ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
ΔΙΑΤΜΗΜΑΤΙΚΟ ΜΕΤΑΠΤΥΧΙΑΚΟ ΠΡΟΓΡΑΜΜΑ
«ΜΟΡΙΑΚΗ ΒΙΟΛΟΓΙΑ & ΒΙΟΪΑΤΡΙΚΗ»

ΙΔΡΥΜΑ ΤΕΧΝΟΛΟΓΙΑΣ & ΕΡΕΥΝΑΣ
ΙΝΣΤΙΤΟΥΤΟ ΜΟΡΙΑΚΗΣ ΒΙΟΛΟΓΙΑΣ & ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ

Συνοπτική Αναφορά
Μεταπτυχιακής Ερευνητικής Εργασίας

ΣΑΡΡΗΣ ΜΙΧΑΛΗΣ
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Ευχαριστίες

Θα ήθελα να ευχαριστήσω θερμά τον μέντορά μου κύριο Σπηλιανάκη για την εμπιστοσύνη που μου έδειξε και την συνεχή επιστημονική καθοδήγησή του. Ευχαριστώ όλα τα μέλη του εργαστηρίου και ιδιαίτερα τον Πέτρο για την καθημερινή εποικοδομητική αλληλεπίδραση που είχαμε και την φιλία που αναπτύξαμε μέσα από αυτήν.
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Μελέτη της πρωτεΐνης SATB1 ως συμμετέχων μόριο στη διαδικασία των διαχρωμοσωμικών αλληλεπιδράσεων

Περίληψη

Η διαφοροποίηση των παρθένων (naïve) CD4 κυττάρων σε Τ κύτταρα βοηθούς τύπου 1 (Th1) και τύπου 2 (Th2) ρυθμίζεται μέσω επιγενετικών φαινομένων. Έχει αναφερθεί ότι ενδοχρωμοσωμικές και διαχρωμοσωμικές αλληλεπιδράσεις μεταξύ απομακρυσμένων ρυθμιστικών περιοχών συνεισφέρουν στην αναπτυξιακή αυτή μετάβαση, παρουσιάζοντας έτσι έναν πρωτότυπο τρόπο ρύθμισης ο οποίος τονίζει τη σημασία της ανώτερης τρισδιάστατης δομής της χρωματίνης. Η πρωτεΐνη SATB1 (special AT-rich sequence binding protein 1) είναι μία γνωστή πρωτεΐνη οργανωτής της χρωματίνης η οποία πακετάρει την χρωματίνη σε δομές θηλιάς επιτυγχάνοντας έτσι την συντονισμένη έκφραση των υποκείμενων γονιδίων. Στην εργασία αυτή αναφέρουμε την έκφραση της SATB1 πρωτεΐνης σε παρθένα CD4 κύτταρα και δείχνουμε ότι συμμετέχει ως πρωτεΐνη «αρχιτέκτονας» στο περίπλοκο φαινόμενο των διαχρωμοσωμικών αλληλεπιδράσεων απομακρυσμένων γενετικών τόπων. Περαιτέρω, αναφέρουμε το συνεντοπισμό των πρωτεϊνών SATB1 και Lamin-A στον πυρήνα των CD4 κυττάρων και των θυμοκυττάρων.

SATB1 as a candidate player in the process of interchromosomal interactions

Abstract

Epigenetic phenomena regulate the differentiation of naïve CD4 cells to Th1 and Th2 cell lineages. Intra and interchromosomal interactions between distal regulatory regions have been reported to contribute to this developmental switch, exhibiting a novel mode of regulation highlighting the importance of higher order chromatin structure. SATB1 (special AT-rich sequence binding protein 1) is a well known chromatin organizer protein that packages chromatin into loops to coordinate the expression of the underlying genes. Here we report SATB1 expression in naïve CD4 cells and show that this architectural protein participates to the complex phenomenon of interchromosomal interaction of genomic loci. Moreover we report the colocalization of SATB1 with Lamin-A in the nucleus of CD4 cells and thymocytes.

Introduction

A critical event of adaptive immunity is the differentiation of naïve $CD4^+$ T cells into effector T helper 1 (Th1) or Th2 cells upon antigen presentation by antigen Presenting Cells (APC). Th1 cells promote cell-mediated immunity by producing inflammatory cytokines such as LTb, IFNγ and lymphtoxin-α which stimulate macrophages, cytotoxic T cells (Tc or CTL cells) and certain opsonic or complement-fixing IgGs. Cell-mediated immunity targets intracellular bacteria and viruses. Th2 cells produce interleukin 4 (Il4), Il5, Il13 and promote antibody production by B cells, enabling humoral immunity and immunity against extracellular parasites. Deregulation of the Th1 lineage functions can cause autoimmune diseases whereas deregulation of the Th2 lineage functions causes allergies.

Th cell differentiation depends on polarizing cytokine signals during the activation phase by antigens. Signaling is mediated by members of the STAT family (Signal Transducers and Activators of Transcription). Moreover, ligands for the Notch pathway contribute to the instruction of both lineages. Key transcription factors in each cell lineage act as lineage-determining master regulators orchestrating the accessibility and expression of the defined subsets of cytokine genes (Glimcher & Murphy,2000; Mosmann and Coffman, 1989; Murphy

and Reiner, 2002; Wilson et al. 2009). The transcription factor T-box-containing protein expressed in T cells (T-bet) is the master regulator of the Th1 differentiation program whereas GATA binding protein 3 (GATA3) controls the development of the Th2 cell lineage. Furthermore, the Th2 cytokine locus which is expressed in Th2 cells and the IFNg and LT.TNF loci which are expressed in Th1 cells, undergo structural and epigenetic changes during differentiation. Regulatory elements such as promoters, enhancers, insulators, locus control regions (LCR), non-coding sequences (CNSs) and hypersensitive to DNaseI sites (HS, RHS) perform a complex role as their occupancy by transcription factors and their epigenetic status (histone acetylation and methylation, DNA methylation) play key roles in the process of differentiation. Histones in the chromatin of cytokine promoters for example, are hypo-acetylated in naïve T cells, but upon TCR stimulation, H3 and H4 are acetylated, irrespective of the polarizing conditions.

Chromatin structure is an important determinant governing the activation or silencing of genes and is finely regulated by epigenetic processes-modifications. Moreover, the last years , spatial (3D) organization of chromatin has emerged to be a key contributor of gene regulation. Looping events between distal regulatory DNA sequences are correlated with gene expression levels (typical examples being the Hbb, Igf2-H19, Igk, HoxB1 and Th2 cytokine locus). In the CD4 T cell differentiation system, long range intrachromosomal interactions exemplify higher order chromatin structure formation, leading to the coordinate regulation of cytokine genes. Intrachromosomal interactions between regulatory regions have been reported in TH2 loci (Spilianakis et. al Nature Immunology 2004) forming a proposed "prepoised initial core chromatin conformation", in which the Th2 LCR and the promoters of interleukin genes participate in this structure. This poised conformation gives to the interleukin genes the ability of rapid response to stimuli for transcriptional activation, when the Th2 specific transcriptional factors bind to this "ready to shoot" conformation. In TH2 cells it has also been reported that the global chromatin organizer SATB1 (special AT-rich sequence binding protein 1) mediates the looping event of the core chromatin configuration, coordinating the expression of cytokine genes (Cai et al. Nature Genetics 2006). Similarly, IFNg locus is remodeled during the differentiation of naïve CD4 to Th1 cells, by means of being packaged into loops that result in distal cis chromosomal interactions mediated by CTCF and cohesins (Hadjur et al, Nature 2009). Finally, intrachromosomal interactions that combine gene circularization with long-range promoter–enhancer interactions, are reported in lympfotoxin-TNF loci (LT.TNF) , in which TNFa activation involves a combination of both circularization of the TNFa gene itself and looping that brings

into close proximity distal enhancers with the TNFa promoter. So, it seems that the "looping out" of the DNA between distal regulatory elements facilitates protein-protein interactions involved in transcriptional regulation. Evidence for such phenomena has been gained by in vivo methods such as chromosome conformation capture (3C) assays which assesses the spatial proximity of DNA-bound proteins in their native chromatin context and FISH analysis which enables visualization of certain genomic regions in regard to the nuclear 3D space.

