Studying the Interacting Partners of RNA Polymerase III transcription factors, TFIIIB and TFIIIC

A

DISSERATION REPORT

SUBMITTED FOR THE DEGREE OF

Masters of Technology in Biotechnology

Under the guidance of:

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in this thesis entitled "**Studying the Interacting Partners of RNA Polymerase III transcription factors, TFIIIB and TFIIIC**" in partial fulfilment of the requirement for the award of the degree of Masters of Technology in Biotechnology, is an authentic record of work done by me, under the guidance of Dr. M S Reddy, Professor, Department of Biotechnology, Thapar University and Dr. Purnima Bharagava, CCMB, Hyderabad. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

Ishita Nanda

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CERTIFICATE

This is to certify that the thesis entitled "**Studying the Interacting Partners of RNA Polymerase III transcription factors, TFIIIB and TFIIIC**" submitted by Ishita Nanda in partial fulfilment of the requirement for the award of Degree of Master of Technology in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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Ishita Nanda has successfully completed her thesis entitled " Studying the Interacting Partners of RNA Polymerase III transciption factors: TFIIIB and TFIIIC" within her tenure which commenced in Jan 2014 and ended mid June 2014. The research work embodied in this thesis has been carried out by her in my lab at the Centre for Cellular and Molecular Biology, Hyderabad.

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SUMMARY

In all living cells, reading and copying of the genetic information (DNA sequence) into RNA molecule is carried out by multi-subunit DNA-dependent RNA polymerase complexes. In prokaryotes, entire gene repertoire is transcribed by a single type of RNA polymerase. However, in eukaryotes, transcription machineries and regulation of transcription are much complex. Eukaryotes use three types of RNA polymerases for the transcription of their large gene repertoire. Each type of eukaryotic RNA polymerase is dedicated to the synthesis of a particular class of RNA species: RNA polymerase I synthesizes ribosomal RNAs; RNA polymerase II synthesizes messenger RNA molecules which later get translated into protein molecules; RNA polymerase III synthesizes transfer RNA and a few non-coding RNA molecules.

Out of the three, RNA Polymerase III is the least studied in the model organism used which is *S.Cerevisiae.* RNA Pol III has two basic transcription factors, TFIIIB and TFIIIC, each having 3 and 6 subunits respectively. The thesis focuses on RNA Pol III interactome in *S. cerevisiae*. The work highlights the interacting partners of Transcription Factors IIIC and IIIB by checking their interaction with their subunits, TFC1 and BRF1 respectively. The target proteins SPT16, SWC5, PAF1, MAF1. TAF14 and TAF12 that have been selected, are known to play a role with RNA Pol II transcription also.

To approach these objectives, techniques of Immunoprecipitation and Co-immunoprecipitation are used. The yeast strains were doublly tagged by transforming the TFC1-FLAG and BRF1-FLAG background strains with the HA tagged target protein. These strains were then grown under specific conditions and their lysates were obtained followed by pulldown with specific antibodies. Finally, Western blotting was done to procure the blots.

The results were analysed for each protein based on the bands that appeared on the blots. Some proteins interacted with both the subunits of RNA Pol III, whereas some proteins interacted with either TFIIIB and TFIIIC. In brief, PAF1, TAF12, SPT16 are seen to interact with both the subunits which portrays that they have a role to play in RNA pol III transcription. Protein MAF1 showed interaction with TFIIIB and TFIIIC, though in nutrient starved condition only. TAF14 is seen to have interaction with TFIIIB only whereas SWC5 showed interaction with TFIIIC and a weak interaction with TFIIIB.

Since these proteins are established to play a role in RNA Polymerase II transcription, their interaction with TFIIIB and TFIIIC will allow us to understand whether they also play a role in RNA Pol III transcription which is not well studied. To get an insight of their exact mechanism in RNA Pol III transcription, further studies need to be carried out focussing on the connection of each protein with TFIIIB and TFIIIC separately.

