Mechanism of regulation of *SATB1* during T helper cell differentiation

By

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Certificate

This is to certify that this dissertation entitled “Mechanism of Regulation of *SATB1* during T helper cell differentiation” towards the partial fulfilment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Mr. Rahul Biradar at IISER Pune under the supervision of Prof. Sanjeev Galande, Professor, Department of Biology during the academic year 2017-18.

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Declaration

I hereby declare that the matter embodied in the report entitled “Mechanism of Regulation of *SATB1* during T helper cell differentiation” are the results of the work carried out by me at the Department of Biology, IISER, Pune, under the supervision of Prof. Sanjeev Galande and the same has not been submitted elsewhere for any other degree.

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Acknowledgement

First, I would like to express my deep gratitude to my principal investigator Prof. Sanjeev Galande, IISER Pune, for giving me an opportunity to work in his laboratory. I am so thankful for his guidance throughout this project. I have learned a lot through my interactions with him which has definitely boosted my growth as a researcher.

I am indebted to Dr. Satyajeet Khare, SG Lab, who has come to my aid whenever I needed help out of sticky spots and silly mistakes. His valuable insights on my work and his unwavering encouragement have been so important during the past year. He has been a teacher, a mentor, and a friend. This project would have not been possible without his presence.

My heartfelt thanks to Dr. Ameya Sathe, SG Lab, for providing advice and suggestions whenever I needed them. I am grateful for his time and patience while teaching me basic lab techniques - I can say I've learned from the best. I also want to extend my thanks to Dr. Rini Shah, SG Lab, for her constructive criticism over the past year. Discussions with her have always opened my mind to better understanding of both my work and science in general.

I extend my sincere gratitude towards my TAC member Dr. Vineeta Bal, IISER Pune, for her time and crucial comments and suggestions during this project.

I have grown as a person over the past year and my lab family has a big role to play. Working in such a scientifically enriching environment has provided me with a good platform and prepared me for the next stage of my career. It is a great privilege to express my deep sense of gratitude to SG and KK Lab members for their help throughout. The lab has been my home away from home, and I know I have developed some friendships for life. I have learned science, ethics, and vital life skills from them. I will really miss lab treks in and around Pune, our *chai*-time conversations, our badminton sessions, and our all-nighters in the lab. I feel blessed to have had worked with such wonderful colleagues who have also been friends and teachers to me.

I would like to thank Department of Biology, IISER Pune for providing all facilities, instruments and chemicals for the project.

Lastly, I'd like to thank my family. They may not have known or understood details about my work, or have given me scientific insights, but they have believed in me more than anyone else. And that has kept me going through sunshine and clouds with bravery and optimism.

Rahul Biradar

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Abstract

SATB1 is a chromatin organizer protein enriched in T cells. It plays crucial role in differentiation of naïve CD4+ T cells. Transcriptome analysis performed in our laboratory suggested that multiple promoters are involved in *SATB1* expression (P1, P2 and P3) in mouse and human. Specifically, P2 promoter is increased in expression upon differentiation of CD4+ T cells into Th2 phenotype. Interestingly, expression of P1 promoter increased and P3 decreased upon activation of TCR signaling alone. We extended these observation to an *in vitro* system, the Jurkat cell line. Our finding in Jurkat cells suggests that different transcription factors downstream of TCR signaling and cytokine signaling may play a role in regulation of *SATB1* expression. We previously showed involvement of *STAT* family of transcription factors in regulation of P2 promoter expression. Here we show the role of NF-κB in the regulation of *SATB1* P3 promoter expression by chemical inhibition of NF-κB in Jurkat cells subjected to polarizing conditions. However, upon NF-κB inhibitor treatment no significant effect was observed on *SATB1* P1 promoter. We speculate AP1 family transcription factor FOS may regulate *SATB1* P1 promoter.

Preliminary data showed that *STAT6* play crucial role in *SATB1* P2 promoter expression upon cytokine signaling activation. Motif analysis and overlaying on genome show STAT6 binding to upstream putative enhancer. Hence, we perform a chromosome conformation capture (3C) assay to study this promoter-enhancer interaction. So far, we have successfully standardized 3C assay.

This study shows an interplay of direct effector transcription factors of TCR and cytokine signaling in *SATB1* alternative promoter expression.

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Introduction

T-cells form the cell mediated adaptive immune system in jawed vertebrates [Hirano M et al., 2011]. T-cells develop in the thymus and enter peripheral blood. The naive T-cells in the peripheral blood undergo further differentiation into different T-helper subtypes such as Th1, Th2, Th17 etc. in response to an antigenic stimulus provided by antigen presenting cells [Zhu X et al., 2009]. These helper T cells facilitate different sets of immune responses based on the distinct sets of cytokines secreted [Kaiko G et al., 2008]. *SATB1* (Special AT-rich binding protein 1) is one of the various factors that plays a crucial role during $CD4^+$ T-cell differentiation. *SATB1* is a T lineage-enriched chromatin organizer and global gene regulator [Cai S et al., 2003]. Multiple studies have elucidated a crucial role of *SATB1* in cytokine gene expression and immune function [Ahlfors H et al., 2008, Notani D et al., 2010 and Kondo M et al., 2016].

SATB1 organizes distinct chromatin loops in T-cells (figure 1) by tethering matrix attachment regions to nuclear matrix at fixed distances [Galande S et al., 2007].

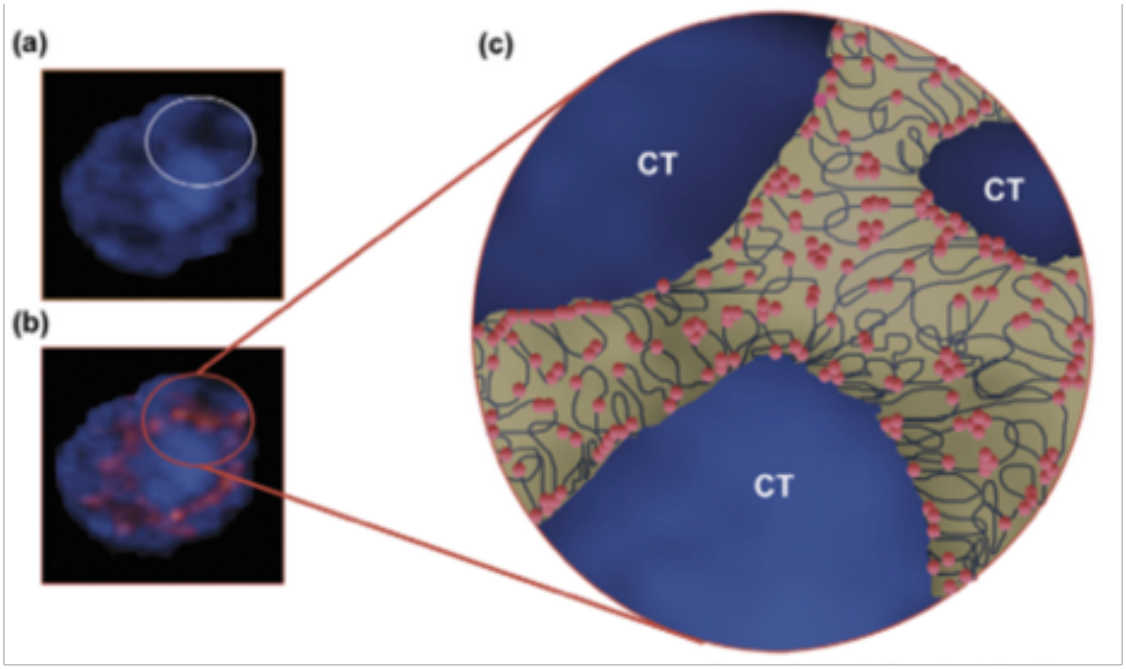

Figure 1: SATB1, a global chromatin organizer protein- (A) and (B) mouse thymocyte stained with DAPI and anti-SATB1 antibody to reveal cage like structure of SATB1. (C) artistic depiction of nucleus where SATB1 is shown to bind and maintain chromatin loop between highly condensed chromosome territories.
Source: Galande S et al., 2007

SATB1 knockout mice do not survive more than three weeks post-partum. The conditional knockout of *SATB1* in hematopoietic cells shows autoimmune dysfunctioning due to poor development of T cells. Thus, *SATB1* plays a crucial role by maintaining immune tolerance by regulating Treg cell differentiation [Kondo M et al., 2016]. *SATB1* regulates Th2 differentiation by recruiting β-catenin through Wnt dependent manner [Notani D et al., 2010]. *SATB1* regulates initial expression of *GATA-3* which promotes production of IL4 and drives Th2 differentiation [Notani D et al., 2010, Yu Q et al., 2009]. *SATB1* represses *IL5* expression during early Th2 differentiation by direct binding to promoter of *IL5* [Ahlfors H et al., 2008].

Structurally, *SATB1* shows a PDZ (**p**ost synaptic density protein, **d**rosophila disc large tumor suppressor, **z**onula occludens-1 protein) like signaling domain, a cut repeat containing domain (CD) and a homeodomain (HD) (figure 2). The PDZ like domain at N terminal region provide a dimerization interface and also interact with other proteins [Burute M et al., 2012].