However the modes of gene regulation are not limited on intrachromosomal interactions. Interchromosomal interactions have been reported in naïve CD4 cells (Spilianakis et al 2005) between the hypersensitive region RHS6 located in the Th2 Locus Control Region (LCR) on mouse chromosome 11 and the IFNg gene on mouse chromosome 10. This phenomenon has a functional significance on the regulation of cytokine gene expression as the interaction of these loci is likely to hold the IFNg and Il4-5-13 gene complex together in a poised state ready for rapid transcriptional activation upon specific developmental lineage commitment. In a proposed model, during naïve CD4 to Th1 or Th2 differentiation, interchromosomal interactions diminish and intrachromosomal ones are favored.

As already mentioned, certain proteins have been implicated in the looping events of the TH2, IFNg and TNF loci. SATB1 is one of those reported, playing a key role in the formation of intrachromosomal interactions in the TH2 locus of Th2 cells coordinating the expression of interleukin genes (Cai et al, Nature Genetics 2006) and moreover organizing together with PML isoforms the loopscape conformation of MHC I locus in Jurkat cells (Kumar et al, Nature Cell Biology 2006). SATB1 is predominately expressed in thymocytes forming a cage-like pattern surrounding heterochromatin. It is proposed as a global chromatin organizer tethering the AT rich DNA sequences in the nuclear matrix and characterizing those regions as MARs (Matrix Attachment Regions) (de Belle et al. JCB 1998). Apart from its architectural role, it acts as a transcriptional regulator recruiting chromatin modifiers, while its binding sites are hot spots for epigenetic modifications linking its function with the histone code. It can act either as a repressor or an activator depending on its post-translational modifications and its protein interactors (Kumar et al., Mol Cell 2006). Knock out mice for SATB1 arrest thymocyte development on the Double Positive (DP, $CD4^+CD8^+$) stage while they do not live more than 3 weeks. Moreover, loss of SATB1 leads to deregulation of many cytokine genes highlighting its importance in T cell development. It has been reported as a target of the Wnt signaling pathway, interacting with β-catenin to affect T helper cell growth and differentiation (Notani et al, Plos 2010). Recently, SATB1 has been identified as an initiator factor for Xist-mediated

silencing in ES cells (Agrelo et al., 2009) and moreover its balance with regard to SATB2 contributes to the plasticity of Nanog expression with consequences to ES cell pluripotency (Savarese et al Gen&Dev 2009).

Lamins are components of the nuclear lamina, a fibrous layer on the nucleoplasmic side of the inner nuclear membrane which forms a framework for the nuclear envelope. Lamin A and C are products of the same gene (*LMNA*) and present in equal amounts in the lamina of mammals. In Drosophila, Lamins apart from being members of the nuclear lamina, have been characterized as nuclear matrix proteins binding to insulators and leading to the formation of chromatin loops. (Byrd and Corces, JCB 2003)

Highlighting the role of SATB1 as a chromatin organizer mediating the formation of intrachromosomal interactions on the TH2 locus of the TH2 clone cell line (D10.G4.1) , we investigated its role in primary naïve CD4 cells. We questioned if it participates in the formation of the proposed "prepoised core chromatin conformation". Moreover we tested if mammalian Lamin-A, as a protein of nuclear matrix, together with SATB1 plays a role in the regulation of interchromosomal interactions. Finally we assessed the role of SATB1 in T lymphocyte differentiation in a developmental context, from thymocytes to Th1 and Th2 cell lineages.

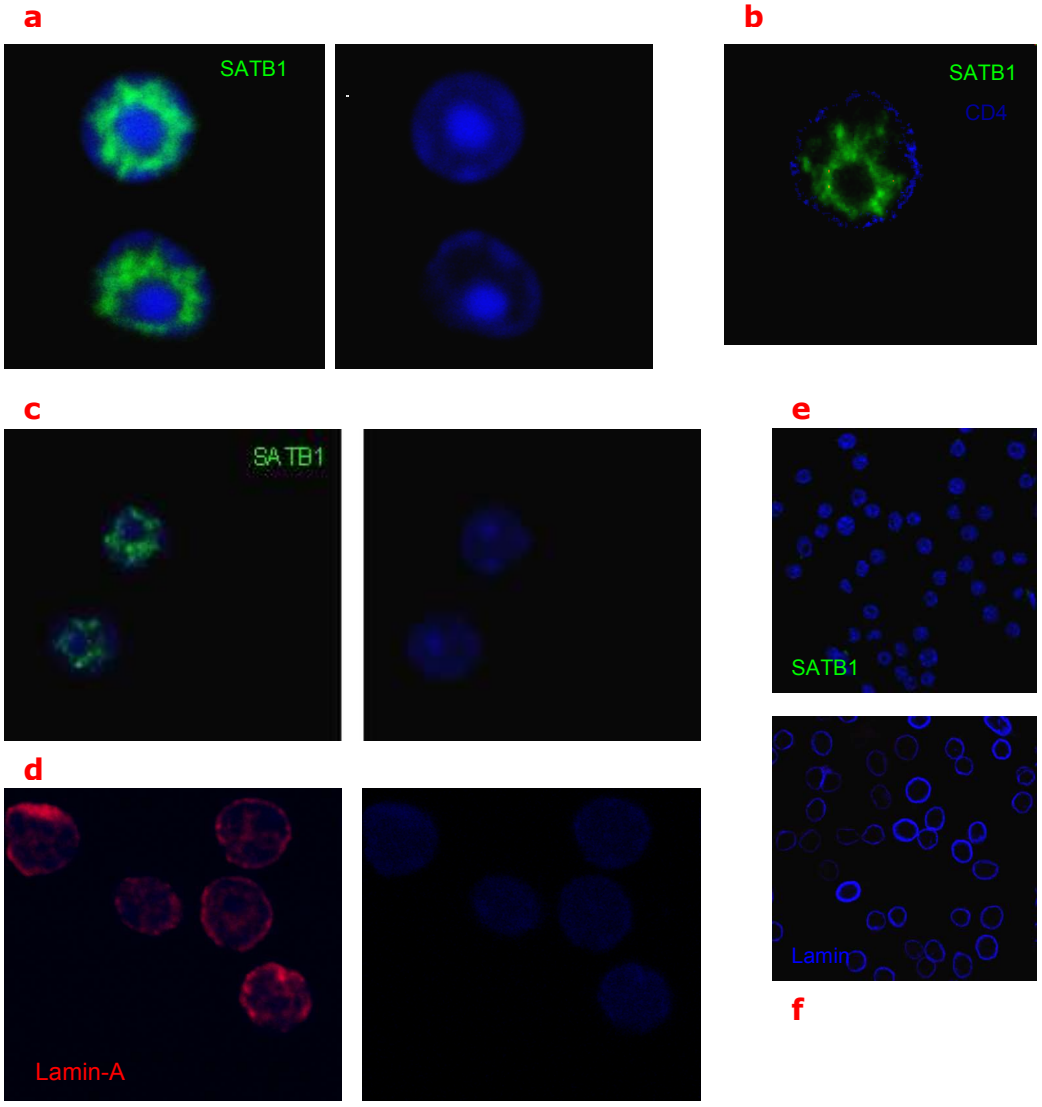
Results

Thymocytes and CD4 cells express both SATB1 and Lamin-A in a cage like pattern

Initially we wanted to study the expression profile of SATB1 in thymocytes as reported in Cai et al (Nature Genetics 2003). We removed thymi from young (4-6 week old) C57BL/6 mice and isolated the whole (heterologous) cell population, referred as thymocytes. Immunostaining with an antibody to SATB1 (anti-SATB1) showed that SATB1 is expressed in the vast majority of thymocytes in a characteristic cage-like pattern circumscribing heterochromatin (fig.1a). Moreover, in a similar way, we examined the expression pattern of Lamin-A with an antibody against Lamin-A protein (anti-Lamin-A) (fig3a). Interestingly, Lamin-A was expressed in a novel pattern, as apart from its well known perinuclear pattern it was also observed intranuclearly, forming a cage-like pattern. To test whether these two proteins colocalize, we performed double immunostaining, both for Lamin-A and SATB1 and indeed we observed that these two proteins partially colocalize(fig3a). We then examined whether primary CD4 T cells, not

