T_{conv} cells. The genomic SATB1 locus in T_{reg} cells is enriched in a repressive mark, H3K27me3, with a concomittant reduction in permissive marks, H3K4me3 and H4Ac, while T_{conv} cells showed an opposite histone landscape, enrichment in permissive and decrease in repressive modifications.

This pattern of histone modification raised the question whether FOXP3 is directly involved in the differential chromatin remodeling from a permissive to a repressive state in T_{reg} cells. It is well documented that chromatin remodeling and epigenetic modifications at cytokine loci are presumably the consequence of upregulation or activation of particular lineage specific transcription factors. Transcription factors can affect epigenetic modifications directly via recruitment of chromating remodeling complexes or indirectly by competing for the binding sites of these complexes on key regulatory elements [324-326]. For example T-bet recruits histone H3K4 methyltransferase and H3K27 demethylase complexes to the IFN-γ locus through its interaction with retinoblastoma-binding protein 5 (RbBp5) and Jumonji domain containing 3 (JMJD3) [327]. GATA3 has been reported to compete with a methyl CpG-binding domain protein-2 (MBD2) for binding to a particular methyl CpG in the IL-4 locus. Furthermore, in T_{conv} cells a deficiency in the DNA methyltransferase Dnmt 1 or the CpG binding protein 2 leads to IL-4 gene transcription in a GATA3 independent fashion [299].

Interestingly, inhibitory binding of FOXP3 to the promoters of IL-2 and IFN-γ results in deacetylation of histone 3 at these promoters [328]. In contrast, FOXP3 binding to GITR, CD25 and CTLA-4 is correlated with increased histone acetylation [328]. Further evidence that FOXP3 and its associated proteins modify chromatin structure comes from studies on the effect of FOXP3 on the IL-4 promoter and a cis-regulatory element within the IL-4 locus. FOXP3 induction in Th2 cells is associated with a significant reduction in the levels of acetylated histone 3 in the regulatory elements of the IL-4 promoter and enhanced levels of repressive monomethylation of histone 3 at lysine 9 [329].

Taken together, our study shows for the first time that SATB1 expression in T_{reg} cells is tightly regulated by FOXP3 at different levels. At transpcriptional level, we could demonstrate direct occupancy of regulatory elements at the genomic SATB1 locus by FOXP3.

At the post-transcriptional level, we have identified a set of FOXP3-driven miRNAs that bind to 3`UTR of SATB1 which might lead to transcript destabilization. Finally, regarding SATB1 chromatin landscape, we observed that in T_{reg} cells the SATB1 locus is enriched in repressive histone marks, whereas in T_{conv} cells permissive histone marks are predominant. Although, the direct involvement of FOXP3 in defining the epigenetic marks at SATB1 locus has not been addressed in our study, the evidence provided by other researchers suggests that FOXP3 might also directly or indirectly regulate the chromatin remodeling of the SATB1 locus similar to other FOXP3-regulated loci.

6.5 Sustained high SATB1 expression in T_{reg} cells

The expression pattern and regulation of SATB1 observed in T_{reg} cells lead us to investigate the functional consequences of SATB1 over-expression in T_{reg} cells. For this purpose we transfected T_{reg} cells with a construct encoding for SATB1 ($SATB1^{high}$ T_{reg} cells) and evaluated their transcriptional profile and their function in contrast to wild type T_{reg} cells ($SATB1^{low}T_{reg}$ cells).

6.5.1 SATB1 expression in T_{reg} cells induces transcriptional T_{eff}-cell programs

Genome wide transcriptional profiling of T_{reg} cells revealed that these cells have a specific transcriptional signature that clearly reflects their functional features as keepers of central tolerance and modulators of the immune response. To elucidate the global impact of high SATB1 expression on T_{reg} cells, we compared the transcriptional profiles of $SATB1^{high}T_{reg}$ and $SATB1^{low}$ T_{reg} cells. Strikingly, SATB1 overexpression led to the expression of genes associated with T_{conv}-cell activation. As an example, the expression of effector cytokine genes like IFN-γ, IL-4 and IL-17, signature cytokines for Th1, Th2, and Th17 cells respectively, was prominently upregulated in $SATB1^{high}$ T_{reg} cells, while the expression of these genes was repressed in control $SATB1^{low}T_{reg}$ cells.

Under physiological circumstances it is well-known that the production of proinflammatory cytokines subverts the regulatory function of T_{reg} cells and if armed with effector cytokines production like IL-17 and IFN-γ, T_{reg} cells could contribute to tissue damage [152, 330, 331]. Furthermore, it has been described that sustained expression of FOXP3 in mature T_{reg} cells is necessary for maintenance of the T_{reg}-cell phenotype and suppressive function [286]. Loss of FOXP3 or its diminished expression in T_{reg} cells leads to acquisition of T_{eff}-cell properties including production of cytokines promoting an immune response such as IL-2, IL-4, IL-17,

and IFN-γ [110, 287, 332-334]. Interestingly, we found that SATB1^{high} T_{reg} cells produced large amounts of the Th1 and Th2 signature cytokines IFN-γ and IL-4 at steady state despite their apparently normal expression of FOXP3. Moreover, expression of characterisitic T_{reg}-cell genes is well preserved albeit the expression of several *bona fide* T_{eff}-cell genes. Several studies have reported that nT_{reg} cells cultured under Th1 or Th17 polarizing conditions co-express FOXP3 and proinflammatory cytokines [264, 335, 336]. This observations can be explained , at least in part, that in T_{reg} cells the TBX21 and GATA3 loci show both repressive and permissive histone marks and the IFN-γ and IL-4 loci only show minimal H3K27me3 [264]. Because of the bivalent histone modifications of proinflammatory cytokine genes, SATB1 induction in T_{reg} cells could mediate, directly or indirectly, a shift towards gene expression and induce transcription of these genes even in the presence of FOXP3 inhibition. How SATB1 orchestrates the expression of Th2 cytokine genes is in line with the idea of hijacking of T_{reg} cell program by SATB1. Upon Th2-cell activation, SATB1 is induced and binds to multiple SATB1 Binding Sites (SBSs) in the Th2 cytokine gene cluster and folds chromatin into many small transcriptionally active chromatin loops. These loopscape structures promote the coordinate and efficient expression of multiple genes in a cluster by bringing distal regulatory sequences into close proximity [190]. Supporting this notion, SATB1 could competitively drive gene expression through remodeling the chromatin as SATB1 recruits chromatin remodeling complexes to the anchored sites and thereby regulates the status of histone modifications and nucleosomal positioning over long-distances of DNA [199]. Likewise, the implication of interchromosomal interaction in IFN-γ expression and regulation has been already described [337] which might include SATB1to play a role in establishing this regulatory network.