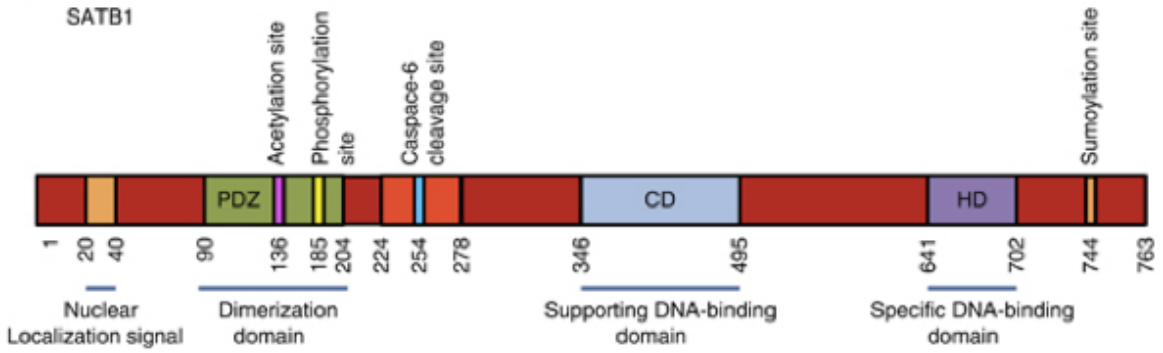

Figure 2: Domain structure of *SATB1*- schematic of *SATB1* protein shows functional domain and key amino acid residues.
Source: Burute M et al., 2012.

Transcriptome analysis on publically available data [Hu G et al., 2013] performed in our laboratory shows presence of three different transcription start site (TSS) at *SATB1* locus in human suggesting three different promoters for *SATB1* gene expression. The figure 3 below shows the schematic for full length *SATB1* gene along with three promoters and three isoforms for *SATB1* (P1, P2 and P3).

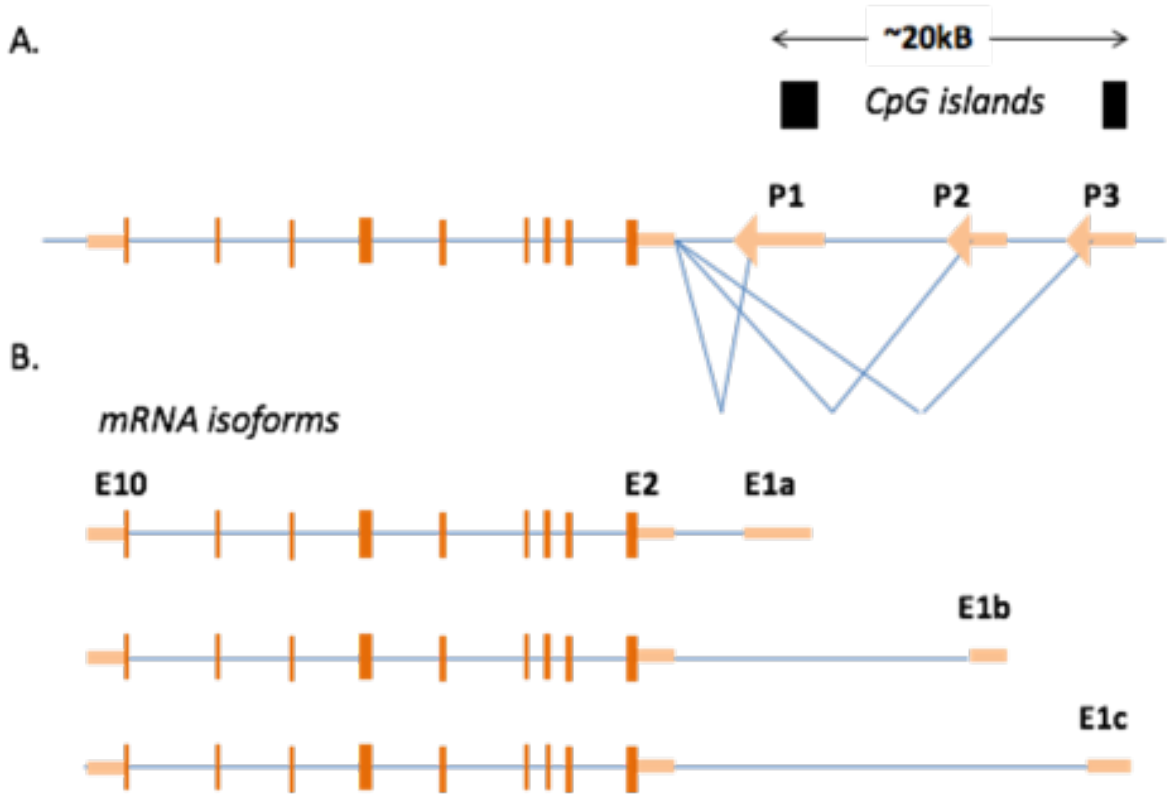

Figure 3: Schematic of *SATB1* full length gene and isoforms – (A) Schematic of human *SATB1* full length with ten exons. Regulatory region spans approximately 20kb distance. The P1 and P3 promoters overlap with CpG islands. (B) shows alternatively spliced mRNA isoforms. The coding DNA sequence (CDS) starts from exon 2. Alternative first exons 1a, 1b and 1c form part of 5'UTR.

Background

Previous work has shown that a *STAT* family transcription factor *STAT6* regulates *SATB1* expression in Th2 cells [Ahlfors H et al., 2010]. ChIP-Seq analysis on available data [Wei et al., 2010] suggested differential occupancy of STAT6 on *SATB1* TSSs with occupancy at P2 promoter but not P1. Stat6 knockout mice also show overall defect in Th2 differentiation specifically affecting P2 promoter expression [Khare et al., manuscript in preparation]. Moreover, Jurkat cells subjected to Th2 polarizing conditions expressed P2 promoter and the expression was affected when cells were treated with inhibitor of JAK3 (kinase responsible for phosphorylation and activation of *STAT6* transcription factor) (figure 4). These results suggested a direct role of *STAT6* in expression of the P2 promoter. Interestingly, STAT6 Motif identification from ChIP and overlaying on the genome [Machanicl and Bailey., 2011], showed direct Stat6 binding site upstream of the P3 promoter but not at the P2 promoter [Khare et al., manuscript in preparation]. Hence, we speculate that *STAT6* interaction is mediated via looping of this putative upstream enhancer element with the P2 promoter of *SATB1* (figure 5). Thus, we aim to study promoter-enhancer interaction in *SATB1* expression and its chromatin conformation in regulating expression. An alternative *in vitro* system is used to investigate the mechanism of *SATB1* regulation in T cell differentiation.

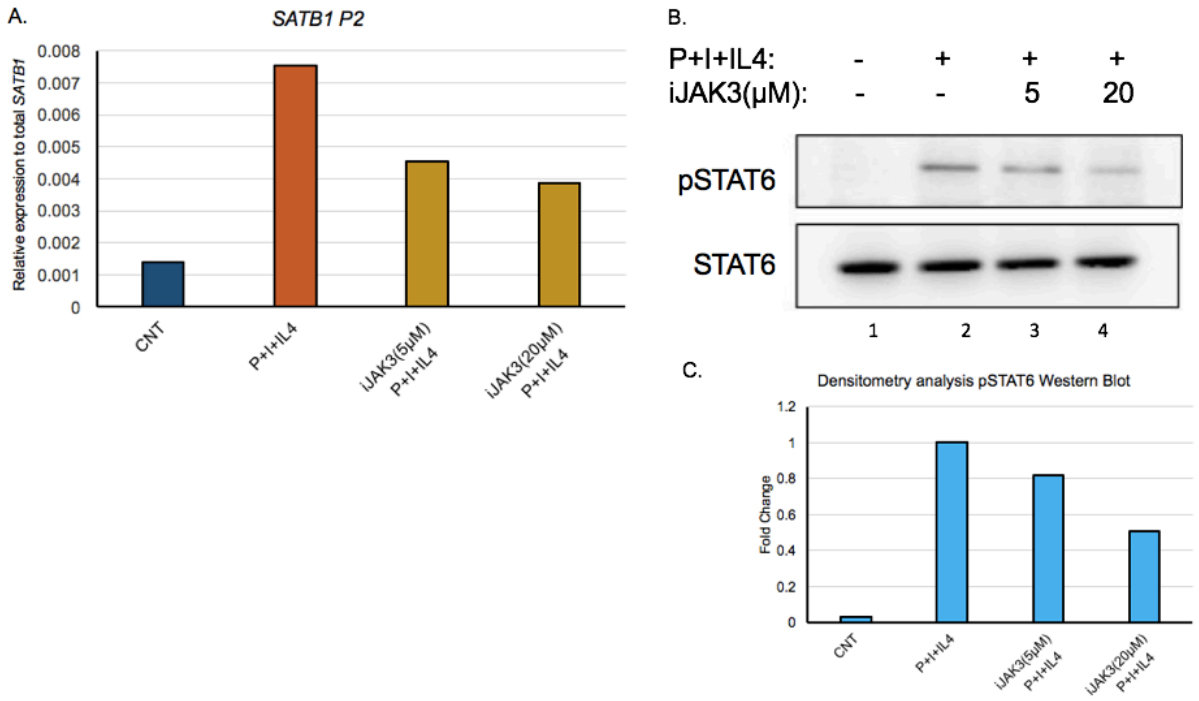

Figure 4: STAT6 regulates *SATB1* P2 promoter expression- (A) expression of *SATB1* P2 promoter in control (CNT), PMA, Ionomycin and IL4 (PIIL4) treated Jurkat and JAK3 inhibitor treated (iJAK3-5μM, iJAK3-20μM) Jurkat cells; P2 promoter shows decrease in expression with the increase in JAK3 inhibitor concentration (B) western blot for phosphorylated Stat6 (pStat6) and Stat6 in the same samples. pStat6 level decreased (lane 3 and 4) with the increase in JAK3 inhibitor concentration. (C) densitometry analysis of pSTAT6 blot confirms decrease in phosphorylation in JAK3 inhibitor treated samples (N=2).