ever reported before, express SATB1. Lymph nodes and spleens were removed from young (4-6 week old) C57BL/6 mice, and CD4 cells were isolated with a negative selection kit (Invitrogen). We have observed that CD4 cells isolated from such young mice (4-6 weeks) are actually in their vast majority naïve (~85-90%) as tested by FACs sorting (data not shown). Immunostaining with anti-SATB1 antibody, showed that SATB1 is expressed in a cage-like pattern, similar to that observed in thymocytes, though in slightly lower expression levels in terms of protein, as tested by western blot analysis (fig.2). Similarly, lamin-A appeared with the same cage-like pattern as in thymocytes and again, SATB1 partially colocalized with lamin-A (fig.3b). Moreover, we examined whether macrophages express SATB1 and Lamin-A. Therefore immunostaining (fig.1e) and western blot analysis (fig.2) were performed in the RAW 264.7 mouse macrophage cell line. As shown in fig 1e and 2, SATB1 is not expressed in RAW 264.7 cels, while Lamin-A staining revealed the well described perinuclear pattern. So SATB1 and lamin-A are expressed both in CD4 cells and in thymocytes in a cage like pattern.

Figure 1. Thymocytes (a) and CD4 cells(b,c) express SATB1 in a cage-like pattern while RAW cells (e) do not. Lamin-A is expressed both in thymocytes (see 3a) and in CD4 cells (d) in a cage-like pattern but not in RAW (f) where it is expressed only perinuclearly



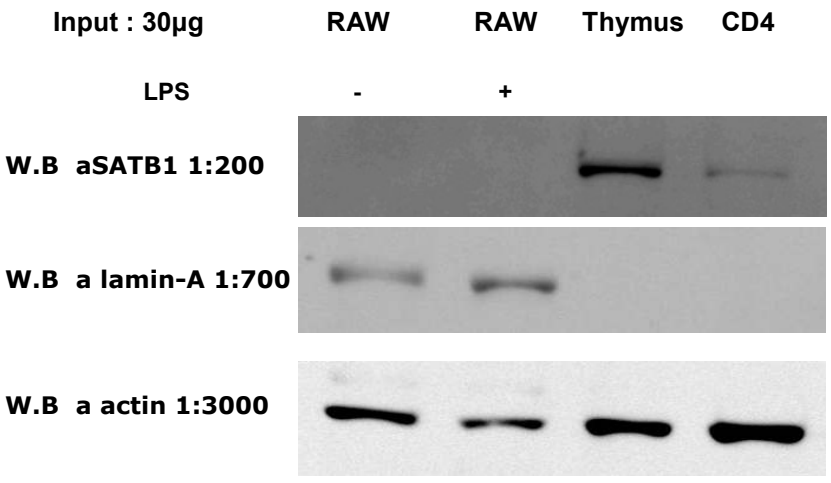


Figure 2 Western Blot analysis of SATB1 and Lamin-A in CD4 cells, thymocytes and RAW cells.

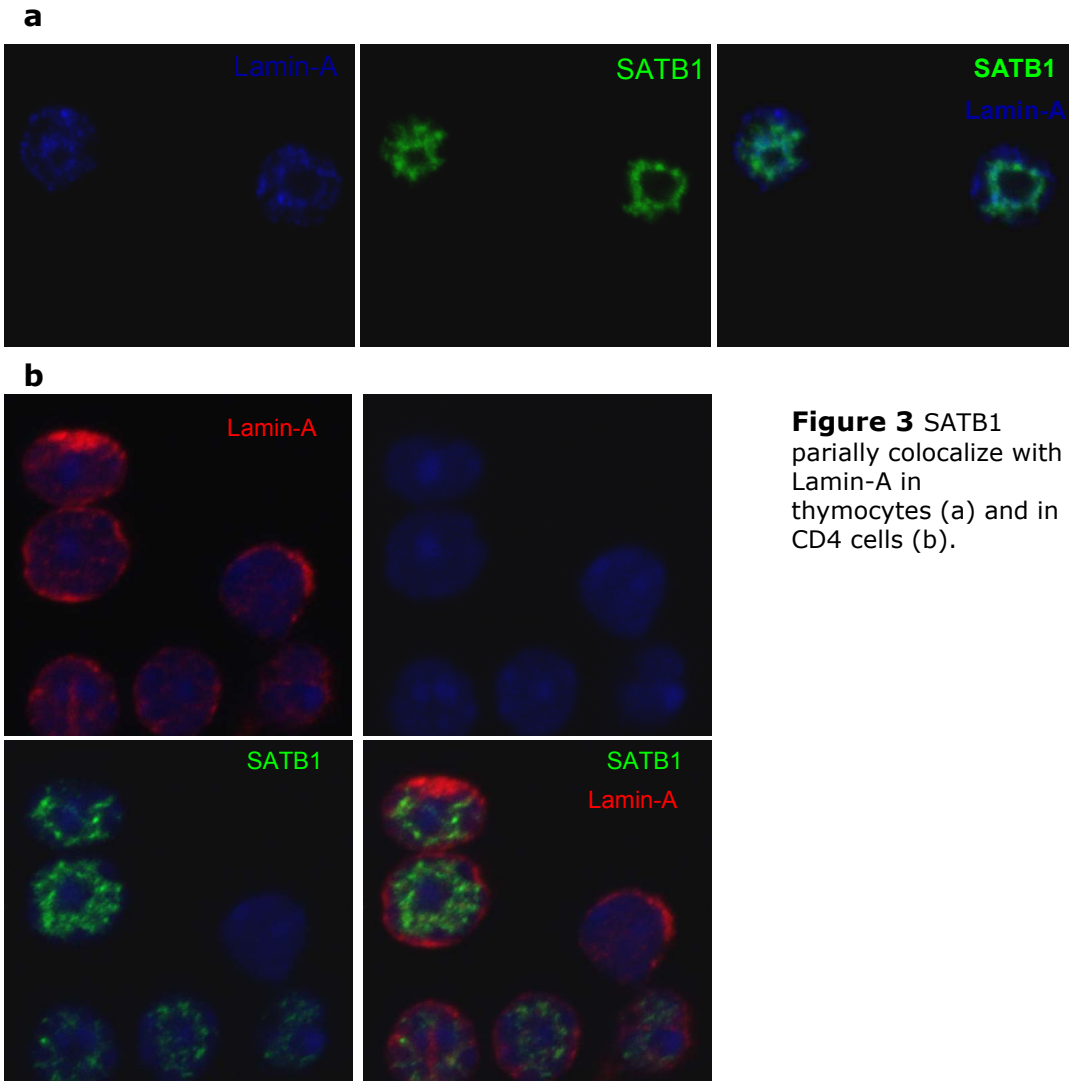


Figure 3 SATB1 parially colocalize with Lamin-A in thymocytes (a) and in CD4 cells (b).

It is important to mention that Lamin-A although it is highly expressed in both CD4 cells and thymocytes, as observed by immunostaining assays, western blot analysis presents contradictory results; Lamin-A is expressed only in RAW cells and is absent in whole cell extracts obtained from CD4 cells and thymocytes. A possible explanation of this could be that Lamin-A is the nuclear matrix protein, in contrast to SATB1. It is reported (Penman et al., JCB 1984) that proteins of nuclear matrix and intermediate filaments are not solubilized under common whole cell extract procedures as the one used for the production of our whole cell lysates. In contrast, perinuclear Lamin-A of RAW cells is extracted and solubilized, therefore is detected by western blot analysis of whole cell lysates.

If this hypothesis holds true, we must comment on the fact that SATB1 is indeed extracted in whole cell lysates under the same conditions in which Lamin-A is not. Moreover, CSK (low salt buffer) treated cells lost their cage-like pattern of SATB1 as revealed by immunostaining (data not shown). Therefore SATB1 may not be a nuclear matrix protein as reported (Cai et al. Nature Genetics 2003).

