Characterization of the oligomeric complex formed by SATB1 and SATB2 proteins and their interactors

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled “Characterization of the oligomeric complex formed by SATB1 and SATB2 proteins and their interactors” towards the partial fulfillment of the BS-MS dual degree program at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Adesh Wadate at Indian Institute of Science Education and Research under the supervision of Dr. Sanjeev Galande, Professor, Department of Biology, during the academic year 2022-2023.

Prof. Sanjeev Galande

(Signature)

Committee:

Prof. Sanjeev Galande

Dr. Siddhesh Kamat

This thesis is dedicated to my family

Declaration

I hereby declare that the matter embodied in the report entitled “Characterization of the oligomeric complex formed by SATB1 and SATB2 proteins and their interactors” are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Prof. Sanjeev Galande and the same has not been submitted elsewhere for any other degree

Adesh Wadate

Date: 10-04-2023

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Contributions

Contributor name	Contributor role
Prof. Sanjeev Galande, Dr. Dipika Yadav	Conceptualization Ideas
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-	Funding acquisition

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Abstract

The Special AT-rich sequence Binding proteins SATB1 and SATB2 play a significant role in many biological processes ranging from the development of an organism to disease. SATB1 and SATB2 show 61% sequence identity in the amino acid sequence and share the conserved domains. The individual role of these proteins has been studied extensively. However, their role as a complex has not been explored yet. Previously, it has been shown that N-terminal ULD-mediated oligomerization of SATB1 is vital for the high DNA binding affinity and differential regulation of gene expression. Based on these observations, we aimed to identify and characterize the higher-order complex forms of SATB1 and SATB2. Towards that, we found that SATB1 and SATB2 form a complex in cell-based assays. Next, due to failure in the cloning of SATB1, we focused on understanding the oligomeric forms of SATB2. For that, we took biochemical approaches to standardize the expression and purification of SATB2 in the bacterial expression system. Finally, we observed the potential of SATB2 to self-associate using purified proteins by Native Gel Electrophoresis, indicating the higher oligomeric forms of SATB2.

Chapter 1 Introduction

Eukaryotic cells exhibit multiple levels of the organization to package their large DNA into the tiny nucleus, starting from individual beads on strings to the entire chromosomes. Inside the nucleus, the DNA is wrapped around histone proteins to form a complex known as chromatin, which shows a distinct 3D architecture. Moreover, Non-histone proteins contribute to chromatin architecture, which is crucial for regulating gene expression and determining cellular identity.

A critical aspect of this 3D chromatin organization is the formation of higher-ordered chromatin loops, which occur when specific AT-rich DNA sequences called Matrix Attachment Regions (MARs) or Scaffold Attachment Regions (SARs) binds to the nuclear matrix or scaffold (Gasser and Laemmli, 1986). These MARs contain sequences composed primarily of As, Ts, and Cs, excluding Gs on one strand of DNA(Dickinson *et al.*, 1992). Additionally, they contain regions of 100-150 base pairs that unwind under negative helical strain, known as Base Unpairing Regions (BURs).

Studies have shown that MARs are frequently found near regulatory regions such as promoters and enhancers, which highlights the significance of understanding the role of MAR-binding proteins in transcriptional regulation(Cockerill and Garrard, 1986). Therefore, a comprehensive and mechanistic understanding of the proteins that bind to MARs is critical to unraveling the complex mechanisms by which 3D chromatin organization regulates gene expression.

1.1 The SATB family of MAR binding proteins

SATB1 and SATB2 function as global chromatin organizers. They specifically bind to AT-rich regions of DNA designated as MARs. Both proteins show well-characterized domains at the protein level. These domains are identified as the N-terminal ubiquitin-like domain (ULD), the middle CUT domains consisting of the CUT-like domain (CUTL), CUT1 and CUT2 domains, and C-terminal Homeodomain(Dickinson *et al.*, 1992; Wang *et al.*, 2014). The middle CUT domains and C-terminal Homeodomain are the DNA-binding domain of SATB family proteins. Moreover, the CUT domains of SATB1 have been shown to facilitate the recognition of specific DNA sequences and mediate the homeodomain binding to its target sites(Purbey *et al.*, 2008). The N-

terminal domain of SATB1 protein enables it to form complexes with other proteins and is indispensable for the DNA binding ability of other domains(Galande *et al.*, 2001; Purbey *et al.*, 2008).

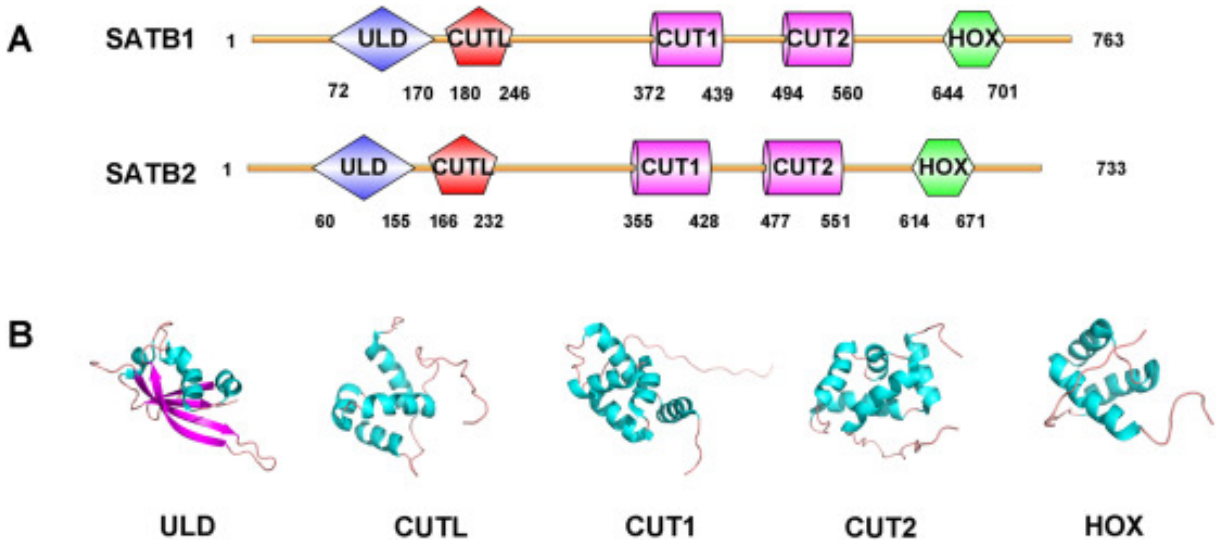

Figure 1. Human SATB1 and SATB2 protein domain structure. (A) The organization of SATB1 and SATB2 domains in humans is shown in the diagram. Five domains, including the ubiquitin-like domain (ULD), the CUT repeat-like domain (CUTL), and three other domains (CUT1, CUT2, and HOX), are highly similar in both proteins. (B) The structures of the five domains are shown in graphics, including the HOX (PDB code 1WI3), CUTL (PDB code 2L1P), CUT1 (PDB code 1wiz), CUT2 (PDB code 2CSF), and ULD (PDB code 3TUO) domains. The graphics are reproduced from a publication by Huang et al. in 2022. PDB : Protein Data Bank

SATB family proteins facilitate chromatin organization and higher-order chromatin structure formation. They bind to MARs and recruit other chromatin-remodeling proteins to these regions, enhancing chromatin loops and 3D chromatin structure(Alvarez *et al.*, 2000; Kohwi-Shigematsu *et al.*, 2012).

SATB proteins show tissue-specific expression patterns. Previous studies have shown their significance in many biological processes, including development, differentiation, and immune response. Depending on the post-translational modifications and the other interacting partners, these proteins can act as transcriptional activators or repressors context-dependent.

Therefore, studying the molecular mechanisms and functions of SATB family proteins is essential for understanding the regulation of gene expression and chromatin organization, which may have implications for various biological processes and diseases.

1.2 A brief introduction to SATB1 and SATB2

SATB1 is a well-studied protein highly expressed in thymocytes and plays a critical role in their differentiation process (Alvarez et al., 2000). Recent research indicates that SATB1 regulates chromatin organization during T-cell development by binding to matrix attachment regions (MARs) and associating with the histone deacetylase of the nucleosome remodeling deacetylase (NURD) complex. This complex, in turn, recruits other chromatin remodeling complexes, such as ACF and ISWI, which are involved in modifying chromatin structure (Yasui et al., 2002; Pavan Kumar et al., 2006). These findings suggest that SATB1 plays a crucial role in shaping the chromatin architecture of developing T cells, ultimately influencing their gene expression and function.

A prior investigation in our laboratory discovered that the phosphorylation of SATB1 affects its ability to bind to matrix attachment regions (MARs) and act as a transcriptional regulator. Consequently, along with chromatin remodelers, SATB1 interacts with chromatin modifiers, such as HDAC1 and PCAF, in a way that depends on phosphorylation, leading to either repression or derepression of downstream genes, respectively(Pavan Kumar et al., 2006).

Additionally, another study from our lab found that during Th2 lineage commitment, the deacetylation of SATB1 resulted in increased DNA binding affinity of SATB1. Due to elevated Wnt signaling, the deacetylated SATB1 upregulated transcription factors specific to the Th2 lineage by recruiting beta-catenin and p300 to their target sites (Purbey et al., 2009).

Furthermore, in addition to its expression in thymocytes, SATB1 is also expressed in various regions during the central nervous system (CNS) development. Previous research has demonstrated that SATB1 plays a critical role in the differentiation and maintenance of medial ganglionic eminence-derived interneurons, which help to maintain a balance between excitatory and inhibitory signals in the developing cortex(Close *et al.*, 2012).

Moreover, SATB1 has been linked to Parkinson's disease, where it functions to prevent cellular senescence in post-mitotic dopaminergic neurons by directly suppressing the expression of the pro-senescence factor p21(Riessland *et al.*, 2019). These findings suggest that SATB1 has multiple functions in different developmental contexts and disease processes, underscoring its diverse roles in cellular and biological processes.

In addition to its involvement in developmental processes, numerous investigations have highlighted the role of SATB1 in tumor progression and metastasis (Naik and Galande, 2019). Studies have revealed that SATB1 is upregulated in breast cancer cells, where it modifies chromatin organization and triggers the activation of metastatic genes while concurrently downregulating tumor suppressor genes. Given these observations, several studies have proposed SATB1 as a promising biomarker for cancer prognosis and a potential target for cancer therapy.

SATB2 is a second member of the SATB protein family and was initially discovered as a mutated gene in individuals with cleft palate (FitzPatrick et al., 2003). Notably, human and mouse SATB2 locus mutations have been linked to craniofacial patterning defects, developmental delays, and cognitive and behavioral abnormalities (Dobreva et al., 2006; Huang et al., 2022). Additionally, increased expression of SATB2 has been observed in differentiating neurons compared to mature neurons, indicating its essential role in neuronal differentiation. SATB2 plays a critical role in determining the identities of callosal and upper-layer neurons in the neocortex(Alcamo *et al.*, 2008; Britanova *et al.*, 2008).

SATB2 exhibits high expression in pre-B cells and binds to the MAR sequences that flank the enhancer of the endogenous immunoglobulin μ heavy chain gene, thereby enhancing its expression(Dobreva *et al.*, 2003). Like SATB1, SATB2 has been examined as a potential prognostic indicator for cancer (Roy et al., 2020). Notably, while a decrease in SATB2 expression is associated with cancer progression, an increase in SATB1 expression leads to tumor progression and metastasis.

Based on the studies mentioned above, it appears that both SATB1 and SATB2 exhibit tissue-specific expression patterns. Several studies have demonstrated that during CNS development, these proteins are differentially expressed, suggesting that they may be involved in regulating distinct sets of genes at the chromatin level. While some evidence suggests that they may work together in a coordinated manner as a complex to control gene expression, this concept requires further exploration. Therefore, this

study aims to investigate whether SATB1 and SATB2 interact with each other and, if so, the nature of the complex they form both in vitro and in vivo.