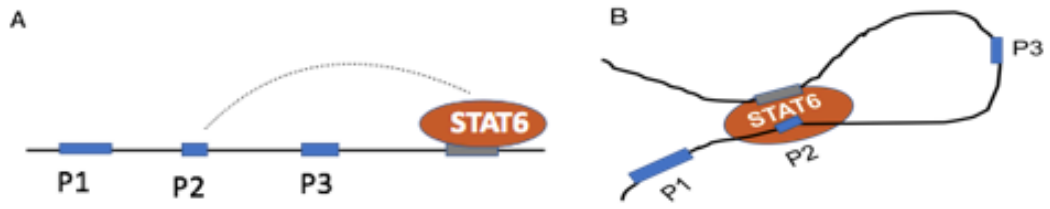

Figure 5: STAT6 mediates *SATB1* promoter enhancer looping - (A) schematic of STAT6 binding to putative enhancer upstream of *SATB1* promoters and (B) STAT6 mediated looping of upstream enhancer with *SATB1* P2 promoter.

Objectives

1. Identification of transcription factors regulating *SATB1* P1 and P3 promoters.
2. To study the regulation of *SATB1* P2 promoter expression by putative enhancer upstream of *SATB1* gene.

Materials and methods

Cell culture

Jurkat cells (ATCC #TIB152) were used and cultured in RPMI1640 media was used with 10%FBS and Anti-anti. Cells were cultured at 37°C and at 5% CO2. Jurkat cells were activated using PMA (working 0.1 µg/ml; Sigma, Cat. No- P1585), Ionomycin (working 1µM, Sigma, Cat. No- I0634) with or without IL4 (working 50ng/ml, MACS, Cat. No-130-095-373) [Ghosh P et al., 1996]. Cells were incubated for 24hrs-48hrs and then cells were harvested for further RNA and protein analysis.

RNA isolation, cDNA Synthesis and quantitative PCR (qRT-PCR)

RNA isolation was carried out using Qiagen column extraction kit [RNeasy kit, Cat. No- 74106] or Trizol method. 0.5µg of RNA samples was processed for cDNA synthesis using high capacity cDNA synthesis kit (Applied Biosystem, Cat. No-431688). Expression profile of SATB1 alternative promoter and different transcription factors (Table1) was studied by performing Quantitative real time PCRs using Applied Biosystems ViiA™ 7 Real-Time PCR System using KAPA SYBR Green with low ROX. The program used was after initial 95°c for 2 min, 40 cycles of denaturing at 95°c for 15 seconds, annealing at 63°c for 15 seconds and extension at 72°c for 30 seconds. ΔCt values were calculated using the formula: $\Delta Ct = (Ct_{Target} - Ct_{internal\ control})$. The fold change in gene expression were calculated using the formula, fold difference = $(2^{(Ct\ Target\ -\ Ct\ internal\ control)} / 2^{(Ct\ control\ -\ Ct\ loading\ control)}$. This fold change was plotted for samples.

Table 1: List of RT-PCR primers used along with expected amplicon size and sequence details

Sr.No.	**Name**	**Amplicon Size**	**Sequence**
1	18s rRNA forward	101	CGCCGCTAGAGGTGAAATTCT
2	18s rRNA reverse		CGAACCTCCGACTTTCGTTCT

3	*SATB1* forward	217	GAAGAGGAAGGCTTGGGAGT
4	*SATB1* reverse		ATGCTCCTCCTTGCAATCAT
5	*SATB1* P1 forward	250	CCTTCAGGTCTGCTGCTTTT
6	*SATB1* P1 reverse		CCCTTCGGATCACTCACATT
7	*SATB1* P2 forward	206	TGCTAGCAGTGCCAGAGAGA
8	*SATB1* P2 reverse		CCCTTCGGATCACTCACATT
9	*SATB1* P3 forward	160	AGCCGTTCTTGGTTTCAGG
10	*SATB1* P3 reverse		CCCTTCGGATCACTCACATT
11	*IL2* forward	283	CCTCAAGCTCAATAAGCATTTTAAG
12	*IL2* reverse		GTGGGGATACAAAAGTAACTCAG
13	*NFAT* forward	226	GTGCCACAACCTTCAGACCT
14	*NFAT* reverse		GTCTTCCACCTCCACATCGT
15	*NF-κB1* forward	112	ATGTATGTGAAGGCCCATCC
16	*NF-κB1* reverse		ATAACCTTTGCTGGTCCCAC
17	*FOS* forward	122	CTACCACTCACCCGCAGACT
18	*FOS* reverse		TGGTCGAGATGGCAGTGAC

Immunoblotting

Proteins were quantitated by BCA method. Equal amounts of proteins were separated on a 10% polyacrylamide gel and transferred on a PVDF membrane. The blots were blocked in 5% BSA (Sigma, Cat. No- A9647) made in TBST. Primary antibodies used are total STAT6 (1:1000 dilution, Cell signaling, Cat. No. 9362), phospho STAT6 (1:1000 dilution, Cell signaling, Cat. No. 9361), ß-Actin (Cat. No. ABClonal AC004) or/and ɣ-Tubulin (1:2000 dilution, Sigma T6557). Mouse and rabbit HRP secondary antibody was used at 1:5000 and 1:8000 dilution respectively. The blots were imaged on LAS4000.

NF-κB inhibitor Assay

Jurkat cells were plated on day one at 0.2 million/ml density and cultured as mentioned earlier. On day two cells were activated using PMA, Ionomycin and/or IL4. After 24hr

activation on day three NF-κB inhibitor (TOCRIS, Cat. No- CID2858522;) treatment was given for next 24hr. Cells were harvested on day four after 24hr of inhibitor treatment. The cells were processed for RNA isolation and cDNA synthesis. Promoter specific transcript expression of *SATB1* was quantified by qRT-PCR.

Bacterial Artificial Chromosome Design and (BAC) Isolation

BAC (RP11.158G18) construct containing human *SATB1* gene locus was ordered from CHORI (Oakland, CA, US). Since the Nde1 restrictions sites were distributed equally in *SATB1* primers region 3C primers were designed at unidirectional to these sites as shown in figure 6; A as Anchor (A), T1, T2, T3 and T4.

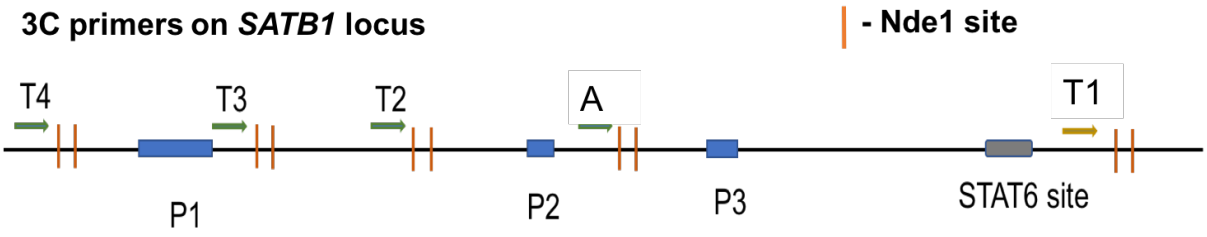

Figure 6: Schematic of 3C primer design at SATB1 promoter region – schematic shows unidirectional 3C primers (A-anchor, T1, T2, T3 and T4) designed at *SATB1* promoters (P1, P2 and P3) and putative enhancer region along with restriction site for NdeI and unidirectional 3C promoters.

A glycerol stock of E. Coli Dh5α containing BAC was obtained. LB agar plates with chloramphenicol (10 µg /ml) were used to streak the glycerol stock. A single colony was inoculated into 3 ml LB broth (primary culture) containing chloramphenicol (10 µg /ml) and grown for 8-10 hrs in incubator/shaker at 37°C. A 0.5% of this primary culture was inoculated into 500ml LB broth (secondary culture) containing chloramphenicol and grown at 37°C with shaking for overnight. Bacterial culture was pelleted by centrifugation at 5000g for 10 min. A phaseprep BAC isolation kit from Sigma (MA0100) was used to isolate BAC from bacteria.

BAC Library preparation

BAC isolation was confirmed by restriction digestion using Age1-HF (NEB, Cat. No- R3552S) and Nde1 (NEB, Cat. No- R0111S) at 37°C for 4hr. The digested BAC was purified by Phenol Chloroform Isoamyl alcohol (PCI) extraction (Invitrogen, Cat. No- 15593-049) and loaded on 1% agarose gel to compare with the expected digestion pattern obtained from Advanced Plasmid Editor (ApE) software. Also, BAC isolation was confirmed by PCR using *SATB1* sequence specific primers. For BAC library preparation 20 µg of BAC template was processed for restriction digestion and ligation. Digestion of BAC was performed by adding 1200U of Nde1 (NEB, Cat. No- R0111S) restriction enzyme and incubating overnight at 37°C. The digested BAC was purified by PCI extraction. The ligation of digested BAC was performed by adding 42 µl of ligation mix (22 µl of 10X ligation buffer to a final 1 X concentration and 20 µl of 10mM ATP) and 19 µl of T4 DNA ligase (NEB, Cat. No- M0202L). This ligation reaction was incubated at 16°C overnight. Heat inactivation of the reaction performed at 65°C for 15 min. A PCI extraction was performed to purify ligated BAC template. The digested and ligated samples were run on 1% agarose gel to confirm ligation. A qRT-PCR was performed using 3C primers (Specific to looping interaction) on BAC.