This hypothesis should be further verified with more delicate biochemical expresiments. So far, halos on slide preparations, in which high salt buffer extracts the DNA loops and the non-matrix proteins confirm this hypothesis. Immunostaining assays on CD4 cells treated with halos on slide preparation were unable to stain SATB1 (data not shown).

SATB1 and Lamin-A expression profile in Th1 and Th2 cells.

We further wanted to investigate the SATB1 expression profile in Th1 and Th2 cell lineages. Therefore, we isolated CD4 cells and cultured them in vitro under Th1 and Th2 polarizing conditions as described in Materials and Methods. Snapshots in a time dependent manner during Th1 and Th2 differentiation were taken by fixing the cells and performing immunostaining assays in order to examine the pattern and expression levels both for SATB1 and Lamin-A (fig.4 & 5). As observed, after 6 hours under Th1 conditions the pattern is gradually being altered from the characteristic cage-like of CD4 unstimulated cells, into a more diffused intranuclear one, while after 12 hours and on, the expression level is diminished. Similarly under Th2 conditions the pattern follows the same as observed in Th1, as it is gradually turned to a diffused one. However, in contrast to Th1 polarizing conditions, CD4 cells under Th2 polarizing conditions show a significant increase in SATB1 expression levels. Th_o condition, in which no polarizing conditions are performed, but the cells are only stimulated, as their TCR complex encounter the dimmer of anti-CD3 and CD28, exhibits the same pattern and expression levels as those observed under Th2 polarizing conditions.

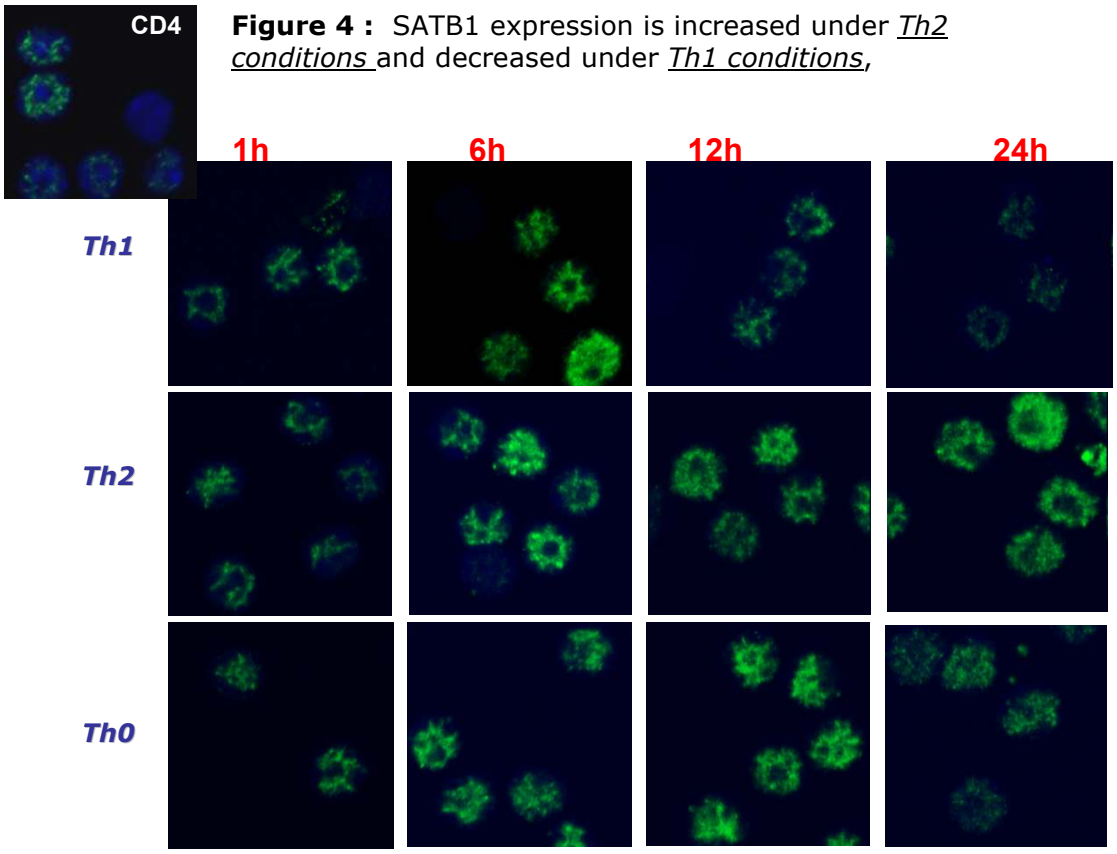
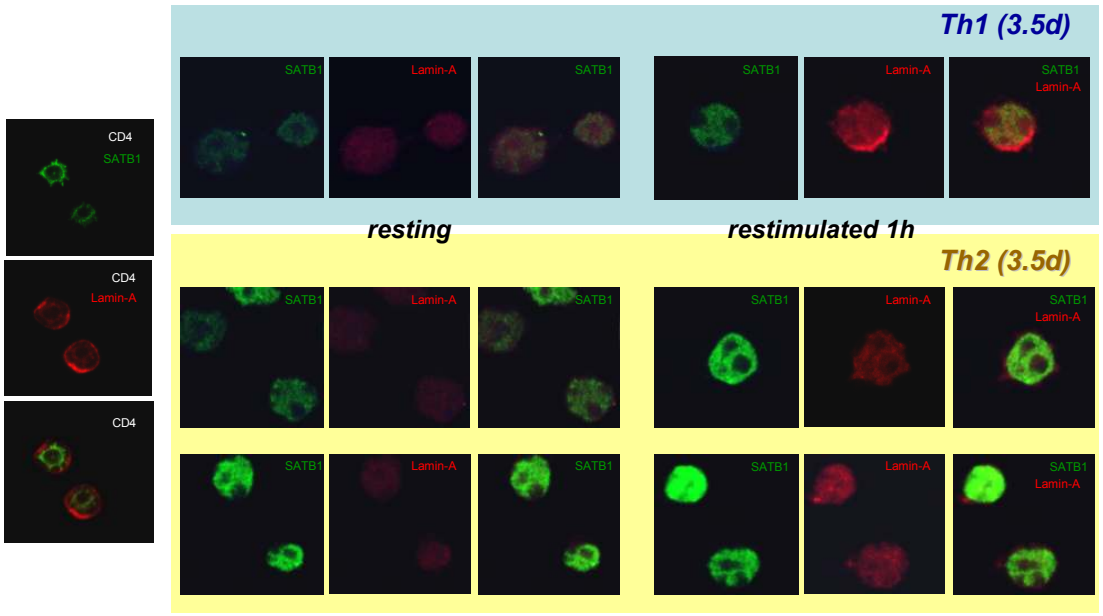


Figure 4 : SATB1 expression is increased under *Th2 conditions* and decreased under *Th1 conditions*,

When the same polarizing conditions are employed for 3.5 days, the cells differentiate to Th1 and Th2 cell lineages. Immunostaining for SATB1 revealed not only a different pattern in both Th1 and Th2 cells compared to CD4 unstimulated cells, but also completely different expression levels between Th1 and Th2 cells. In particular, when both Th1 and Th2 cells were restimulated the expression was increased compared to the non-restimulated Th1 and Th2. Moreover, SATB1 is upregulated in Th2 cells and downregulated in Th1 compared to naïve CD4, while in both lineages the pattern is turned to a diffused one, excluded from a particular region, likely to be the nucleolus (can be verified with immunostaining against fibrillarin). As far as Lamin-A is concerned, immunostaining showed that it's pattern is altered from the cage-like and perinuclear one in a diffused intranuclear one. All in all, SATB1 is downregulated in Th1 cells and upregulated in Th2 cells while it's pattern is a diffused intranuclear one in both lineages, resembling that of Lamin-A.

Figure 5 : SATB1 and Lamin-A expression during CD4 differentiation



Interchromosomal interactions take place on the cage-like pattern of SATB1 in naïve CD4 cells.

