Role of chromatin organizing protein SATB1 in Insulin like growth factor-1 (IGF-1) signaling

Thesis submitted by

Dipeshwari J Shewale

Department of Biological Sciences

Indian Institute of Science Education and Research

Under the guidance of

Mentor: Dr. Manjunath G.P

Department of Biological Sciences

Indian Institute of Science Education and Research

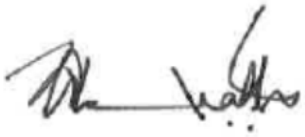
Advisor: Dr. Vidita Vaidya

Department of Biological Sciences,

Tata Institute of Fundamental Research, Mumbai

CERTIFICATE

This is to certify that this dissertation entitled ".**Role of chromatin organizing protein SATB1 in Insulin like growth factor-1 (IGF-1) signaling**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by "Dipeshwari Janardhan Shewale at Indian Institute of Science Education and Research (IISER),Pune" under the supervision of "Dr. Manjunath G.P, Scientist C, Department of Biology, IISER-Pune " during the academic year 2015-2016.



Signature of the Supervisor **Date:25.03.2015**
(Dr.Manjunath G.P)

DECLARATION

I hereby declare that the matter embodied in the dissertation report entitled **"Role of chromatin organizing protein SATB1 in Insulin like growth factor-1 (IGF-1) signaling"** are the results of the investigation conducted by me at the Department of biology, Indian Institute of Science Education and Research (IISER) Pune, under the guidance of Dr. Manjunath G.P, Scientist C, IISER Pune and the same has not been submitted elsewhere for any other degree.

Signature of the Student **Date: 25.03.2015**

(Dipeshwari Janardhan Shewale)

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ABSTRACT

Calorie restriction as a regime to regulate organismal life span has been known to be effective across a wide range of model organism including humans. Insulin and Insulin like growth factor (IGF) mediated signaling is one of the primary sensors of metabolic flux in several model organisms and is invoked during regulation of organismal life span by calorie restriction. Extensive research over the past two decades indicates that calorie restriction mediated increase in life span is caused by the down regulation of the IGFR mediated signaling. The down regulation of the IGFR pathway prevents phosphorylation of the FOXO family of transcription factors by both Akt/PI3K and JNK pathways. Phosphorylation leads to the retention of these factors in the cytosol due to their association with protein chaperones. The loss of the phosphate groups on the FOXO's leads to the disruption of their association with protein chaperones followed by their translocation into the nucleus.

The transcriptional activity resident downstream of IGF signaling is mediated via Forkhead family (FOXO1) transcription factors (FOXO). FOXO1 regulates the transcription of various insulin responsive (IR) genes by its association with a conserved DNA element called the Insulin Responsive Sequence (IRS). In addition, this processes is also likely to involve the participation of unknown transcriptional activators and/or repressors. We report the discovery of Special AT-rich Binding (SATB) proteins as novel repressors of transcription from IRS sequences. Our study shows the physical interaction between global chromatin organizing SATB1 protein and insulin responsive sequence (IRS) present. We also demonstrate a possible feedback mechanism in the form of a FOXO3a footprint on SATB1 promoter indicating that SATB proteins and IGF signaling regulate each other.

1. INTRODUCTION

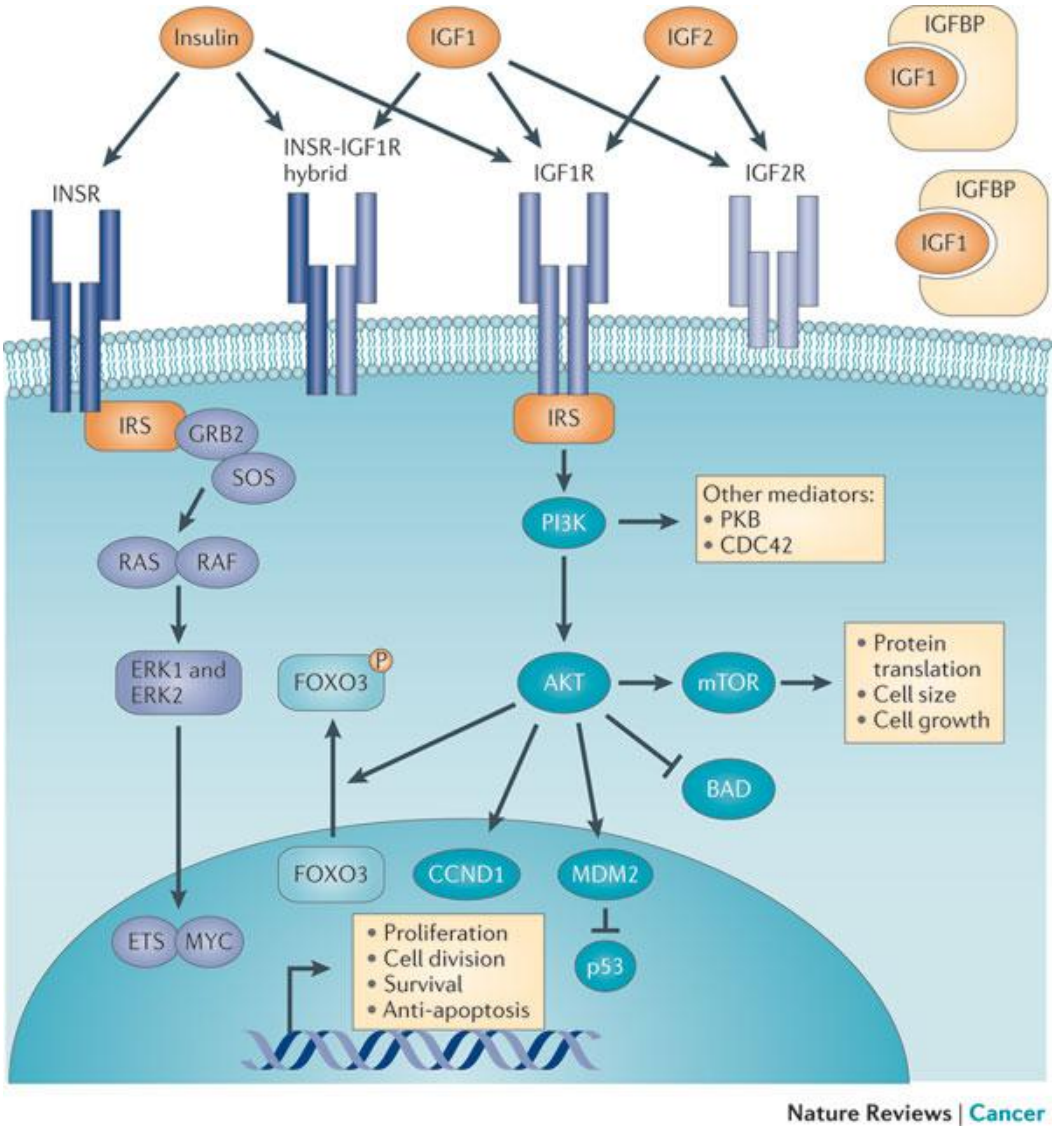
Calorie restriction as a regime to regulate organismal life span has been known to be effective across a wide range of model organism including humans. Insulin and Insulin like growth factor (IGF) mediated signaling is one of the primary sensors of metabolic flux in several model organisms. It is invoked during regulation of organismal life span by calorie restriction.

Extensive research over the past two decades indicates that calorie restriction mediated increase in life span is caused by the down regulation of the IGFR mediated signaling. The down regulation of the IGFR pathway prevents phosphorylation of the FOXO family of transcription factors by both Akt/PI3K and JNK pathways. While there does not seem to be a universal mechanism for the phosphorylation mediated localization of these transcription factors, it is observed in most cases that phosphorylation leads to the retention of these factors in the cytosol due to their association with protein chaperones. The loss of the phosphate groups on the FOXO's leads to the disruption of their association with protein chaperones followed by their translocation into the nucleus.

Calorie restriction decreases the risk of most of the age related pathologies and results in extension of lifespan by affecting different signaling pathways like Insulin/IGF-1 signaling pathway, AMPK pathway, Target of rapamycin (TOR) pathway and the Sirtuin pathway. CR is found to be an important player in tumor suppression in multiple types of cancers but it has drawbacks like lowering of body temperature, hunger continuing for a long time and lowered libido (Speakman and Mitchell, 2011). CR is known to affect the circulating levels of insulin *in vivo* and thus decrease the insulin/ IGF-1 signaling.

Lifespan regulation by IGF-signaling has been conserved during evolution from nematodes to mouse. It is shown in *C elegans* that many single gene mutations in the components of this pathway result in large increase in lifespan. Similarly, in the fly *Drosophila melanogaster*, mutations in both the insulin receptor and its substrate increase lifespan up to 48% in homozygous female flies (Clancy et al., 2001). Pit 1 mutation present in Snell dwarf mice decreases the insulin levels thus decreasing the

activity of IGF-signaling pathway. This results in long lived mice similar to daf-2 mutant



in *C elegans* (Hsieh et al., 2002).

Figure 1: IGF signaling in higher Eukaryotes Binding of Insulin like Growth Factor (IGF) to its receptor (IGFR) results in its dimerization and self phosphorylation. This results in the activation of PI3 kinase, which in turn activates the Akt signaling. The resultant phosphorylation of FOXO's and their association with protein chaperones 14-3-3. The FOXO proteins are unable to enter the nucleus and are retained in the cytoplasm. In the absence of IGF-IGFR interaction, FOXO's are dephosphorylated and dissociate from 14-3-3, translocating into the nucleus where they transcribe genes

responsible for proliferation, cell division, survival and apoptosis. Figure adapted from (Khandekar et al., 2011)

FOXO proteins control the transcription of genes involved in metabolism, cell growth and homeostasis, DNA repair so we see extension in lifespan as a result of inhibition of insulin /IGF-1 signaling (Daitoku and Fukamizu, 2007). But exact molecular mechanisms need to be elucidated.

Mutations in Growth hormone (GH) signaling in yeast, prevents it from accumulation of DNA damage and has important role in reducing insulin resistance and cancer in mice. Observations from humans with mutation in GH receptor gene showed reduced risk of cancer and diabetes than control. They exhibited reduced RAS, Protein kinase A and TOR signaling with increase in superoxide dismutase-2 gene expression, which causes lifespan extension in model organisms (Guevara-Aguirre et al., 2011). Dwarf mice having mutations in components of IGF1 signaling show extension in lifespan and resistance to cancer. Naked mole rats are long lived and resistant to cancer. High molecular mass Hyaluronan stimulates CD44 cells to arrest cell cycle progression and repress mitogenic pathway in naked mole rats. It also induces high sensitivity to contact inhibition in these rats thus making them resistant to cancer (Gorbunova et al., 2014). Calorie restriction reduces invasion and growth of tumor cells implanted in VM mouse as compared to mice fed on ad libitum in case of most aggressive human cancer known as glioblastoma multiform (Shelton et al., 2010). Some of the gram positive and negative bacterial pathogens are known to infect *C elegans* using same virulence factors by which they affect humans. It is seen that daf-2 mutant and age-1 mutant live two times longer than wild type and are resistant to these pathogens. This pathogen resistant phenotype is suppressed by daf-16 which proves that the resistant phenotype is regulated by IGF signaling pathway (Garsin et al., 2003). It is better to stay away from pathogens than stimulating costly immune response on infection with pathogen.

Insulin signaling is known to regulate stress response in *C.elegans.* Worms with mutation in IGF receptor exhibit behavioral avoidance of pathogen by reducing the uptake of bacteria in pathogen rich areas (Hasshoff et al., 2007). Heterozygous knockout mice for igfr(+/-) lives 26% longer than wild type and it shows increased

resistance to oxidative stress which is hallmark of aging. This study indicates that IGF signaling may be controlling lifespan in mammals via reduction in oxidative stress (Holzenberger et al., 2003). Above studies show that disease burden, oxidative stress is reduced and pathogen resistance is increased in organisms with mutations in IGF signaling pathway and can together lead to lifespan extension.