Table 2: List of SATB1 sequence specific primers and 3C primers with amplicon size and sequence.

Sr. No.	**Primer Name**	**Amplicon size**	**Sequence**
1	*SATB1*_3C_Anchor_F		ACTCCAGGTCCCTGGCTAAT
2	*SATB1*_3C_T1_F	185	TTTTGCATTCAACTACATCATGTCT
3	*SATB1*_3C_T2_F	207	CTTTGTGCAAAGGGGTTTTG
4	*SATB1*_3C_T3_F	251	CTCTTAAAGGCCCCACCTCT
5	*SATB1*_3C_T4_F	175	TGGTTGCCTCATCCTAGAGC
6	*SATB1*_Mid_F	117	GAGGGCGTTATACTGGGTGA

7	*SATB1*_Mid_R		GGTGGTTACCAGTTGCCTGT
8	*SATB1*_Distupstr_F	136	GCAGGAGGGAGATTGCTAAG
9	*SATB1*_Distupstr _R		AGCCATCTGACACAACAGGA
10	*SATB1*_Dist _F	99	TCAAGTTGTCCTCTTTCCCCA
11	*SATB1*_Dist _R		TTCTCTTCCTTCCCTTGGCCC
12	*SATB1_Prox_F*	147	TGTGCCCTTATCCATTCCAT
13	*SATB1*_Prox_R		GAGTTTCAGGTCGGTTTTGC

Chromosome conformation capture (3C)

Following protocol of 3C was referred from Current Protocol [Miele, A. et al 2006) and Nature Protocol [Helene et al, 2007]. The protocol was broken down into 3 main steps. SDS treatment was standardized and arbitrary name was given as summarized in table below.

Step 1

Jurkat cells were plated at 0.2 million/ml density and cultured as mentioned earlier. On day one, cells were activated using PMA, ionomycin and/or IL4. After 48 hr of activation cells were harvested and centrifuged at 450 X g for 10 min. 2-10 X 10^6 cells were used per experiment. The cell pellet was resuspended in complete RPMI medium and crosslinked using formaldehyde 1% or 2% final concentration, (Pierce, Cat. No-28908). Formaldehyde was added dropwise and cells were incubated for 10 min at room temperature. 125mM glycine added to quench crosslinking reaction and incubated for 5 min at RT followed by 15 min on ice. Cells were centrifuged at 800 X g for 10 min. The cell pellet was resuspended in 1ml of ice cold lysis buffer (10mM Tris.Cl pH8, 10mM NaCl, 0.2% Igepal (NP-40)) and incubated for 10min on ice. Dounce homogenizer was used to isolate nuclei by giving 20 strokes of tight pestle B. Integrity of nuclei was checked in hemocytometer. Two washes of 0.5 ml of 1X restriction enzyme buffer (Cutsmart, NEB Cat.No-B7204S) were given. Nuclei were snap frozen and stored at -80°c or continued to next step.

Step 2

Nuclei from 2-10 million were resuspended in 362 µl of 1.35 X Cutsmart buffer. SDS was added to 0.1-0.3% final concentration.

Table 3: SDS treatment conditions

Sr. No.	Protocol name	SDS concentration	TritonX-100 concentration	Conditions (temperature and time)
1	Current protocol (CP)	0.1% SDS	1%	65°C for 10 min
2	Nature protocol (NP)	0.3% SDS	2%	37°C for 1 hour
3	Combined protocol (SK)	0.3% SDS	2%	65°C for 10 min, 37°C for 50 min

The nuclear suspension was passed 4-5 times through 22G syringe. The SDS treatment conditions were mentioned in table 1. TritonX-100 was added to 1 to 2 % final concentration and mixed by pipetting or passed through syringe. A small aliquot of 20-40 µl was snap frozen and stored at -80°c before setting digestion. Digestion of DNA was performed by adding 400U of Nde1(NEB, Cat. No- R0111S) restriction enzyme and incubated at 37°c for 5hrs. 86 µl of 10% SDS was added and incubated for 30 min at 65°c to inactivate enzyme. A small aliquot of 20-40 µl taken and stored at -80°c. The digested sample processed for ligation. The ligation mix is prepared by adding 745 µl of 10% TritonX-100, 745 µl of 10X ligation buffer (500mM Tris-Cl, 100mM $MgCl_2$, 100mM DTT), 80 µl of 10mg/ml BSA, 80 µl of 100mM ATP and 5960 µl of distill water. 4000U of T4 DNA ligase (NEB, Cat. No- M0202L) was added. The ligation reaction was incubated at 16°c overnight on a rotor.

Step 3:

Reverse crosslink of ligated DNA and protein was performed by adding 25 µl of 20mg/ml Proteinase K (Sigma, Cat. No- P2308) to each sample and incubated at 65°c overnight (10-12hrs) at 900rpm. 25 µl of 20mg/ml Proteinase K was again added to sample and incubated for 2 hrs at 42°c. Samples were treated with 15 µl of 20mg/ml RNases A (final 300 µg). The DNA was purified by performing Phenol Chloroform Isoamyl alcohol (PCI, Invitrogen Cat. No- 15593-049) extraction and ethanol

precipitation. To reduce coprecipitation of DTT equal volume of NFW is added along with 1/10th volume of 3M sodium acetate and 2.5 volume of absolute ethanol. This is stored at -80°c for 30 min. Following centrifugation at 14,000g DNA pellet was washed with 1ml of 70% ethanol for two times. Pellet was air dried and dissolved in 60-100 µl of NFW. The undigested and digested aliquots were processed parallelly for decrosslinking by first increasing volume up to 400µl with Proteinase K buffer (1M Tris-Cl pH8, 0.5M EDTA pH8) and then proportionate amount of Proteinase K and RNase A followed by PCI extraction. The concentration of DNA was measured using Nanodrop. Samples were tested for digestion and ligation on a 0.8% agarose gel. Upon confirming gel images, 3C library was prepared by performing qRT-PCR with 3C primers (primers bind to expected ligation junctions). This ligation frequency is compared with control BAC library to comment on looping.

Statistical Analysis

Statistical significance was calculated between fold change values of control and *NF-κB* inhibitor treated sample with 2 tailed student t-test. F-test was performed for comparison of variance between the samples. A *P*-value less than 0.05 was considered statistically significant.

Result

Objective 1: Identification of transcription factors regulating *SATB1* P1 and P3 promoter.

Identification of transcription factors regulating *SATB1* P1 promoter

Upon activation of Jurkat cells with PMA, Ionomycin and IL4, different transcription factors are activated. Figure 7 below shows activation in Jurkat cells by PMA (P) and Ionomycin (I) mimics activation of TCR signaling whereas treatment with IL4 leads to activation of Cytokine signaling.

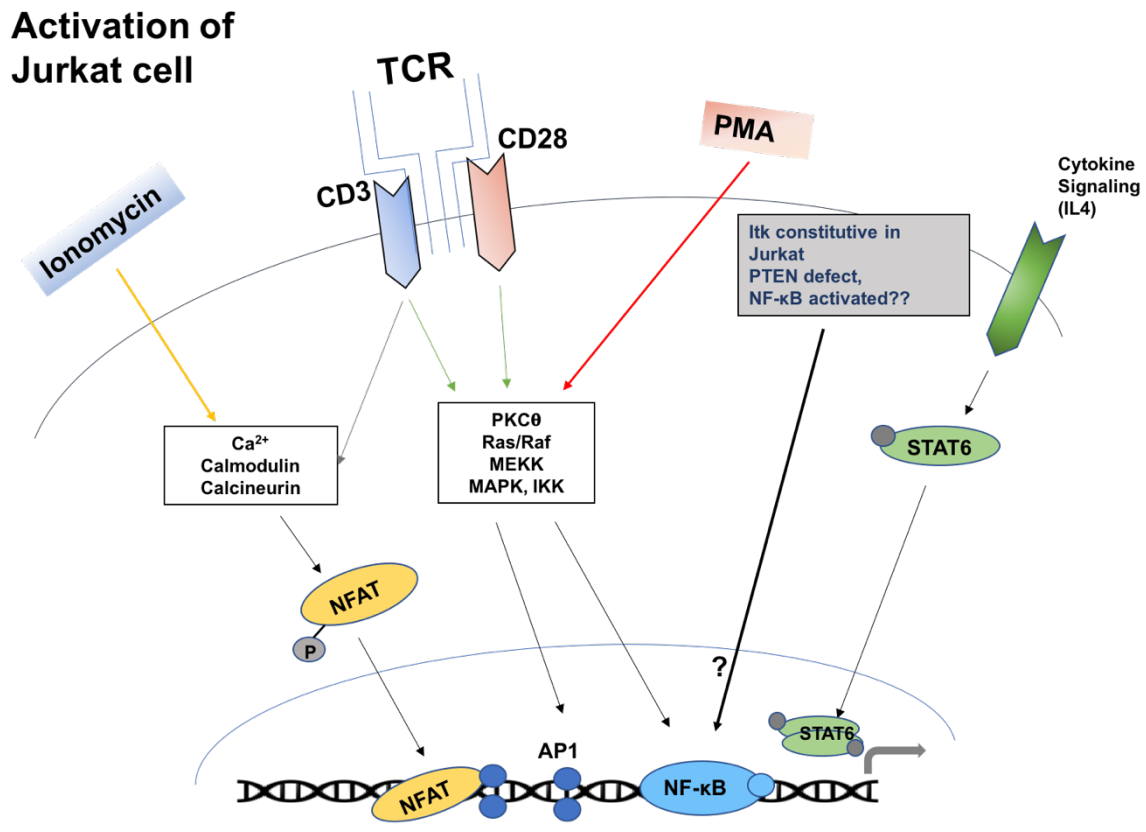

Figure 7: Activation of Jurkat cells and downstream transcription factors- treatment of PMA, Ionomycin and IL4 leads to activation of various transcription factors which regulate effector gene expression. PMA mimics the action of CD28 receptor signaling and activates NF-κB and AP1. Ionomycin mimics CD3 signaling and activates NFAT upon co-activation of CD28 signaling. The cytokine signaling activates STAT family of transcription factors. Deficiency of PTEN in Jurkat cells leads to PI3K activity and constitutive phosphorylation of Akt.