6.5.2 SATB1^{high} T_{reg} cells are functionally defective

Gene expression data prompted us to interrogate the functional properties of SATB1^{high}T_{reg} cells as the edition of T_{reg} cell transcriptional program with T_{eff} cell transcriptional program could negatively affect the suppressive function of SATB1^{high} T_{reg} cells. Determining the suppressive activity of a regulatory T-cell population *in vitro* is often the first step in analyzing T_{reg} cell function. The benefits of this assay include ease and simplicity of use and high reliability. In 1998, two groups showed that $CD4^+CD25^+$ T-cells potently suppressed *in vitro* proliferation of other $CD4^+$ and $CD8^+$ T-cells when both populations were co-cultured and stimulated with specific antigen or polyclonal TCR stimulators such as CD3 mAb in the presence of antigen-presenting cells (APCs) [102, 338]. In our *in vitro* suppression assays, we

observed impaired suppressive activity of SATB1^{high} T_{reg} cells on the proliferation of co-cultured naive CD4^{+} T-cells compared to SATB1^{low} T_{reg} cells which showed full suppressive activity under the same experimental conditions. In light of gene expression data obtained for SATB1^{high} T_{reg} cells, this functional impairment further support a skewing of SATB1^{high} T_{reg} cells also towards functional T_{eff} cell program and failure of FOXP3 to prevent excessive polarization of T_{reg} cells and acquisition of effector cytokines.

In vivo function of T_{reg} cells is determined by assessing their capacity to suppress tissue inflammation. Several models are used for this purpose. The T-cell transfer model of adoptive colitis was one of the first murine models to convincingly and reproducibly demonstrate the existence of T_{reg} cells [266, 339]. In this model, the transfer of naive T-cells expressing high levels of CD45RB induces a Th1-mediated colitis when transferred into an immunodeficient host. Concomitant transfer of T-cells with low expression of CD45RB prevents development of colitis [267, 340]. It has now been demonstrated that the regulatory capacity of CD45RB^{low} T-cells is mediated by the CD4^{+}CD25^{+}FOXP3^{+} fraction and that transfer of these T_{reg} cells not only prevents colitis initiation, but also can reverse established disease [104, 341]. In our mouse model, the function of SATB1 transduced T_{reg} cells is severely impaired as the co-transfer of SATB1^{high} T_{reg} cells with CD4^{+}CD25^{-}CD62L^{high} T_{eff} cells failed to prevent colitis development. However, wild-type or control vector transduced T_{reg} cells effectively suppressed the development of disease as judged by both body weight loss and histological analysis of colon tissues. This *in vivo* observation confirmed the detrimental effect of abundance of SATB1 on the functional integrity of T_{reg} cells. One has to ask the question how SATB1 drives such a dramatic shift in cell-fate and overrides T_{reg} cell regulatory programs. In fact, nT_{reg} cells are relatively stable in a healthy immune system. However, reversal of T_{reg} cells function is not uncommon and multiple studies have shown T_{reg} cells instability, particularly, under non-physiologic settings such as lymphopenic and inflammatory conditions [249, 331]. Furthermore, highly polarized settings such as infection and inflammation are likely to contribute in a synergistic manner to the failure of T_{reg} cells to maintain and appropriately control tissue damage. The described mechanisms of impaired regulation in T_{reg} cells can be grouped into three main etiologies: inadequate numbers of T_{reg} cells, defects in T_{reg} cell function, and resistance of T_{eff} cells to suppression [342]. In general, exposure of T_{reg} cells to proinflammatory signals appears to abrogate their suppressive function. These signals act either directly on T_{reg} cells dampening T_{reg} cell function or indirectly on effector cells rendering them resistant to the T_{reg} cell mediated suppression. IL-1,

IL-6, TNF-α, IL-7, and IL-15 are among the factors have been repoted to modulate T_{reg} cell function either alone or in combination with other factors [343]. As an example, inflammatory signals delivered by cytokines like TNFα decrease T_{reg} cell activity in rheumatoid arthritis. Although, T_{reg} cells circulate in normal numbers, they have decreased activity *ex vivo* [344]. Besides the negative signals initiated by cytokines like TNF-α, T_{reg} cells also receive inhibitory signals via the TCR or TLR-2 [345-348]. Indeed, it has been reported that suppressive T_{reg} cells require much lower antigen concentration than that required for T_{eff} cells activation [349]. In line with this findings downstream TCR activation molecules like Akt and NF-κB have shown to reduce T_{reg} cell function and thus their expression and function appears to be tightly regulated [350]. This suggests that TCR signaling in T_{reg} cells can result in a negative feed-back inhibiting T_{reg} cell mediated suppression. On the effector cell side, more often chronic but also acute exposure of effecter cells to antigens has been reported to render the cells hyperactive by shifting the balance of the downstream signaling molecules like NF-κB and PKB towards activation, thereby increasing the threshold for suppression and overcoming regulatory activity [351].