Genetics of CR mediated lifespan extension is extensively studied but very little is known about the epigenetic regulation of this phenomenon. In CR mediated lifespan extension expression of many different genes is regulated in a coordinated manner. In *C elegans* these genes responsible for DNA repair, homeostasis, reduction in oxidative stress and many more functions are controlled by Daf-16 and RNAi of these genes shows that they are players in distinct functions of Daf-16 (Greer et al., 2007; Lee et al., 2003). This implicates the role of higher order chromatin organization in this process.

Aging may be controlled by epigenetic factors like DNA methylation, histone modifications and micro-RNA expression. Sir2 is a histone deacetylase, which is major player in lifespan extension in yeast and its inactivation leads to reduction in replicative lifespan of yeast. Sir2 requires NAD as cofactor and its orthologs in worms and flies also lead to increase in lifespan. Overexpression of Sir2.1 in *C elegans* increases the mean lifespan by up to 50%. It is also known that DNA methylation in different human tissues decreases with age (Berdasco and Esteller, 2012; Longo and Kennedy, 2006). Heterochromatin is marked by enrichment of H3Kme3 and Heterochromatin Protein-1 (HP-1) and it is seen that heterochromatin is lost in late passage fibroblasts of humans. This loss in heterochromatin and nucleosome may lead to transcriptional deregulation and changes in DNA repair which leads to aging. Similar changes in heterochromatin were seen in *Drosophila melanogaster* (O'Sullivan and Karlseder, 2012; Wood et al., 2010). Recent study in *C elegans* proposes that DAF-16 (FOXO) control transcription of several IR genes to promote stress resistance and longevity by recruiting BAF-like subclass of ATP-dependent chromatin remodeler SWI/SNF (Riedel et al., 2013).

There is strong co-relation between proteins involved in aging and age-related pathologies, it is therefore, likely that above-mentioned chromatin organizing proteins are involved in age-related pathologies such as cancer and diabetes. SATB1 is global

chromatin organizer that brings together different gene loci and recruits multiple chromatin remodeling enzymes to control gene expression. It is involved in repression of tumor suppressor genes and up regulation of genes necessary for metastasis in breast cancer cells. SATB1 knockdown using RNAi in aggressive cancer cells leads to reversing of tumor progression and metastasis and opposite effect is seen on ectopic expression of SATB1(Han et al., 2008). SATB1 levels are higher in number of prostate cancer cells as compared to non-tumorigenic cells and it increases the invasive potential and motility of the cells by decreasing the E-cadherin expression in the cells (Shukla et al., 2013).

SATB1 and its binding partners CREB binding protein (CBP) and p300 are induced in mouse hypothalamus upon nutritional deprivation. SATB1 and CBP can be used as markers for age and they are shown to decrease with aging and diabetes in five different strains of mice. It is shown that cbp-1 RNAi in adult *C elegans* blocks lifespan extension by dietary restriction and *dve*1 RNAi in daf-2 mutant background partially attenuates the lifespan extension seen due to the mutation in daf-2 (Zhang et al., 2009). Consistent with these findings, we have demonstrated that both SATB1 and SATB2 act as robust repressors of transcription from Insulin Responsive Sequences (IRS) in human cell lines and that DNA binding domains of SATB1 are essential for this transcriptional activity. In addition we have also demonstrated physical interaction of SATB1 with human IRS sequences *in vitro* as well as DAF-16 homologs FOXO1, FOXO3a and FOXO4 *in vivo.*

The mechanism by which chromatin remodelers are able to influence aging is poorly understood. Regulation of ageing and age related pathologies is regulated by the perturbing the transcriptional status of several genes most of which are regulated by the Forkhead family of transcription factors (FOXO). One possible mechanism may involve the modulation of the accessibility of the genes that are transcribed from promoters controlled by Forkhead family of transcription factors by chromatin remodelers. This is especially pertinent in case of the dominant negative mutant of mice that over express the PDZ domain of SATB1. These mice display several phenotypic characters that mimic those observed in long-lived mutants in mice. These include enhanced life span as well as accumulation of visceral fat. Interestingly though, unlike the Ames and Snell

dwarf mice, these mice are not sterile. The over expression of PDZ domain may directly influence the interaction between FOXO transcription factors and SATB1 by a titration effect. It is also possible that the presence of stoichiometric excess of PDZ domain in the cellular pool may disrupt other protein-protein interactions and perturb the cellular machinery resulting in the long-lived phenotype observed in the *satb1* dominant negative mice in as yet undefined manner.

This study aims to address the mechanism by which SATB1 and SATB2 regulate transcription in response to insulin signaling. The specific topics to be addressed are

1. To find different binding partners of Insulin responsive sequence in presence and absence of IGF signaling in Human Embryonic Kidney (HEK293T) cells.
2. What domains of SATB1 are involved in regulating IGF mediated gene expression?

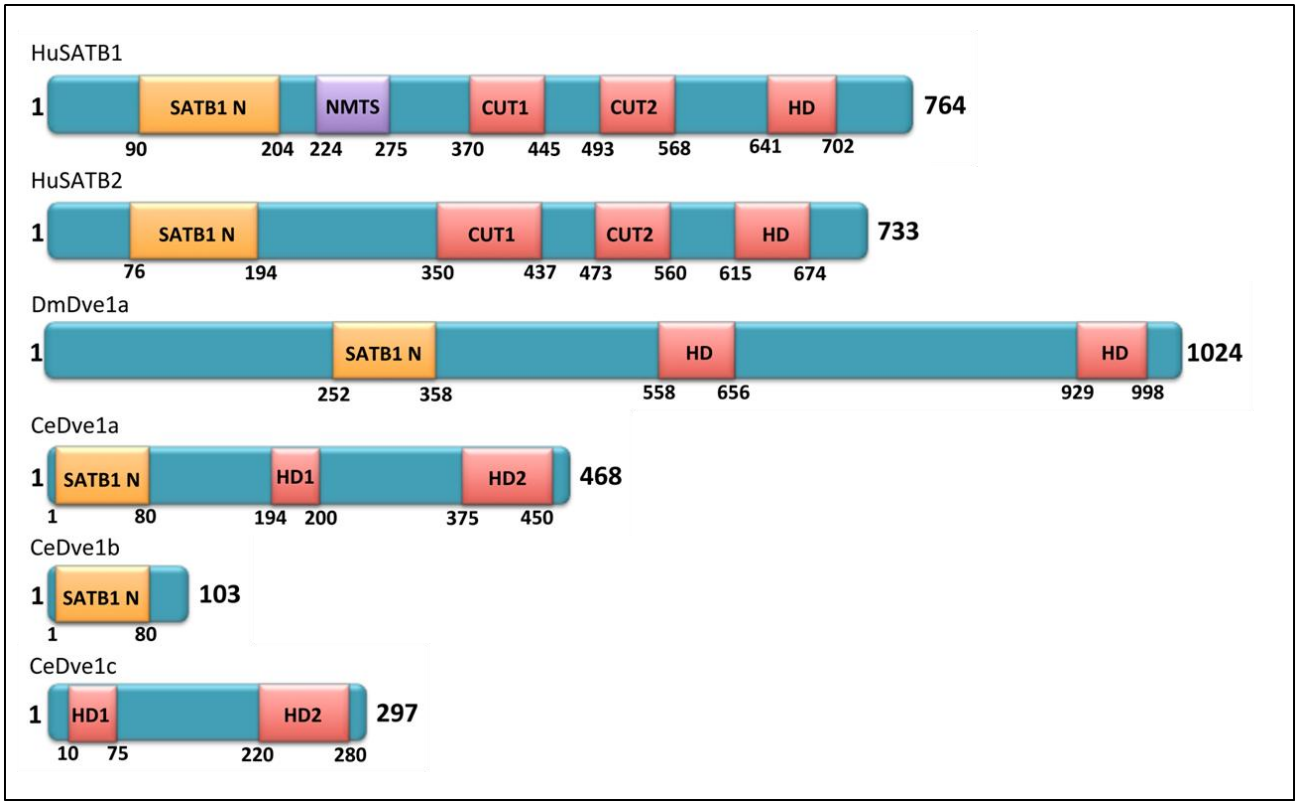


Figure 2: Domain organization of SATB family proteins. SATB family of proteins has conserved protein-protein interaction domains called PDZ domain near the N-terminal. Human SATB Proteins contain nuclear matrix targeting sequence, two CUT domains

and a homeodomain (HD) which are necessary for DNA binding activity. CUT domain is absent in fly and worm DVE1 protein.

2. MATERIALS AND METHODS

2.1 Materials:

Oligonucleotides were purchased from SIGMA-GENOSYS and Integrated DNA technologies. DNA polymerase, restriction endonucleases and DNA ligase were bought from New England Biolabs (NEB). Fine chemicals were purchased from SIGMA-ALDRICH, Invitrogen and Roche. Animal tissue culture media components were obtained from Invitrogen. Composition of all the buffers used in the study is given in Appendix A.

2.2 Isolation of Nuclear Lysate:

Nuclear lysate was prepared from Human Embryonic Kidney (HEK) 293T cells using a protocol reported earlier (Abmayr et al., 2006).Cells were washed with ice cold Phosphate buffer saline (1XPBS) for twice and scraped in fresh 1X PBS. Cells were then centrifuged at 3000rpm, 4˚C for 10 minutes. The supernatant was discarded and packed cell volume (PCV) was determined using graduation mark on the falcon. Cells were suspended in 5 PCV of hypotonic buffer and centrifuged at 3000rpm for 5 minutes. The cell pellet was again suspended in 3 PCV of hypotonic buffer and incubated on ice for 20 minutes to swell. Cells were then transferred to a glass dounce homogenizer and homogenized with 10 up and down strokes with the help of loose pestle. Cells were shifted to 1.5ml micro centrifuge tube and centrifuged at 100g for 5 minutes. Supernatant was discarded and cells were suspended in 1ml 1XPBS. Nuclei were isolated by centrifugation at 3300g for 15minutes. To extract the nuclear lysate mix the nuclear pellet in half of the packed nucleus volume (PNV) of low salt buffer and high salt buffer consecutively. They are then rotated at 10 rpm on an end-to-end rocker. After 1 hour the nuclear lysate was extracted by centrifugation at 25000g for 45 minutes and the pellet was discarded. The lysate was then dialyzed against dialysis buffer for 3 hours. Protein concentrations were determined using bicinchoninic acid assay (BCA assay) kit (Pierce).

2.3 Electrophoretic mobility shift assay (EMSA):

Oligonucleotides with 3 tandem repeats of Insulin responsive sequence (3XIRS) and IgH matrix associated region (IgH MAR) were synthesized using solid phase synthesis (Table 1). Duplex probes were assembled from oligonucleotides and purified on PAGE.

Assembly of nucleoprotein complex was performed in a reaction volume of 20µL. Increasing concentration of nuclear lysate was incubated with a fixed concentration of cy5 labeled duplex probe in the presence of 20mM Tris-HCl (pH7.5), 1mM MgCl2. This reaction was incubated on ice for 15 minutes and 5ul of loading dye (glycerol and EDTA) was added to terminate the reaction. The sample was then loaded on 7.5% native page and ran at 100V, 4˚C for 3 hours in 0.5X TAE. Bromophenol blue was loaded on either side of the samples to track the mobility of the oligonucleotides. Increasing concentration of the probes (3XIRS and 3XIgHMAR) was loaded on 7.5% Native Page and 4uL of probe was used for further reactions. The probes were then incubated with increasing concentration of nuclear lysate from 2µg to 10 µg (Figure 3) and at the highest concentration the probe was completely utilized. Typhoon Trio $^{+}$ Variable mode Imager was used to image the EMSA gels.