We observed, that resting Jurkat cells predominantly express *SATB1* P3 promoter. Upon activation of TCR signaling, expression of *SATB1* P3 promoter decreased significantly whereas increase in P1 promoter expression was observed. Interestingly, upon activation of cytokine signaling, *SATB1* P3 promoter increased significantly (figure 8A).

As shown in figure 7, Jurkat cells do not express *PTEN* and constitutive express *ItK*, *PI3K* and *Akt* [Shan X et al., 2000; Xu Zheng et al., 2002]. Thus, we speculate NF-κB is constitutively active in resting Jurkat cells and regulates *SATB1* P3 expression. To test this hypothesis NF-κB inhibitor assay was performed in activated Jurkat cells. The expression profile of total *SATB1*, *SATB1 P3* and *IL2* normalized to resting Jurkat sample shown in figure 8 below. *IL2* is a NF-κB target gene (figure 8D).

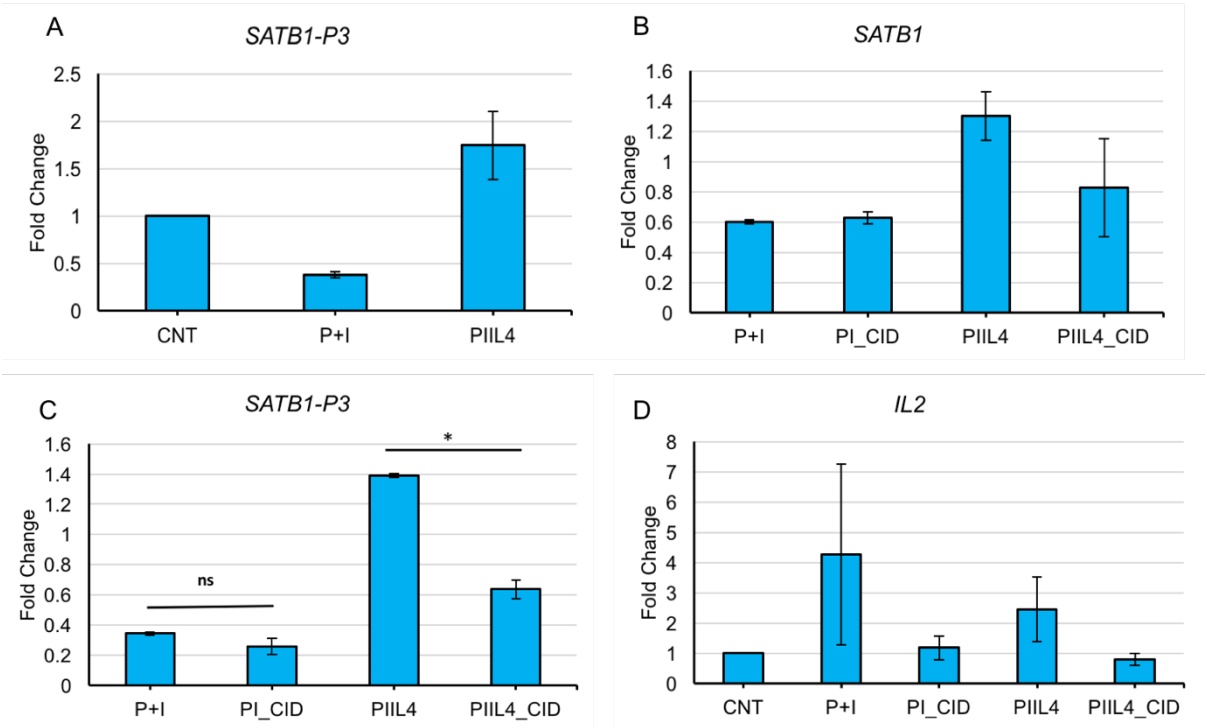

Figure 8: Effect of NF-κB inhibitor (CID) on SATB1 P3 promoter – (A) expression profile of *SATB1* P3 promoter plotted in resting, P+I treated and P+I+IL4 treated Jurkat cells. P3 promoter expression decreased in P+I treatment whereas it increased in P+I+IL4 treated Jurkat cells (N=2). Figure (B), (C) and (D) shows expression of total *SATB1*, *SATB1* P3 and *IL2* transcript expression in Jurkat cells treated with P+I, P+I and NF-κB inhibitor, P+I+IL4 and P+I+IL4 and NF-κB inhibitor (N=2). No significant change is observed in expression of P3 promoter in Jurkat cells treated with P+I upon addition of NF-κB inhibitor, whereas *SATB1* P3 expression significantly reduced in P+I+IL4 treated Jurkat upon NF-κB inhibitor treatment. Panel D show *IL2* expression used as a positive control for NF-κB inhibitor treatment.

NF-κB inhibitor (CID2858522 abbreviated as CID) treatment in P+I treated Jurkat cells did not show any significant decrease of *SATB1 P3*. However, significant decrease in expression of *SATB1* P3 promoter transcript was observed in NF-κB inhibitor treated cells subjected to polarization conditions (figure 8C). This result suggests that NF-κB regulates *SATB1* P3 promoter expression. This regulation is direct or indirect effect of NF-κB at *SATB1* P3 promoter region need to be tested in future.

Identification of transcription factors regulating *SATB1* P1 promoter.

Resting Jurkat cells express *SATB1* P1 promoter and the expression is maintained in TCR and cytokine activated Jurkat cells. The NF-κB inhibitor treatment showed it did not affect *SATB1* P1 expression. As shown in figure 7, NFAT and AP1 are downstream effector of TCR regulate expression of multiple gene. We quantified expression of NFAT and AP1 family factor FOS in resting, PMA treated and P+I treated Jurkat cells along with *SATB1* P1 promoter expression. *SATB1* P1 promoter expression increased upon treatment of PMA alone in Jurkat cells however expression of *NFAT* did not change upon PMA treatment (figure 9). Thus, we think that NFAT may not regulate P1 promoter.

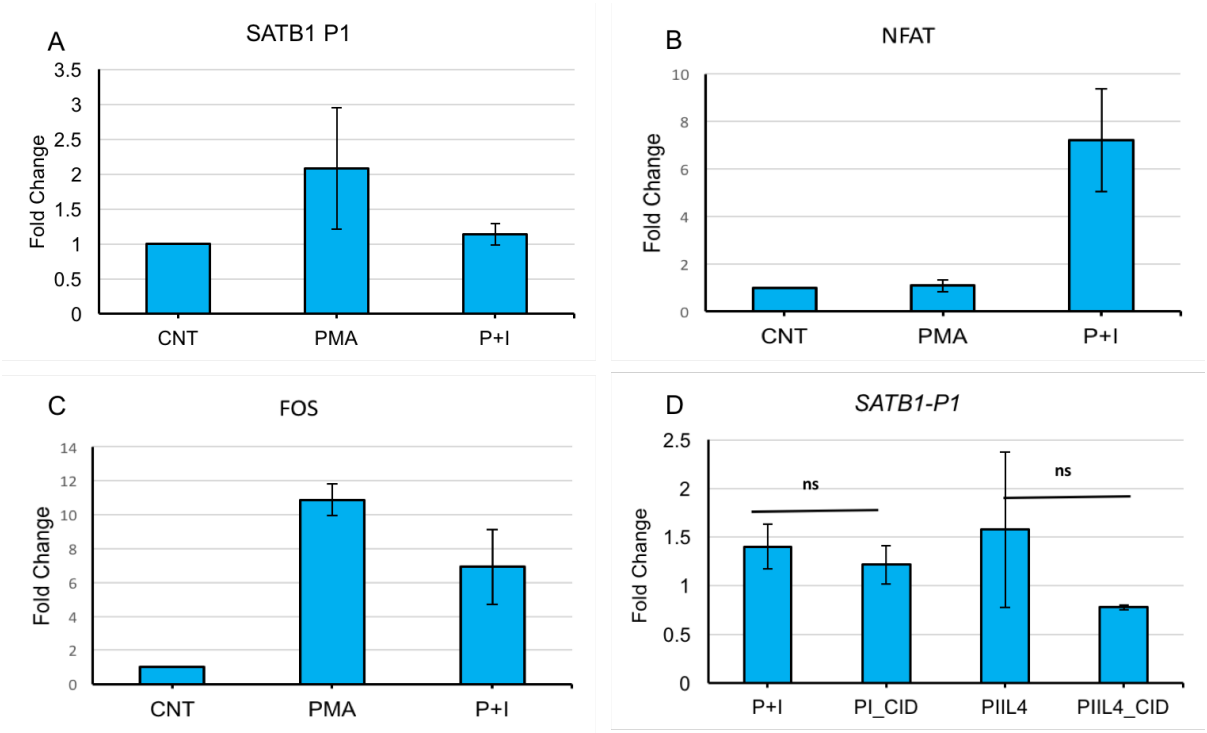

Figure 9: Activation of Jurkat cells and downstream transcription factors – qRT-PCR was performed and gene expression was plotted. Figure (A), (B) and (C) show expression profile of SATB1 P1 promoter, key transcription factors downstream of TCR signaling (NFAT and AP1 family factor FOS) plotted for untreated control, PMA activated and PMA and Ionomycin (P+I) activated Jurkat cells respectively (N=3). (D) No significant change was observed in SATB1 P1 promoter expression upon NF-κB inhibitor treatment in P+I and P+I+IL4 activated Jurkat cells(N=2).