1.3 Role of oligomerization in the regulation of gene expression

Protein complexes and oligomerized states of proteins, such as homomers or heteromers, have been implicated to have conformation-specific roles in gene regulation. Oligomeric complexes, formed by the association of multiple protein subunits, play a crucial role in gene regulation and are implicated in various diseases. Oligomerized proteins can regulate transcription, repair, apoptosis, and other evolutionary processes. In the context of gene regulation, oligomeric complexes can function as transcription factors that bind to specific DNA sequences and activate or repress the expression of target genes. Oligomeric complexes can also function as co-activators or co-repressors by interacting with other transcription factors and modifying chromatin structure (Bhambhani *et al.*, 2011).

However, dysregulation of oligomeric complexes can lead to various diseases. For example, mutations in the oligomeric complex-forming proteins can disrupt their normal function, leading to abnormal gene expression and the development of diseases such as cancer, autoimmune disorders, and neurodegenerative diseases. Additionally, abnormal aggregation of oligomeric complexes can lead to the formation of toxic protein aggregates that can contribute to the pathogenesis of diseases such as Alzheimer's and Parkinson's disease(Choi and Gandhi, 2018).

Therefore, understanding the regulation and function of oligomeric complexes is critical for understanding the molecular basis of development and disease and also for developing targeted therapies.

1.4 The important role of oligomerization of SATB1

Initially, it was believed that the N-terminal domain of SATB1 resembled a PDZ domain based on the results obtained from peptide sequence similarity analysis (Galande et al., 2001). However, subsequent structural studies revealed that the N-terminal domain was more akin to a ubiquitin-like domain (ULD) (Wang et al., 2012). During an investigation into the fate of SATB1 in thymocytes and T-cell apoptosis, it was discovered that the N-terminal domain has an oligomerizing function. The study

identified a specific region within the N-terminal domain that affects the DNA binding affinity of the C-terminal domains. This study also demonstrated that SATB1 dimerizes using a classical yeast two-hybrid assay and provided the first evidence that SATB1 can form dimers and that its dimerization is critical for its function (Galande et al., 2001).

The full-length SATB1 protein interacts with DNA through the minor groove, as observed in previous studies. Further investigations demonstrated that the Homeodomain (HD) binds through the minor groove, whereas the CUT Domain (CD) binds through the major groove(Dickinson *et al.*, 1992; Yamaguchi *et al.*, 2006; Purbey *et al.*, 2008). These findings helped to elucidate the mechanism of SATB1-DNA interaction based on the structure of SATB1's binding site. In Their study, Purbey et al. identified a palindromic consensus binding sequence for SATB1, in which two AT-rich half-sites are arranged in an inverted manner and flank a central cytosine or guanine (TATTAGTAATAA). The study proposed that the N-terminal dimerization domain bridges the DNA binding regions of the two SATB1 monomeric subunits so that they bind in an antiparallel fashion to the inverse palindromic consensus binding element, which includes a conserved homeodomain binding sequence (TAAT). This study highlighted the significance of the consensus binding motif and protein dimerization as crucial properties for a high-affinity DNA-protein interaction (Purbey et al., 2008).

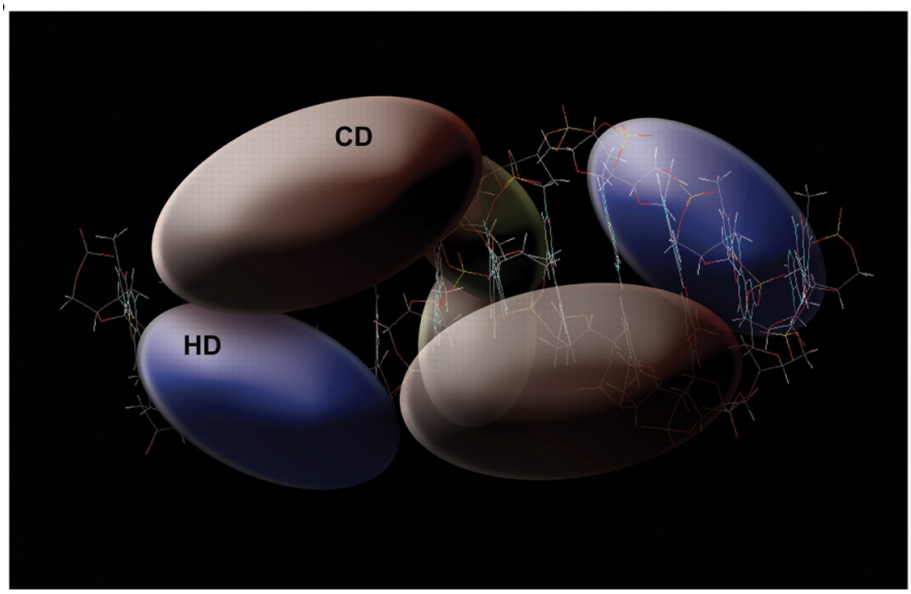

Figure 2. Model showing SATB1's attachment of the consensus DNA element. The image demonstrates that one monomer is present on one side of DNA while the

other monomer is present on the opposite side. The minor groove is recognized by HD, and the major groove is recognized by CD. The dimerization function is performed by the N-terminal PDZ, which links the two monomers together. (Adapted from (Purbey *et al.*, 2008))

In vitro studies by Wang et al. revealed that SATB1 forms a homotetramer and can simultaneously bind to two DNA fragments, suggesting that tetramerization of SATB1 plays a crucial role in organizing higher-order chromatin architecture by recruiting various chromatin remodeling factors. This process enhances the anchoring of specific DNA sequences in close proximity. Additionally, Zheng et al. demonstrated that disrupting the tetramerization of SATB1 significantly affected the promoter binding activity of associated genes and the recruitment of other interacting partners. Their study also revealed the contribution of dimer and tetramer forms of SATB1 in differential binding to partners, suggesting that SATB1 regulates gene expression by interacting with different partners depending on its oligomerization state.

1.5 Rationale

Since SATB1 and SATB2 proteins are pleiotropic factors, they exhibit functional diversity. These proteins either co-express or differentially express in certain tissues to regulate various physiological functions in a context-dependent manner. Therefore, it is essential to dissect the association behavior of these two proteins to understand the differential regulatory role of proteins.

Further, the characterization of homomeric forms of SATB1 protein is reported as compared to SATB2. As such, both proteins show conserved functional domains, which indicate that the higher oligomeric forms of SATB2 protein could also exist to control its regulatory role. Hence, this study was undertaken to elucidate the nature of oligomers formed by SATB1 and SATB2 and the functional significance of the different oligomeric forms *in vivo*.

1.6 Objectives of the study

1) To investigate the oligomer complexes formed by SATB1 and SATB2 proteins using bacterial and mammalian expression systems.

2) To identify differential associations of the complex with known chromatin modelers.

3) Identification of target genes regulated by their oligomeric form complexes.

Chapter 2 Materials and Methods

2.1 Restriction-free cloning

We used a restriction-free PCR-based cloning strategy to insert human SATB1(NM_001195470.3) and SATB2(NM_001172509.2) in vectors (kindly provided by Prof. Thomas Pucadyl's lab). We used pET15b_EGFP, pET15b_mCherry, and pET15b_EYFP expression vectors for bacterial expression and purification of the proteins. For mammalian expression vectors, we used pCMV10

2.2 PCR amplification and PCR cleanup

We used the Human Embryonic Kidney cell line HEK293T as a source for human SATB1 and SATB2 genes. HEK293T shows a good expression of SATB1 and SATB2. We did RNA isolation of harvested HEK293T cells using the standard Trizol method. The cDNA was prepared from 1ug of isolated RNA using an iScript cDNA synthesis kit. The cDNA was used to amplify SATB1 and SATB2 using RF-specific primers and Prime Star GXL PCR kit.

The PCR cycle used to amplify the genes is as follows:

- ➔ Hot start at 95℃ for 5 minutes
- ➔ Denaturation at 95℃ for 30 seconds
- ➔ Annealing at 60℃ for 45 seconds
- ➔ Extension at 68℃ for 2 minutes 30 seconds

} 35 Cycles

- ➔ Final Extention at 68℃ for 5 minutes

The required plasmids were amplified using the RF-specific primers and Prime Star GXL PCR kit.

The PCR cycle used to amplify the vectors is given below:

- ➔ Hot start at 95℃ for 5 minutes
- ➔ Denaturation at 95℃ for 30 seconds
- ➔ Annealing and Extension at 68℃ for 10 minutes

} 35 Cycles

- ➔ Final Extention at 68℃ for 5 minutes

The amplified PCR products were purified by agarose gel electrophoresis, followed by PCR cleanup using NucleoSpin gel and a PCR cleanup kit.

The purified inserts and vectors were mixed in a ratio of 1:3 (Concentration of vector: Concentration of insert). The mixture was digested DNA was incubated with 1ul of DpnI enzyme in the NEB cutsmart buffer overnight at 37℃.

Table 2. List of primers

Primer Name	Sequence 5' to 3'	Insert/ Vector
Hu_SATB2_pET15b F	CTGGTGCCGCGCGGCAGCATGGAGCGGCGGAGCGAG	Insert
Hu_SATB2_pET15b R	CGGATCCTCGAGCATATGTCTCTGGTCAATTTCGGC	Insert
Hu_SATB2_pET15b F	GCCGAAATTGACCAGAGACATATGCTCGAGGATCCG	Vector
Hu_SATB2_pET15b R	CTCGCTCCGCCGCTCCATGCTGCCGCGCGGCACCAG	Vector
Hu_SATB1_pET15b F	CTGGTGCCGCGCGGCAGCATGGATCATTTGAACGAG	Insert
Hu_SATB1_pET15b R	CGGATCCTCGAGCATATGGTCTTTCAAATCAGTATT	Insert
Hu_SATB1_pET15b F	GACCATATGCTCGAGGATCCGGTGAGCAAGGGC	Vector
Hu_SATB1_pET15b R	CTCGTTCAAATGATCCATGCTGCCGCGCGGCACCAG	Vector
Hu_ SATB2_pEGFPN1 F	GCTACCGGACTCAGATCTCATGGAGCGGCGGAGCGAGAGC	Insert
Hu_ SATB2_pEGFPN1 R	GCAGAATTCGAAGCTTGAGCTCTCTCTGGTCAATTTCGGC	Insert
Hu_ SATB2_pEGFPN1 F	GCCGAAATTGACCAGAGAGAGCTCAAGCTTCGAATTCTGC	Vector
Hu_ SATB2_pEGFPN1 R	GCTCTCGCTCCGCCGCTCCATGAGATCTGAGTCCGGTAGC	Vector
Hu_ SATB2_pcmv10 F	CTTGCGGCCGCGAATTCAATGGAGCGGCGGAGCGAGAG	Insert
Hu_ SATB2_pcmv10	CGATATCAGATCTATCGATTATCTCTGGTCAAT	Insert

R	TTCG	
Hu_ SATB2_pcmv10 F	CGAAATTGACCAGAGATAATCGATAGATCTGA TATCG	Vector
Hu_ SATB2_pcmv10 R	CTCTCGCTCCGCCGCTCCATTGAATTCGCGGC CGCAAG	Vector

2.3 Transformation and Plasmid isolation

The overnight Dpn1 digested DNA was transformed into DH5α competent cells. The DH5α cells were grown on LB agar supplemented with 100ug/ml of either ampicillin or kanamycin, depending on the antibiotic resistance marker of the vector. Further, Transformed colonies were grown in 1.5 ml LB media supplemented with 100ug/ml of appropriate antibiotics. Plasmid isolation was done by the standard alkaline lysis method. Plasmid purification was done by the phenol-chloroform-isoamyl alcohol purification method. Further, the probable clones were confirmed by performing restriction enzyme digestion and monitoring their expression in suitable expression systems like BL21 (DE3), Rosetta, and HEK293T cells.