We next examined whether SATB1 participates in the process of interchromosomal interactions (Spilianakis et al. Nature 2005). We performed immunoDNA FISH analysis to test whether TH2, IFNg and LT.TFN locus, separately, colocalize with SATB1. Those loci have been reported (Spilianakis et al. Nature 2005 and unpublished data) to participate in interchromosomal interactions. In particular in has been shown that a particular region of the 200kb TH2 locus, RHS6, on mouse chromosome 11 interacts with the IFNg locus on mouse chromosome 10 in naïve CD4 cells. Moreover, the TH2 locus interacts also with the LT.TNF locus on mouse chromosome 17 (Spilianakis unpublished data). ImmunoDNA FISH analysis showed that 81% of the TH2 loci, colocalize with SATB1 (fig.6a,7). Concerning the IFNg loci, 71% of them reside in the SATB1 cage like pattern, while TNF loci exhibit 93% colocalization with SATB1 (fig.7). These results showed it is likely that interchromosomal interactions take place within the SATB1 network, implicating a possible direct or indirect role of this protein in the regulation of this phenomenon.

Moreover, if the hypothesis mentioned above, in regard with the absence of SATB1 from nuclear matrix, holds true, we can explain our observations in which TH2 and IFNg loci localize on the loop fragment of halos on slide treated CD4 cell and thymocyte nuclei (fig 8). In halos treated CD4 nuclei, both TH2 and

IFNg loci localize on the loop fragment at approximately 70%. Those results combined with i) the obesrvation that these loci mostly colocalize with SATB1 ii) the presence of SATB1 and simultaneous absence of Lamin-A using western blot analysis of whole cell lysates and iii) the observation that halos are not stained with anti-SATB1, support the hypothesis that SATB1 may not be a nuclear matrix protein.

To gain more insight about the colocalization of loci with SATB1, we performed immuno double DNA FISH analysis for both the TH2 and the LT.TNF loci which revealed that the vast majority of the interchromosomally interacting loci colocalize with SATB1 (fig.9). We scored 30 events of interchromosomal interactions between TH2 and LT.TNF loci of which 27 appeared to take place in the SATB1 pattern yielding a 90% colocalization. So interchromosomal interactions take place in the pattern of SATB1, implying a possible active role of this protein in the regulation of this phenomenon.

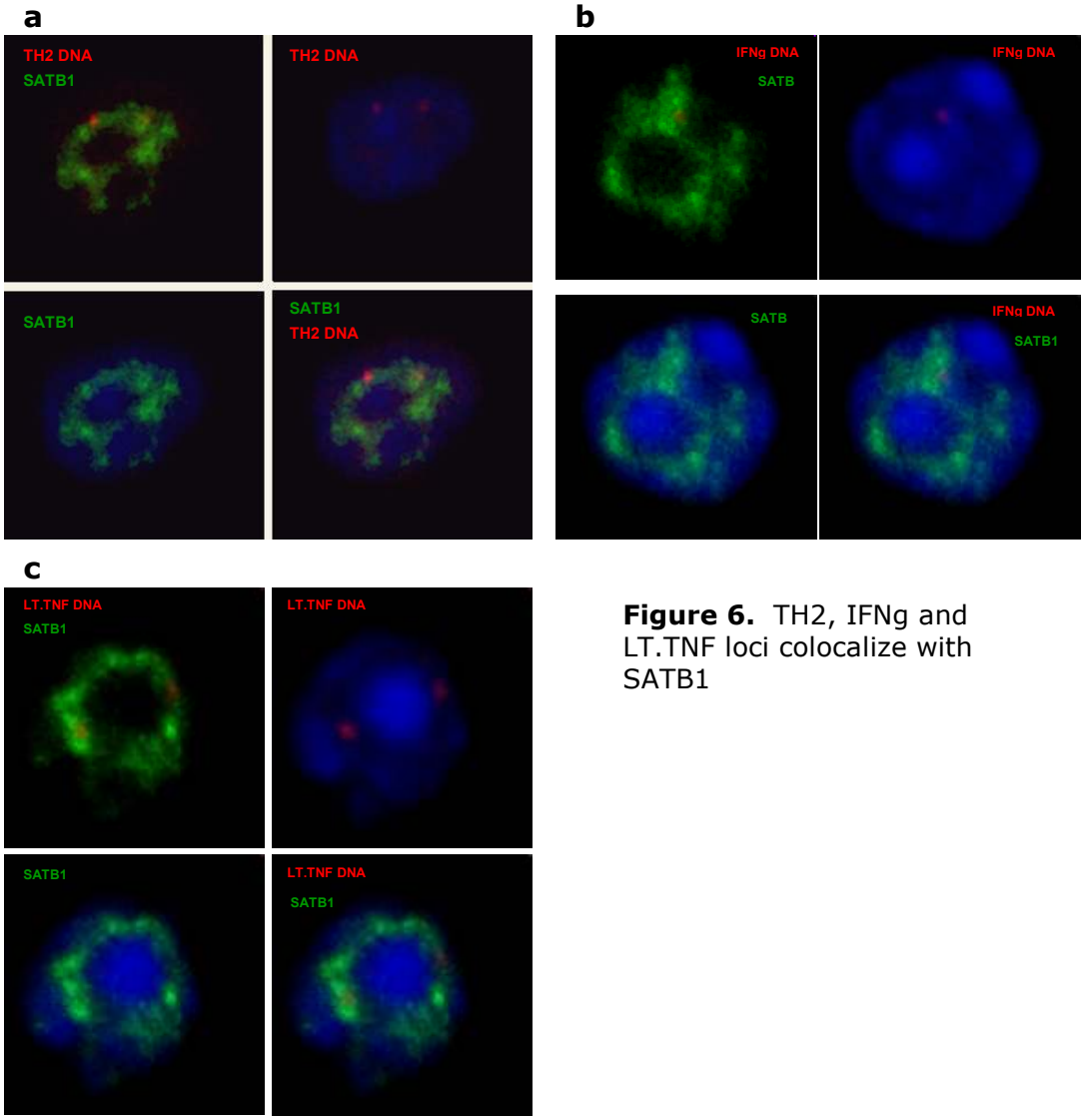


Figure 6. TH2, IFNg and LT.TNF loci colocalize with SATB1

Figure 7. TH2, IFNg and TNF loci localization with SATB1 percentages

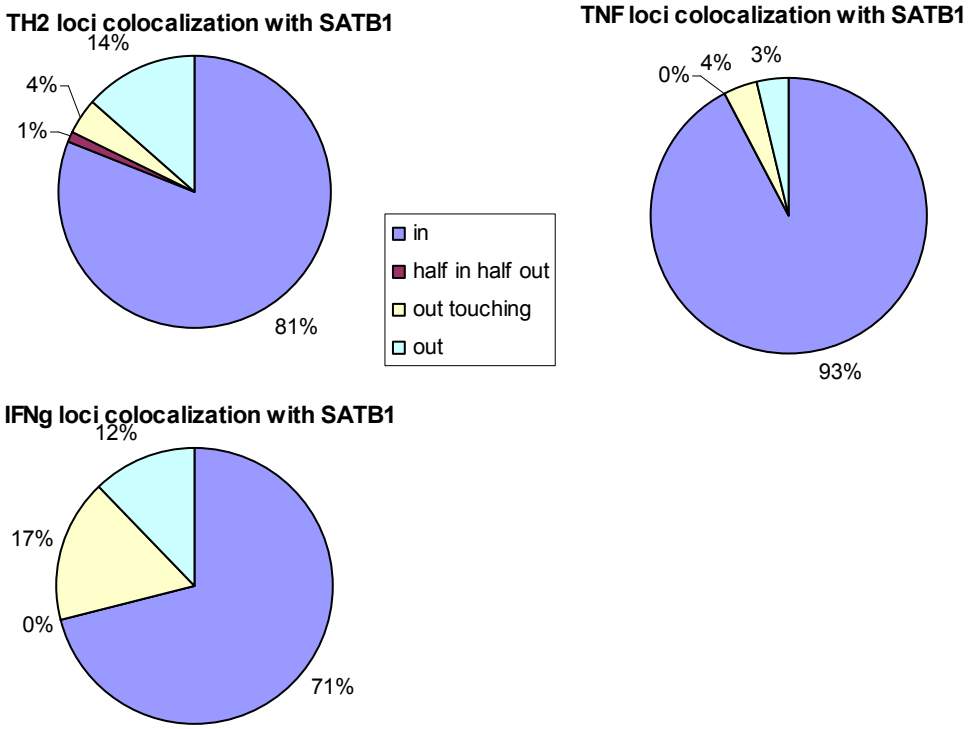


Figure 8. TH2 and IFNg loci localize on the loop fragment of halos treated CD4 cell nuclei