2.4 Western blotting:

The EMSA gel containing two reactions of 10ug nuclear lysate with 4 ul of 3XIRS and 3XIgHMAR was transferred on nitrocellulose membrane for 1 hour 30 minutes at 0.6 ampere. The blot was then blocked in 5% skimmed milk for 1 hour and was probed with anti-SATB1 antibody (ab49061/abcam,1:5000 dilution) at 4˚C overnight. Three TBS-T (Transfer buffer saline with 0.1% Tween 20) washes of 7 minutes each were given to remove anything binding non-specifically to the blot. Blot was then probed with secondary anti-rabbit antibody for 1 hour at room temperature. Blot was again given three washes as mentioned above. It was developed using Millipore developing reagents on ImageQuant™ LAS 4000.

2.5 Transfection of HEK 293T cells

SATB1 and SATB2 knockdown was achieved by transfecting HEK293T cells with constructs expressing short hairpin (sh) RNA against SATB1 and SATB2 as well as for overexpression of SATB1 and SATB2. Cells were seeded

in a 6-well plate and were allowed to form an adherent layer overnight. Liposome mediated transfection was done when the cells were approximately at 60% confluent using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. 2.5 µg of plasmid and 2.5µl Lipofectamine™ 2000 was used for each transfection. Cells were starved for for 30 minutes using OptiMEM followed by addition of liposomes. Liposomes were removed after 8 hours of incubation at 37°C and replaced with complete DMEM. Cells were allowed to grow for 48 hours and whole cell lysate was prepared using RIPA buffer.

2.6 Chromatin Immunoprecipitation (ChIP)

2.6.1 Preparation of chromatin

Chromatin was prepared using 90% confluent T-75 flask containing HEK293T cells. Cells were fixed by adding formaldehyde to final concentration of 1%. Formaldehyde was added directly to the culture media in drop wise manner and the flask was incubated for 10 minutes at room temperature (RT) with constant shaking. Glycine was added to final concentration of 150 mM to quench excess of cross-linker. Cells were incubated for 10 minutes at RT with constant agitation and media was aspirated after 10 minutes. Cells were washed twice with ice cold PBS and scraped off in 10ml PBS with protease inhibitor complex. They were harvested at 1500rpm for 10 minutes at 4˚C and the pellet was suspended in 1.6 ml of cell lysis buffer for ChIP. Cells were incubated for 15 minutes on ice and the cell nuclear pellet was obtained by centrifugation at 1000rpm for 10minutes at 4˚C. Nuclear pellet was suspended in 2 mL of nuclear lysis buffer for ChIP and incubated for 20 minutes on ice. Chromatin shearing was performed by sonication using a focused sonicator (Covaris-S2). The conditions were as follows:

Duty cycle	5
Intensity	4%
Cycles per burst	200

Number of cycles	20
Temperature of the water bath	6-8˚C

Chromatin was obtained by centrifuging these sonicated samples at 14000rpm for 20 minutes at 4˚C. Chromatin was transferred in 1.5 ml tubes and stored at -80˚C. Before freezing, 50µl of chromatin was taken in fresh 1.5 ml tube and the volume was made up to 300µl using sonication buffer. Crosslinks were reversed at 65˚C overnight using 20µL 5M NaCl and 1mg/ml RNase. Chromatin was deproteinised using 20 µg proteinase K in 20mM of TrisCl ph7.9 and 1mM EDTA at 42°C for 1 hour. DNA was extracted using Phenol-Chloroform and precipitated with ice-cold ethanol. Concentration of the chromatin was determined spectrophotometricaly. Size of the chromatin was established by resolving 1.0 µg of chromatin on a1% agarose gel using a 1kb ladder as a reference.

2.6.2 Pre clearing and Immunoprecipitation

35 µg of chromatin was used for each immunoprecipitation. The volume of the reaction mixture was made up to 1ml using chromatin dilution buffer. The solution was cleared using unsaturated magnetic beads (10µl slurry washed with ChIP buffer) for 2 hours. Cleared chromatin solution was incubated with 10µL anti-FOXO3A antibody (Cell signaling, cat. Number). Rabbit IgG was used as a negative control. The chromatin was allowed to interact with antibody overnight at 4˚C. Immune complex was isolated using magnetic affinity beads (), the complex was washed thrice with low salt buffer, thrice with high salt buffer and thrice with a buffer containing Lithium chloride. This was followed by three washes with Tris-EDTA, pH8.0. The chromatin was eluted in 150µL of elution buffer twice and the samples were pooled. The reaction volume was made up to 300µL using ChIP buffer where necessary. The cross links were reversed as described earlier.

Binding of FOXO3A on SATB1 promoter was established by PCR amplification using primers for a region upstream of transcription start site of the SATB1 gene.

2.7 Cloning and purification of SATB1/2 deletion constructs

SATB1 is global chromatin organizer and transcription factor integrating higher order chromatin architecture with gene regulation. To check which domains of SATB1/2 are important in regulating expression of IR genes different N and C-terminal domains and some important domains like CUT, HOX(DNA binding domain), and NMTS (Nuclear Matrix Targeting Sequence) were deleted. Amino terminal SUMO fusions are targeted to nuclear periphery so we created K233R and K350R, two lysine deletions at SUMO conjugation sites of SATB2. Other SATB1/2 domains were deleted based on the structure of the protein. SATB1/2 and its different constructs were amplified using Q5 High Fidelity DNA polymerase by overlap extension PCR. Preparatory PCR was performed for SATB1 and its constructs. The PCR products obtained were purified by Phenol: Chloroform: Isoamyl alcohol (25:24:1) extraction and precipitated using chilled ethanol and sodium acetate pH 5.2.

These SATB1 constructs were gel eluted using Qiagen MinElute gel extraction Kit and they were double digested using BamH1 and EcoR1 enzymes and 5 X NEB buffers 2 along with pTriEX. SATB1 constructs were ligated to pTriEX (mammalian expression vector) using Quick ligase (NEB). DH5α competent cells were transformed using this ligation mix and were grown on ampicillin plates. Insert release was checked for 5 colonies from each construct by digesting with BamH1 and EcoR1. Mini preparation of DNA was done for all the 14 constructs and DNA was obtained and sent for sequencing. The SATB1 constructs were transformed in electro-competent cells by electroporation and then maxi preparation was done to obtain super coiled DNA for transfection.

All the agarose gel images obtained during cloning and purification are given in Appendix B

2.8 Codon optimization, cloning and purification of SATB1

SATB1 coding sequence from human genome was examined for occurrence of rare codons that were likely to influence its expression in bacteria. Graphic Codon usage analysis was performed using online tool graphic codon usage analyzer (http://gcua.schoedl.de/sequential_v2). Several rare codons were identified on the basis of this analysis.

Oligonucleotides were synthesized using solid phase synthesis (1st Base) and coding sequence optimized for expression in *E. coli* was assembled using overlap extension PCR (OEP). The amplicon obtained from gene synthesis was ligated into bacterial expression vector pET17b (Novagen) using RE sites NdeI and BamHI. Identity of the construct was confirmed by insert release upon digestion with NdeI and BamHI. The clones were used to transform *E. coli* BL21 (DE3) Star competent cells. Transformed cells were selected on double antibiotic selection of ampicillin and chrloramphenicol. Two colonies from each clone were analyzed for expression of SATB1 upon addition of IPTG on a pilot scale. Clone number five showed robust expression of SATB1 and was sequenced to ensure the absence of mutations.

E. coli Rosseta strain harboring plasmid pET17b-HuSATB1 was cultured in Luria-Bertani broth (1ml) containing 50 µg/ml ampicillin at 37°C. At mid exponential phase (A600 ~0.4), SATB1 expression was induced by the addition of isopropyl-1-thio--D-galactopyranoside to a final concentration of 1 mM. Cells were further incubated for 12hours at 25ºC and collected by centrifugation at 6000 g for 10 min. All subsequent steps were performed at 4°C unless indicated otherwise. Bacterial cells were resuspended in 50 mM Tris-HCl (pH 8) containing 10% (w/v) sucrose, (4 ml per gram of wet cell paste) quickly frozen, and stored at -20°C. To the cells thawed overnight on ice. Lysozyme was added to a final concentration of 200 mg/ml, and the suspension was incubated at 4°C for 30 min. The cells were disrupted by sonication (Vibra Cell Sonicator, Sonics and Materials, Inc., Danbury, CT) in a pulse mode (50% duty cycle) for 30 min. The cell lysate was clarified by centrifugation at 25,000 rpm in a Beckman JLA25.5 rotor for 1 hour.

HuSATB1 was precipitated from the clarified cell lysate by ammonium sulfate fractionation (25% saturation) over one hour at 4^{o}C. The precipitate was collected by centrifugation at 14000 rpm in a Beckman JLA 25.5 rotor for 30 minutes. The precipitate was dissolved in half the original volume of the cell lysate in buffer A [20mM TrisCl (pH 8), 250mM NaCl, 1mM EDTA, 10% (v/v) glycerol and 1mM 2 mercaptoethanol]. The sample was dialyzed against three changes of buffer A at every 6 hours. The dialysate was applied to a 20ml Heparin Sepharose column equilibrated in buffer A. The eluate was pooled and applied to a 20ml Q-sepharose column equilibrated in buffer A. The column was washed until the A_{280} of the eluate was nearly zero. The bound proteins were eluted with a linear gradient of 250mM to 500mM NaCl in buffer A over 20 column volumes. HuSATB1 eluted between 400 and 450mM NaCl. The samples were pooled, concentrated and applied to a size exclusion column (S-300; 120ml). The proteins were resolved in 1.5 column volumes of buffer A at the flow rate of 1 ml/min. The peak fractions were analyzed on 10% SDS PAGE and fractions containing a homogenous solution of SATB1 were pooled. The pooled samples were dialyzed against storage buffer containing 20mM TrisCl (pH 8), 100mM NaCl, 20% Glycerol and 1mM DTT. The proteins samples were quantified and stored at -80^{o}C.

3. RESULTS

3.1 Physical Interaction of nuclear proteins with 3XIRS and 3XIgH MAR

The results of our experiments showed interaction of 3XIRS and 3XIgHMAR with protein present in the nuclear lysate isolated from HEK293T cells. A dominant complex of molecular weight in the range of a few mega Daltons was visible as a complex that failed to enter the gel. In addition, several smaller complexes were visible especially when higher concentration of nuclear lysate was used. We were unable to resolve the nucleoprotein complex present in the wells further by altering the percent of PAGE, or ionic conditions (Figure 3).