2.4 Protein expression

After confirming the clones, we transformed them into the BL21 DE3 and/or Rosetta (DE3) strain of E.coli host with the appropriate antibiotics. Following a 12-hour incubation at 37℃, a single colony was inoculated into 10 ml of LB media supplemented with 100ug/ml of antibiotics to establish a primary culture. The next day, a secondary culture of 1 liter of LB media supplemented with antibiotics was initiated with 10 ml of the primary culture. We induced protein expression using isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM for 16 hours at a shaking speed of 180 RPM once the OD600 of the secondary culture reached a value within the range of 0.5-0.6. Prior to IPTG induction, we set aside a small volume of the secondary culture as an uninduced control. To analyze protein expression, we confirmed and analyzed the protein expression profile by running SDS-Poly-Acrylamide Gel Electrophoresis (SDS-PAGE), followed by both coomassie staining (0.1% Coomassie brilliant blue R250, 50% methanol, 40% deionized water, 10% glacial acetic acid) and western blotting

2.5 Protein Purification

At the end of 16 hours of protein induction, cells were collected by centrifugation (4000 RPM, 30 min at 4℃). The pellet was washed with PBS and suspended in 50 ml of 6X-histidine buffer, which contained 50 mM Tris pH 8.0, 150 mM NaCl, 0.2% Triton-X 100, 2mM PMSF, 1mM DTT, 10mM imidazole, and 10 µg/ml of lysozyme. The suspension was incubated at 4℃ for 20 minutes on a rotator, and the viscosity was reduced by sonication (total 8 minutes with pulse of 2 seconds and rest of 6 seconds at amplitude of 60%). The lysate was centrifuged at 14000 X g at 4℃ for 30 minutes, and the supernatant was then mixed with Ni-NTA agarose beads for six hours at 4℃. The beads were washed three times with lysis buffer containing 50mM (first wash) and 100mM (remaining two washes) imidazole at 1150 X g for 5 minutes. The bound protein was eluted using different concentrations of imidazole (ranging from 200 mM to 500 mM) in the 6X-histidine buffer. The protein quality of the eluent was assessed using 8-10% SDS-PAGE, followed by both coomassie staining (0.1% Coomassie brilliant blue R250, 50% methanol, 40% deionized water, 10% glacial acetic acid) and western blotting

2.6 Western blotting

The protein samples were subjected to electrophoresis on a 7.5-10% SDS-PAGE gel in SDS running buffer and subsequently transferred onto a PVDF membrane using a wet transfer protocol. The PVDF membrane was then blocked using 5% skimmed milk prepared in 1X TBST (50mM Tris, 150mM NaCl, and 0.1% Tween-20) for an hour at room temperature. Next, the membrane was incubated with the primary antibody, diluted as per standard protocol, overnight at 4°C. The membrane was then washed three times with 1X TBST for 10 minutes each time and subsequently incubated with the appropriate secondary antibody for an hour at room temperature. After incubation, the membrane was washed again three times with 1X TBST to minimize non-specific signals. Finally, the proteins were probed with the ECL western blotting substrate and visualized using ImageQuant LAS 4000 software.

2.7 Cell culture and transfection

HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum and 1% Penstrep, and maintained at 37 °C in a humidified

incubator with 5% CO2. For transfection, cells were transfected with Invitrogen Lipofectamine 3000 reagent following the manufacturer's protocol, and harvested after 48 hours by removing the culture media and resuspending them in phosphate buffered saline (PBS). The resuspended cells were pelleted by centrifugation (2500 RPM, 5 min at 4℃).

For protein analysis, the pellet was lysed in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP40, 0.25% sodium deoxycholate, and 0.1% SDS) supplemented with Protease Inhibitor Cocktail (PIC) and incubated at 4℃ on a rotator for 1 hour. The lysate was then centrifuged at 14000 RPM at 4℃ for 30 min, and the supernatant was collected in separate microfuge tubes. The protein sample was prepared by adding 6X SDS PAGE loading dye.

For performing RNA isolation, the pelleted cells were homogenized in the Trizol reagent. RNA isolation was performed using the standard Trizol method.

2.8 Co-immunoprecipitation assay

This assay used FLAG-tagged SATB1, GFP-tagged SATB1, and His-tagged SATB2. All the constructs were transfected in HEK293T cells in six different combinations. The cells were harvested after 48 hours. The cell lysed in BC150 buffer (20% Glycerol, 20mM Tris-HCl; pH ~ 8.0, 2mM EDTA, 150mM KCl) supplemented with 10% NP40. The input sample was collected before the addition of the α-FLAG primary antibody in the cell lysate. Samples were incubated overnight on the rotor at 4℃. The complex bound to the antibody was precipitated by using Protein A beads. After washing the beads three times with BC150 buffer , the sample was eluted in 6x SDS loading dye to run it on the SDS-PAGE, followed by western blotting.

2.9 Native PAGE

We made a 6.5% polyacrylamide gel to separate the purified proteins. The gel was run at 80 volts in cold conditions (4℃) for 3-4 hours. After the run, we stained the gel with a staining solution (50% methanol, 40% distilled water, and 10% glacial acetic acid) overnight at 4℃. The next day, we replaced the staining solution with a destaining solution (50% distilled water, 40% methanol, and 10% glacial acetic acid). We kept it in the destaining until the clear protein bands became visible.

Chapter 3 Results and Discussion

3.1 Identification of SATB1 and SATB2 complex using co-immunoprecipitation assay

Since SATB1 and SATB2 coexpress in certain tissues and can regulate a similar set of target genes, we were curious to know if they interact with each other. Toward this, we employed the strategy of co-immunoprecipitation assay to study the interactions of SATB1 and SATB2 in HEK293T cells. In this assay, the protein of interest is captured using an antibody specific to that protein from the total protein in the whole-cell lysate. If the second protein of interest is forming any complex with the captured protein, it will also get captured along with the first protein. In the end, the association of two proteins is confirmed by the signal detected for a second protein indirectly probed in the western blot analysis.

Before performing the coimmunoprecipitation assay, we had to standardize the transfection of SATB1 and SATB2 expressing mammalian expression constructs in the Human Embryonic Kidney HEK293T cell line. We used Flag-tagged SATB1, GFP-tagged SATB1, and His-tagged SATB2 constructs for this assay. We observed a good expression of all the proteins in HEK293T cells on transfecting two micrograms of DNA (Figure 3 A-C).

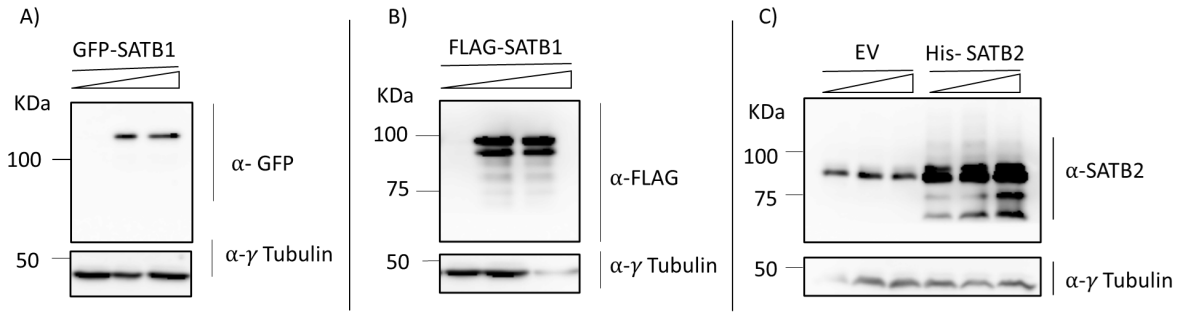

Figure 3. Standardization of transfection efficiency for SATB1 and SATB2 expressing plasmids. A) Standardization of transfection efficiency using increasing DNA concentration of GFP-tagged SATB1, immunoblotted with α-GFP and α-γ-tubulin

antibody. B) Standardization of transfection efficiency using increasing DNA concentration of FLAG-tagged SATB1, immunoblotted with α-FLAG and α-γtubulin antibody. C) Standardization of transfection efficiency using increasing DNA concentration of His-tagged SATB2, immunoblotted with α-SATB2 and α-γtubulin antibody. KDa: KiloDalton

Once the standardization of the transfection efficiency was done, we proceeded with the co-immunoprecipitation assay. For that, FLAG-tagged SATB1, GFP-tagged SATB1, and His-tagged SATB2 were transfected alone as well as with each other to understand the association of SATB1 and SATB2 in a complex. The cells were harvested after 48 hours of transfection, followed by lysis of cells and co-immunoprecipitation assay. Further, western blot analysis was done to examine the interaction of proteins. The input sample was collected before starting the co-immunoprecipitation assay for confirmation of overexpressed factors (Figure 4 A Lane 1-6). Consistent with earlier reports, our results confirmed that SATB1 can self-associate (Figure 4 B Lane 5) as we detected that GFP-tagged SATB1 could interact with FLAG-tagged SATB1. Similarly, we also observed that SATB2 is forming a complex with SATB1 (Figure 4 B Lane 6)

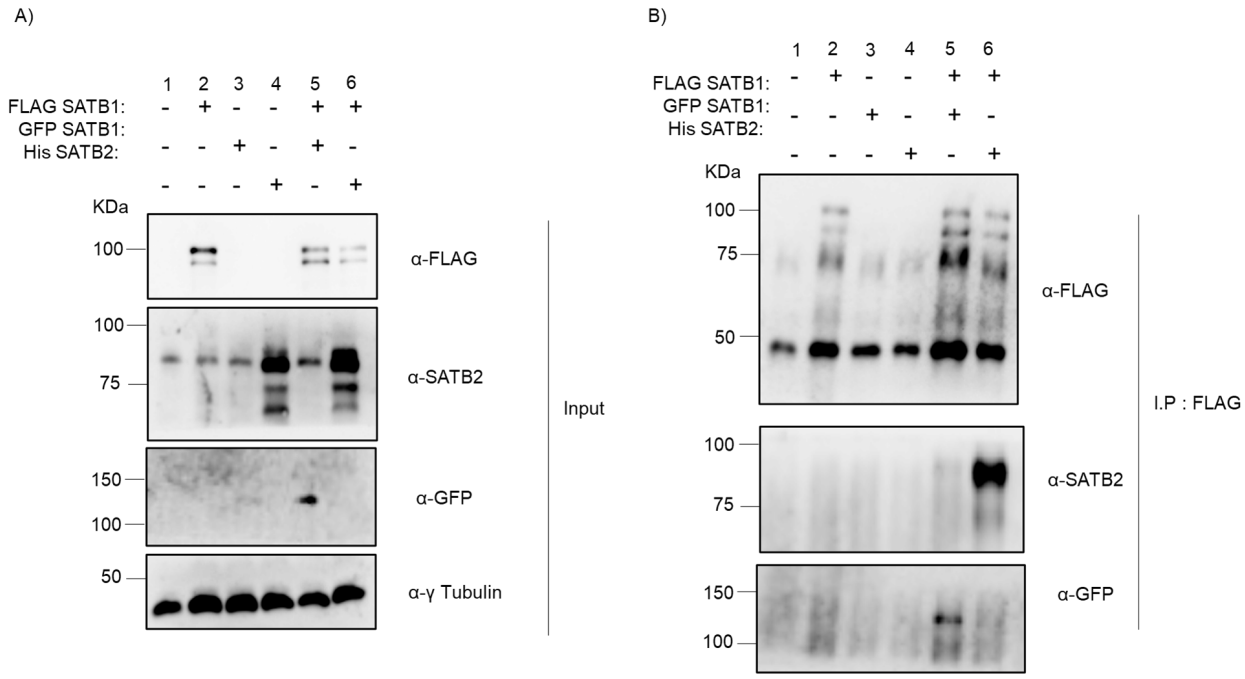

Figure 4. Co-immunoprecipitation of SATB1 and SATB2 protein. A) Input samples (before immunoprecipitation) for all the conditions were run on SDS-PAGE gel and immunoblotted with the specific antibodies. B) The immunoprecipitated

samples were run on the SDS-PAGE gel and immunoblotted with specific antibodies. KDa: KiloDalton

3.2 Cloning of human SATB1 and SATB2 protein

Previous studies have reported the role of oligomeric forms of SATB1 in regulating the expression of various target genes. Similarly, based on the results of the Co-IP assay, we speculated that SATB1 and SATB2 could be present in the cells in different oligomeric forms. Moreover, it is possible that these hetero-oligomeric complexes might function differentially than the reported homo-oligomers of SATB1. To further investigate the oligomeric complex formed by these proteins, we decided to express and purify human SATB1 and SATB2 proteins using the method of heterologous protein expression in bacterial systems and affinity chromatography, respectively.