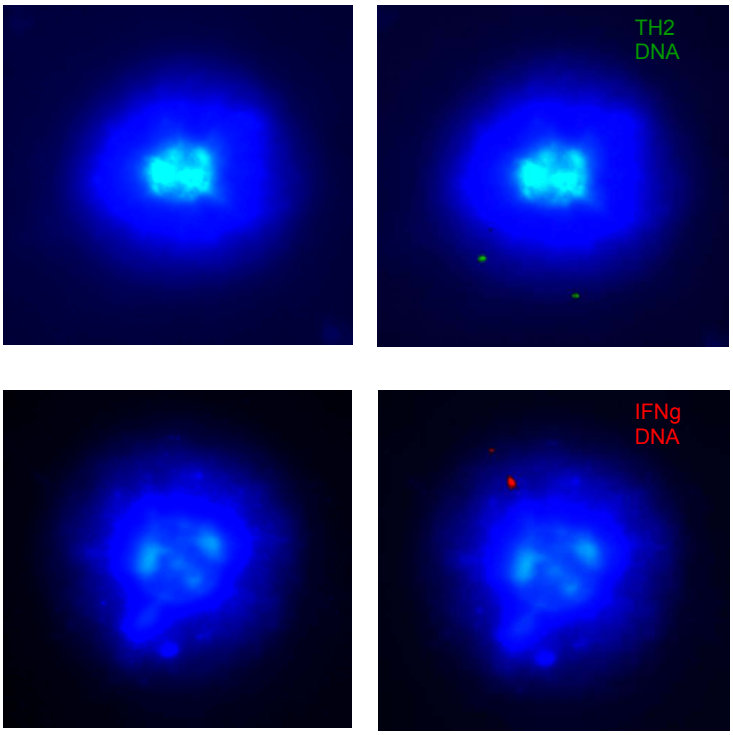
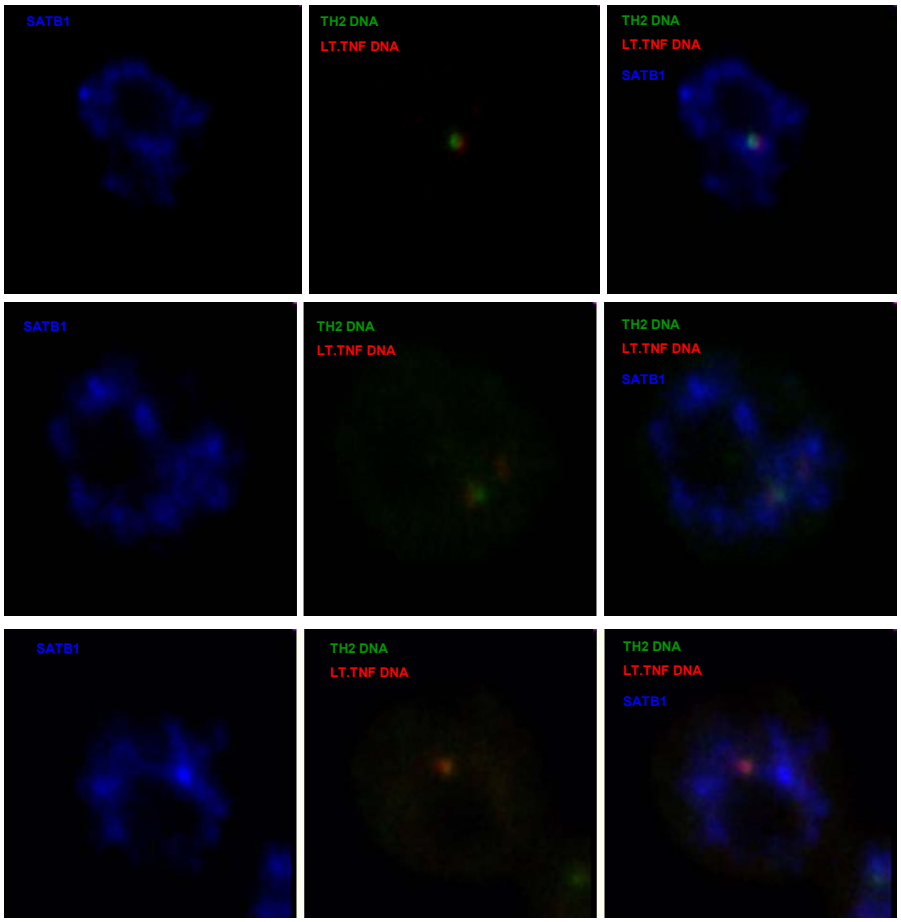


Figure 9: Interchromosomal interactions take place on SATB1 stained regions



Identifying SATB1-bound sequences in the 200kb TH2 cytokine locus.

It has been reported that SATB1 binds to nine SATB1 Binding Sites (SBSs) and to the CNS-1 and CNS-2 in activated D10.G4.1 forming a higher-order packaging of chromatin on TH2 cytokine locus (Cai et al 2006) (fig.10A). This loopscape seems to play a functional role on the coordinated expression of the TH2 cytokine genes. We investigated whether SATB1 binds to the TH2 locus in thymocytes and in CD4 naïve cells . It has been observed (Spilianakis unpublished data) that interchromosomal interactions are formed in earlier than naïve T cell developmental stages, in particular thymocytes apart from those observed in naïve CD4 cells (Spilianakis et al 2005). We started identifying SBSs on the TH2 locus in thymocytes and in naive CD4 cells. It is known that SATB1 specifically binds Base Unpaired Regions (BURs) in thymocytes (de Belle et al. 1998) and tethers these regions to the nuclear matrix (Matrix Attachment Regions). We used two online web tools, EMBOSS and Genomatix, for the in silico prediction of MARs on the TH2 locus. We then performed ChIP assays targeting

the in silico predicted MARs and other critical regulatory regions. We identified so far, five SBSs, namely SBS-No1, SBS-No2 till SBS-No5 (fig.10). SBS-No1 is located at 45.509-45.770 bp region of the TH2 locus (BAC182) where 3 in silico-predicted MARs reside. It is noteworthy that it is observed only in thymocytes and not in CD4 cells implicating an alteration in the loopscape of TH2 locus during the differentiation of thymocytes to single positive CD4 cells. SBS-No2 is located in the RHS6 region of the TH2 LCR locus. SBS-No3 is located at 48.650-48.879bp and SBS-No 4 at 9.636-9.818bp of the TH2 locus, as have also been reported in activated D10.G4. from Cai et al. Finally, SBS-No5 is located at 30.874-32.347bp of the same locus. SBS-No4 and SBS-No5 have only been investigated in the TH2 locus of naïve CD4 cells.

So SATB1 binds on at least 3 sites on the TH2 locus of murine thymocytes and on at least 4 sites of naïve CD4 cells with the SBS-No3 being of immense importance as it is known that RHS6 is the cis regulatory region on the TH2 locus that interacts with IFNg/LT.TNF loci interchromosomally.