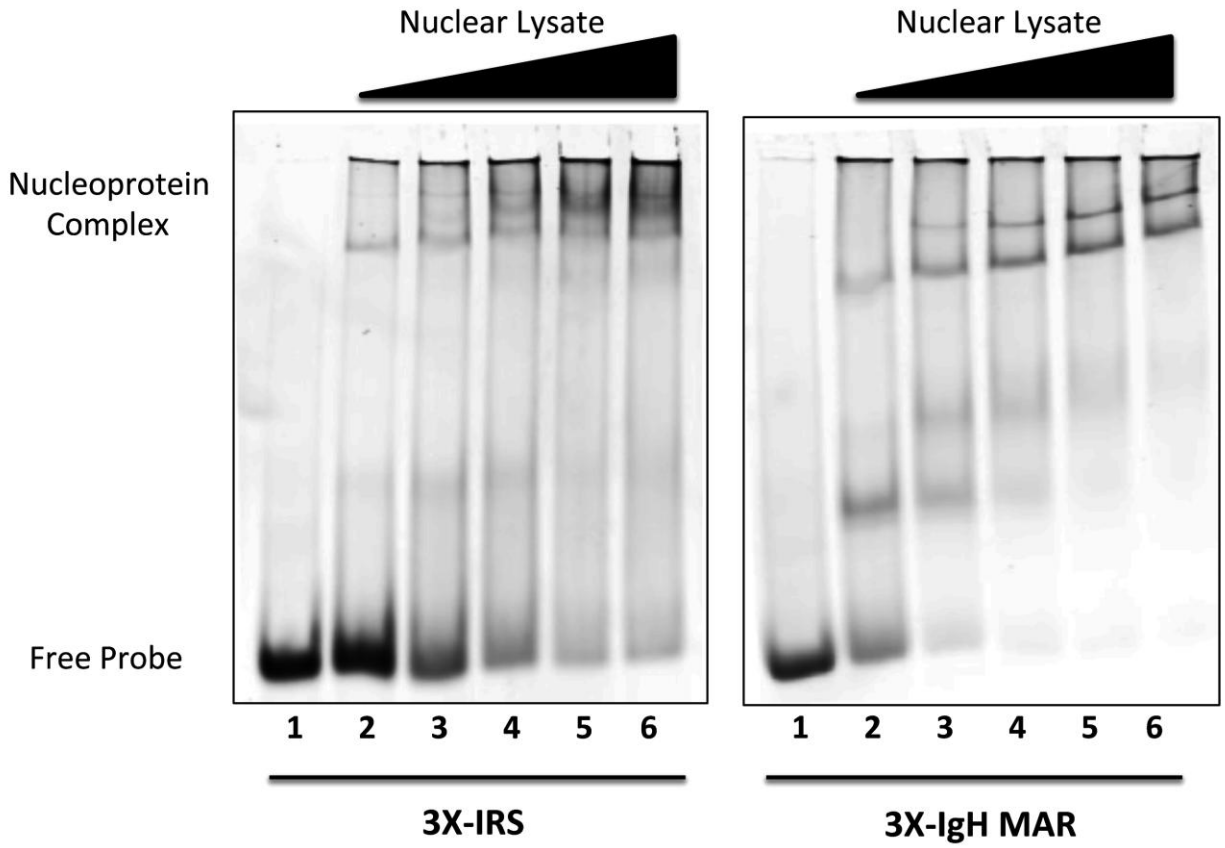


Figure 3: Nuclear proteins present in HEK 293T lysate interact with 3X IRS sequence. Interaction Insulin Responsive Sequence (IRS) and IgH Matrix Associated Region (IgH-MAR) with nuclear proteins isolated from Human Embryonic Kidney (HEK) 293T cells. Tandem repeats of IRS and IgH MAR sequences labeled with Cy5 at 5' end was incubated with increasing concentrations of nuclear lysate on ice. The

nucleoprotein complexes were separated from the free probe by electrophoresis on a 7.5% polyacrylamide gel in 0.5X TrisAcetate EDTA (TAE).

3.2 SATB1 is present in the nucleoprotein complex with 3XIRS

In order to detect the presence of SATB1 in the nucleoprotein complex formed in the presence of 3X IRS, we transferred the DNA-protein complexes to a nitrocellulose membrane and probed with anti-SATB1 antibody (Rabbit polyclonal raised in house). We were able to detect the presence of SATB1 in the large complex that failed to enter the gel but not in the complexes that migrated relatively fast.

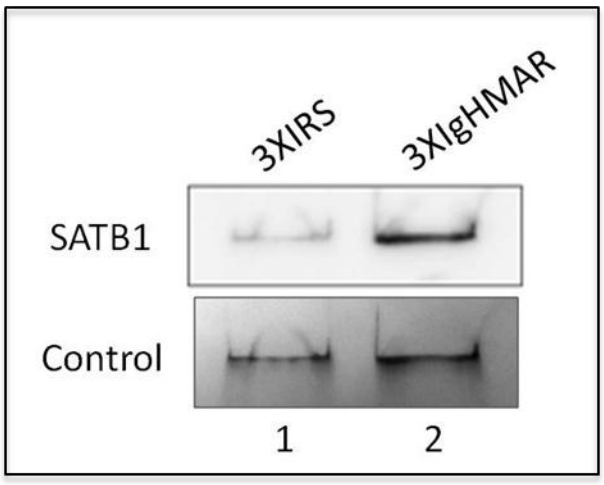


Figure 4: SATB1 is present in the nucleoprotein complex assembled at both 3X IRS and 3X IgH MAR. Nucleoprotein complexes were assembled by incubation of nuclear lysate with Cy5 labeled oligonucleotides. The complexes were blotted on to nitrocellulose membrane and probed with anti-SATB1 antibody. Staining with Ponceau was used to detect total proteins.

3.3 SATB proteins act as repressors of transcription from IRS sequence: We assayed for the effect of SATB proteins on transcription from IRS sequences using the firefly luciferase system. Coding sequence for firefly luciferase was cloned downstream of 3X IRS and transfected along with overexpression constructs for SATB1 and SATB2 or constructs that expressed short hairpin (sh) RNA against SATB1 and SATB2. We observed that the luciferase activity was reduced drastically in the presence of both

SATB1 and SATB2. Alternately, reducing the levels of SATB1 or SATB2 resulted in elevated levels of transcription from 3X IRS sequence.

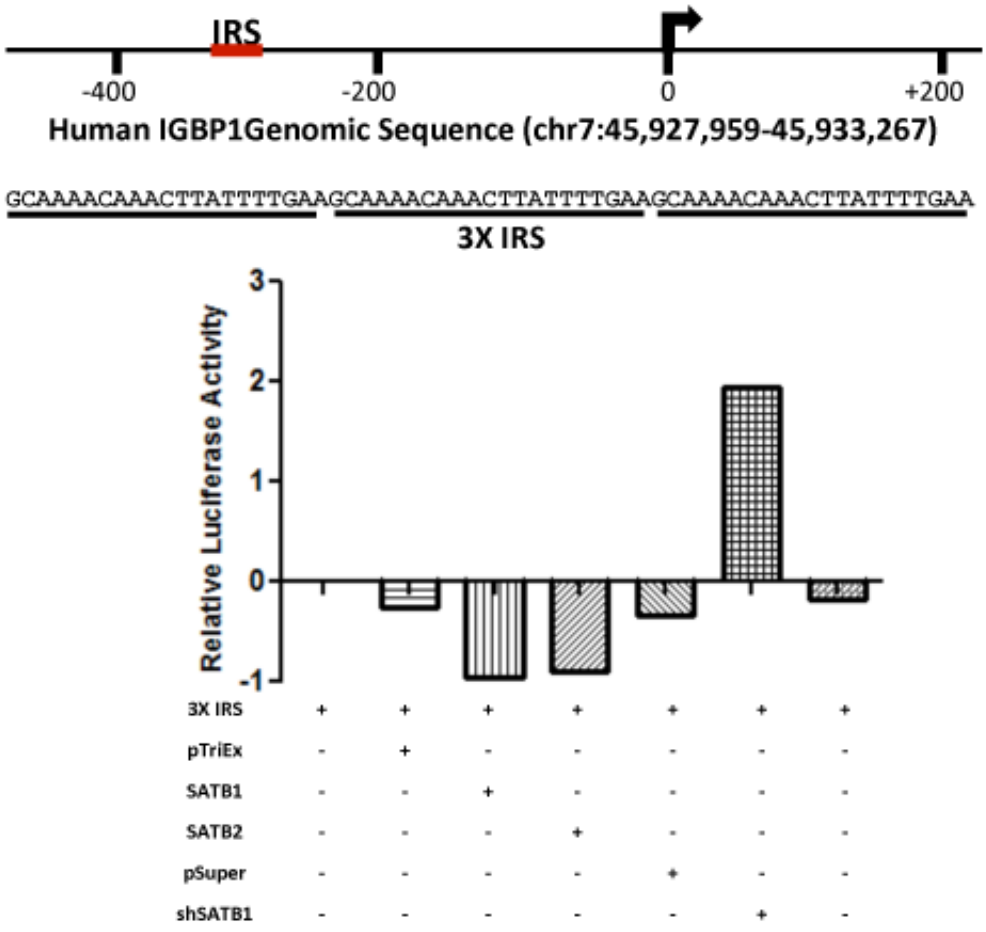


Figure 5: SATB proteins act as repressors of transcription from IRS sequence. Schematic representation of the IRS sequence upstream of IGBP1 gene present on chromosome 7 in the human genome **(Top Panel).** Luciferase activity was used as a measure of transcription from a construct containing three tandem repeats of IRS upstream of firefly luciferase gene. Constructs for overexpression or knockdown were co transfected with the luciferase construct and luciferase activity was measured 48

hours after transfection. pTriEx-3-Neo and pSuper plasmids were used as transfection controls **(Bottom panel).**

3.4 DNA binding domain of SATB1 is essential for its transcriptional repression activity. We performed Luciferase mediated transcription assay to determine the SATB1 domains responsible for transcriptional repression. 3X IRS :: Luciferase construct was co transfected with SATB1 variants cloned in pTriEx-3-Neo. Luciferase activity was measured 48 hours after transfection. We found that DNA binding domains of SATB1 were essential for its ability to repress transcription from 3X IRS sequence. Constructs expressing only the N-terminal region acted as dominant negative alleles and resulted in higher luciferase activity when compared to the control.

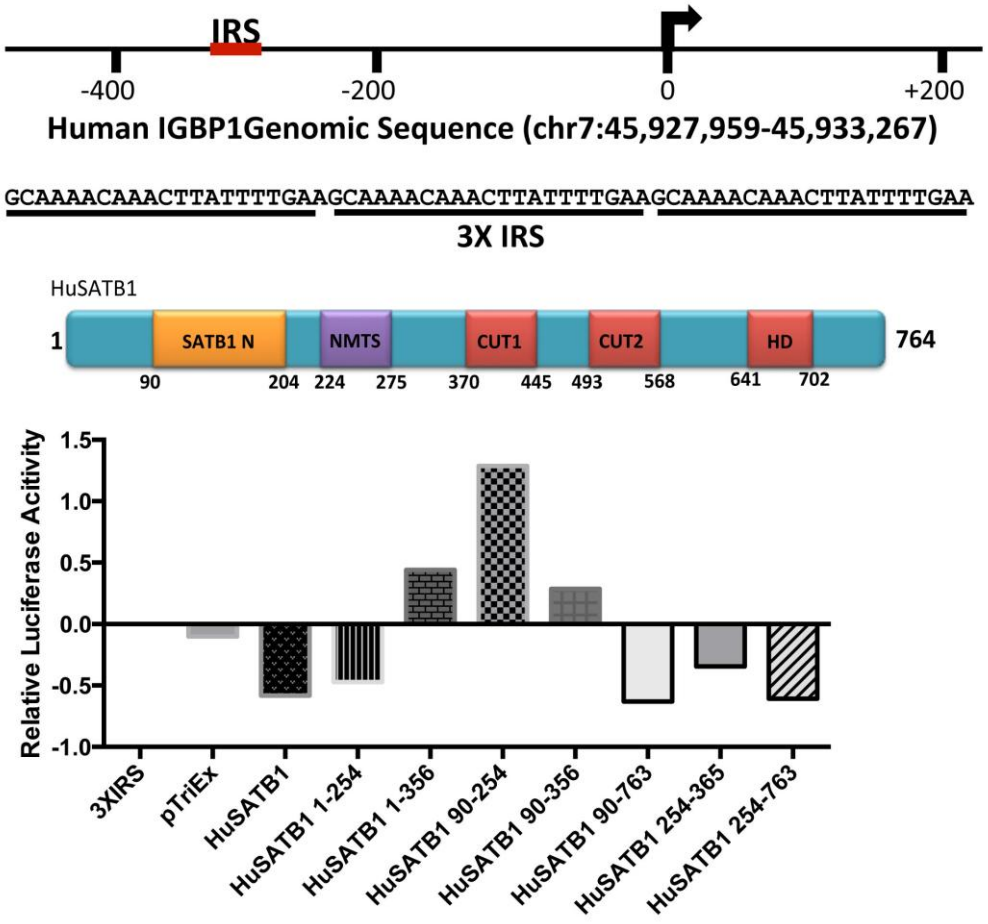


Figure 6: DNA binding domains of SATB1 are essential for its transcriptional activity. Schematic representation of the IRS sequence present upstream of the IGBP1

gene present on chromosome 7 in human genome **(Top panel).** Schematic representation of the domain organization in Human SATB1 **(Central panel).** Luciferase activity from 3X IRS sequence was measured in the presence of SATB1 variants. Luciferase activity was normalized using the activity in the presence of 3XIRS::Luciferase construct alone **(Bottom panel).d**