Together, we have shown that induction of SATB1 in T_{reg} cells resulted in adaptation of a subset of T_{eff} cell associated gene expression with almost intact T_{reg} cell transcritional signature. These findings suggest that SATB1 overrides FOXP3 program and render T_{reg} cells permissive for proinflammatory cytokine production. Functionally, $SATB1^{high}$ T_{reg} cells showed attenuated suppressive function *in vitro.* Furthermore, *in vivo*, they failed to maintain tissue homeostasis in $RAG^{-/-}$ mice leading to the onset of colitis. Therefore, inhibition of SATB1 protein expression is indispensable for maintaining suppressive function and preventing excessive effector polarization of T_{reg} cells. Although our study clearly showed that high expression level of SATB1 resulted in malfunctioning T_{reg} cells, the detailed molecular mechanism behind the defective suppressive function in our study is still elusive and it is opened for further inverstigations. Hence, such knowledge might uncover some aspects of T_{reg} cell functional defects associated with several autoimmune diseases. Finally, this study have improved our understanding how FOXP3 counteracts the effector inflammatory program to maintain T_{reg} cell mediated self-tolerance and provided evidence that SATB1 represents a novel molecular switch between effector and regulatory immune function. It is tempting to propose SATB1 as an attractive molecular therapeutic target that might potentially modulate immune response in immunologically mediated pathologies such as autoimmunity and inflammatory disorders. Therefore, additional studies should follow to

define the molecular mechanisms underlying its functions and regulation in other effector immune cells.

6.6 Introduction of a model of SATB1 regulation in T_{reg} cells

Our data support a model in which SATB1 is differentially expressed in T_{reg} cells and T_{conv} cells. On the one hand, in T_{reg} cells, SATB1 expression is suppressed and reciprocally correlated with FOXP3 levels. The expression of SATB1 is tightly regulated at several levels, all under direct or indirect control of FOXP3. At transcriptional level, direct binding of FOXP3 to the genomic SATB1 locus is associated with gene transrepression which might be mediated either via competition with transactivators or through recruitment of silencing complexes. FOXP3-associated miRNAs represent post-transcriptional negative regulators of SATB1 through destabilization of the transcripts by binding to its 3´UTR. Predominance of repressive histone modifications at the SATB1 locus in T_{reg} cells render it inaccessible to the transcription machinery. Functionally, SATB1 derepression in T_{reg} cells is associated with curtailed suppressive function and acquisition of T_{eff} cell characteristics as production of proinflammatory cytokines (Fig. 36, a). On ther other hand, in T_{eff} cells, where FOXP3 is repressed, SATB1 expression is highly expressed and futher induced after physiological stimulation. This induction is coupled with production of effector cytokines like IL-2, IL-4, IL-5, and IL-13 and might result in other so far not yet clearly described functions. Furthermore, the SATB1 expression pattern in T_{eff} cells correlates with histone modifications as the SATB1 locus is highly enriched for permissive histone marks (Fig. 36, b).

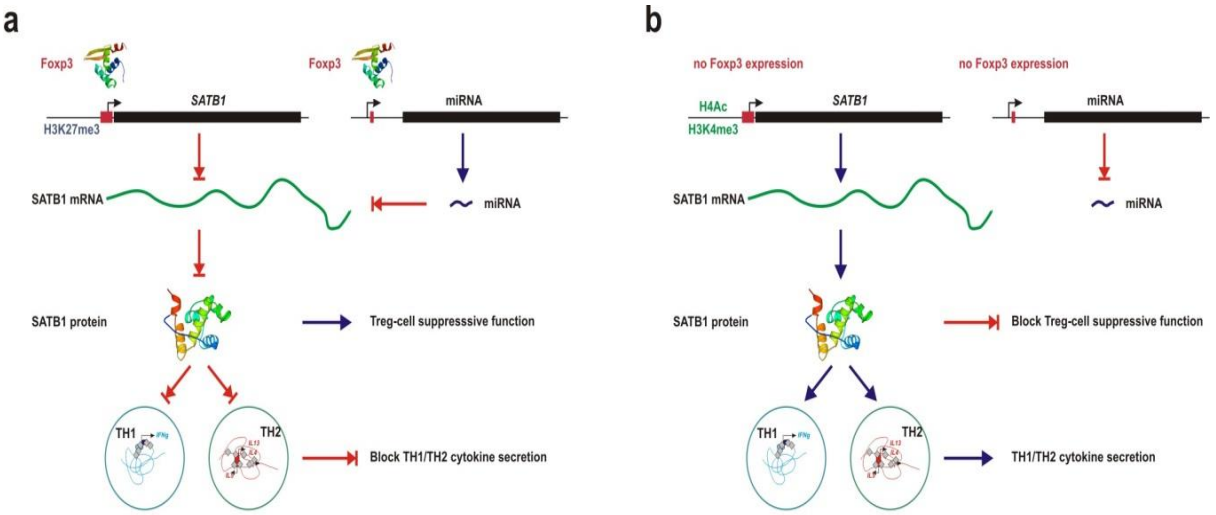

Figure 36. Proposed model for the mutual regulation of SATB1 in T_{reg} cells and T_{conv} cells.

(**a**) In T_{reg} cells SATB1 is downregulated and the master regulator FOXP3 negatively regulates SATB1 expression either directly by physical interaction with the genomic SATB1 locus or indirectly through specific binding of FOXP3-induced miRNAs to the SATB1 3´UTR. Moreover, the genomic SATB1 locus is enriched in repressive histone marks which might stabilize negative expression pattern of SATB1 in these cells. (**b**) In T_{conv} cells, the expression of SATB1 is upregulated as SATB1 expression is required for effector functions like production of proinflammatory cytokines. Repressed FOXP3 expression in addition to permissive histone modifications favoring SATB1 gene accessbility might explain, at least partially, the high basal and inducible expression of SATB1 in T_{eff} cells.