LIST OF ABBREVIATIONS

dNTP	Deoxy-ribonucleoside tri-phosphate
EDTA	Ethylene diamine tetracetic acid
PMSF	Phenyl methylsulfonyl-fluoride
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase chain reaction
YEP	Yeast extract and peptone medium
OD	Optical Density
A_{600}	Absorbance at 600 nm
RNAP I	RNA Polymerase I
RNAP II	RNA Polymerase II
RNAPIII	RNA Polymerase III
RT	Room temperature
° C	Degree Celsius
µg	Microgram
µL	Microliter
kDa	Kilo Daltons
mg	Milligram
min	Minutes
mL	Milliliter
NEB	New England Biolabs
TBE	Tris boric acid EDTA
TGS	Tris Glycine SDS
TBST	Tris Boric acid Saline Tween 20
MLB	Modified lysis buffer
MQ	MilliQ
TE	Tris EDTA

IP	Immunoprecipitaion
Co-IP	Coimmunoprecipitaion
PVDF	Polyvinyline Fluoride
LCMS	Liquid chromatography mass spectrometry
TFIIIC	Transcription Factor III C
TFIIIB	Transcription Factor III B

1. INTRODUCTION

Transcription is the first stage of the expression of genes into proteins, in which a particular segment of DNA is copied into RNA by the enzyme RNA polymerase (RNAP). During transcription, a DNA sequence is read by an RNA polymerase, which produces a complementary, anti-parallel RNA strand called a primary transcript or a mRNA (messenger RNA). In contrast to DNA polymerase, RNAP includes helicase activity; therefore no separate enzyme is needed to unwind DNA (James et al., 2004).

Transcription is carried out by RNA polymerases and some accessory proteins called transcription factors. Much of the complexity in animal and plant cells can be attributed to the evolution of elaborate systems made up of short (6 to 8 base pair) *cis*-regulatory DNA sequences or motifs, as well as the Transcription factors that bind to the motifs, interact with each other to form complexes, and recruit RNA polymerases (Levine and Tjian, 2003). The mode of action of Transcription factors is to recognize and bind to a segment of DNA in the promoter and or enhancer region. It causes a change in the conformation, or three-dimensional structure of a transcription factor that accompanies DNA binding (Latchman, 1997).

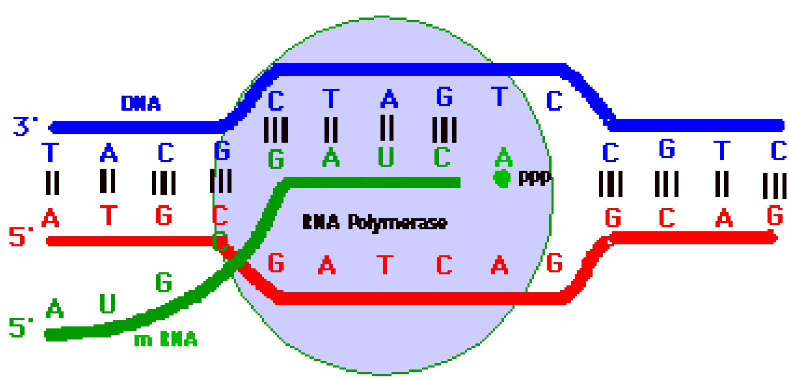

Figure 1.1 Basic mechanism of transcription (Google image).

In bacteria, the RNA Polymerases catalyze the synthesis of mRNA and non coding RNA, while eukaryotes have multiple types of nuclear RNAP, each responsible for synthesis of a distinct subset of RNA. RNA polymerase I synthesizes a pre-rRNA 45S (35S in yeast), which matures into 28S, 18S and 5.8S rRNAs which will form the major RNA sections of the ribosome. RNA polymerase II synthesizes precursors of mRNAs and most snRNA and microRNAs. This is the most studied type, and, due to the high level of control required over transcription, a range of transcription factors are required for its binding to promoters. RNA polymerase III synthesizes tRNAs, 5S rRNA and other small RNAs found in the nucleus and cytosol. RNA polymerase IV synthesizes siRNA in plants. RNA polymerase V synthesizes RNAs involved in siRNA-directed heterochromatin formation in plants.