3.5 SATB1 interacts physically with Forkhead family transcription factors. In order to test if there was physical interaction between FOXO proteins and SATB1 we performed co immunoprecipitation. We found that SATB1 was precipitated along with FOXO proteins. We used Rabbit IgG as a negative control and CBP, a known interacting partner of SATB proteins as a positive control. We found SATB1 in immune complex isolated using antibodies against FOXO1, FOXO3a and FOXO4 indicating that SATB1 interacts with all three isoforms of FOXO *in vivo.*

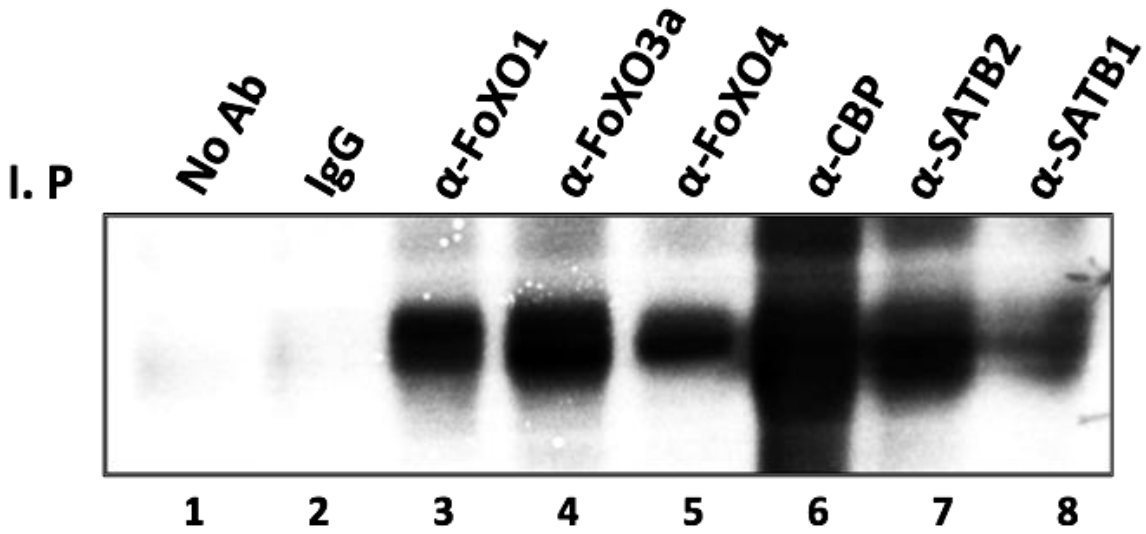


Figure 7: SATB1 physically interacts with Forkhead family transcription factors *in vivo.* Nuclear lysate isolated from HEK 293T cells was incubated with appropriate antibodies overnight at 4°C. The immune-complex was isolated and resolved on a 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with anti-SATB antibody (Rabbit polyclonal raised inhouse). Immuno precipitation with anti-CBP and anti-SATB1 antibody was used as a positive control.

3.6 FOXO3A binds SATB1 promoter; potential feedback loop between IGF signaling and SATB1. Chromatin Immunoprecipitation (IP) performed with FOXO3A antibody showed the presence of FOXO binding site on SATB1 promoter. IP with rabbit IgG which was our negative control did not show any amplification with primers against SATB1 promoter as compared to the positive control which is 10%Input and FOXO3A IP.

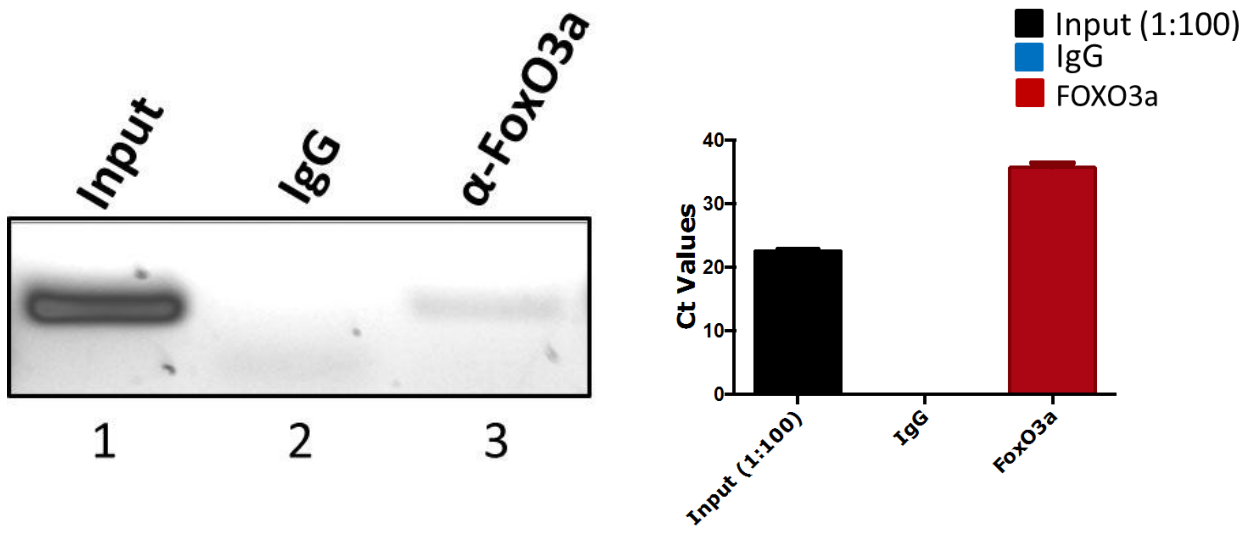


Figure 8: FOXO3a binds SATB1 promoter and may potentially regulate its transcription in response to IGF signaling. Chromatin immunoprecipitation (ChIP) was performed using HEK 293T cells. The cross-linked chromatin was incubated with either Rabbit IgG or anti-FOXO3a antibody (Cell Signaling catalog number) overnight. The DNA-protein crosslinks were reversed and the purified DNA was used as a template for PCR reaction using primers flanking the SATB1 promoter. Both input and chromatin immunoprecipitated with anti-FOXO3a antibody showed amplification of a 200bp fragment, whereas the immunoprecipitate isolated using IgG showed no amplification.

3.7 Optimization of codons for Bacterial expression and purification of HuSATB1. Codon usage analysis in the coding sequence for Human SATB1 was performed using online tool graphic codon usage analyzer. We identified several codons that are poorly represented in *Escherichia coli*. In order to improve the expression of human SATB1 in bacterial expression systems we optimized codon usage by synthesizing the optimized orf using overlap extension PCR. The optimized orf showed higher expression of SATB1 when compared to the native sequence and we were able to purify SATB1 to near homogeneity.

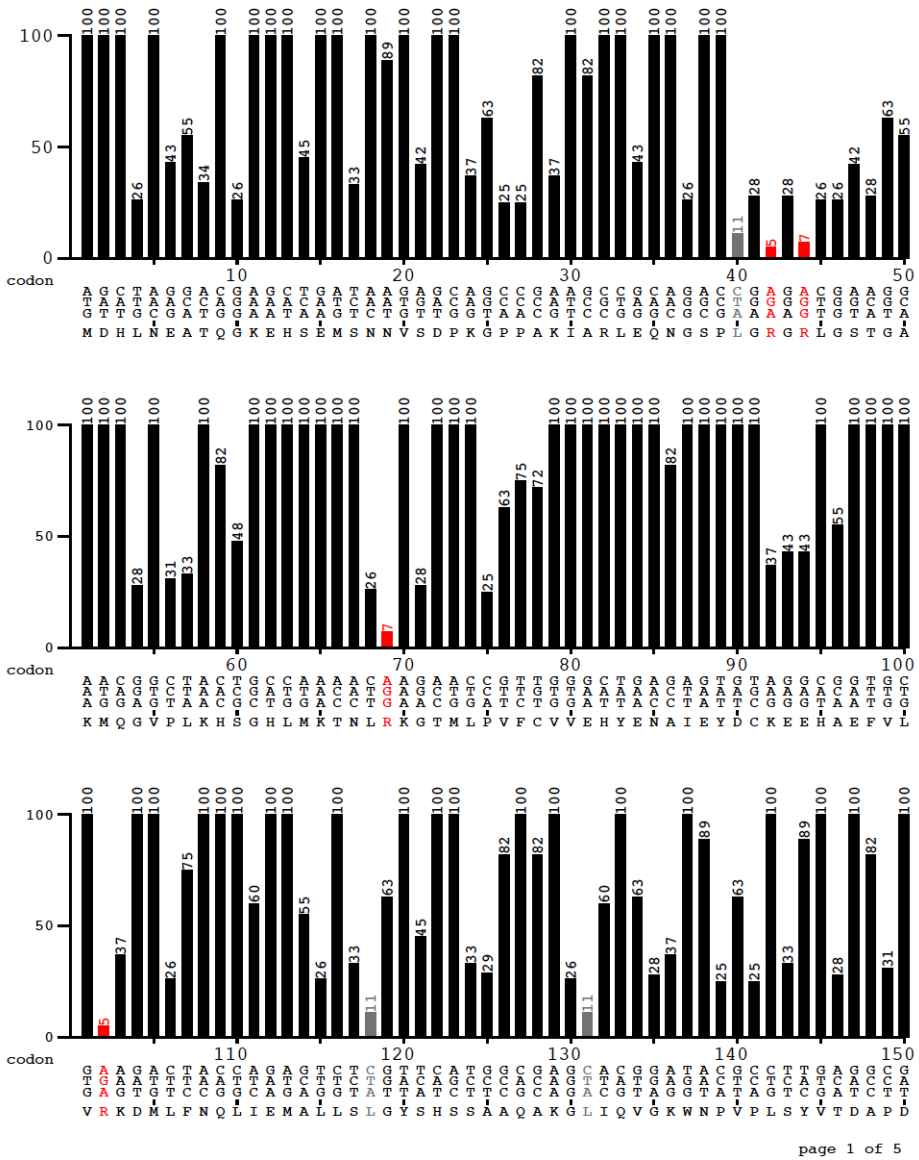


Figure 9: Codon usage analysis by Graphical Codon Usage Analyzer (GCUA). The native coding sequence for human SATB1 was obtained from UCSC genome browser

and analyzed for codon usage frequency. Codon usage was represented using an adaptive index, 100 being the most optimum codon and any codon with an adaptive index of less than 10 was classified as suboptimal.

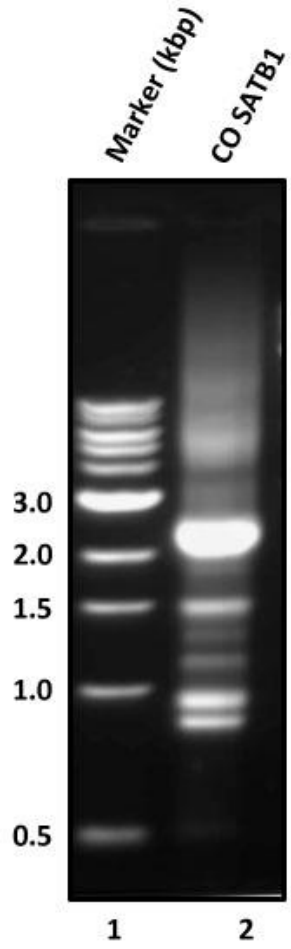


Figure 10: Assembly of artificial SATB1 coding sequence adapted for bacterial expression. Overlapping oligonucleotides were synthesized using solid phase synthesis. The complete coding sequence was assembled using overlap extension PCR. The PCR was catalyzed by Thermococcus kodakaraenis (KOD) DNA Polymerase. The artificial gene was cloned in bacterial expression vector pET17b (Novagen) and used to transform *E. coli* BL21 (DE3) Star cells for protein expression.