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8 Appendices

Table 1. FOXP3 binding regions

Tiling array region	start	stop	length (bp)	FOXP3 binding motifs	MAT score	p-value ($-10\log_{10}$)	FOXP3 binding region	FOXP3 binding motifs	start	stop	Length (bp)
B1	-19933	-18711	1223	9	7.05	154.29	**BR1**	7	-19337	-18711	626
B2	-17526	-15898	1629	15	5.25	90.42	**BR2**	6	-16970	-16592	379
B3	-15767	-14637	1131	7	5.16	87.58	**BR3**	2	-15692	-15439	254
B4	-13165	-12161	1005	5	6.43	130.41	**BR4**	4	-22566	-12161	406
B5	-12160	-11018	1143	8	4.89	79.92	**BR5**	4	-11490	-11217	274
B6	-11017	-9639	1379	14	6.02	115.69	**BR6**	6	-10161	-9818	345
B7	-8491	-6303	2189	13	6.88	147.33	**BR7**	4	-7085	-6865	221
B8	-5995	-4369	1627	15	5.47	97.25	**BR8**	5	-5181	-4896	286
B9	-4356	-1861	2496	27	12.86	483.42	**BR9**	4	-3947	-3697	251
							BR10	5	-3110	-2710	401
B10	-854	1465	2320	13	7.88	189.63	**BR11**	1	+269	+669	401
B11	+2895	+6146	3252	29	7.96	193.47	**BR12**	3	+3336	+3736	401
B12	+6505	+7653	1149	9	5.59	101.04	**BR13**	1	+6303	+6502	200
							BR14	3	+6653	+6769	117
B13	+11598	+13006	1409	12	6.93	149.36	**BR15**	7	+12260	+12760	501
B14	+20356	+22256	1901	20	11.38	381.62	**BR16**	6	+21031	+21431	401

Table 2. human qPCR oligonucleotides

SATB1 Forward	CGATGAACTGAAACGAGCAG
SATB1 Reverse	CGGAGGATTTCTGAAAGCAA
FOXP3 Forward	ACCTACGCCACGCTCATC
FOXP3 Reverse	TCATTGAGTGTCCGCTGCT
IL-5 Forward	GGTTTGTTGCAGCCAAAGAT
IL-5 Reverse	TCTTGGCCCTCATTCTCACT
IFN-g Forward	CACTGAAGAAATCTTTCAGGGAAT
IFN-g Reverse	CCGTCTTTCTTCTCCACACTTT
IL-17A Forward	TGGGAAGACCTCATTGGTGT
IL-17A Forward	GGATTTCGTGGGATTGTGAT
CTLA4 Forward	TTCATCCCTGTCTTCTGCAA
CTLA4 Reverse	AGTGGCTTTGCCTGGAGAT
GITR Forward	AGGCCAGGGGGTACAGTC
GITR Reverse	AAGGTTTGCAGTGGCCTTC
PDE3B Forward	AACAATGGTATAAGCCTCATTATCAA

PDE3B Reverse	CGAGCCTCATTTAGCACTGA
CD25 Forward	ACGGGAAGACAAGGTGGAC
CD25 Reverse	TGCCTGAGGCTTCTCTTCA
CD127 Forward	AAAGTTTTAATGCACGATGTAGCTT
CD127 Reverse	TGTGCTGGATAAATTCACATGC
CD73 Forward	TGATCCTCCCAAACTTCCTG
CD73 Reverse	AACCACGTTGATATCTTGGTCA
CD39 Forward	AAGGCCACAGCTGGCTTAC
CD39 reverse	GGAAAGGCGATGCTTTAAATAC
GPR83 Forward	TCTGCCAGAAATTATTTACCTTCA
GPR83 Reverse	AGCTGGCTCAGGGAAGTCT
b-2 microglobulin Forward	TTCTGGCCTGGAGGCTAT
b-2 microglobulin Reverse	TCAGGAAATTTGACTTTCCATTC

Table 3. siRNA

FOXP3 sense	GCACAUUCCCAGAGUUCCUdTdT
FOXP3 antisense	AGGAACUCUGGGAAUGUGCdTdT
control sense	UUCUCCGAACGUGUCACGUdTdT
control antisense	ACGUGACACGUUCGGAGAAdTdT
SATB1 siRNA pool 1 sense	GAAGGAAACACAGACAUUA
SATB1 siRNA pool 1 antisense	UAAUGUCUGUGUUUCCUUC
SATB1 siRNA pool 2 sense	GGAAUGCUCUGAAGGACUU
SATB1 siRNA pool 2 antisense	AAGUCCUUCAGAGCAUUCC
SATB1 siRNA pool 3 sense	GCAAAUGUCUCAGCAGCAA
SATB1 siRNA pool 3 antisense	UGCUGCUGAGACAUUUGC
SATB1 siRNA pool 4 sense	UCAGAAAUGUCUAACAAUG
SATB1 siRNA pool 4 antisense	CAUUGUUAGACAUUUCUGA

Table 4. Mouse qPCR oligonucleotides

SATB1 Forward	5′-AGGAGTGCCCCCTTTCAC-3′
SATB1 Reverse	5′-TGCTGCTGAGACATTTGCAT-3′
Foxp3 Forward	5′-ACCACACTTCATGCATCAGC-3′
Foxp3 Reverse	5′-CCAGTGGCAGCAGAAGGT-3′
IL-5 Forward	5′-CAAACTGGTAATGTAGCCAAGGAT-3′
IL-5 Reverse	5′-CTGTAACCTCAGCCTTCAGGA-3′
IL-6 Forward	5′-GCTACCAAACTGGATATAATCAGGA-3′
IL-6 Reverse	5′-CCAGGTAGCTATGGTACTCCAGAA-3′
IFN-γ Forward	5′-CCTTTGGACCCTCTGACTTG-3′
IFN-γ Reverse	5′-AGCGTTCATTGTCTCAGAGCTA-3′
β-actin Forward	5′-CTAAGGCCAACCGTGAAAAG-3′
β-actin Reverse	5′-ACCAGAGGCATACAGGGACA-3′