Figure 10A. Schematic representation of the TH2 cytokine locus with the positions of SATB1-binding sequences (SBS-C1 to SBS-C9), figure 2a from Cai et al. Nature genetics 2006,

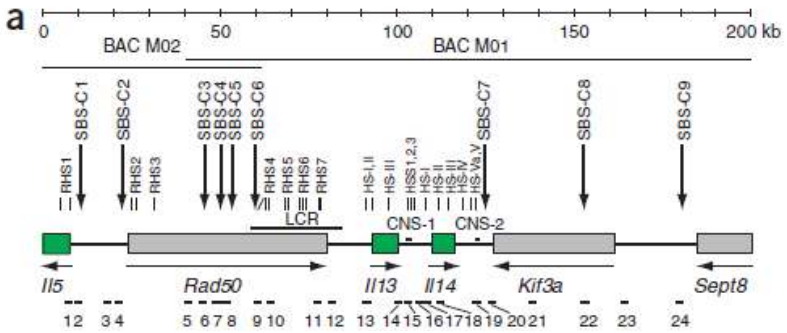


Figure 10B. SATB1 binds on at least 3 regions on the TH2 locus of murine ***thymocytes***

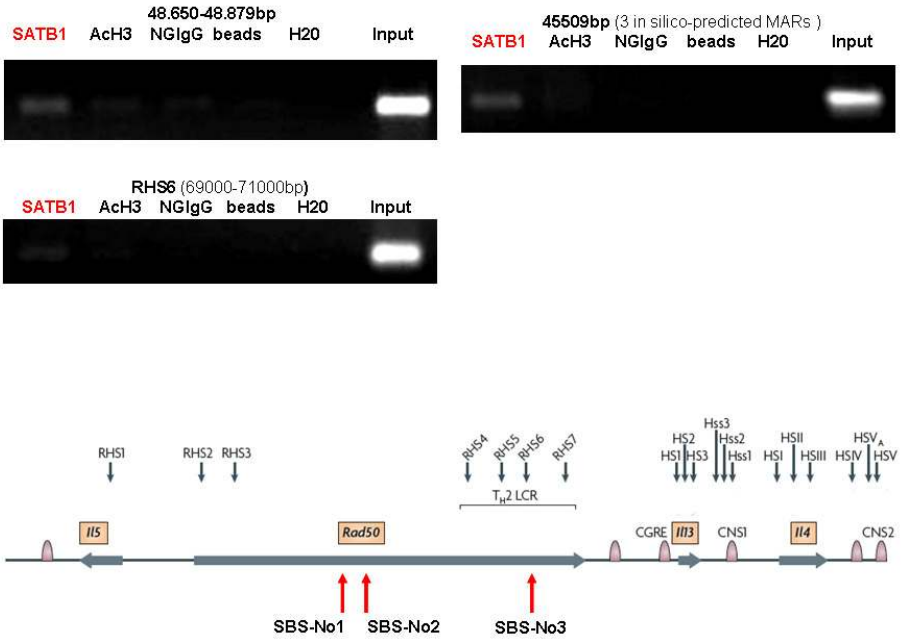
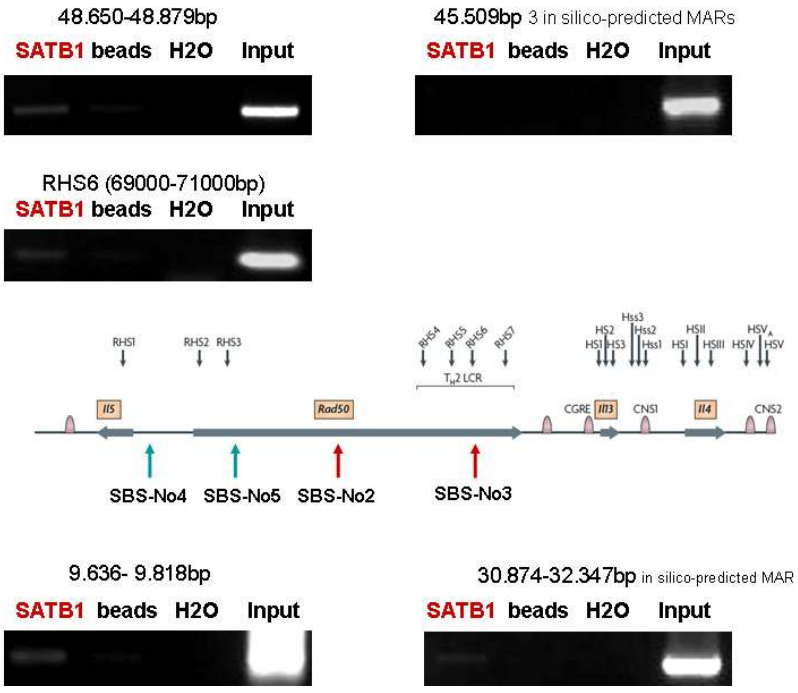


Figure 10C. SATB1 binds on at least 4 regions on the TH2 locus of murine ***naïve CD4 cells***



Discussion

SATB1 seems to be a very pleiotropic molecule. Its repertoire of functions evolve early in development, as is expressed in ES cells in a diffused pattern regulating the expression of Nanog (Savarese et al.2009) and it acts as a silencing factor for Xist (Agrelo et al.2009). It is crucial for T cell development since its absence results in the arrest of T cells in the DP stage in the thymus (Alvarez et al. 2000). In Th2 cells SATB1 regulates the expression of cytokine genes on the Th2 locus by means of forming a higher ordered chromatin structure enabling coordinated expression of the genes. In a similar way, SATB1 interacts with PML upon IFNg treatment to organize the loopscape of MHC I locus. Its pleiotropy is not limited in the different roles at different cell types. It can be switched from an activator to a repressor depending on its post-translational modifications (Kumar et al Mol Cel 2006). Moreover, SATB1 globally reprogrammes gene expression during metastasis by tethering hundreds of gene loci onto its regulatory network, assembling them with chromatin modifying and transcription factors (Hye-Jung Han et al, Nature 2008). Here, we report that naïve CD4 cells, a cell type which is transcriptionally inactive but poised, expresses SATB1 at moderate levels in a cage-like pattern supporting the view that this interchromatin territory of SATB1 includes genes that are transcriptionally poised (Galande et al. 2007 Cur Op Gen Dev) We also give the first evidence that interchromosomal interactions take place on this cage like pattern of SATB1. It seems that in naïve CD4 cells, cells with a limited differentiation potential but not terminally differentiated, this cage like pattern of SATB1 recruits areas with gene clusters that must be ready to be turned on quickly, when the decision for terminal differentiation is taken. In this role, Lamin-A may also participate, exhibiting a novel cage-like pattern, and pulling SATB1 and the gene clusters of choice in the nuclear matrix compartment for transient transcriptional rest. Maybe this is the reason why terminally differentiated cells exhibit no intranuclear cage-like pattern of Lamin-A and SATB1. Lamin-A in those cells is only perinuclear, neighbouring with heterochromatin and hosting transcriptionally poised or repressed genes (although this is not a rule). In those cells SATB1 exhibits a diffused intranuclear pattern as there is no longer need for massive transcriptional rest, and SATB1 can act either as a repressor or activator "at will". On the contrary, in plyripotent ES cells (non-differentiated) Lamin-A is absent (Constantinescu et al. 2006 Stem Cells) as there is no need for massive transcriptional repression but the transcriptional program must be delicate while in the same cell type SATB1 also exhibits a diffused intranuclear pattern. This interplay of Lamin-A and SATB1

patterns further supports the view for a functional and structural interaction of these proteins acting as global genome organizers and regulating the developmental potential of the cells.

Elucidating SATB1 and Lamin-A binding sites on the TH2, IFNg and TNF loci in different cell types of T cell development and confirming its role in loopscape formation by means of ChIP loop assays will unravel the role of these proteins as chromatin organizers and coordinators of cytokine gene transcription. Furthermore, verification of Lamin-A and SATB1 interaction and characterization of their role by means of biochemical and drug-treatment experiments in different T cell types will reveal the nuclear organization of T cells in a developmental context. Finally, using disease mouse models and performing the same studies will give us information as to how structural changes of T cell nucleus can contribute to pathological conditions emanating from improper development of these cells.

Materials and Methods

Mice and Cell Cultures

C57BL/6 mice were used for the isolation of thymi, spleens and lymph nodes. Experiments were also carried out on RAW 264.7 which is a mouse leukemic monocyte macrophage cell line.

Isolation and differentiation of naïve CD4 T cells.