RNA polymerase I transcribes all rRNA genes except 5S rRNA. 18S, 28S and 5.8S rRNA genes are present as a single transcription unit found in tandem repeats of 100 or more copies. Each transcription unit contains promoter elements such as core promoter (CP), upstream control element (UCE) and enhancer. CP is recognized by core factor (CF) and UCE is recognized by upstream activating factor (UAF). Pol I transcription is coupled to ribosome assembly and takes place in the nucleolus (Grummt, 2003).

RNA polymerase II transcribes all protein coding genes and many non coding RNA genes like snRNAs. Amongst the three polymerases, Pol II has the least number of subunits but highest number of transcription factors (TFIIA, TFIID, TFIIB, TFIIE, TFIIF and TFIIH) indicating the enormity of transcription complex assembly. In general, Pol II promoters can be put into two categories: Focused promoters and dispersed promoters. Focused promoters have either a single transcription initiation site or a distinct cluster of initiation sites within a short span of nucleotides. Dispersed promoters are more common in vertebrates wherein the transcription initiation sites may be dispersed in a window of 50-100 nucleotides, in CpG islands (Muller et al., 2007; Juven-Gershon et al., 2008).

The genes transcribed by RNA Pol III fall in the category of "housekeeping" genes whose expression is required in all cell types and most environmental conditions. Therefore the regulation of Pol III transcription is primarily tied to the regulation of cell growth and the cell cycle, thus requiring fewer regulatory proteins than RNA polymerase II. Under stress conditions however, the protein Maf1 represses Pol III activity (Vannini et al., 2010).

In the process of transcription (by any polymerase) there are three main stages: *Initiation*, requiring construction of the RNA polymerase complex on the gene's promoter; *Elongation*, the synthesis of the RNA transcript; *Termination*, the finishing of RNA transcription and disassembly of the RNA polymerase complex.

The types of RNAs transcribed from RNA polymerase III includes, Transfer RNAs, 5S ribosomal RNA, U6 spliceosomal RNA ,RNase P and RNase MRP RNA, 7SL RNA (the RNA component of the signal recognition particle), SINEs (short interspersed repetitive elements), Several small nucleolar RNAs and Several gene regulatory antisense RNAs.

Pol III genes can be divided into three classes according to their initiation

Class I: Example 5S rRNA

1. TFIIIA binds to the intragenic 5S rRNA control sequence, the box C.
2. TFIIIA serves as a platform that replaces the A and B Blocks for positioning TFIIIC in an orientation with respect to the start site of transcription that is equivalent to what is observed for tRNA genes.
3. Once TFIIIC is bound to the TFIIIA-DNA complex the assembly of TFIIIB proceeds as described for tRNA transcription. (Ciganda et al., 2011)

Class II: Example tRNA genes

1. TFIIIC binds to two intragenic control sequences, the box A and box B.
2. TFIIIC acts as an assembly factor that positions TFIIIB to bind to DNA at a site centered approximately 26 base pairs upstream of the start site of transcription. TFIIIB is the transcription factor that assembles Pol III at the start site of transcription. Once TFIIIB is bound to DNA, TFIIIC is no longer required. TFIIIB also plays an essential role in promoter opening (Oettel et al., 1998).

Class III: Example U6 snRNA

1. SNAPc (SNRNA Activating Protein complex) binds to the PSE (Proximal Sequence Element) centered approximately 55 base pairs upstream of the start site of transcription.
2. SNAPc acts to assemble TFIIIB at a TATA box centered 26 base pairs upstream of the start site of transcription. The TFIIIB for U6 snRNA transcription contains a smaller Brf1 paralogue, Brf2 (Dieci et al., 2007).
3. TFIIIB is the transcription factor that assembles Pol III at the start site of transcription.

To study the molecular biology revolving around RNA polymerase III transcribed gene, the model organism we have selected is *Saccharomyces cerevisiae.*

S. cerevisiae is a species of yeast. It is one of the most intensively studied eukaryotic model organisms in molecular and cell biology, much like *Escherichia coli* as the model bacterium. *S. cerevisiae* cells are round to ovoid, 5–10 micrometers in diameter (Fig.1.2). It reproduces by a division process known as budding, therefore is also known as budding yeast (Feldmann et al., 2010).