Table 5. ChIP-PCR oligonucleotides

SATB1 BR1 Forward	GAACAGTAGTACATTCATATACTGG
SATB1 BR1 Reverse	TTAAGATGCCTATTCATGCTATTCC
SATB1 BR2 Forward	GAAAGTTATGTTTCAGCATGATGAG
SATB1 BR2 Reverse	CTGGTATTTACTTCAGGAAGGTAAG
SATB1 BR3 Forward	TCAACACATTAACACACATTATTTC
SATB1 BR3 Reverse	ATAACTTGAAAGCATAAAAATAGGC
SATB1 BR4 Forward	CAAACAGAGCACTCCCCTGAG
SATB1 BR4 Reverse	GGAAAGAACGCATAGAAGGTCAATC
SATB1 BR5 Forward	TCTCATTTCCTCTTGTTTATTGTTG
SATB1 BR5 Reverse	ATAATAAGGTTAAGTAGGCTTCTCC
SATB1 BR6 Forward	TTCTACTGTGGCATTATTTATCAAC
SATB1 BR6 Reverse	GCATATCCAGTTACCTATCTTGTAG
SATB1 BR7 Forward	TCTCCCAAAACAAACATAAACATTG
SATB1 BR7 Reverse	TTTCCCTAATTACTTGGCATAGAT
SATB1 BR8 Forward	AAAGTGTTATGGTGGTAAGCATTG
SATB1 BR8 Reverse	CAGAGAAAGCATTTAGAATTCGAGTG
SATB1 BR9 Forward	ATAGTCATTCTTAGATGCCTTTATG
SATB1 BR9 Reverse	CTGTTGGGCAAAATAAAATTTAAAC

SATB1 BR10 Forward	ATTGGTGGCTTTACAGAGCTTAAT
SATB1 BR10 Reverse	AATTTGCTGGTAGGGAAAAGAGAA
SATB1 BR11 Forward	GTTTCAGGTCGGTTTTGCTAATTT
SATB1 BR11 Reverse	GATTGTGCCCTTATCCATTCCATA
SATB1 BR12 Forward	TAGGCAGCTGTTTCTTCAAACTAA
SATB1 BR12 Reverse	CTGAAACTGCCCTACAACTAAGAA
SATB1 BR13 Forward	GCAGTAGAAAGGTGGGTTCTTC
SATB1 BR13 Reverse	GCAATGAATGCAGAATTACCTTT
SATB1 BR14 Forward	GCCTGTACTGTACCCAGTC
SATB1 BR14 Reverse	CATATTCAATGGCGTTTTCATAATG
SATB1 BR15 Forward	AGGTCAGCTATTACTTCTACCAGC
SATB1 BR15 Reverse	GGAAGGTAAAAATGCGCAAATCAG
SATB1 BR16 Forward	TTTTGTAGATGCTCTTGCTACCTC
SATB1 BR16 Reverse	TCATATGATCTACATGCTTGCGTT
SATB1 -15kb ChIP Forward	AGCAAGATTTAATTGCTGGTCCTG
SATB1 -15kb ChIP Reverse	TGCAATTGAAAAGACAAAGCCCTA
CTLA4 Forward	AAGGCTTTCTATTCAAGTGCCTTC
CTLA4 Reverse	TGTTCAGGTCTTCAGGAAGTAGAG
PDE3B Intron 1 BS2 Forward	TATGCATTCCGTATGCGTGGTAG
PDE3B Intron 1 BS2 Reverse	AATGAACCTACAACAAGCAGCCT
PDE3B Intron 10 Forward	GTTTTGTGTGCAAACATGGGTCT
PDE3B Intron 10 Reverse	TGGAAAATGTTCTCAAGGTGGCT
IL7RA Promoter Forward	CAGGGAATATCCAGGAGGAA
IL7RA Promoter Reverse	TGTGTGAGCCAGTGTGTATGAA
AFM Intron 1 Forward	GCAGAACCTAGTTCCTCCTTCAAC
AFM Intron 1 Reverse	AGTCATCCCTTCCTACAGACTGAGA
hsa-miR-155 Forward	TGCTAATCGTGATAGGGGTTTTTG
hsa-miR-155 Reverse	GCCTGAAGTCTAAGTTTATCCAGC
hsa-miR-21 Forward	CACCACAGGTAAGACTTTAATCCG
hsa-miR-21 Reverse	AAATGTGAGTGAGAGCTGTGAATG

Table 6. Oligonucleotides for cloning of the 3′UTR of human *SATB1*	
SATB1 3′UTR Forward	5′-ATACTCGAGGATAAAAGTATTTGTTTCGTT-3′
SATB1 3′UTR Reverse	5′-ATAGCGGCCGCACGATACAAACAATTTTAA-3′

Table 7. Oligonucleotides for mutagenesis of the 3′UTR of human *SATB1*	
miR-155 Forward	5′-TAATTACCTTCTGTGATTATGATT-3′
miR-155 Reverse	5′-GCTTGTTTGAGGCTCCGGAA-3′
miR-34a	5′-CTTTTATTTCCAAAATAAAAACAAATTTGAATTACCCGTGTGCCATATAATACAAGGCATTTGTTGGCATATG-3′
miR-21 I	5′-GTAAAAATTGCATACAACAATAAGAGTGATCGATATAGTATGAATTGCTTGGATAACATAGAGCACTTTTTA-3′
miR-21 II	5′-CATTCGTGATCTATTAGTTTTATTTACCTATCGATATTTGCATGATAGTAAAAATTGCATACAACAATAAGAGTG-3′
miR-18a	5′-GCAAACATCAATTATTTTCACATTAATTGCATAATTTTCATTCGTGATCTATTAGTTTTATTTACCTAAGCTTATTTGCATG-3′
miR-7	5′-TACAGATGTAGCTTTAAAATTGATTGTAAACCAAACCTTGACACATTGCAAACATCAATTATTTTCACATTAAT-3′