To visualize the oligomers easily, we decided to use the fluorescently tagged fusion proteins. Therefore we used the pET15b bacterial expression vectors, which have an N-terminal 6X-Histidine tag and a C-terminal fluorescent tag. We made a plan to clone SATB2 in GFP and mCherry-containing vectors and SATB1 in a YFP-containing vector.

Similarly, for overexpression of SATB2 in cell-based systems, we chose GFP and FLAG tags containing mammalian expression vectors to clone SATB2 specifically.

Bacterial expression system vectors		Mammalian expression system vectors
pET15b-EGFP	N-terminal 6XHis tag	pEGFP-N1
pET15b-mCherry		pCMV10
pET15b-EYFP		

Table 2. Bacterial and mammalian expression vectors.

Initially, we were using the restriction enzyme-based cloning method. However, due to failure in procuring a successful clone, we decided to use the restriction-free PCR-based cloning method for cloning SATB1 and SATB2 in desired expression vectors. The restriction-free cloning method does not require restriction enzymes. However, it involves a step where we use the DpnI enzyme to digest the parent plasmid, which could reduce the false colonies after transformation

3.3 Restriction-free cloning:

In Restriction-free (RF) cloning, the gene of interest and the destinated plasmid is amplified using primers designed specifically for RF cloning. These primers are designed in such a way that the amplified products will be flanked by a sequence that will facilitate homologous recombination (van den Ent and Löwe, 2006; Jacobus and Gross, 2015).

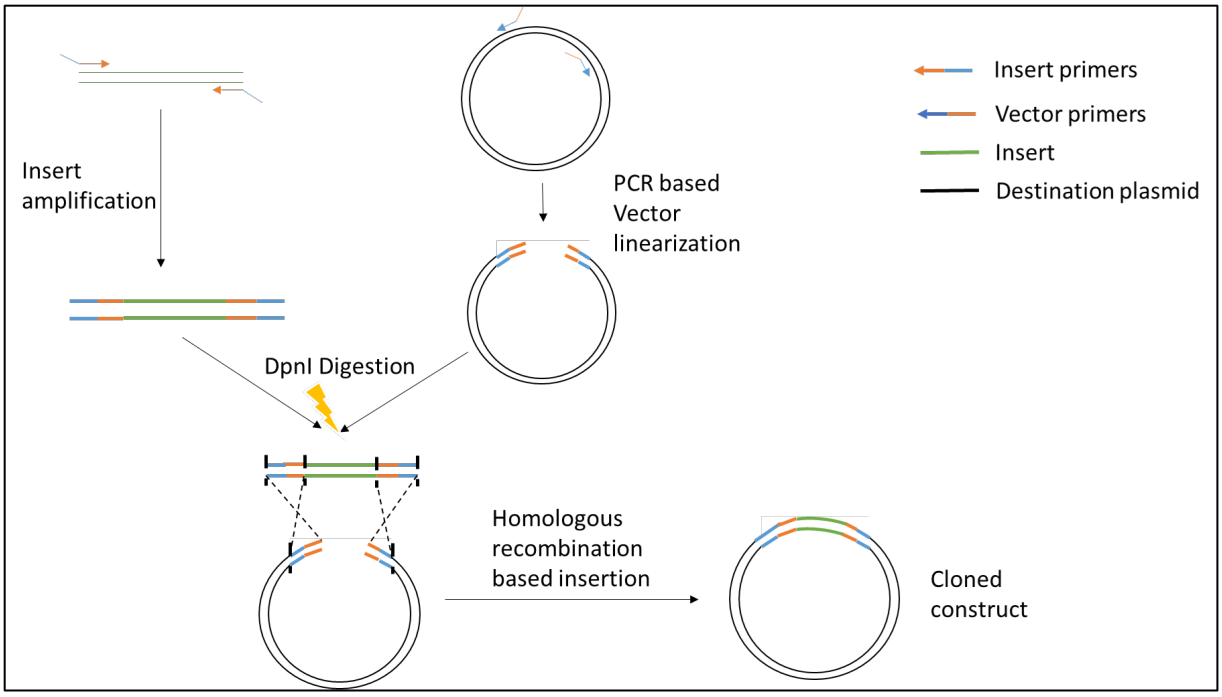

Figure 5. Schematic representation of restriction-free cloning.

PCR showed successful amplification of SATB1 and SATB2 genes for cloning in available pET vectors (Figure 6 A). In classical cloning, we use a restriction enzyme to linearize the vector, whereas, in the Restriction-free method, specific primers are used to linearize the vector by PCR. We did not get vector amplification at our first attempt. To get a successful amplification, we optimized many parameters. Finally, it worked when we used a 2-step PCR cycle where we kept annealing and extension at 68 degrees. Both pET15b_EGFP and pET15b_mCherry vectors were linearized successfully (Figure 6 B).

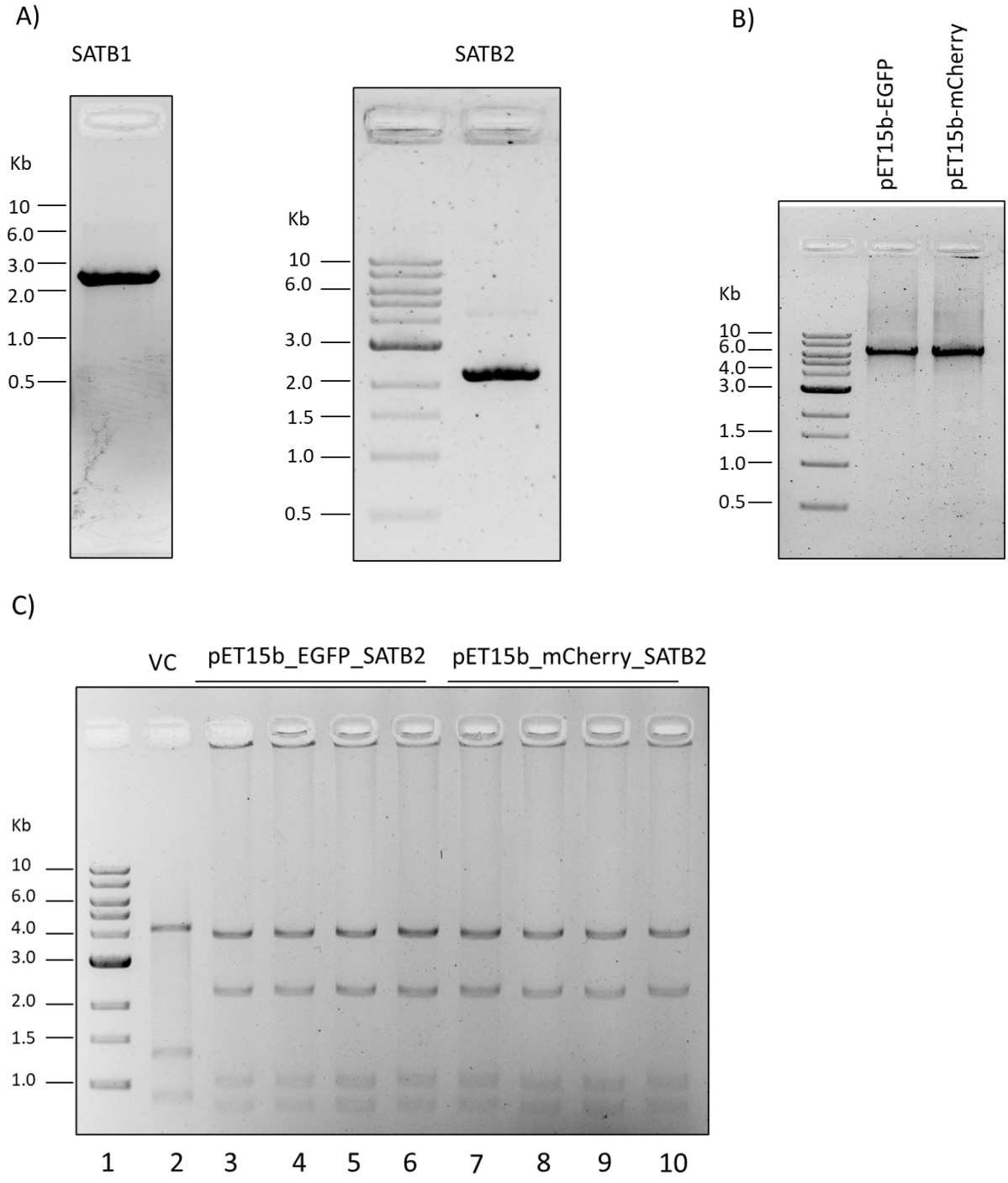

Figure 6. Restriction-free cloning of SATB1 and SATB2 for bacterial expression system. A) PCR amplification of the SATB1 (2388 bp) and SATB2 (2202 bp) genes for cloning in bacterial expression vectors. B) PCR amplification of bacterial expression vectors pET15b_EGFP (6425 bp) and pET15b_mCherry (6416 bp). C) Diagnostic digestion of pET15b_EGFP_SATB2 and pET15b_mCherry_SATB2 with EcoRV and XhoI Enzymes. Lane 1: DNA marker, Lane 2: pET15b_mCherry vector control (VC), Lane 3 - 6: Digested pET15b_EGFP_SATB2, Lane 7 - 10: Digested pET15b_mCherry_SATB2. All PCR products were checked on 0.8-1% agarose gel. Kb: Kilo bases

The amplified vector and insert were directly mixed in a standard 1:3 ratio and subjected to overnight DpnI digestion. When we transformed the digested mixture into DH5α competent cells, we did not get a single bacterial colony. We speculated that the purification of amplified products before transformation might increase the chances of obtaining a clone. Therefore, we followed the same protocol, but we purified amplicons using the gel purification method this time. The transformation was successful, as we found bacterial colonies on the agar plate after 12 hours of incubation.

The plasmids were isolated from transformed bacterial colonies using the alkaline lysis method of plasmid isolation. Further, we screened the plasmids using agarose gel electrophoresis and restriction digestion method. The confirmatory digestion of pET15b_EGFP_SATB2 and pET15b_mCherry_SATB2 with restriction enzymes showed bands at the expected sizes (Figure 6 C). Unfortunately, we could not linearize the pET15b_EYFP vector even after modulating different parameters.

For the mammalian expression system, pEGFP_N1 and SATB2 were amplified using the same restriction-free cloning method (Figure 7 A). But, upon confirmatory digestion, we observed an upward shift of approximately 100 bp in the bands of digested pEGFP_N1_SATB2 constructs (Figure 7 B). Nevertheless, we transfected these plasmids in HEK293T cells to check the expression of GFP-tagged SATB2. The absence of the fluorescence signal confirmed the clone as a false positive. Similarly, we could not procure the pCMV10_SATB2 clone due to the lack of confirmation of the positive clone.