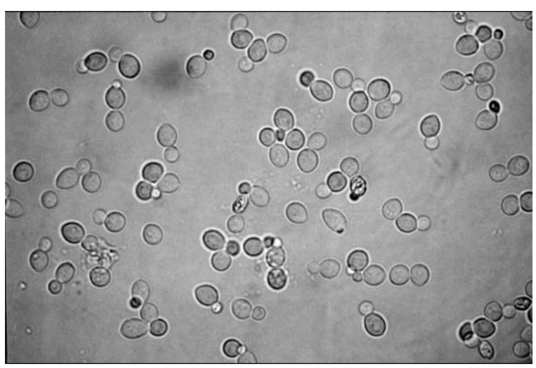

Figure. 1.2 Microscopic view of budding yeast (Google image).

S. cerevisiae contains many different strains used in laboratories for research purpose. *S. cerevisiae* is small with a short generation time (doubling time 1.25–2 hours at 30 °C or 86 °F) and can be easily cultured . These are all positive characteristics in that they allow for the swift production and maintenance of multiple specimen lines at low cost. *S. cerevisiae* divides with meiosis, allowing it to be a candidate for sexual genetics research. *S. cerevisiae* can be transformed allowing for either the addition of new genes or deletion through homologous recombination. Furthermore, the ability to grow *S. cerevisiae* as a haploid simplifies the creation of gene knockouts strains.

As a eukaryote, *S. cerevisiae* shares the complex internal cell structure of plants and animals without the high percentage of non-coding DNA that can confound research in higher eukaryotes. Aforementioned qualities of the model organism *S. cerevisiae* make it a strong research economic driver (Boekhout et al., 2003).

SCOPE OF THE STUDY

Intense studies have been carried out on RNA Polymerase I and II with their transcription factors. My work primarily focuses on RNA Polymerase III interactome, using *S.Cerevisiae* as the model organism.

The background of my work was obtained by checking the interactome of TFIIIC and TFIIIB, subunits of RNA Pol III. A large scale Immunoprecipitation was performed to obtain the interacting partners of TFIIIC and TFIIIB. Several unique peptides were obtained corresponding to their proteins. A few proteins were selected for validating the interactions with BRF1 and TFC1, subunits of TFIIIB and TFIIIC respectively.

Interestingly, the proteins selected are found to play a role in RNA Polymerase II transcription. This area has not been studied earlier, hence the key objectives of my work included:

1) Constructing doubly tagged strains in *S.cerevisiae* to study the individual interaction.

2) Validation of the selected interacting partners of TFIIIC and TFIIIB through biochemical assays.

- Interaction of the selected proteins with TFC1 subunit of TFIIIC was checked.
- Interaction of the selected proteins with BRF1 subunit of TFIIIB was checked.

2. REVIEW OF LITERATURE

RNA polymerase III is the most complex among the three polymerases as it contains highest number of subunits. Pol III is made up of 17 subunits while pol I and pol II are made up of 14 and 12 subunits respectively. *In vitro* transcription system with all recombinant factors is established for Pol III transcription (Ducrot et al., 2006). Pol III transcribes all tRNA genes, 5S rRNA gene, U6 snRNA gene (abbreviated as SNR6), 7SL RNA gene and some other non coding RNA genes. Pol III genes are scattered throughout the genome and accumulating evidences indicate that the Pol III transcription happens at or near the nucleolus (Haeusler and Engelke, 2006).

Yeast pol III has 6 unique subunits namely Rpc17, Rpc31, Rpc34, Rpc37, Rpc53 and Rpc82. Out of the 17 subunits of Pol III, the largest subunits RPC160 and RPC128 show remarkable similarities with the two largest subunits of Pol II-RPB1 and RPB2. Pol I and Pol III share the subunits AC40 and AC19 which are homologous to Pol II subunits RPB3 and RPB11 (Geiduschek and Kassavetis, 2001; Huang and Maraia, 2001; Schramm and Hernandez, 2002). Such a high homology between subunits of different polymerases underlines conservation of their function and structure.

There are two general transcription factors for RNAP III: the multi subunit TFIIIC and the three subunits TFIIIB.