Table 8. bisulphite sequencing oligonucleotides	
SATB1 CpG A Forward	TCTCCCTGTGCCACCCAG
SATB1 CpG A Reverse	ACCACAATGGCACTAGGAC
SATB1 CpG B Forward	TAACTCCTCATAATTTAAAA
SATB1 CpG B Reverse	GGGAGTTATTAGGAAGTGGT
SATB1 CpG C Forward	CTCTCCCTATACCACCCAA
SATB1 CpG C Reverse	TATTATAATGGTATTAGGAT
FOXP3 Forward	TGTTGTAGGATAGGGTAGTT

FOXP3 Reverse	CATAAAAATAATCTATCTAA

Table 9. oligonucleotides for filter retention

SATB1 BR1 motif 3 sense	GATGTAATAAGCATGTTTACAATATTTTATTTCAGTC
SATB1 BR1 motif 3 antisense	GACTGAAATAAAATATTGTAAACATGCTTATTACATC
SATB1 BR1 motif 3 (mutated) sense	GATGTAATTCAATTACGAATTGTATTTAATTTCAGTC
SATB1 BR1 motif 3 (mutated) antisense	GACTGAAATTAAATACAATTCGTAATTGAATTACATC
SATB1 BR2 motif 1 sense	TTAGATTTGTAATGTAAACAGTGTGCCTAAGAG
SATB1 BR2 motif 1 antisense	CTCTTAGGCACACTGTTTACATTACAAATCTAA
SATB1 BR2 motif 1 (mutated) sense	TTAGATTTAGTGTGGATAAATCATGCCTAAGAG
SATB1 BR2 motif 1 (mutated) antisense	CTCTTAGGCATGATTTATCCACACTAAATCTAA

Table 10. miRNA binding motifs in the 3′UTR of human *SATB1*

motif	wild-type	mutated
miR-155 motif at +333	AGCATTA	AGCTAAT
miR-34a motif at +538	CACTGCC	CACACGG
miR-21 motif I at +886	ATAAGCT	ATATCGA
miR-21 motif II at +932	ATAAGCT	ATATCGA
miR-18a motif at +961	CACCTTA	CACGAAT
miR-7 motif at +1012	GTCTTCC	GTCAAGG

Table 11. Oligonucleotides for cloning of the Foxp3 binding regions of human *SATB1*

Foxp3 BR9 Forward	5′-ATATGGTACCATGCTCATTTATTCTGTT-3′
Foxp3 BR9 Reverse	5′-ATATAAGCTTGAAATAAAATATTGTAAACA-3′

Foxp3 BR10 Forward	5′-ATATGGTACCGCTATTAGAGTGATATATT-3′
Foxp3 BR10 Reverse	5′-ATATAAGCTTGTTCATGTAAAAATAAAG-3′
Foxp3 BR11 Forward	5′-ATATGGTACCGTGTTTCTGTTTCTAGATAG-3′
Foxp3 BR11 Reverse	5′-ATATAGATCTGAGTTTCTTTTGAGTTACACGT-3′
Foxp3 BR12 Forward	5′-ATATGGTACCGACTGAATTAAATATAATGG-3′
Foxp3 BR12 Reverse	5′-ATATAAGCTTTCCAAGTTGTTAAACTGCA-3′
Foxp3 BR13 Forward	5′-ATATGGTACCTAATTTGTTTGGACACAA-3′
Foxp3 BR13 Reverse	5′-ATATAAGCTTGCAATGAATGCAGAATTAC-3′
Foxp3 BR14 Forward	5′-ATATGGTACCCACAGTGAATCTCTTGTG-3′
Foxp3 BR14 Reverse	5′-ATATAAGCTTCTATCAAAAAGATGAAGAAGAA-3′

Table 12. Foxp3 binding motifs at the human SATB1 locus

motif	wild-type	mutated
Foxp3 BR9 motif I + II	ATTCTGTTTATCATTTT	CGTGCGGCCGCAGGACA
Foxp3 BR9 motif III + IV	ATGTTTAC	GGTCCGTG
Foxp3 BR10 motif I	GTAATGTAAACAGTGTG	TGTCCTGCGGCCGCACG
Foxp3 BR10 motif II	CAAAATGTTTATCTGAC	TCGTCCCTGCAGGCCGG
Foxp3 BR10 motif III	TGAAAAATAATTCTTGT	CGTGGCTGGCGAAGAGC
Foxp3 BR10 motif IV	AATATTCTTTATTTTTA	GCTGGCGGCGCCGCGCG
Foxp3 BR10 motif V	CTTTATTTTTACATGAA	GGCGCCGCGCGTGATTG
Foxp3 BR11 motif I	ATAATTGTTGTTACTGA	CGTGCGGCCGCAGGACA
Foxp3 BR12 motif I	TTAAATATAATGGTTTT	CCGGCGCGCCACCGCGA
Foxp3 BR12 motif II	GGTAATGTTATTTTTTG	CATCCCTGCAGGGACCA
Foxp3 BR12 motif III	TAGCAGTAAAAAATC	GCTCGCGGCCGCCGA

	TG	GT
Foxp3 BR13 motif I	ATGTTTGC	CGGTCCGTG
Foxp3 BR14 motif I + II	GATTATTTATTT	TCGGCGGCCGCG
Foxp3 BR14 motif III	GATTGTTGGTTT	CCGGCGCGCCAC

Table 13. Oligonucleotides for mutagenesis of the Foxp3 binding motifs of the human *SATB1* locus