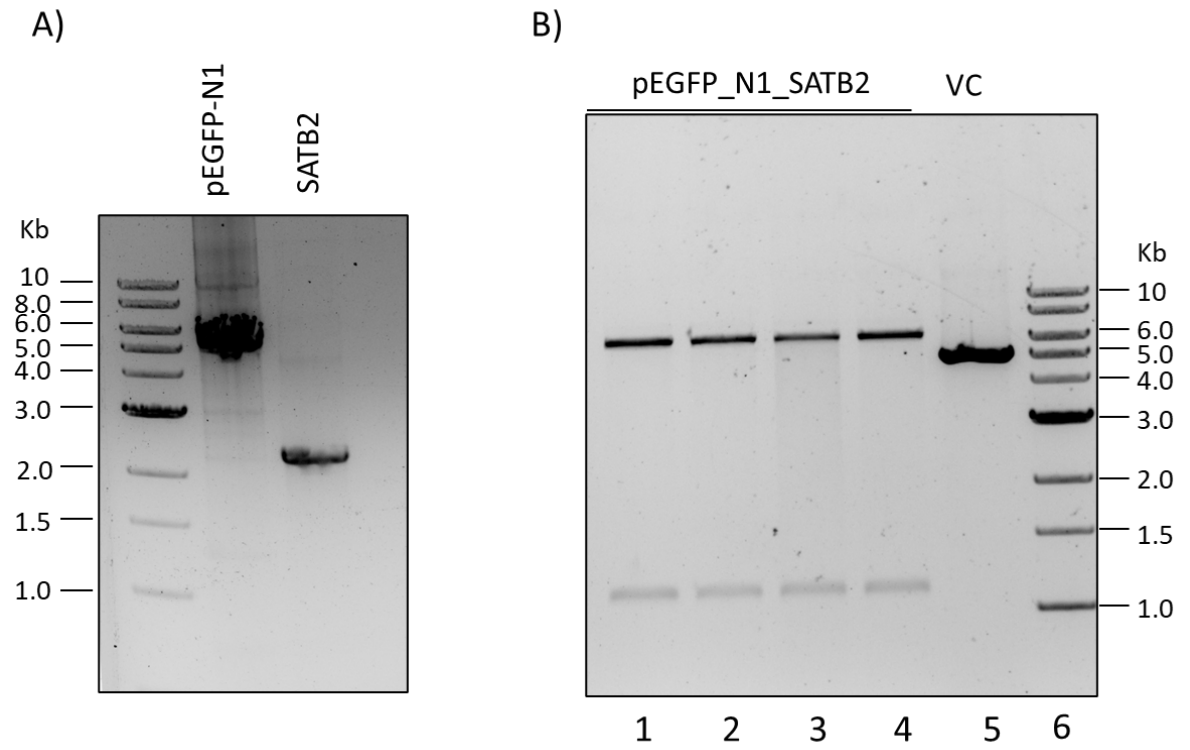

Figure 7. Restriction-free cloning for mammalian expression system. A) PCR amplification of SATB2 and mammalian expression vector pEGFP_N1 (4733 bp). B) Diagnostic digestion of pEGFP_N1_SATB2 and vector control with EcoRI and EcoRV Enzymes. Lane 1 - 4: Digested pEGFP_N1_SATB2, Lane 5: Digested pEGFP_N1 vector control (VC), Lane 6: DNA marker. All PCR products were checked on 0.8-1% agarose gel. Kb: Kilo bases

3.4 Standardization of expression and purification of SATB2

3.4.1 Standardization of protein expression

We proceeded with the positive clones of SATB2 for expressing them in a suitable host. Initially, we used the BL21 DE3 strain of E.Coli to express the proteins, but we did not observe substantial expressions of them. Hence, we decided to express these clones in BL21-derived Rosetta-competent cells, which enhances the expression of eukaryotic proteins by providing the codons rarely used in prokaryotic systems like E.coli. Further, we standardized the suitable temperatures for protein expression. We chose lower temperatures, such as 25℃, to enhance the stable expression of the proteins. We observed better expression of SATB2 protein in Rosetta cells induced at 25℃ in comparison to 18℃ (Fig . 8A-B compare lanes). Hence, 25℃ was chosen for induction of protein expression for downstream purification of proteins.

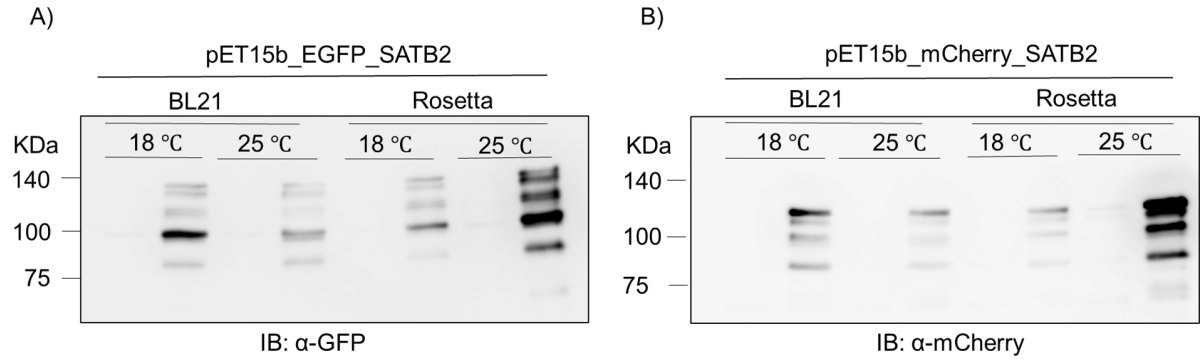

Figure 8. Protein expression in BL21 DE3 and Rosetta competent cells at different temperatures. A) Standardization of protein expression of pET15_EGFP_SATB2 in bacterial strain BL21 and Rosetta at 18℃ and 25℃. Immunoblotted with α-GFP antibody. B) Standardization of protein expression of pET15_mCherry_SATB2 in bacterial strain BL21 and rosetta at 18℃ and 25℃. Immunoblted with α-mCherry antibody. IB: Immunoblot. KDa: KiloDalton

Since we observed low molecular weight bands along with the expected high molecular weight band of SATB2 protein, we speculated that these bands could be the result of contaminating non-specific bacterial proteins or the degradation of overexpressed proteins.

In order to reduce the low molecular weight bands, we employed different strategies. First, we standardized different IPTG induction times of the protein. We harvested bacterial cells after 8hrs, 12hrs, and 16 hrs of induction (Figure 9A). These harvested samples were run on SDS-PAGE to compare the expression and degradation at different time points. Here, we observed that induction for 16 hours shows a high percentage of degradation than other two time points (Compare lane 2,4&6). But Overall, we did not see any significant effect of induction time on the degradation pattern of this protein. Second, we tried to reduce the degradation using inhibitors of proteases during the lysis step of purification. We used different inhibitors such as PMSF, PIC, Phostop, and NaB to counteract the degradation, but western blot analysis again showed no significant difference in these conditions (Figure 9B compare 2&4). We always observed the lower molecular bands when we probed with SATB2 antibody.

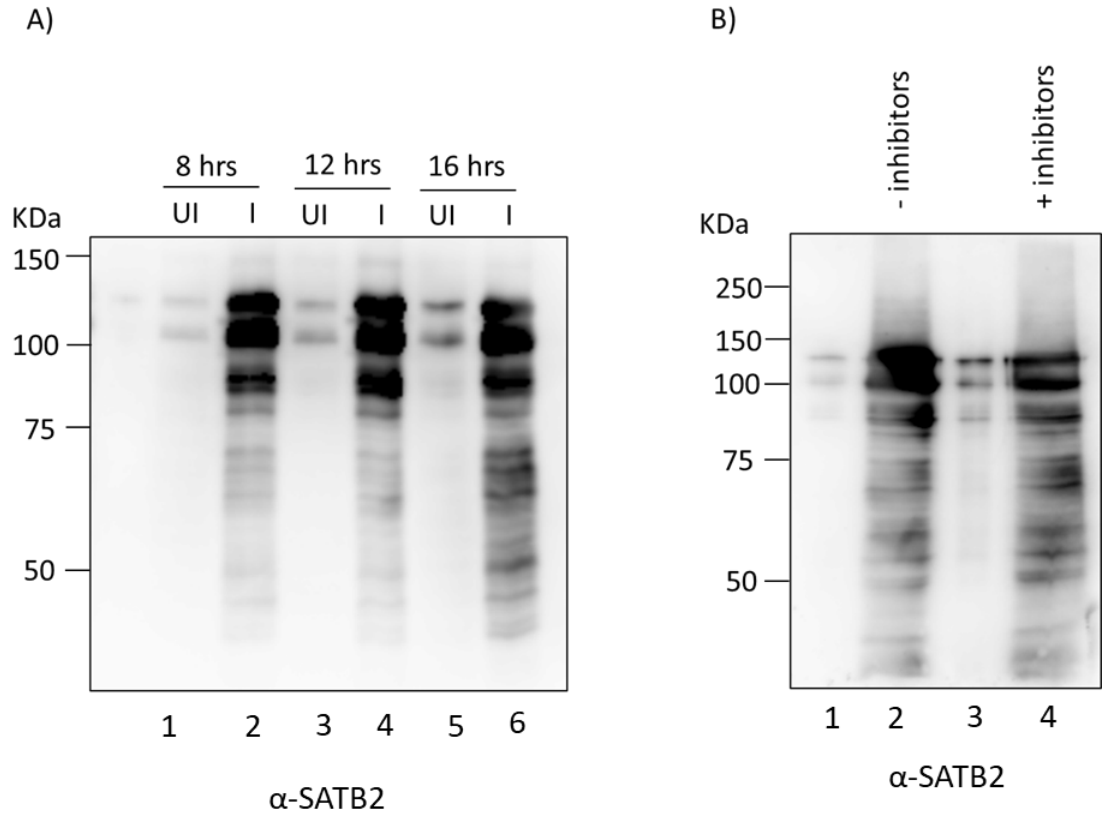

Figure 9. Protein degradation of SATB2 in different conditions. A) Protein degradation was monitored at expression level after 8hrs, 12hrs, and 16hrs of IPTG induction. Immunoblotting was done using α-SATB2 antibody. UI: Uninduce, I: Induced B) Protein degradation was analyzed in the presence and absence of inhibitors during the lysis stage. Immunoblotting was done using α-SATB2 antibody. KDa: KiloDalton

Finally, instead of focusing on the degradation, we shifted our attention toward enhancing the protein expression to increase the yield of the protein. We expressed our clones in different growth media to see the effect of growth media on the expression of the protein. We used standard Luria-Bertani (LB), Terrific Broth (TB), SOB, and 2X-YT media. Preliminary results showed better expression in LB and TB as compared to SOB and 2X-YT (Data not shown here)

Other approach we investigated to increase the expression of SATB2 was ethanol. It was reported in a study where the authors showed that adding ethanol at the time of IPTG induction in the range of 1% to 4% enhances the expression of the protein(Chhetri *et al.*, 2015). The mechanism by which it enhances the expression is still not clear. The probable mechanism that is mentioned in the study is that ethanol treatment mimics the heat shock response, which might activate the chaperones, which will help in the stabilization of the protein. They have also mentioned that it could

increase the solubilization of protein. Nevertheless, when we used the different percentages of ethanol as suggested in the paper, we observed a relatively high expression in LB added with 1% ethanol (Figure 10A lane 4)

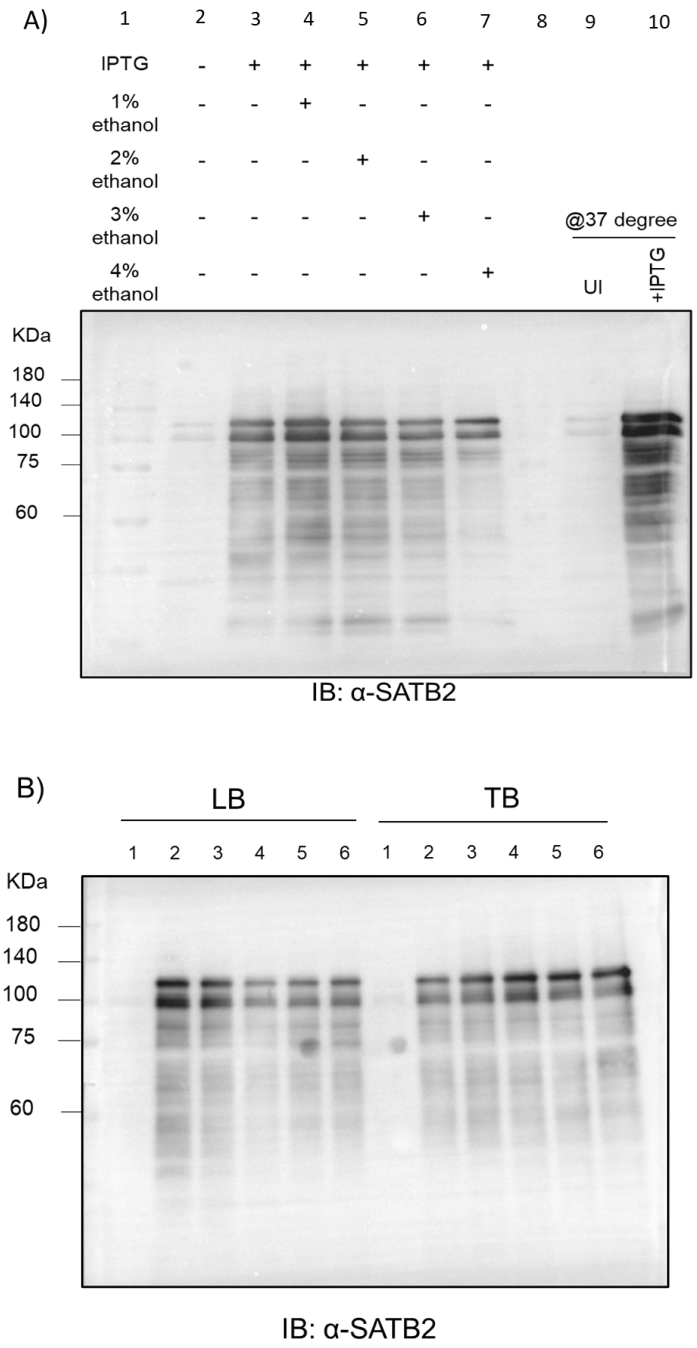

Figure 10. Protein expression enhancement using different media and ethanol. A) Lane 2-7: Protein expression in LB with different percentages of ethanol, Lane 10: Protein expression at 37℃ for 4 hours. B) Comparison of protein expression in LB and