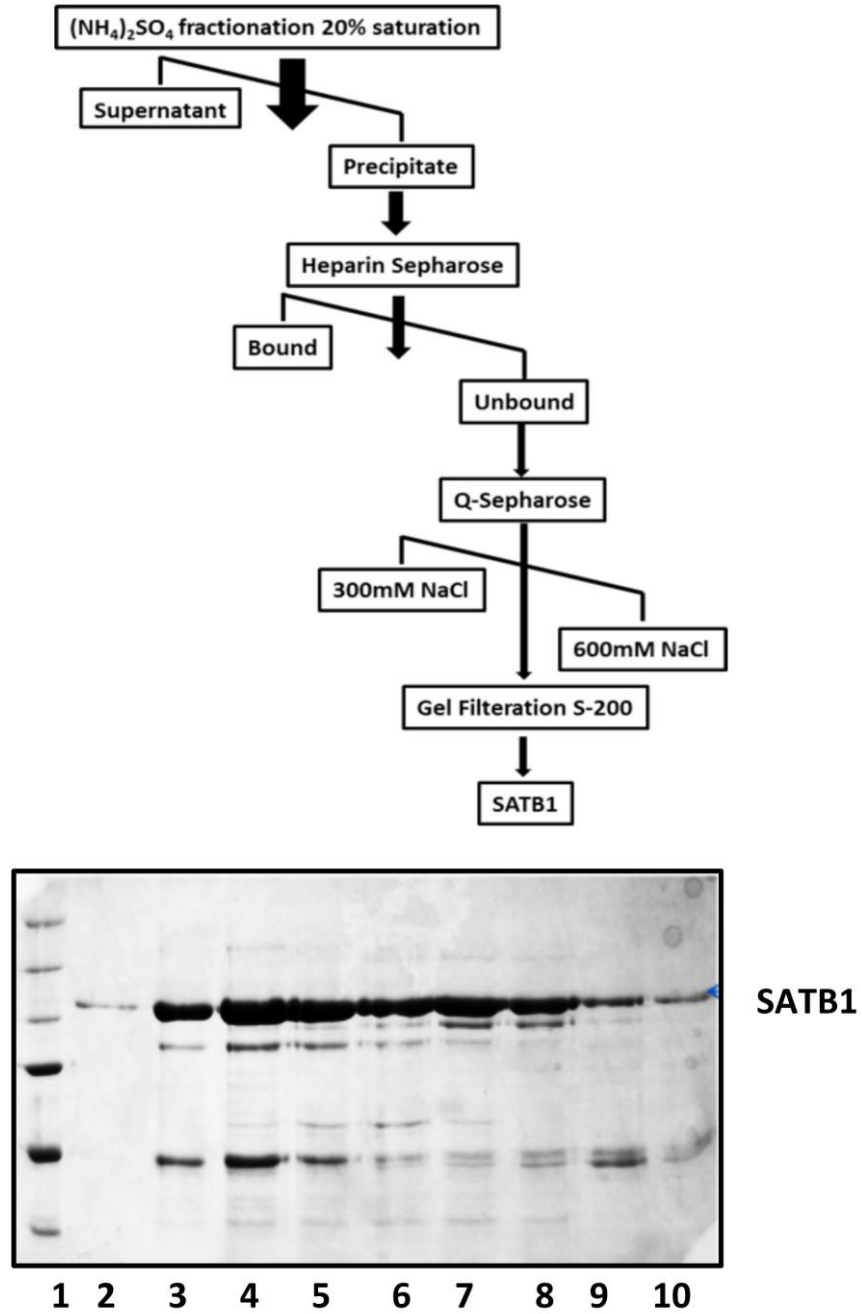


Figure 11: Purification of Human SATB1 expressed in *Escherichia coli.* Purification scheme for SATB1 overexpressed in *E. coli* (Top Panel), a combination of selective fractionation, ion exchange and size exclusion chromatography was used to purify SATB1 from bacterial cell free lysate. **(Bottom Panel)** Partial purification of SATB1 protein, the image shows the eluates obtained after size exclusion chromatography using sephadex-200 resin

4. DISCUSSION

Our study establishes SATB1 as a bonafide member of IGF signaling. SATB1 is both a physical and functional interacting partner of IGF signaling. Our studies indicate that while SATB1 regulates transcription in response to IGF signaling, its own transcription may be regulated by IGF signaling, thus providing a cellular feedback loop. The presence of SATB1 in the nucleoprotein complex assembled using IRS sequence as well as its role as a transcriptional repressor indicate that SATB1 alone or as a part of a large complex may be recruited to these sequences and act to suppress leaky transcription from IR sequence. The presence of FOXO3a binding site on SATB1 promoter as well as its validation by ChIP using FOXO3A antibody indicates that SATB1 levels may be regulated in response to IGF signaling.

. IGF-1 signaling is major pathway involved in aging and it regulates different genes necessary for DNA repair, tumor suppression, cell survival and homeostasis. SATB1 is also involved in regulating genes involved in major age-related pathologies like breast and prostate cancer (Han et al., 2008; Shukla et al., 2013). As IGF signaling also plays a key role in tumor suppression in various types of cancers the two show a strong correlation. SATB1 levels are strongly co-related with age in the hypothalamus of five different strains of mice and its expression decreases with age in mice. These studies are validated by RNAi experiments with DVE-1 (worm ortholog of SATB1) in daf-2 mutant background that showed that loss of DVE-1 function attenuates the extension in lifespan caused by daf-2 mutation.

SATB1 is a global chromatin organizer and it can regulate several genes by recruiting different chromatin modeling enzymes to the promoter of a gene. It can act as repressor or activator for a gene based on its binding partners. SATB1 represses the gene expression when it interacts with HDACs like CBP and activates it when it interacts with HATs like PCAF (Pavan Kumar et al., 2006). Recent study on Embryonal Carcinoma Cells (ECCs) shows that Akt phosphorylates SATB1 at serine 47 residue which leads to repression of Nanog gene causing differentiation of ECCs. In this instance SATB1 is shown to be phosphorylated by Akt in IGF1-PI3K dependent manner and phosphorylation of SATB1 protects it from apoptotic cleavage in these cells (Chen et al., 2013). This supports our hypothesis that SATB1 might be repressing IR genes in

the presence of IGF1 signaling as it will lead to phosphorylation of SATB1 by Akt and then it can repress the IR genes by interacting with co-repressors like CBP/p300.

We propose to investigate the levels of IR genes under varying conditions of SATB1/2 expression by using real time quantitative PCR. We will express SATB1 and perform immunoprecipitation with anti-SATB1 antibody and immunoblot to check for co-repressors in the presence and absence of IGF1. The identity of the proteins bound to IRS can be established by oligo affinity purification followed by mass spectrometry. This will be an unbiased approach to find the interactors of SATB1 in this process.

Our second main result from Chromatin immunoprecipitation of FOXO3A shows that SATB1 has a FOXO3A binding motif on its promoter. Our result is supported by transfac analysis which was performed for SATB1 using bioinformatics tools which predicted the presence of FOXO3A binding site on SATB1 promoter. From these observations it can predicted that FOXO3A might be involved in regulating the transcription of SATB1 as it is physically binding to its promoter. We can check this by monitoring the levels of SATB1 in the presence of knockdown and over expression of FOXO3A. To investigate if this regulation is mediated by IGF signaling we can perform the same experiment in presence and absence of IGF1. In the presence of IGF1 we can check the levels of phosphorylated SATB1 and FOXO3A and see how they change with activation by IGF1 at increasing time points. IGF1 is known to activate IGF1 signaling in mammalian cells in about few minutes, therefore, we can look at the change in phosphorylated SATB1 over two minutes to 60 minutes.

We have established the functional relationship of SATB1 with IR genes by luciferase assay we can now move on to check which domains of SATB1 are necessary for repression. We have created different deletion mutants in which there are some N-terminal and C-terminal deletions and internal deletions of particular domains of SATB1 like protein-protein interaction (PDZ) domain, Nuclear matrix targeting sequence (NMTS) and DNA binding domains like Homeodomain (HD), CUT domain1, CUT domain 2. We have cloned these constructs in mammalian expression vector and these will be transfected in HEK293T cells and luciferase assay will be performed as explained previously. These experiments can also be performed in presence and absence of IGF-11.

IGF-1 signaling pathway is the major pathway involved in calorie restriction mediated lifespan extension of several model organisms from worms to humans. IGF1 binds to its receptor and activates PI3K-Akt pathway and FOXO proteins are phosphorylated which leads to their nuclear exclusion. Our study gives new insight about the regulation of IR genes by chromatin organizing protein SATB1. This protein acts as a transcriptional repressor of insulin responsive genes and it physically interacts with IRS present approximately 300bp upstream to all insulin responsive genes. We also show that expression of this protein might be regulated by IGF-1 signaling through FOXO3A as it binds to the SATB1 promoter sequence. IGF signaling is involved in different age-related pathologies like cancer, diabetes, neurodegenerative diseases.

In conclusion, we report the discovery of SATB proteins as a regulator of gene expression in response to IGF signaling. The results of our studies indicate that SATB proteins and their interacting partners may play a crucial role in preventing expression of insulin responsive genes in the presence of IGF signaling and hence reduce transcriptional noise making IGF signaling more sensitive to changes in the metabolic flux.