Foxp3 BR9 motif I + II	5′-TAAGGACTGACATGGCCTTGGTGTCCTGCGGCCGCACGAAA TGAGCATGGTACCGGCCAGTTAGGCCAGAG -3′
Foxp3 BR9 motif III + IV	5′-TATACCCTCTAGTGTCTAAGCTTGAAATAAAATATTCACGGACCGCTTATTACATCATTTTACAGAAATAAATAAGAC -3′
Foxp3 BR10 motif I	5′-GCTCTGTAAAGCCACCAATACACTCTTAGGCGTGCGGCCGCAGGACAAAATCTAAAGAAAAGCCAAAAGGTAAAGCAGAAAAGTG-3′
Foxp3 BR10 motif II	5′-GAATTTGCTGGTAGGGAAAAGAGAAAAATCACCGGCCTGCAGGGACGATTACACATAAAAAAGCAACAAGTTAGCACAAGTACTGAGATAAAAGGG-3′
Foxp3 BR10 motif III	5′-CCATTTTAGCACCCTGAAGGCAGTTAATCATTAACAAAAAGCTCTTCGCCAGCCACGGAATTTGCTGGTAGGGAAAAGAGAAAAATCAGTCAG-3′
Foxp3 BR10 motif IV + V	5′-GCTGGAAGTCGAGCTTCCATTATATACCCTCTAGTGTCTAAGCTTGCAATCACGCGCGGCGCCGCCAGCACAATGTAACCCAAAATAGAAATGCCTCATTTATCGCTTAGG-3′
Foxp3 BR11 motif I	5′-CTAAGACCATATATGATAATTACTGAATAAAACAGTGAACCGTGCGGCCGCAGGACATTCCTTTAAAAGGAA

	AGAAAATCTCGAAATTCTAGTTTTTTAGG-3′
Foxp3 BR12 motif I	5′- CACCTTTTGAATGGACCCTGCCAAAACTTCGCGGTGG CGCGCCGGTTCAGTCGGTACCGGCCAGTTAGGCCAG AGAAATG-3′
Foxp3 BR12 motif II	5′- CTGCTAATGAATTTGCTCTTAAGAAAATAAGCCATGG TCCCTGCAGGGATGAAACTTCTACATAAGATGTCCAA GATCTCTGAAACTGCCCTAC-3′
Foxp3 BR12 motif III	5′- CCCAATTAAAACAAAAATAAAAAACTAGAATCATAC TCGGCGGCCGCGAGCATGAATTTGCTCTTAAGAAAA TAAGCCATGGTCCCTGCAGGGATG-3′
Foxp3 BR13 motif I Forward	5′-ATACTGTATACTACTCATAGCAAA-3′
Foxp3 BR13 motif I Reverse	5′-CACGGACCGAACTCACCATTTATCTTCAG-3′
Foxp3 BR14 motif I + II	5′- GGAAATATTAGCCATGACGCGGCCGCCGAACAAGAG ATTCACTGTGGGTACCGGCCAGTTAGGCC-3′
Foxp3 BR14 motif III	5′- CTATCAAAAAGATGAAGAAGAAGAGATGGAGTGGC GCGCCGGTTCAGAAATACAGCTTGGGGGTGGC-3′

Table 14. Oligonucleotides for miR RNAi targeting human SATB1 and Foxp3

SATB1 miR RNAi sense	5′- TGCTGATTCACTGCATACTGCTGGTTGTTTTGGCCA CTGACTGACAACCAGCAATGCAGTGAAT-3′
SATB1 miR RNAi antisense	5′- CCTGATTCACTGCATTGCTGGTTGTCAGTCAGTGGC CAAAACAACCAGCAGTATGCAGTGAATC-3′
Foxp3 miR RNAi sense	5′- TGCTGAAAGCACTTGTGCAGACTCAGGTTTTGGCCA CTGACTGACCTGAGTCTACAAGTGCTTT-3′

Foxp3 miR RNAi antisense	5′-CCTGAAAGCACTTGTAGACTCAGGTCAGTCAGTGGCCAAAACCTGAGTCTGCACAAGTGCTTTC-3′

Table 15. Histone ChIP-PCR oligonucleotides

SATB1 -25kb Forward	TTAATGGCGGTAAGTAGTAAGTTC
SATB1 -25kb Reverse	TAAGACCAGAGGAATCAGAGATC
SATB1 -24kb Forward	GCCCATGTGCTTTCTATACAAGAC
SATB1 -24kb Reverse	ACTCATCTGCTTCCCCAAGTTATC
SATB1 -23kb Forward	TCCCTCCTGCTTCCATAGTTTC
SATB1 -23kb Reverse	TTGGCAAGAAGTGTAGTTAGGTTC
SATB1 -22kb Forward	GCACACACACTACAGTAACC
SATB1 -22kb Reverse	CGCACTCCTCCTCTTGTC
SATB1 -21kb Forward	GTCCATGCCTGAGTGAGTTCTG
SATB1 -21kb Reverse	ACGAGGAGTGGGTGCTACG
SATB1 -20kb Forward	TGACCTCAGAAGACCAACAG
SATB1 -20kb Reverse	GAGAAAGTTCGCCAAGGAAG
SATB1 -19kb Forward	GGATTTCCCTCCTAAAGTAC
SATB1 -19kb Reverse	GGTCTTCTTTCTCTATCTTCC
SATB1 -17.5kb Forward	ACCTTCAGATATTTCAGAGAGCAG
SATB1 -17.5kb Reverse	AAGCACAAACCACACAAAACTG
SATB1 -13.5kb Forward	AGCAGTTAATCACAGAGGTAGTTC
SATB1 -13.5kb Reverse	TTGTCTAAGTAAGCCTAAGTCCAG
SATB1 -9.5kb Forward	TGTGTAGCATAGACTGGACTGTAG
SATB1 -9.5kb Reverse	GCCAAGCCTCAGAGAAACAATG
SATB1 -6.5kb Forward	TTGTGCTGCTTTAAGAGTGTAGAG
SATB1 -6.5kb Reverse	TCCGCCAGTGACTGTGTTAG
SATB1 -4kb Forward	TCCCGTGATCTTTGTTTAGTGGTG
SATB1 -4kb Reverse	GCCAGGCAATGTCACCTCAAG
SATB1 -3kb Forward	TCAGGGTGCTAAAATGGTAGAAC
SATB1 -3kb Reverse	CACTTCTGCTGTAATCTAGGGTAG
SATB1 -2kb Forward	CCGCCTCACTCTAATCAAG
SATB1 -2kb Reverse	AAAGCCGAAAGACAAGTAAC
SATB1 -1kb Forward	CTTCTGATCTTCCTCCTCCTC