Lymph nodes and spleens were removed from 4-6 week-old C57BL/6 mice and were used to make single cell suspension. For immunostaining assays CD4 cells were isolated with the Dynal® Mouse CD4 Negative Isolation Kit (Invitrogen) while CD4 cells for Chromatin preparation or whole cell lysates were isolated with CD4 (L3T4) MicroBeads from Miltenyi Biotec. 350.000 naïve CD4 T cells were incubated in Click's Medium (suplemmented with 10%FBS, 00,5mM β-merca, 100μg/mL Pen/Strep, 2mM Glutamine and 25mM Hepes pH 7.2) and stimulated with plate-bound mouse anti-CD3 (145-2C11) and anti-CD28. Cells were collected 1h, 6h, 12h, 24h and 3.5days later . For Th1 conditions, CD4 cells were stimulated with IL-2 (20U/mL), anti-Il4 (10μg/mL, rat antimouse IL4, clone 11B11, BD 554384) and IL-12 (3,5 ng/mL), while for Th2 conditions CD4 cells

stimulated with IL-2 (20U/mL), IL-4 (1000U/mL) and anti-IFNg (10µg/mL, rat monoclonal anti-mouse IFNg, clone XMG1.2).

Western Blot

Thymocytes, CD4 and thymocytes cells were isolated and washed with 1xPBS. Whole cell extracts were prepared by incubating cells in lysis buffer (containing 10 mM Tris-HCl, pH 8, 170 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitors) for 20 min in ice and performing three freezing-thawing treetments. Extracts were subjected to SDS-PAGE. Western blotting analysis was performed using anti-SATB1 (goat polyclonal, E15-Sc5990), anti-LaminA (rabbit polyclonal, abcam26300), anti-Actin A and anti-Nucleophosmin (mouse monoclonal, ab10530)

Chromatin Immunoprecipitation

$30x10^6$ primary cells were used to prepare soluble chromatin solution (SCS). For chromatin immunoprecipitation a proportion of SCS corresponding to $3\text{-}4x10^6$ cells were used . After preclearing of SCS, 5µg of goat polyclonal anti-SATB1 (Santa Cruz goat polyclonal, E15-Sc5990) was added. For the immunoprecipitation of AcH3, 2µg of anti-AcH3K14 (rabbit polyclonal IgG, Upstate 06-911) was used while as a negative control precipitation took place with 5 µg of NGIgG (Santa Cruz,, sc-2028). Primers for the PCR-in gel analysis were selected so as to amplify the in silico-predicted MARs on TH2 locus, selective SBSs as reported in Cai et al. Nature Genetics 2006, and selective hypersensitive sites such as RHS6 designed with the NIH tool, Primer Blast.

Immunostaining.

Cells were prepared on coverslips in a way to permit the maintenance of the three-dimensional structure of the cells. 12 mm coverslips were coated for 10 min with poly-L-lysine (SIGMA, Poly-L-lysine Hydrobromide) and then washed and air-dryed. Cells were permitted to attach onto poly-L-lysine-coated coverslips and were fixed with 4%PFA in 1x PBS for 10 min, washed three times with 1 x PBS (5 min per wash) and permeabilized with 0.5% Triton X-100 in 1xPBS for 5min and washed again three times with 1 x PBS on ice. Blocking was performed with 0,4% acetylated BSA (Ambion, AM2614) in 4xSSC for 30min on a hybrislip placed in a humified chamber. The first antibody/ies (1:200 anti-SATB1 SantaCruz: E-15 sc-5990, 1:100 anti-Lamin-A (rabbit polyclonal, abcam26300), antiCD4 (APC Rat

anti-mouse CD4, SB L3T4) diluted in detection buffer (4x SSC, 0.1% acBSA, 0.01% Tween20) were incubated for 1h at room temperature place on a hybrislip in a humified chamber. After 3 washes with 4xSSC/0.1% Tween 20 (5min each, RT), the secondary antibodies (Donkey antiGoat (DaG) 488nm alexa Fluor, DaG 647nm Alexa Fluor, Donkey antiRabbit 546nm Alexafluor) diluted in detection buffer were incubated for 45min at room temperature placed on a hybrislip in a humified chamber. After 3 washes, samples were incubated in TOPRO3 (1:6000 dilution in 2xSSC, 647nm, Invitrogen) for 1 min and washed with 2xSSC for another 1 min. Finally there were mounted with Prolong Gold with DAPI (Invitrogen). Samples were visualized using a confocal Microsope (BioRad).

ImmunoDNA FISH

Cells were prepared as mentioned above and the immunostaining part was done first in exactly the same way as mentioned above. After the 3 washes of the sample it was not stained with TOPRO3. Instead samples were further washed two times with 1x PBS, and then fixed with 2%PFA in 1xPBS, 10 min at RT in order to stabilize-fix the complex of protein-1st antibody-2nd antibody-fluorophore. The samples were washed 3 times with 1xPBS (5min each wash) and then permeabized again for 5 min with 0.5% Triton X-100 in 1xPBS. After 3 washes with 1x PBS, samples were immersed in 70-80-95-100% EtOH for 3 min each. Then they were quickly drained, and denaturated for 7min at 70% formamide in 4xSSC at 73^{o}C. Upon denaturation, samples were quickly placed on ice, formamide was removed and 70-80-95-100% EtOH was placed 3 min each on ice. The preparation of probes was as follows : 3μL (~100ng) of the probe* were mixed with 1 μl mouse cot-1 DNA (Roche) and dried in a speed vac. Lyophilized DNA molecules were resuspended in di-Formamide and reconstituted for 20 min at 37^{o}C. Denaturation took place at 95^{o}C for 10 min and then probes were placed on ice for no more than 3 min. The probe mix consisted of 7 μl master mix (60% formamide, 2xSSC, 30% dextran sulphate), 1 μl mouse cot-1 DNA (Roche) and 3 μl of each probe*. The mix was placed on the sample, sealed with rubber cement, and denatured for additional 5 min at 73^{o}C. Then hybridization took place for 16 h at 37^{o}C in a humified chamber. The samples were washed 3 times with 2xSSC (prewarmed at 37oC, 5 min each) and then stained with TOPRO3 (1:6000 dilution in 2xSSC, 647nm, Invitrogen) for 1 min and washed with 2xSSC for another 1 min. Finally they were mounted with Prolong Gold with DAPI (Invitrogen). Samples were visualized using a confocal Microscope (BioRad).

*Probe preparation: Two micrograms of BAC DNA of interest was labelled using a nick translation kit (Roche), according to the manufacturer's instructions, and either 0.05mM Spectrum Orange (Vysis) or 0.025mM pectrumGreen-dUTP (Vysis). The probe was incubated overnight (15 h) at 16^{o}C and then purified using the PCR purification kit (Qiagen) and eluted in 30 µl of water. The probe mix consisted of 7µl master mix (60% formamide, 2xSSC, 30% dextran sulphate), 1 µl mouse cot-1 DNA (Roche) and 2 µl of each probe.

Nuclear Halo Preparation

CD4 cells were were permitted to attach onto poly-L-lysine-coated coverslips. Then they were immersed in 0.5% Triton X-100 in CSK buffer for 4 min at 4°C, and then CSK buffer was gently drained from the slides. After 0.5% Triton X-100 treatment, the membrane and soluble proteins were removed from T cells. Then, 2 M NaCl buffer (2 M NaCl, 10 mM Pipes, pH 6.8, 10 mM EDTA, 0.1% digitonin, 0.05 mM spermine, 0.125 mM spermidine, 1 mg/ml aprotinin, 1 mg/ml leupeptin, 1.2 mM phenylmethylsulfonyl fluoride) was added and allowed to incubate for 3 min at 4°C. After this extraction of the histones and soluble nonhistone proteins by 2 M NaCl buffer, the DNA loops were released from the nuclei and formed a nuclear halo. The process of gentle draining and refilling was repeated with sequential rinses in 10x, 5x, 2x, and 1x PBS, followed by another series of rinses with 10, 30, 50, 70, and 95% ethanol. The slides were air dried at room temperature for 30 min. The nuclear halos were fixed by baking slides at 70°C for 2 h in an oven. Preps were either stained with DAPI or proceeded for indirect DNA FISH labeling.

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