Since expression of *SATB1* P1 promoter did not show any significant change upon NF-κB inhibitor treatment in TCR and Cytokine activated cells (figure 9D), NF-κB does not seem to regulate *SATB1* P1 promoter.

AP1 family transcription factor FOS expression increased upon treatment of PMA alone in Jurkat cells which correlates with the increased *SATB1* P1 promoter expression. Thus, we hypothesize FOS may regulate expression of *SATB1* P1 promoter. To confirm this, we plan to study *SATB1* P1 promoter expression upon inhibition of AP1/ FOS.

Objective 2: Regulation of *SATB1* P2 promoter and putative enhancer upstream to *SATB1*

The promoter-enhancer looping can be studied by performing Chromatin Conformation Capture (3C) assay. Careful design of 3C allows to measure interaction frequency of proposed interacting fragments over any random interactions. BAC with the region of interest (human *SATB1* locus) was used as a negative control for the 3C experiment. BAC isolation was performed as described in materials and methods. Figure 10A shows BAC isolation confirmation by restriction digestion using AgeI enzyme. Figure 10B confirms BAC isolation by PCR using sequence specific control primers (Table 2) for the *SATB1* locus as described in materials and methods.

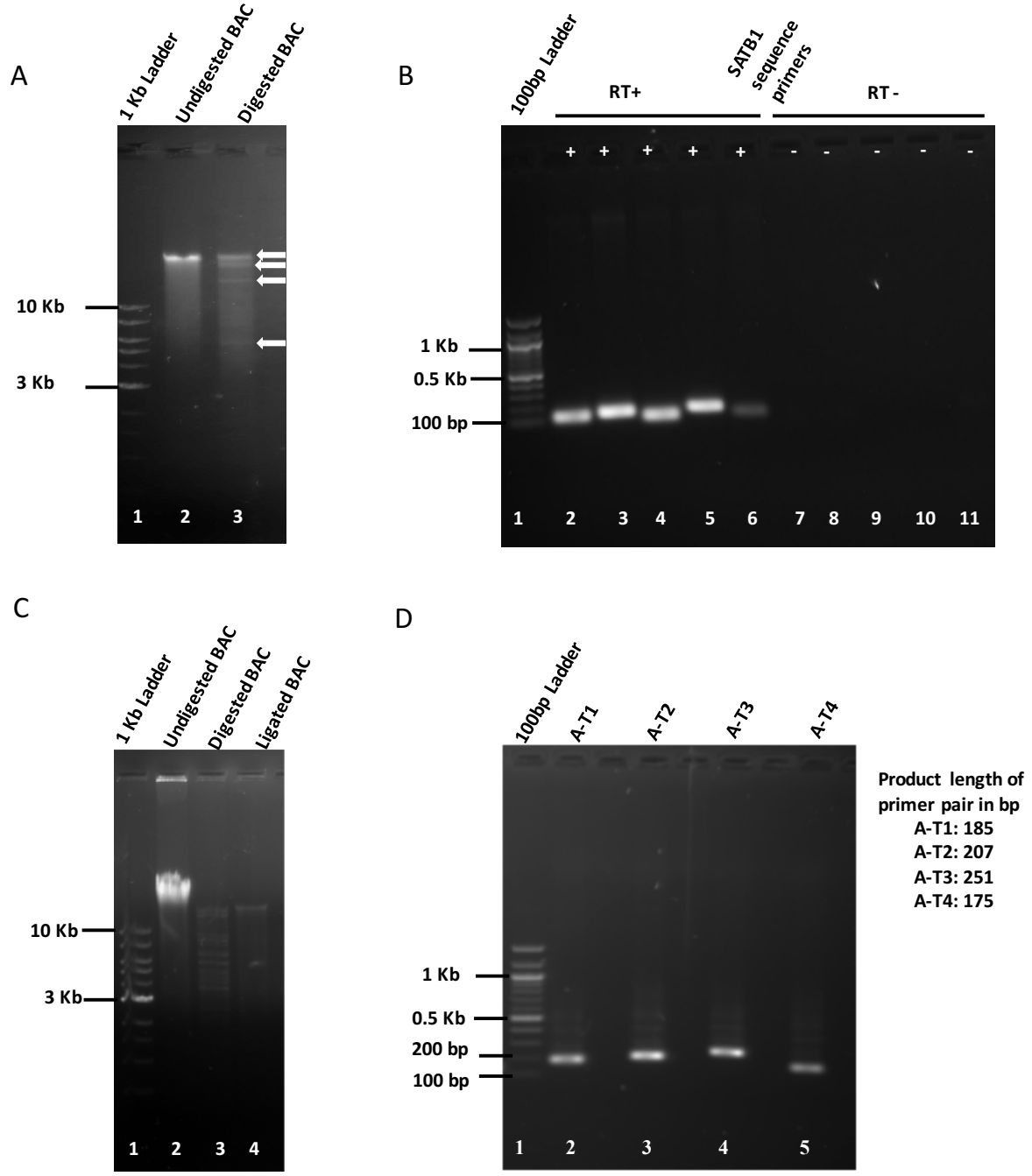

Figure 10: Confirmation of BAC isolation and preparation of BAC library – BAC DNA samples run on 1% agarose gel and stained with EtBr. (A) BAC isolation was confirmed using RE digestion of BAC sample. Expected digested bands indicated by arrows; Lane1: 1Kb ladder, Lane2: Undigested BAC, Lane3: AgeI digested BAC showing expected digestion pattern. (B) BAC isolation was confirmed by PCR using SATB1 sequence specific primers (lane 2-6). lane1: 100bp ladder, lane 7 to 11: NTC. (C) Confirmation of BAC digestion and ligation. lane 1: 1Kb ladder, lane 2: undigested, lane 3: Nde1 digested BAC lane 4: Ligated. (D) Validation of 3C primer pairs. Lane1: 100bp ladder, Lane2: T1 reverse1-anchor, Lane3: T1-T2 reverse2, Lane4: T1-T3 reverse3, Lane5: T1-T4 reverse4.

The BAC digestion and ligation was performed as described in BAC library preparation in materials and methods (figure 13C). Quantitative PCR performed to prepare BAC library using 3C primers (Table 2) and PCR product was run on agarose gel as shown in figure 10D. The intensity of each band suggests possible P2 primer ligation product is present in equimolar ratios. The Ct values obtained from BAC qPCR with 3C primers act as control for the 3C assay. The Anchor-T1 primer amplicon will be indicative of P2 promoter-enhancer interaction.

Standardization of 3C assay in Jurkat cells

Jurkat cells were processed for 3C assay as described in materials and methods. The outline of 3C assay is shown in figure 11 below.

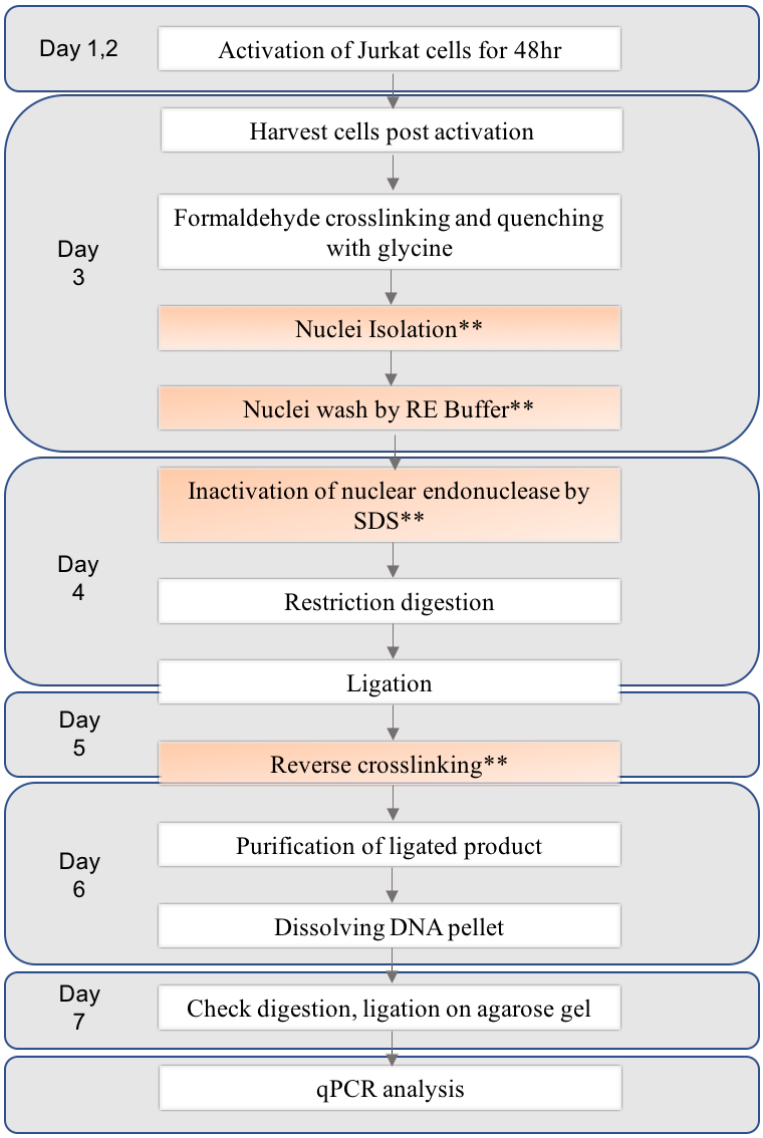

Figure 11: Schematic of 3C assay workflow, (*Standardization steps)

Standardization of nuclei isolation was confirmed by checking nuclei integrity under microscope. A 3C assay was performed according to the Current protocol [Miele A et al., 2006]. Digestion and ligation of genomic DNA was confirmed by agarose gel electrophoresis (figure 12). The expected digestion of human genome by Nde1 restriction enzyme should give a smear around 7 kb region. However, we unexpectedly obtained smear around 500bp region. This unexpected digestion could be due to DNA degradation during inactivation of nuclear endonucleases by SDS treatment.