SATB1 -1kb Reverse	CTCTCCCTGCCCATTGAC
SATB1 0kb Forward	GTCGGTTTTGCTAATTTCACTCAG
SATB1 0kb Reverse	GCTGGATCGCCTCCTTCG
SATB1 +1kb Forward	ATGGGACCTCTATCAACGGTAAAC
SATB1 +1kb Reverse	GGGCAGCAATGTTAATCTGGAAG
SATB1 +2kb Forward	CACATCTTTGGCCCAAAACTTG
SATB1 +2kb Reverse	AGAAGGTAACAAACATCTAACTGC

Table 16. oligonucleotides for miRNAs against human SATB and FOXP3

SATB1 miRNA sense	TGCTGATTCACTGCATACTGCTGGTTGTTTTGGCCACTG ACTGACAACCAGCAATGCAGTGAAT
SATB1 miRNA antisense	CCTGATTCACTGCATTGCTGGTTGTCAGTCAGTGGCCAA AACAACCAGCAGTATGCAGTGAATC
FOXP3 miRNA sense	TGCTGAAAGCACTTGTGCAGACTCAGGTTTTGGCCACT GACTGACCTGAGTCTACAAGTGCTTT
FOXP3 miRNA antisense	CCTGAAAGCACTTGTAGACTCAGGTCAGTCAGTGGCCA AAACCTGAGTCTGCACAAGTGCTTTC

9 Zusammenfassung

In dieser Arbeit wurde SATB1 als neues Effektormolekül für regulatorische T-Zellen identifiziert. SATB1 ist ein nukleäres Protein, das Faktoren rekrutiert, die bei der Gestaltung der Chromatinstruktur entscheidend beteiligt sind und dadurch die Expression zahlreicher Gene reguliert. Durch genomweite Expressionsanalysen regulatorischer und konventionellen T-Zellen konnte eine konstitutive Expression von SATB1 in konventioneller $CD4^+$ T-Zellen beschrieben werden, während die SATB1 Expression in regulatorischen T-Zellen reprimiert wird. Diese Repression in regulatorischen T-Zellen konnte sowohl in Mäusen als auch im Menschen unter stimulierenden und nicht stimulierenden Bedingungen durch mRNA-, Protein- und Einzelzell-Analysen bestätigt werden.

Das zu SATB1 entgegengesetzte Expressionsmuster von FOXP3, dem wichtigsten Transkriptionsfaktor für die Entstehung und Aufrechterhaltung des Phänotyps und der Funktion regulatorischer T-Zellen, ließ eine aktive Beteiligung von FOXP3 an der Regulation von SATB1 vermuten. Einerseits wurde die SATB1 Expression bei gleichzeitiger Induktion von FOXP3 in $CD4^+$ T-Zellen inhibiert. Andererseits erfolgte nach Verlust der FOXP3 Expression in regulatorischen T-Zellen eine Induktion von SATB1. Des Weiteren zeigte sich, dass SATB1 in FOXP3-exprimierenden Thymozyten sehr schwach exprimiert war, während es sehr viel stärker in Thymozyten aus *scurfy* Mäusen exprimiert wurde, die ein mutiertes und somit nicht funktionelles FOXP3 Gen besitzen.

Die Regulation der Genexpression durch FOXP3 durch direkte Bindung an die DNA, wurde bereits beschrieben. Um eine direkte Bindung von FOXP3 am genomischen SATB1 Lokus zu bestätigen, wurden mittels Chromatinimmunopräzipitation (ChIP) genomweite Bindungsanalysen durchgeführt, wodurch multiple FOXP3 Bindestellen im Promoter und in intronischen Regionen des genomischen SATB1 Lokus identifiziert und mittels ChIP PCR validiert werden konnten. Außerdem konnten eine hohe Bindungsspezifität und der Bindungskoeffizient von FOXP3 an verschiedenen SATB1 Motiven bestimmt werden. Des Weiteren konnte die funktionelle Relevanz dieser FOXP3 Bindungsstellen für mehrere Bindestellen gezeigt werden. Zusammengenommen zeigen diese Analysen, eine Regulation der SATB1 Expression durch die direkte DNA-Bindung von FOXP3 erfolgte.

Neben der direkten Repression durch Bindung an den genomsichen SATB1 Lokus kontrollierte FOXP3 die SATB1 Expression auch indirekt auf post-transkriptionaler Ebene durch FOXP3-abhängige MikroRNAs.

Außerdem konnten in konventionellen $CD4^+$ T-Zellen vermehrt permissive und weniger inhibitorische Histonmodifikationen am SATB1 Gen gefunden werden während die CpG Zusammensetzung in konventionellen und regulatorischen T-Zellen am SATB1 Gen keine nennenswerten Unterschiede aufwies.

Des Weiteren wurden die Auswirkungen einer induzierten Expression von SATB1 für die Funktion von regulatorischen T-Zellen untersucht. Induzierte Expression von SATB1 veränderte signifikant das Genexpressionsmuster und die Funktion von regulatorischen T-Zellen, was mit dem Verlust der inhibitorischen Funktion der regulatorischen T-Zellen *in vitro*, der zusätzlichen Produktion von entzündungsfördernden Zytokinen wie IL-4 oder IFN-γ und der Induktion von transktiptionellen T-Effektorzellprogrammen einherging. *In vivo* zeigte sich ebenfalls ein Verlust der suppressiven Funktion SATB1 exprimierender regulatorischen T-Zellen.

Zusammenfassend ist SATB1 ein wichtiges Effektormolekül, dessen Expression durch FOXP3 in regulatorischen T-Zellen supprimiert wird, und mit den regulatorischen Eigenschaften der Zellen eng verbunden ist. Durch eine Überexpression von SATB1 *in vivo* und *in vitro* verlieren regulatorische T-Zellen ihre suppressive Funktion und zeigen einen T-Zelleffektorphänotyp mit entzündungsfördernden Eigenschaften.