TB with different percentages of ethanol content at 25℃. 1: Uninduced, 2: IPTG control, 3: IPTG + 1% ethanol, 4: IPTG + 2% ethanol, 5: IPTG + 3% ethanol, 6: IPTG + 4% ethanol. Immunoblotted with α-SATB2 antibody. UI: Uninduce. KDa: KiloDalton

We also added the ethanol in TB along with LB to compare the expression pattern of the protein in the two media. We observed that with increasing ethanol percentage, expression decreased. Even though there was a relative enhancement in 1% ethanol in LB and 2-3% ethanol in TB, the highest expression was in the sample added with IPTG only (Figure 10B compare lane 2 & rest) . Hence, we went back to the standard LB media for the expression and purification of the protein.

Moreover, in order to increase the expression of the protein, we also induced protein expression at 37℃ for 4 hours in LB. At higher temperatures, we saw a good expression but the same level of degradation (Figure 10B last lane). We still proceeded with the purification, but we observed that after sonication followed by centrifugation, a large fraction of protein remained in the pellet, and there was negligible protein in the supernatant. The possible cause of this difference could be the high protein expression rate at higher temperatures, leading to the formation of protein aggregates and inclusion bodies that hold the protein inside the pellet. We had observed a similar pattern with a significant level of protein in the pellet during the initial standardization stages when we expressed our proteins at 25℃. Hence, the results indicate that the expression at a higher temperature would not enhance the purification process. Hence we used the temperature of 18℃ and kept the induction time for 16 hours after induction for downstream purification process.

3.4.2 Standardization of protein purification

The standardization of protein expression was followed by protein purification. We used an immobilized affinity chromatography method for protein purification, where we used Ni-NTA agarose beads as a solid support, which will bind to the 6X-histidine tag present at the N-terminal of expressed proteins. The purification protocol has four major steps, which include lysis of bacterial pellet, binding of proteins to Ni-NTA beads, washing to remove unbound and non-specific proteins, and elution of the bead-bound protein.

Initially, we gave three washes with histidine buffer containing 20 mM imidazole, and elutions of protein were carried out using a gradient of imidazole concentration from

50 to 500 mM (Figure 11 A-B) When we analyzed the profile of elutions on an SDS-PAGE gel stained with coomassie dye, we again observed low molecular weight bands of unexpected band size along with the expected size of GFP-tagged SATB2. The western blot of same purified samples showed high intensity degraded lower bands and low intensity full length protein band (Figure 11 C&D).

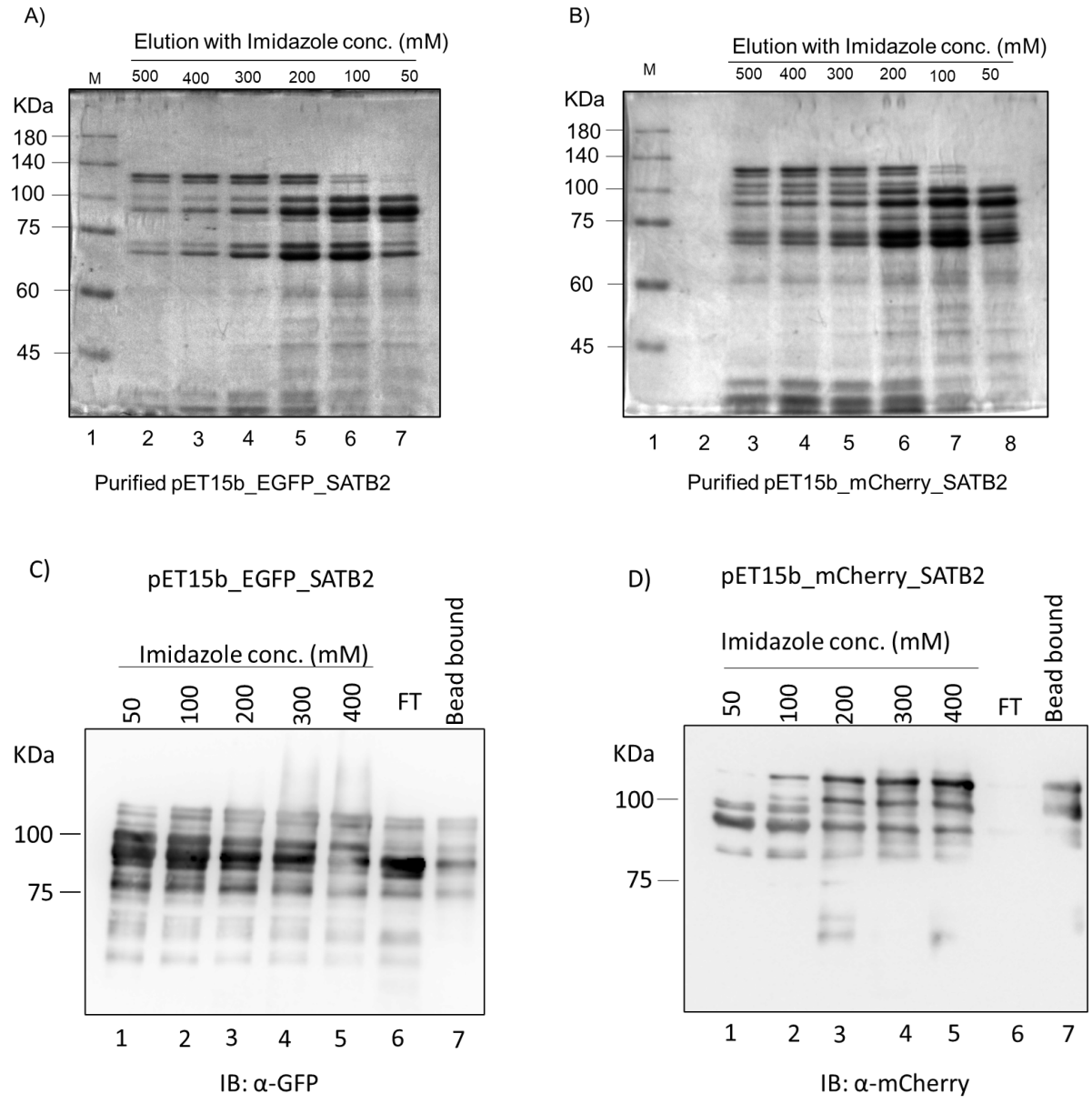

Figure 11. Purification of SATB1 and SATB2 by affinity chromatography A) Coomassie staining was performed to check protein purification of pET15b_EGFP_SATB2 B) Coomassie staining was performed to check protein purification of pET15b_mCherry_SATB2. C) Western blot showing purified pET15b_EGFP_SATB2 immunoblotted with an α-GFP antibody. D) Western blot

showing purified pET15b_mCherry_SATB2 immunoblotted with α-mCherry antibody. KDa: KiloDalton

Additionally, we observed that the GFP-tagged SATB2 showed inefficient binding as significant amount of protein has remained in the flowthrough (Figure 11C, lane 6). It is not the case with the mCherry-tagged SATB2 as we see a negligible amount of protein in the flowthrough (Figure 11D, lane 6). Therefore, we decided to standardize the binding efficiency of GFP-tagged SATB2. We wanted to check the affect of concentration of imidazole on the binding efficiency of the protein, therefore we kept three different conditions with omM, 20mM and 40mM imidazole concentration during the binding stage. Another parameter that we changed was the duration of binding stage. As we wanted to minimize the degradation product and increase binding efficiency of protein we chose three time durations which are 4 hours, 6 hours and 8 hours of binding time.

In another keen observation, the lower molecular bands in the gel are more prominent in the 50mM and 100mM elution samples (Figure 11A lane 6&7, 11B lane 7&8) whereas the expected upper band is not present. This indicates that the elution of intact bound protein starts from 200mM (Figure 11A lane 2-5, 11B lane 3-6) Therefore, we decided to give one wash with 50mM imidazole and the remaining two washes with 100mM imidazole to reduce the lower molecular weight bands and to increase the percentage of the full-length purified protein.

When we analysed the effect of different imidazole concentrations we observed that in 0mM condition the lower molecular weight band is more prominent as compared to the 20mM and 40mM condition. Next, when we compared the 20mM and 40mM conditions, it is evident that the protein is still present in the flowthrough significantly even though the elution profiles are comparable between two conditions. Hence, the 20mM imidazole concentration seemed more promising condition for further purification process (Figure 12 A,B and C).