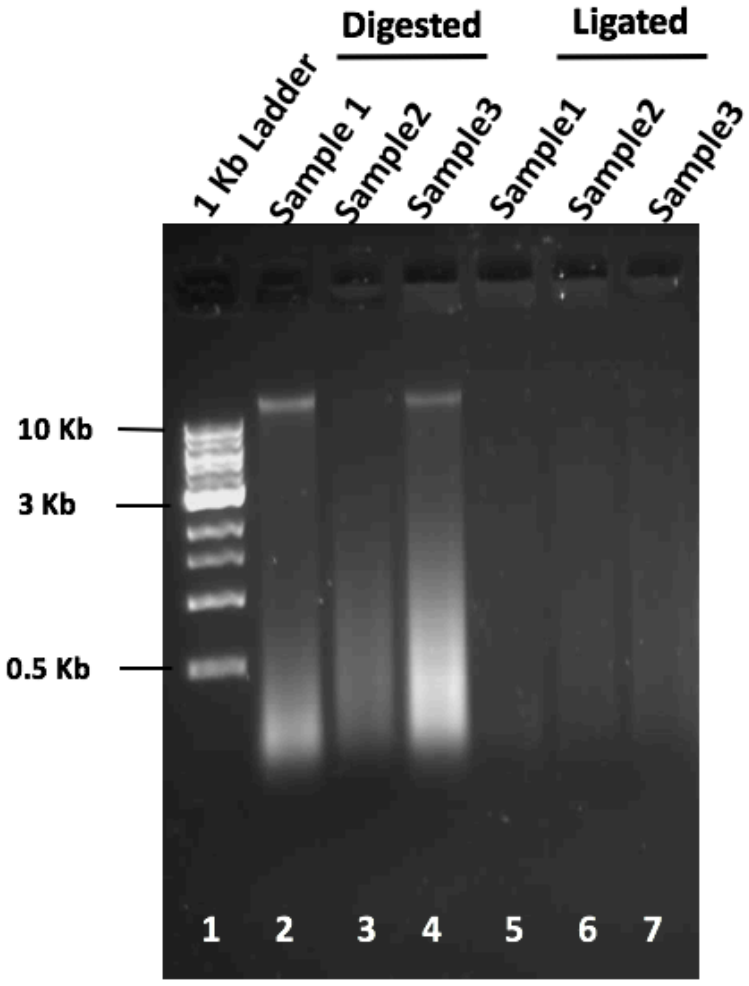

Figure 12: Confirmation of 3C digestion and ligation- An aliquot of digested and ligated sample was run on 1% agarose gel and stained with EtBr. Lane 1: 1kb ladder, lanes 2 to 4: digested samples 1, 2 and 3 respectively, lanes 5 to 7: ligated samples 1, 2 and 3 respectively. Figure shows degradation of DNA in digested and ligated samples.

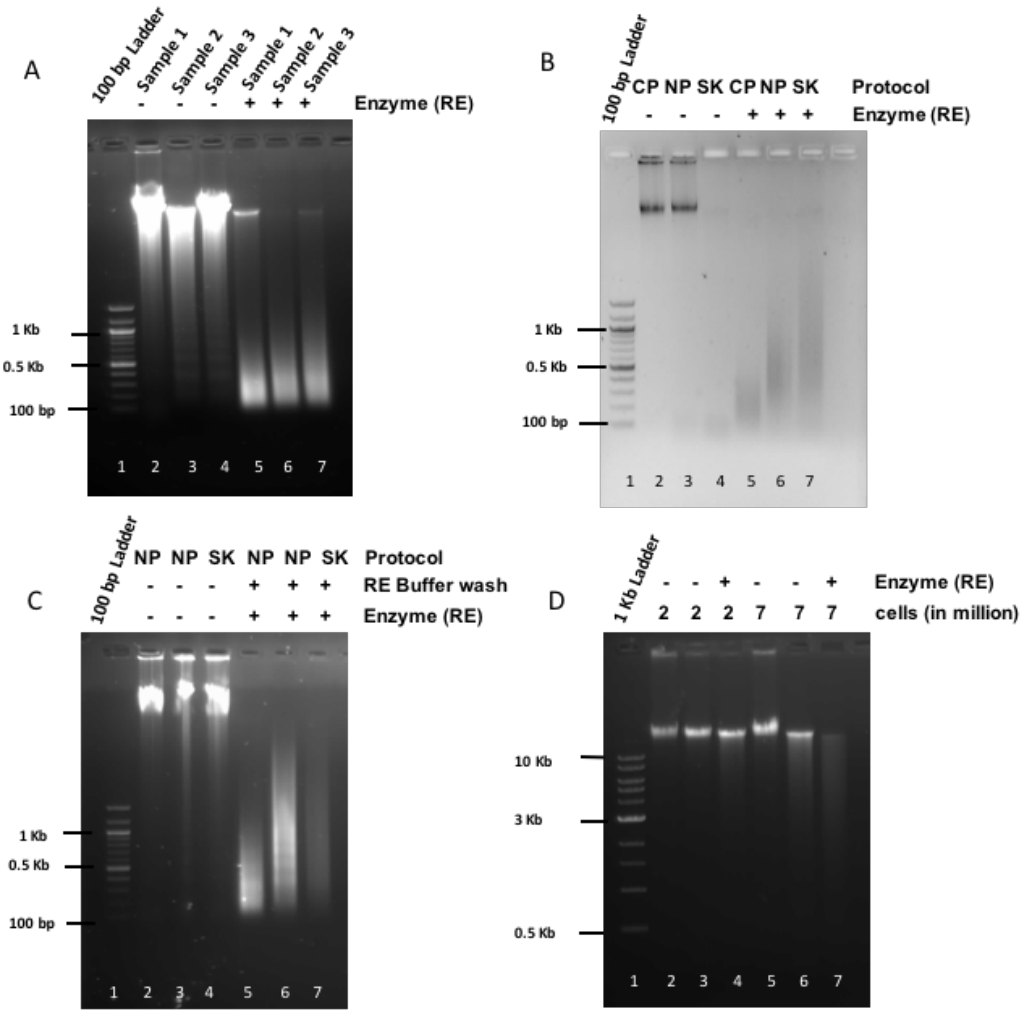

Figure 13: Standardization of SDS treatment for 3C assay- Undigested and digested test sample aliquots was separated on 1% agarose gel and stained with EtBr. (A) SDS treatment condition from current protocol (CP) followed, degradation of DNA by nuclear endonuclease was observed in all samples, lane1: 100bp ladder, lanes 2-4: undigested samples respectively, lanes 5-7: digested samples. (B) three SDS treatment conditions were followed and digestion performed. No DNA degradation was observed in undigested samples whereas NP and SK samples showed improvement over CP protocol. However, the improvement was not sufficient with some DNA degradation. lane1: 100bp ladder, lane2: CP undigested, lane3: NP undigested, lane4: SK undigested lane5: CP digested, lane6: NP digested, lane7: SK digested. (C) Restriction Enzyme Buffer (REB) wash was added to the isolated nuclei, DNA degradation was reduced in all samples but some degradation persisted. lane1: 100bp ladder, lane2: undigested, no REB wash, lane3: undigested, REB wash and SDS treatment at 37°C for 1hr, lane 4: undigested, REB wash and SDS treatment at 65°c for 10min and 37°C for 50min. lane 5-7: digested sample of lane2-4 respectively (D) digestion of 3C sample was performed for 2 million and 7 million nuclei. negligible DNA degradation was observed in all samples. lane1: 1kb ladder, lane2: 2 million undigested, lane3: 2 million undigested and incubated at 37°C, lane4: 2 million digested, lane5: 7 million undigested, lane 6: 7 million undigested and incubated at 37°C, lane7: digested 7 million.

This result suggested standardization of 3C protocol was required. As described in materials and methods, the protocol was divided in three main steps for extensive standardization.

Current protocol for generation of 3C template from mammalian cells was referred to standardize restriction digestion step. In figure 13A, undigested sample showed degradation of DNA. The expected digestion pattern should just start from undigested band. The digestion of undigested sample nearly matched Mnase digestion pattern. Also, Nde1 digested sample showed unexpected digestion ranging from 1kb to 100bp. Thus, these results indicated higher activity of nuclear endonuclease digesting both undigested and Nde1 digested DNA samples. Standardization of SDS treatment before digestion step was needed.