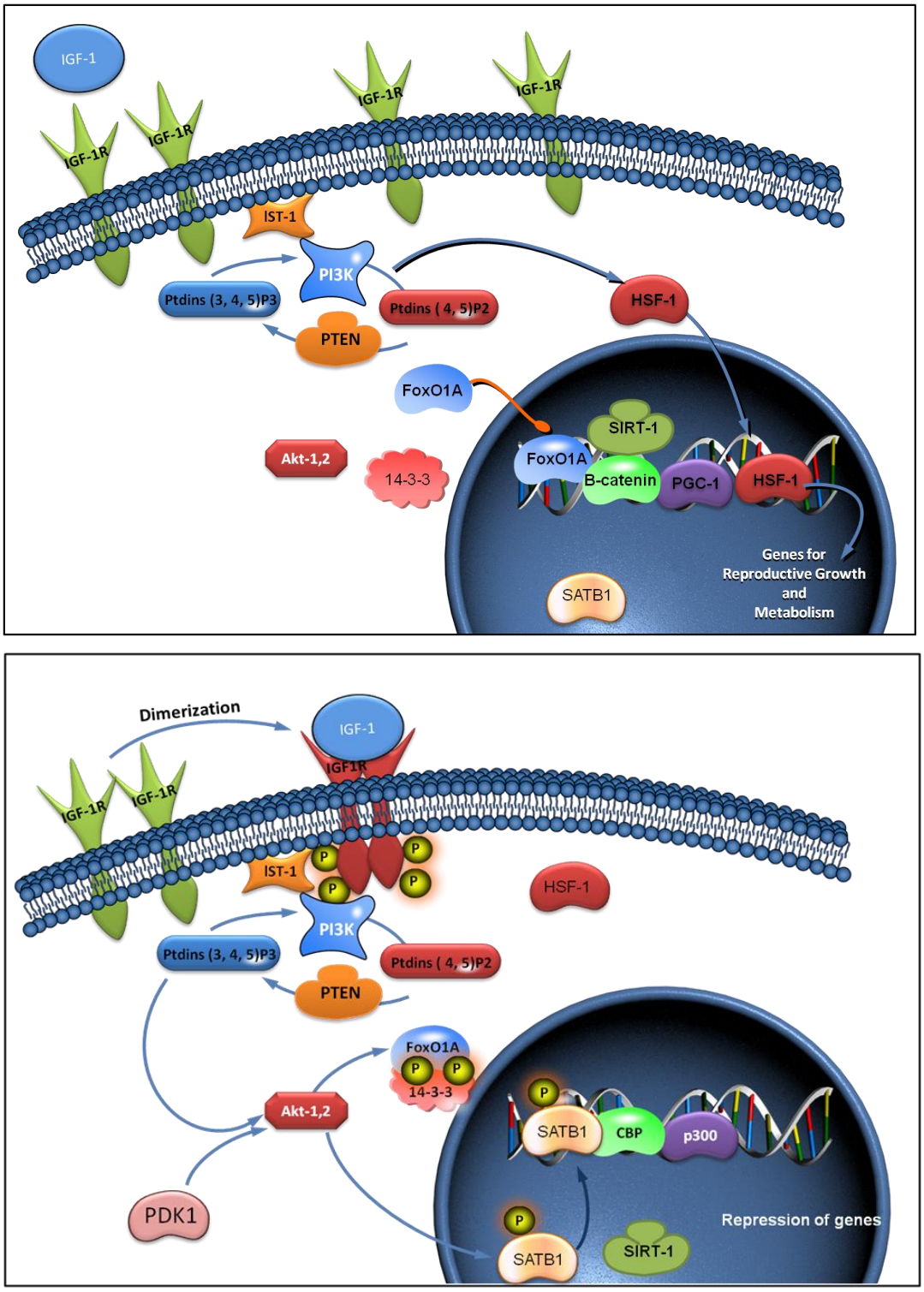


Figure 12: Role of SATB proteins during IGFR signaling in humans. In the absence of IGF, phosphorylation of FOXO1a as well as that of SATB1 occurs. Phosphorylation causes, FOXO1a to be retained in the cytoplasm while allowing SATB1 to assemble a transcriptionally repressive complex at IRS sequence. In the absence of IGF, a net de-

phosphorylation occurs, causing FOXO1a to translocate into the nucleus and disassemble the SATB1 mediated repressive complex to regulate the transcription of IR genes.

REFERENCES

Abmayr, S.M., Yao, T., Parmely, T., and Workman, J.L. (2006). Preparation of nuclear and cytoplasmic extracts from mammalian cells. Curr Protoc Pharmacol *Chapter 12*, Unit12.13.

Berdasco, M., and Esteller, M. (2012). Hot topics in epigenetic mechanisms of aging: 2011. Aging Cell *11*, 181-186.

Chen, B., Xue, Z., Yang, G., Shi, B., Yang, B., Yan, Y., Wang, X., Han, D., Huang, Y., and Dong, W. (2013). Akt-signal integration is involved in the differentiation of embryonal carcinoma cells. PLoS One *8*, e64877.

Clancy, D.J., Gems, D., Harshman, L.G., Oldham, S., Stocker, H., Hafen, E., Leevers, S.J., and Partridge, L. (2001). Extension of life-span by loss of CHICO, a Drosophila insulin receptor substrate protein. Science *292*, 104-106.

Daitoku, H., and Fukamizu, A. (2007). FOXO transcription factors in the regulatory networks of longevity. J Biochem *141*, 769-774.

Garsin, D.A., Villanueva, J.M., Begun, J., Kim, D.H., Sifri, C.D., Calderwood, S.B., Ruvkun, G., and Ausubel, F.M. (2003). Long-lived C. elegans daf-2 mutants are resistant to bacterial pathogens. Science *300*, 1921.

Gorbunova, V., Seluanov, A., Zhang, Z., Gladyshev, V.N., and Vijg, J. (2014). Comparative genetics of longevity and cancer: insights from long-lived rodents. Nat Rev Genet *15*, 531-540.

Greer, E.L., Dowlatshahi, D., Banko, M.R., Villen, J., Hoang, K., Blanchard, D., Gygi, S.P., and Brunet, A. (2007). An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in C. elegans. Curr Biol *17*, 1646-1656.

Guevara-Aguirre, J., Balasubramanian, P., Guevara-Aguirre, M., Wei, M., Madia, F., Cheng, C.W., Hwang, D., Martin-Montalvo, A., Saavedra, J., Ingles, S.*, et al.* (2011). Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans. Sci Transl Med *3*, 70ra13.

Han, H.J., Russo, J., Kohwi, Y., and Kohwi-Shigematsu, T. (2008). SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis. Nature *452*, 187-193.

Hasshoff, M., Böhnisch, C., Tonn, D., Hasert, B., and Schulenburg, H. (2007). The role of Caenorhabditis elegans insulin-like signaling in the behavioral avoidance of pathogenic Bacillus thuringiensis. FASEB J *21*, 1801-1812.

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Géloën, A., Even, P.C., Cervera, P., and Le Bouc, Y. (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature *421*, 182-187.

Hsieh, C.-C., DeFord, J.H., Flurkey, K., Harrison, D.E., and Papaconstantinou, J. (2002). Implications for the insulin signaling pathway in Snell dwarf mouse longevity: a similarity with the C. elegans longevity paradigm. Mechanisms of Ageing and Development *123*, 1229-1244.

Khandekar, M.J., Cohen, P., and Spiegelman, B.M. (2011). Molecular mechanisms of cancer development in obesity. Nat Rev Cancer *11*, 886-895.

Lee, S.S., Kennedy, S., Tolonen, A.C., and Ruvkun, G. (2003). DAF-16 target genes that control C. elegans life-span and metabolism. Science *300*, 644-647.

Longo, V.D., and Kennedy, B.K. (2006). Sirtuins in aging and age-related disease. Cell *126*, 257-268.

O'Sullivan, R.J., and Karlseder, J. (2012). The great unravelling: chromatin as a modulator of the aging process. Trends Biochem Sci *37*, 466-476.

Pavan Kumar, P., Purbey, P.K., Sinha, C.K., Notani, D., Limaye, A., Jayani, R.S., and Galande, S. (2006). Phosphorylation of SATB1, a global gene regulator, acts as a molecular switch regulating its transcriptional activity in vivo. Mol Cell *22*, 231-243.

Riedel, C.G., Dowen, R.H., Lourenco, G.F., Kirienko, N.V., Heimbucher, T., West, J.A., Bowman, S.K., Kingston, R.E., Dillin, A., Asara, J.M.*, et al.* (2013). DAF-16 employs the chromatin remodeller SWI/SNF to promote stress resistance and longevity. Nat Cell Biol *15*, 491-501.

Shelton, L.M., Huysentruyt, L.C., Mukherjee, P., and Seyfried, T.N. (2010). Calorie restriction as an anti-invasive therapy for malignant brain cancer in the VM mouse. ASN Neuro *2*, e00038.

Shukla, S., Sharma, H., Abbas, A., MacLennan, G.T., Fu, P., Danielpour, D., and Gupta, S. (2013). Upregulation of SATB1 is associated with prostate cancer aggressiveness and disease progression. PLoS One *8*, e53527.

Speakman, J.R., and Mitchell, S.E. (2011). Caloric restriction. Mol Aspects Med *32*, 159-221.

Wood, J.G., Hillenmeyer, S., Lawrence, C., Chang, C., Hosier, S., Lightfoot, W., Mukherjee, E., Jiang, N., Schorl, C., Brodsky, A.S.*, et al.* (2010). Chromatin remodeling in the aging genome of Drosophila. Aging Cell *9*, 971-978.

Zhang, M., Poplawski, M., Yen, K., Cheng, H., Bloss, E., Zhu, X., Patel, H., and Mobbs, C.V. (2009). Role of CBP and SATB-1 in aging, dietary restriction, and insulin-like signaling. PLoS Biol *7*, e1000245.

Appendix A: Composition of buffers used in the study

1. Isolation of Nuclear Lysate

   1.1 Phosphate Buffered Saline

   a) 137mM NaCl
   b) 2.7mM KCl
   c) 10mM Na_2HPO_4
   d) 1.8mM KH_2PO_4

   1.2 Hypotonic Buffer

   a) 25 mM TrisCl pH 7.9
   b) 1.5 mM $MgCl_2$
   c) 10mM KCl
   d) 0.1% NP40
   e) 1mM DTT
   f) 0.5 mM PMSF
   g) 1x PIC

   1.3 Wash Buffer

   a) 20mM HEPES pH7.9
   b) 25%glycerol
   c) 1.5mM $MgCl_2$
   d) 0.02 M KCl in low salt buffer and 1.2 M in high salt buffer
   e) 0.2 mM EDTA
   f) 0.2 mM PMSF
   g) 0.5 mM DTT

   1.4 Nuclear Lysate Storage Buffer

   a) 20mM HEPES, pH 7.5
   b) 20% glycerol
   c) 100 mM KCl
   d) 0.2 mM EDTA
   e) 0.2 mM PMSF
   f) 0.5 mM DTT

2. Chromatin Immunoprecipitation (ChIP)

2.1 Sonication buffer :

a) 50 mM Tris-Cl ph 7.9
b) 140 mM NaCl
c) 1mM EDTA
d) 1% Triton X-100
e) 0.1% Sodium deoxycholate
f) 1.0% SDS
g) 0.5 mM PMSF
h) 1x PIC

2.2 ChIP dilution buffer

a) 0.01% SDS
b) 1.1% Triton X-100
c) 1.2 mM EDTA
d) 16.7mM Tris-Cl ph 8.0
e) 167 mM NaCl

2.3 Low salt wash buffer

a) 0.1% SDS
b) 1% Triton X-100
c) 2 mM EDTA
d) 20 mM Tris-Cl ph 8.0
e) 150 mM NaCl

2.4 High salt wash buffer

a) 0.1% SDS
b) 1% Triton X-100
c) 2 mM EDTA
d) 20 mM Tris-Cl ph 8.0
e) 500 mM NaCl

2.5 LiCl wash Buffer

a) 0.25 M LiCl
b) 1% NP40

c) 1% Sodium deoxycholate
d) 1 mM EDTA
e) 10 mM Tris-Cl ph 8.0

2.6 TE buffer -50 ml

a) 20mM TrisCl (pH 8.0)
b) 100mM EDTA

2.7 Elution buffer

a) 1% SDS
b) 0.1 M Sodium Bicarbonate

2.8 Saturated Magnetic Beads

a) Protein-G Magnetic Beads – 1.0 ml (50 % slurry)
b) tRNA (10 mg/ml) 40 µL
c) Fish skin gelatin 1 %

Incubate for 2-3 h at the rocker in cold room. Wash twice with chip dilution buffer.