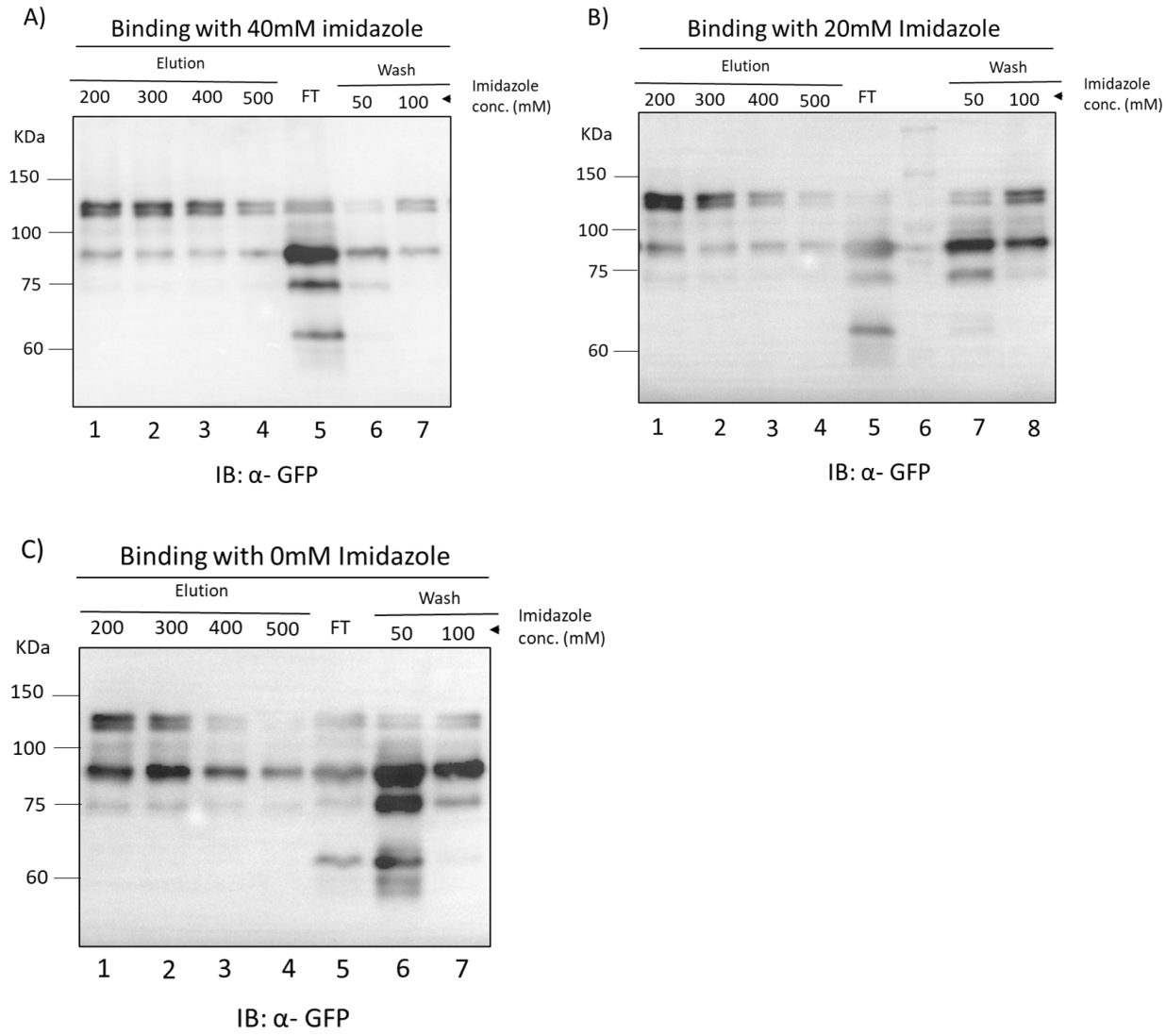

Figure 12. Standardization of binding efficiency of SATB2 protein with different imidazole concentration. A) A western blot showing a protein purification profile of GFP-tagged SATB2 in the condition of 40 mM imidazole concentration during the binding stage. IB: α-GFP antibody. B) Protein purification profile of GFP-tagged SATB2 in the condition of 20 mM imidazole concentration during the binding stage. IB: α-GFP antibody. C) Protein purification profile of GFP-tagged SATB2 inthe condition of 0 mM imidazole concentration during the binding stage. IB: α-GFP antibody.

Furthermore, we analysed the purification profile with different binding time duration. We did not observe much difference in these conditions. If we compare the profile of 4 hours and 6 hours of binding duration, we see bands in the eluted samples in both the conditions but we also see bands in the wash profile of 4 hour condition (Figure 13 A&B). However, we can not conclude much from the present data as it needs further fine tuning.

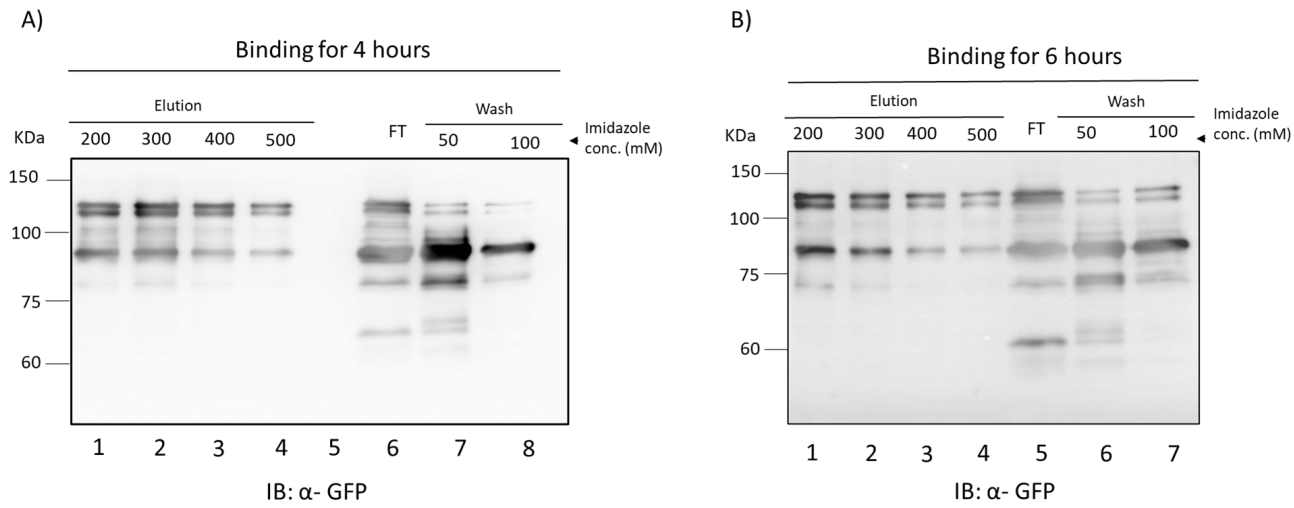

Figure 13. Standardization of binding efficiency of SATB2 for different time duration. A) A western blot showing a protein purification profile of GFP-tagged SATB2 after 4 hours of binding condition. IB: α-GFP antibody. B) A western blot showing a protein purification profile of GFP-tagged SATB2 after 6 hours of binding condition. IB: α-GFP antibody.

Finally, we also purified the pET15b_mCherry_SATB2 protein while considering all the parameters and got a prominent band of our interest of protein in the elutions. The same ladder-like pattern appeared below the expected band, but the intensity was very low this time compared to the bands in previous coomassie-stained gels. We again did a western blot analysis to verify the non-specific lower molecular bands for protein degradation, and we found nearly the same pattern as in coomassie-stained gel (Figure 14 A & B). These results indicated an improved profile of SATB2 protein purification.

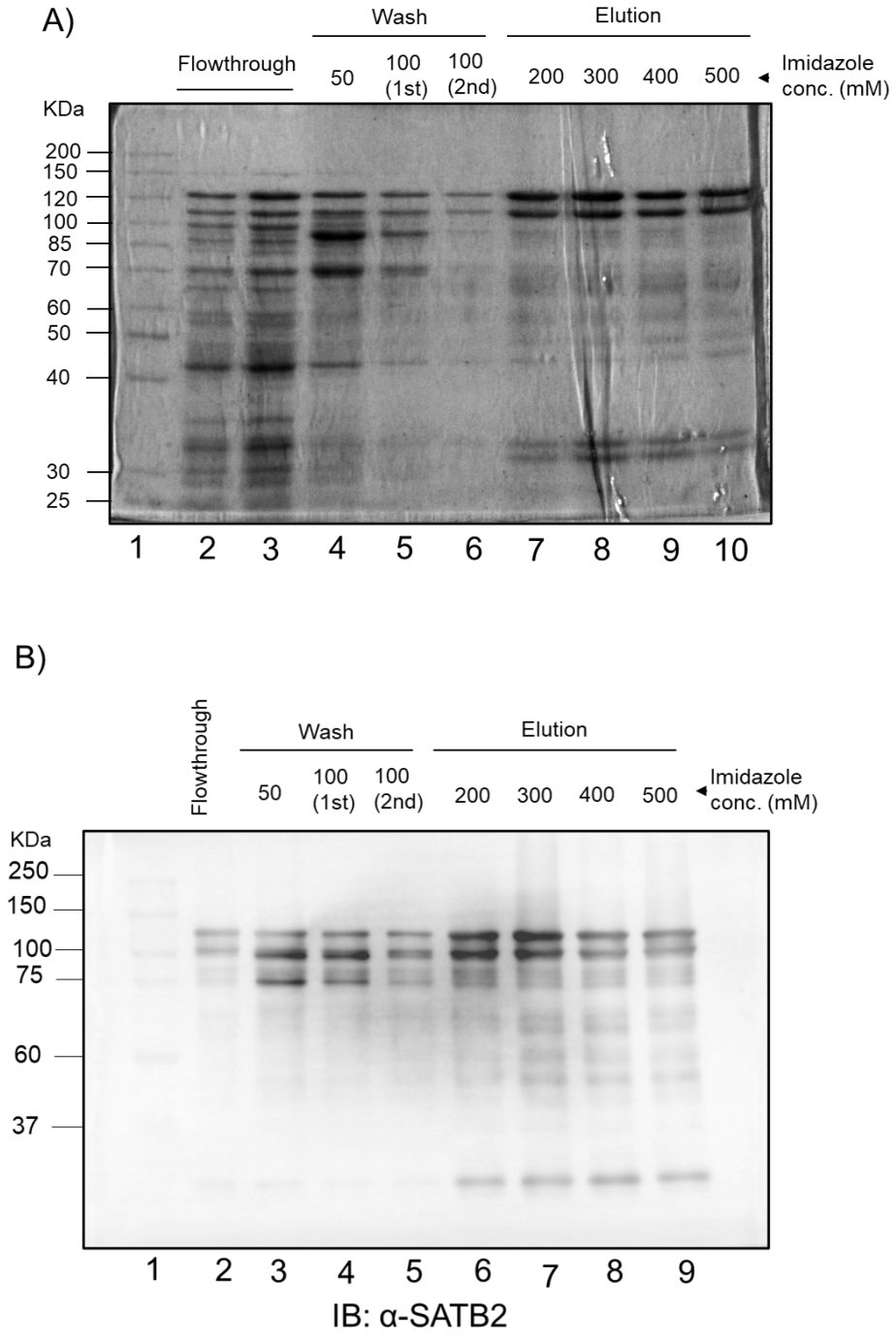

Figure 14. The optimized profile of pET15b_mCherry_SATB2 purification. A) Coomassie-stained gel showing wholesome proteins in a flowthrough, washes, and elutions of purified SATB2 protein. B) Western blot of purified pET15b_mCherry_SATB2 showing the extent and quality of purification. immunoblotted with α-SATB2 antibody. KDa: KiloDalton

3.5 Native PAGE of purified proteins

The native PAGE is a technique used for separating proteins in their native conformation with the help of their intrinsic charge and molecular weight. It is different from SDS-PAGE, where SDS is specifically used for denaturing proteins and making them linear. In Native PAGE, the Protein's globular structure is retained in the absence of SDS and β-mercaptoethanol from the running buffer and sample buffer. Therefore, this technique can be used to study the quaternary structures of the proteins.

In literature, it is not known whether SATB2 exists in any oligomeric form. Therefore, we wanted to understand if SATB2 can self-associate. We performed Native PAGE with the purified SATB2 to check if we could find a similar pattern. A coomassie-stained gel showed a banding pattern which shows an indication of oligomeric states. We used a globular protein BSA as our reference for molecular weight while running the gel. In Native PAGE, the higher oligomer forms of SATB2 are observed, which is interpreted based on the high molecular weight band present than expected size bands (Figure 15 A&B). If the protein is present in different oligomeric forms, it will show bands separated at a distance comprising the molecular weight of a monomer, dimer, trimer and tetramer depending on the nature of oligomeric states. The approximate molecular weight of the bands indicates the possibility of a dimer, trimer, and tetramer.