Standardization of SDS treatment conditions such as concentration, time and temperature of incubation was performed (figure 13B). The standardization conditions were as described in material and methods - (1) Current Protocol (CP), 0.1% SDS at 65°C for 10 min (2) Nature Protocol (NP), 0.3% SDS at 37°C for 1 hour (3) Combined (SK), 0.3% SDS, 10 min at 65°C followed by 50 min at 37°C. The undigested sample for first two conditions did not show degradation but much higher degradation in Nde1 digested pattern (1kb to 100bp). The undigested sample in the third condition had an issue with PCI extraction, but Nde1 digestion pattern of the same sample showed better digestion than previous two conditions. Thus, we selected nature protocol and combined SK protocol as SDS treatment standardization conditions.

Since the RE digestion pattern in above conditions still did not match the expected digestion pattern, thus further standardization was required. RE buffer (1X Cutsmart buffer) wash step was added after the nuclei isolated in the protocol. Figure 13C show improved Nde1 digestion pattern upon RE buffer wash.
So far, the number of cells processed for each 3C standardization were around 10-12 million. Multiple protocols suggest dividing the number of nuclei just before digestion step [Helene H et al., 2006, Louwers M et al., 2009]. The number of nuclei processed for 3C was considerably high earlier and thus SDS amount may not be sufficient to inhibit nuclear endonuclease activity resulting in degradation of genomic DNA. Hence, we divided nuclei after nuclei isolation step into 2 million (2M) and 7 million (7M). RE

buffer wash was given to each set. A modified undigested control was kept and incubated similarly to the digestion conditions but without adding enzyme. Figure 13D showed improved undigested and expected digested pattern. A lesser nuclei number show least degradation of undigested DNA and better digestion pattern by Nde1 digested sample.

After standardizing above mentioned conditions, 3C was performed for resting, P+I and P+I+IL4 treated Jurkat cells, 3 million nuclei were processed. Agarose gel image confirmed ligation of digested DNA (figure 14). However, qPCR analysis of the 3C library did not detect 3C PCR products. Less number of cells may have affected ligation frequency; therefore, this step needs further standardization.

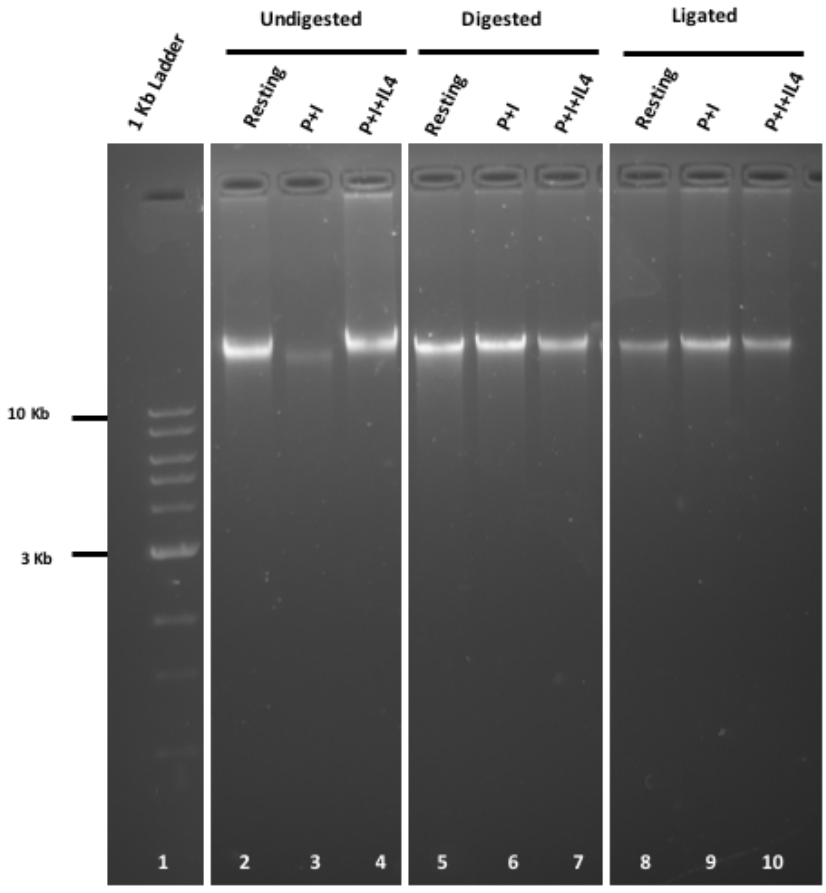

Figure 14: 3C assay- 3 million Jurkat cells were processed for 3C assay. Undigested, digested and ligated samples run on 0.8% agarose gel and stained with EtBr, lane 1: 1kb ladder, lane 2-4: undigested samples1,2 and 3, lane 5-7: digested samples 1,2 and 3, lane 8-10: ligated samples 1, 2 and 3.

Discussion

To study *SATB1* regulation, we have attempted to establish an in vitro Jurkat cell line based system that mimics CD4+ T cell activation and differentiation. Treatment of Jurkat cells with PMA and Ionomycin (P+I) mimics activation of TCR signaling whereas cytokine signaling is activated by treatment of IL4 along with PMA and Ionomycin (P+I+IL4). Transcriptome analysis of primary CD4 T cells and Jurkat cells suggested significant overlap in gene expression between P+I treated Jurkat cells and Th0 cells. A significant overlap was also observed between Jurkat cells subjected to polarizing conditions (P+I+IL4) and in vitro differentiated Th2 cells [Khare et al., manuscript in preparation]. Thus, this system provides a physiologically relevant context to study gene expression.

We found that *SATB1* expression is mediated via alternative promoters in human and mouse *in vitro* differentiated Th2 cells (data not shown) as well as in Jurkat cells subjected to polarizing conditions. Interestingly, use of alternative promoters does not lead to change in sequence of the encoded protein. This suggests that different *SATB1* promoters may respond to different regulatory pathways depending on physiological context, in this case, T-cell activation vs polarization into T-helper phenotype. Thus, we propose that different transcription factors activated under different signaling cascades are involved in regulation of *SATB1* alternative promoters.

Our preliminary data suggest that *SATB1* P2 promoter expression is regulated via cytokine signaling mediated by *STAT6*. Consistent with its known role at enhancers, we find that STAT6 might be involved in looping an upstream putative enhancer with the P2 promoter. To study this interaction, we are performing chromosome conformation capture (3C) assay. We have standardized 3C assay in Jurkat cells and have prepared a BAC library. Since BAC DNA is not involved in looping in vitro, the ligation of digested BAC DNA will be random. This random interaction frequency in the BAC 3C library will be used to normalize interaction frequency obtained from Jurkat 3C sample. The final 3C experiment is yet to be performed.

We performed 3C assay in Jurkat cells and observed degradation of Jurkat DNA after the SDS treatment. This could be because of intra-nuclear endonuclease that remain

active and cleave nuclear DNA. The activity of these nucleases can be reduced by increasing formaldehyde concentration during crosslinking, increasing SDS concentration in nuclear suspension and changing the time and temperature of incubation in SDS buffer. We did not observe significant change in intra-nuclear DNA degradation with change in formaldehyde concentration (data not shown). Reduced DNA degradation was observed at higher SDS concentration. Also, reduced DNA degradation was observed by reducing the number of cells/nuclei per sample processed.

However, with increasing SDS concentration and incubation time of SDS treatment the restriction enzyme activity in digestion step was also adversely affected. Also, reduced cell number could adversely affect frequency of ligation. Therefore, we plan to increase restriction enzyme and DNA ligase concentration in future.

Regulation of *SATB1* P1 and P3 promoter under TCR signaling is currently under study. The resting and cytokine activated Jurkat cells express P3 promoter. We inhibited NF-κB in these activated cells and observed that P3 promoter expression was significantly downregulated whereas no significant effect was observed on *SATB1* P1 promoter. This suggests that NF-κB regulates *SATB1* P3 promoter under polarization conditions. Expression profile of NFAT and AP1 in PMA treated and PMA and Ionomycin (P+I) treated Jurkat cells suggests that NFAT does not regulate *SATB1* P1 promoter. We speculate AP1 family factor FOS may regulate *SATB1* P1 expression but this hypothesis needs to be tested.

Thus, regulation of *SATB1* expression via alternative promoter usage is a tightly regulated and complex process. Our preliminary data shows a discrepancy between the *SATB1* transcript and protein expression in TCR activation state versus polarization state (cytokine signaling activated state) respectively. This is not of any physiological relevance in Th0 cells whereas in non-T helper cells *SATB1* expression is less. It is possible that activation of TCR signaling leads to expression of basal *SATB1* expression via P1 promoter but not P2 promoter since P2 promoter is regulated by cytokine signaling. This hypothesis needs to be tested. Expression of *SATB1* P1 promoter during this state may be a conserved pathway.

High expression of *SATB1* via P2 and P3 promoters is observed in polarizing state. This polarization condition is important for T helper cell differentiation into multiple subtypes such as Th1, Th2, Th17 etc. This differentiation of T-helper cell requires expression of an additional set of effector genes and cytokine genes. Hence, higher expression of a chromatin organizer *SATB1* is required. Thus, utilization of alternative promoter for expression of *SATB1* in this case P2 and P3 promoters provides an extra layer of regulation. This may be an evolved mechanism of regulation of *SATB1* expression in T cell development and differentiation.

Future goals

- Perform 3C assay in activated Jurkat cells to study interaction of SATB1 P2 promoter and putative enhancer.
- Perform AP1 inhibitor assay to delineate its role in SATB1 P1 promoter expression.

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