3. Annealing of oligonucleotides

3.1 SSC buffer

a) 300mM Sodium Citrate pH7.0
b) 1M Sodium Chloride

4. Western blot analysis

4.1 Transfer buffer

a. 10mM Monobasic Sodium Phosphate
b. 10mM Dibasic Sodium Phosphate

4.2 TBS-T

a) 1M Tris-Cl, pH 7.4
b) 1M Sodium chloride
c) 10% Tween 20

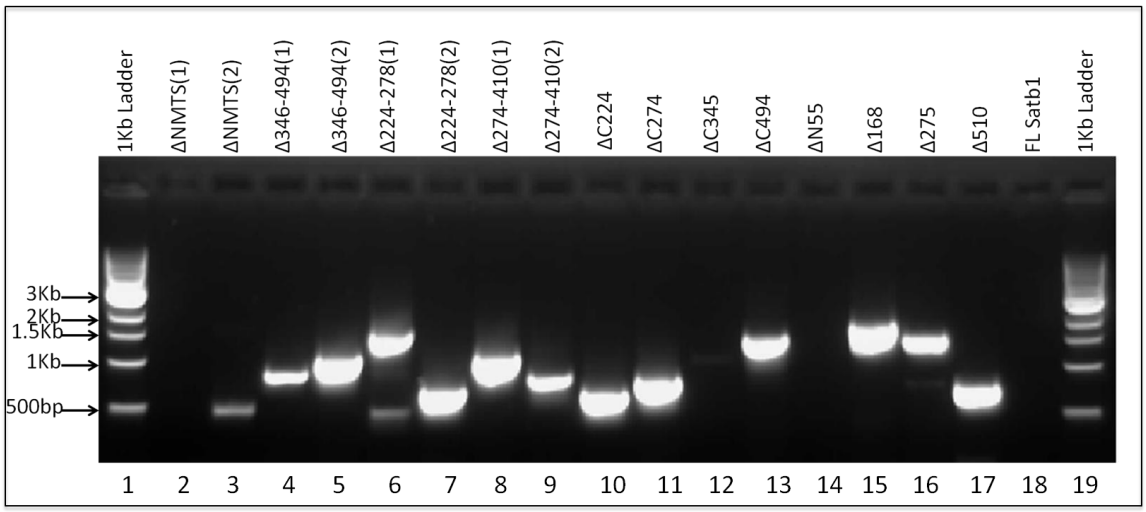
5. Whole cell lysate preparation

5.1 RIPA buffer

a) 1% Sodium deoxycholate
b) 150mM Sodium Chloride
c) 2mM EDTA
d) 0.2mM Sodium orthovandate
e) 10mM Sodium Phosphate, pH 7.2

Appendix B: Agarose gel images generated in the study

(A)



(B)

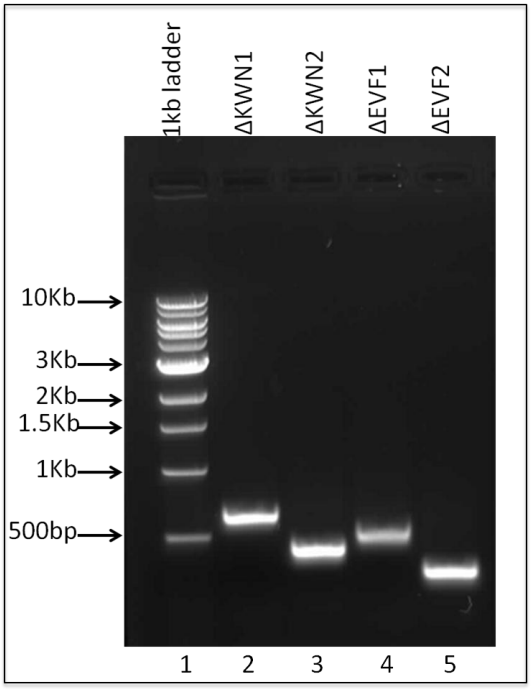
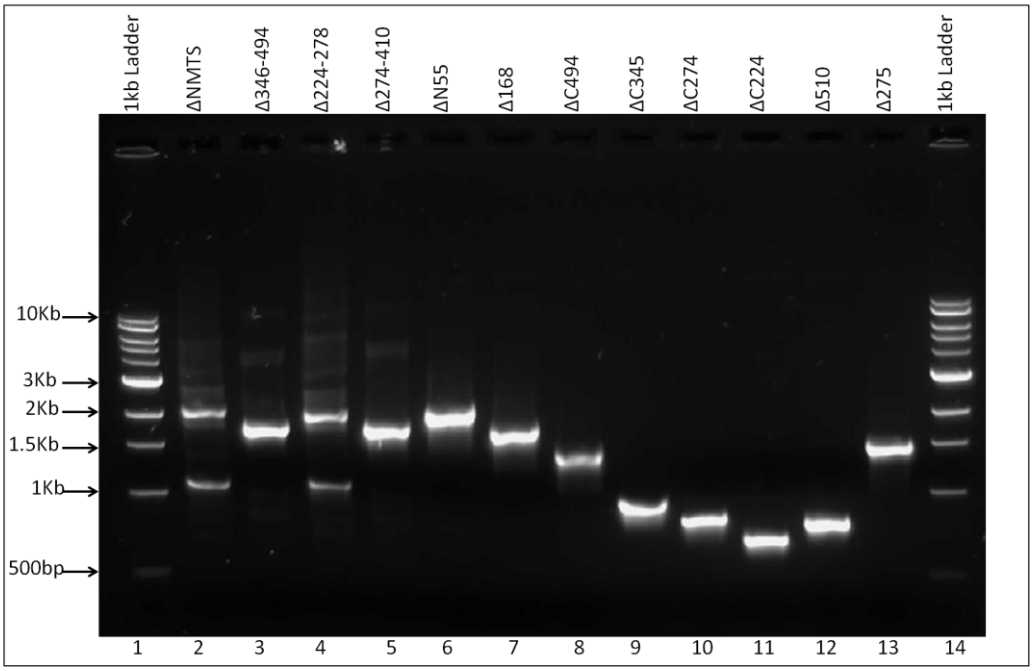


Figure 1: Amplification for SATB1 constructs. N-terminal, C-terminal deletion constructs were amplified and for deletion of each internal domain two fragments were amplified to perform overlap extension PCR.

(A)



(B)

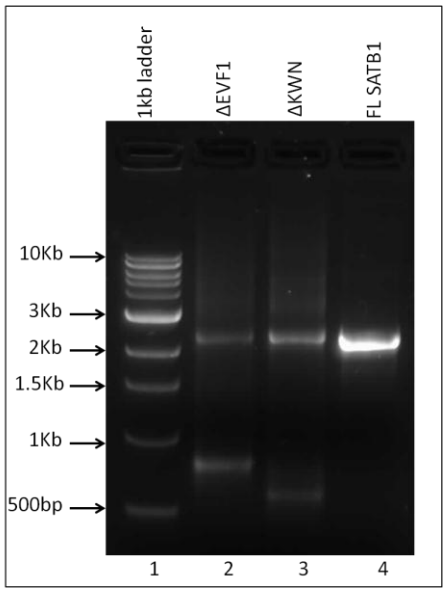


Figure2: Overlap extension PCR and Preparatory PCR of SATB1 variants. Overlap extension PCR was performed for internal deletions using primers for full-length SATB1 and the two fragments for each deletion amplified in Figure1 as a template.

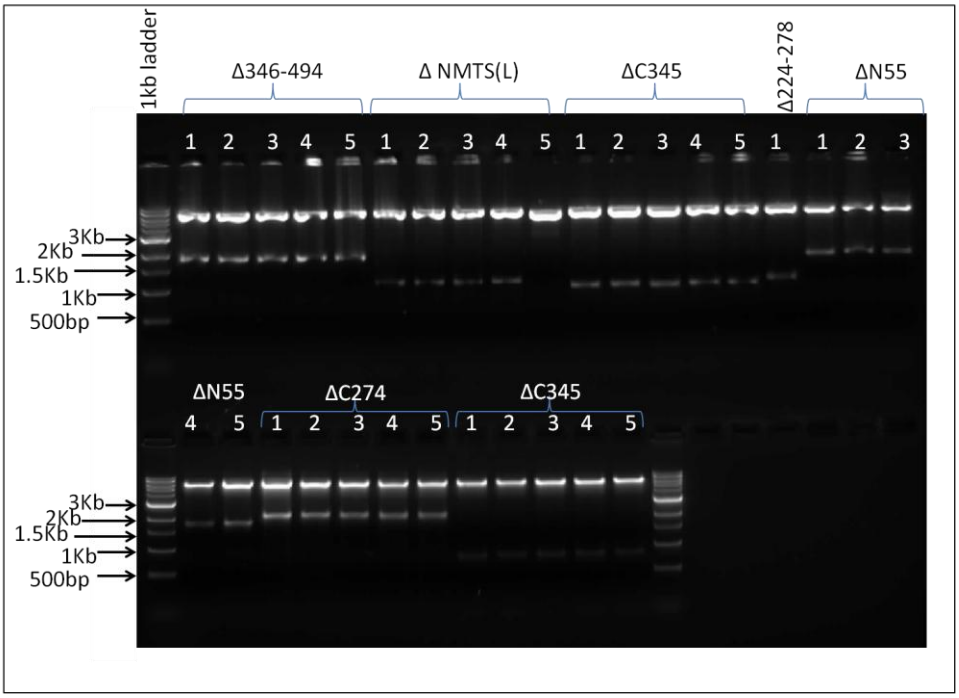
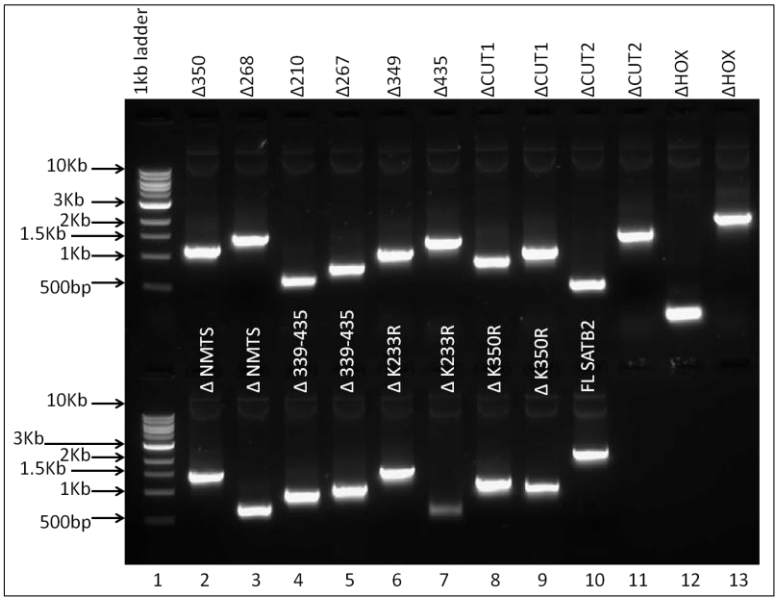


Figure 3: Digestion of plasmid DNA to check for insert release. All the SATB1 constructs were transformed and 5 colonies of each clone were digested with BamH1 and Nde1 to check for insert release.

(A)



(B) **(C)**

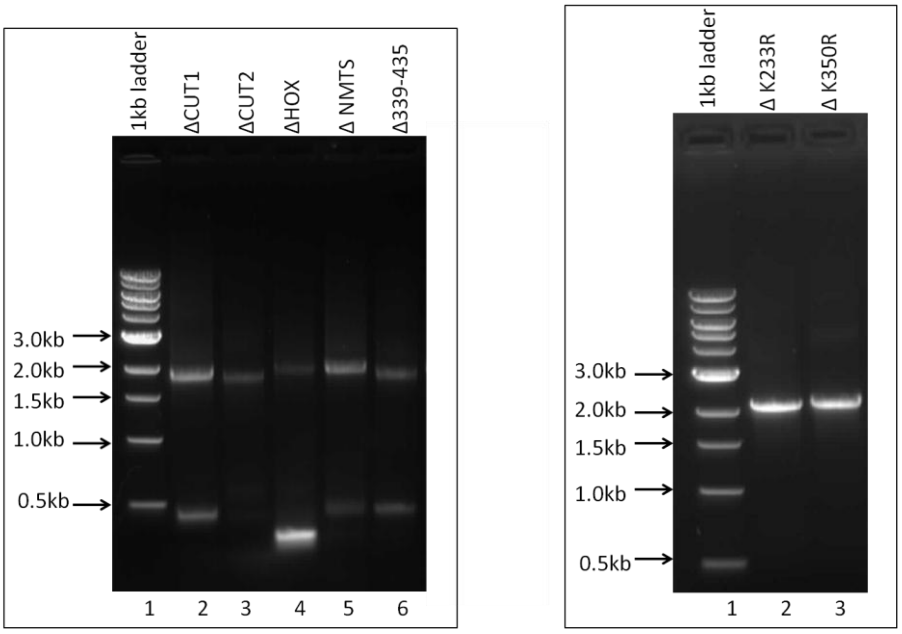


Figure 4: Overlap extension PCR for SATB2 constructs. SATB2 constructs were amplified and overlap extension PCR was performed to gel the internal deletions.