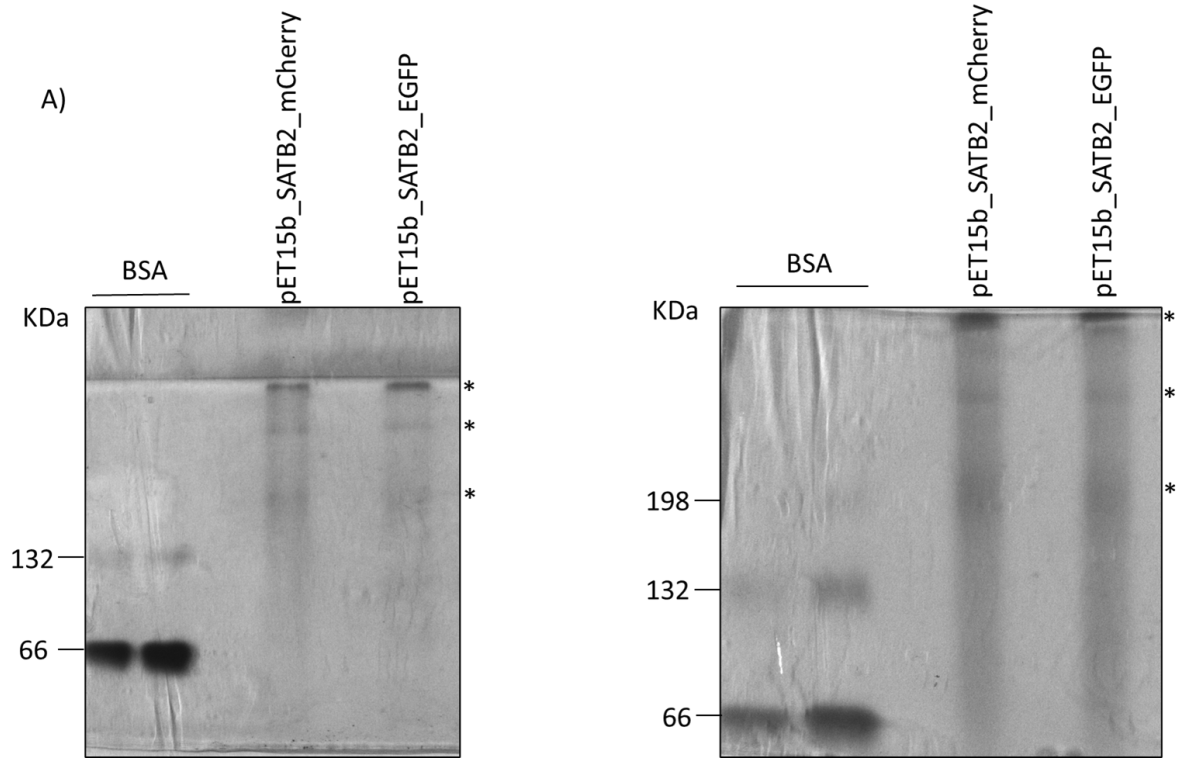

Figure 15. Native PAGE of purified SATB2 protein. A) Both the images show coomassie-stained Native PAGE of pET15b_EGFP_SATB2 and pET15b_mCherry_SATB2. The duplicate data has been shown to rectify the smeary

appearance of the bands in the second image and for better reference of a BSA ladder. KDa: KiloDalton

We also noted the absence of a monomeric band that should be present around the size of 100KDa. It indicates the possibility that the higher oligomeric states of this protein are more prevalent and stable than its monomeric subunit. We would also like to emphasize that when denaturing the protein, we always observed degraded low molecular weight bands instead of getting only one band. Therefore, it is possible that it is more stable in its oligomeric state than in its monomeric form. However, it needs further validation.

For further understanding of the stability of the oligomers, we used a different strategy which involves using β-mercaptoethanol (β-Me). The β-Me acts as a reducing agent and helps in the denaturation of the protein. Formation of self-associated SATB2 is critically dependent on β-Me. For that, we employed different percentages of β-Me to create different denaturation conditions assuming that no β-Me will facilitate higher oligomeric self-associated SATB2. In these conditions, we can expect to see a partial protein denaturation and hence the possibility of observing oligomeric forms in the case of purified proteins.

Initially, we performed this assay using purified SATB2 in the presence of 0% and 6% β-Me under the denaturing conditions. As usual, we saw a monomeric form of SATB2 and similar degradation pattern in a sample denatured with 6% β-Me, whereas in 0% β-Me, we saw a reduced monomeric form at expected size and increased smear present above the expected size of monomer (Figure 16A). This smear could be an indication of retained higher molecular interaction between the purified protein molecules. Hence, we verified it with western blot, where we again saw a smear in a 0% β-Me condition (Figure 16B). Therefore, based on the above observations, we understand that SATB2 could form a higher oligomeric form which seems more prevalent than its monomeric form. Nevertheless, further experimental validations are needed to make an appropriate conclusion from the present data.

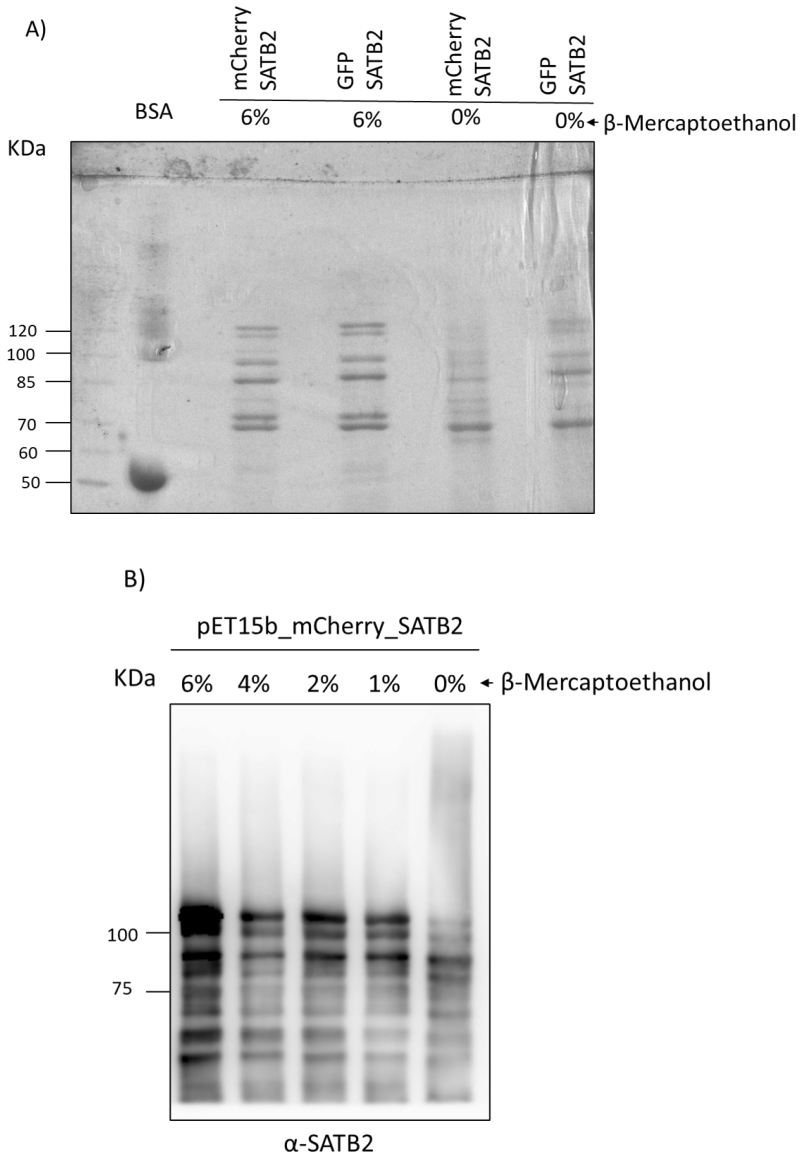

Figure 16. Effect of β-Me on the higher molecular interaction of purified SATB2. A) Comparison of purified pEt15b_mCherry_SATB2 and pET15b_EGFP_SATB2 in the presence of 6% and 0% β-Me. Coomassie brilliant blue R250 was used for staining the gel. B) Partial denaturation of the purified pET15b_EGFP_SATB2 protein was done in different percentages of β-Me. Immunoblotting was done using α-SATB2 antibody. KDa: KiloDalton

3.6 Sequential pulldowns of SATB1 and SATB2

We successfully confirmed that SATB1 and SATB2 are present in a complex from our co-immunoprecipitation study. We wanted to characterize this complex further and study the interacting partners of SATB1 and SATB2 together. Therefore, we decided to do sequential pulldown of these two factors using antibodies and Ni-NTA beads in

non-denaturing conditions. First, we overexpressed FLAG-SATB1 and His-SATB2 in HEK293T cells. We used the α-FLAG antibody to capture the SATB1 protein in the whole cell lysate (Figure 17A). Next, we wanted to use this SATB-enriched pool of proteins to do the second pulldown of His-tagged SATB2 using Ni-NTA agarose beads. The elution of the second pulldown will contain a complex of SATB1 and SATB2 along with the other interacting proteins. Further analysis of this second elution by SDS-PAGE followed by western blotting would give us a profile of known interacting partners reported in the literature.

The immunoprecipitation of FLAG-SATB1 worked, but the elution of FLAG-tagged SATB1 needs to be standardized as a significant amount of SATB1 is still present in the flowthrough and the bead-bound state (Figure 17B). We proceeded further with the amount of the first eluent and performed the second immunoprecipitations using the Ni-NTA beads.

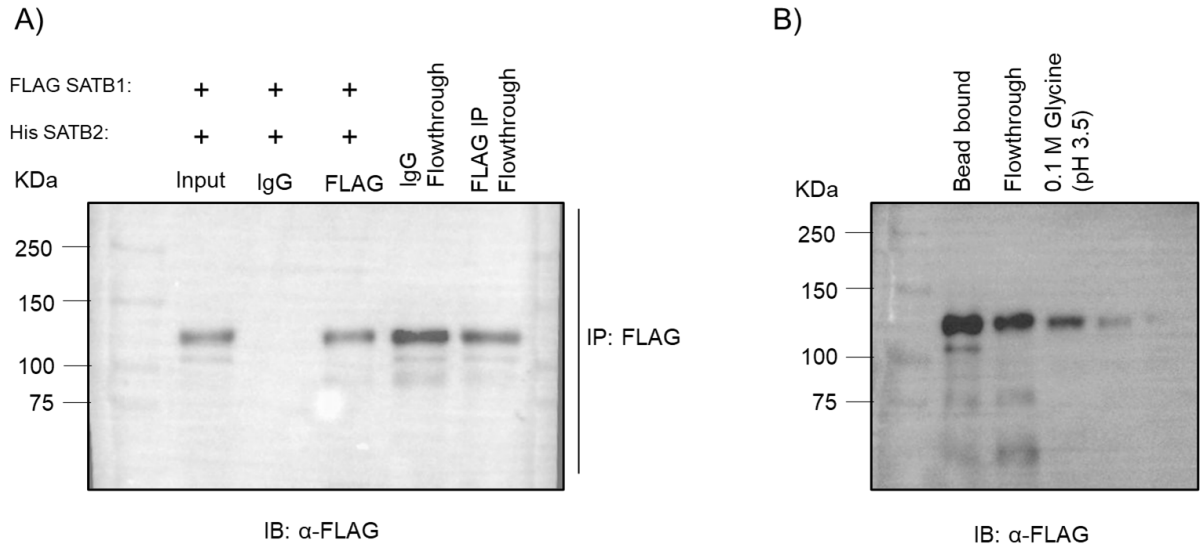

Figure 17. Immunoprecipitation of FLAG-tagged SATB1. A) Immunoprecipitation of FLAG-tagged SATB1 using α-FLAG antibody. They were immunoblotted with α-FLAG antibody. B) Elution of bead-bound FLAG-tagged SATB1 using different glycine concentrations in different pH conditions. KDa: KiloDalton

Unfortunately, we could not gather much information as we did not see a strong signal for the histidine tag or the SATB2 protein. We also did the same study, but in the reverse order, i.e., we did the first immunoprecipitation using Ni-NTA beads to capture His-tagged SATB2. This time we observed that the binding of His-tagged SATB2 is inefficient as we saw a large amount of protein in the flowthrough sample. Hence, We speculated that due to the weak binding of the His-tagged SATB2 to the agarose

beads, we did not see a strong signal for it in the western blot at our first attempt. For further investigation, the elution of flag-tagged protein and binding of histidine tag needs standardization.

3.7 Conclusion and Future Prospects

In this study, we aimed to identify the novel complex formation by SATB1 and SATB2 proteins. Our co-immunoprecipitation assay indicated that SATB1 and SATB2 proteins are present in the same complex in cell-based assays. For *in vitro* characterization of the complex we would perform size exclusion chromatography (SEC) and sucrose-gradient ultracentrifugation. Towards that, we cloned the human SATB2 gene, however, remained unsuccessful in cloning the SATB1 gene for its expression and purification. Currently, SATB1 cloning is in process, and the following experiments will be performed.

There is no report of any oligomeric form of SATB2; hence we focused our attention in identification of SATB2 oligomer formation using the purified proteins. The Native PAGE showed an indication of possible oligomeric states in our study. We are planning to validate these observations by taking various biochemical and biophysical approaches.

We need to determine the interacting regions between two monomers for further structural and functional characterization of the protein complex. If we could alter the interacting regions, we could employ the strategy of amino acid substitution to disrupt the oligomeric association so that it could form only a single species of an oligomer. Using these mutants, we could study the effect of different oligomeric forms on the known target genes. It will help us further elucidate the dynamic mechanism of gene expression regulation by SATB1 and SATB2 protein oligomers.

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