2.1 Transcription factor IIIB

The budding yeast TFIIIB is a hetero-trimeric complex that binds immediate upstream region of the gene and recruits pol III and participates in promoter opening (Kassavetis et al., 2001). TFIIIB complex is composed of TATA binding protein (TBP), TFIIB related factor 1 (BRF1) and BDP1 (Fig.2.1). *S. cerevisiae* TBP and Brf1 make a stable complex termed B' while Bdp1 dissociates from TFIIIB easily, making the B" fraction (hence the name *B d*ouble *p*rime or BDP) (Kassavetis et al., 1991).

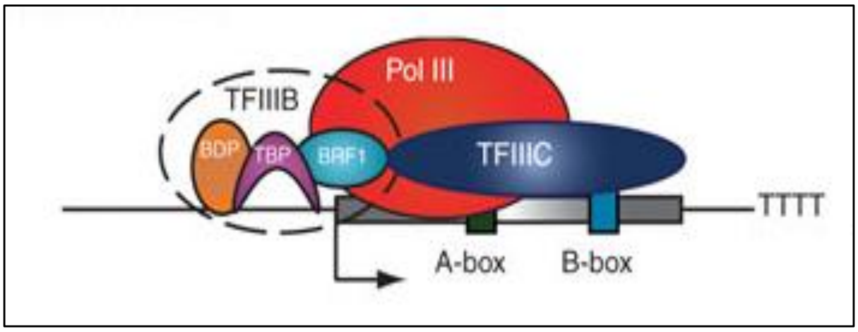

Figure 2.1 Subunits of TFIIIB in RNA Pol III transcription assembly (Andrew et al., 2010)

TBP is a universal factor required by all three polymerases.TBP as a part of TFIIIB bends DNA upstream of the start site. In many other organisms including *S. pombe*, TATA box is an essential/ubiquitous Pol III promoter element (Huang and Maraia, 2001).

Bdp1 is a Pol III specific transcription factor of 594 amino acids with no paralogues in other polymerases (Kassavetis and Geiduschek, 2006). It is loosely bound to the TFIIIB complex indicating that it is recruited after the formation of the stable DNA-TBP-Brf1 complex. Bdp1 determines the physical properties of TFIIIB-DNA complex. Its binding to DNA is not sequence specific and it makes an additional bend between the TBP binding site and transcription initiation site.

The second subunit, Brf1 is a 596 amino acid protein that is important for assembly of TFIIIB. The C-terminal of BRF is unique while N terminus shows homology to pol II factor, TFIIIB, hence named B related factor. Brf1 plays a critical role in holding the TFIIIB complex together by acting as a two sided adhesive that binds to both TBP and Bdp1 although other regions are also involved in binding to these factors (Kassavetis and Geiduschek, 2006). TFIIIB is recruited to Pol III genes by TFIIIC mainly via the interaction between Brf1 and the tetratricopeptide (TPR) repeat of Tfc4 subunit of TFIIIC. This interaction involves three separate TPR elements and three sites in Brf1 (two in the N terminal segment and one in the homology segment II in C terminal half) (Geiduschek and kassavetis, 2001; Schramm and Hernandez, 2002). Many of the protein binding sites in Brf1 are overlapping indicating the assembly of TFIIIB is sequential and is associated with structural reorganization of Brf1. The proposed sequence of assembly is: i) TFIIIC binding to A-B Boxes; ii) interaction between Tfc4 subunit of TFIIIC and Brf1; iii) anchoring the Brf1-TFIIIC complex to DNA by the Brf1 bound TBP; iv) reconfiguration of interaction between TFIIIC and Brf1 to accommodate Bdp1

(Kassavetis and Geiduschek, 2006). Brf1 interacts also with C34 and C17 subunits of Pol III (part of Pol III specific initiation sub-complex and stalk respectively). It is believed that through this interaction, TFIIIB recruits Pol III to its promoters (Werner et al., 1992; Khoo et al., 1994; Wang and Roeder, 1997; Ferri et al., 2000).

Brf1 is the target of most of the cellular mechanisms that regulate Pol III transcription. The central regulator of Pol III called Maf1 acts directly on Brf1 as well as Pol III (Upadhya et al., 2002; Desai et al., 2005). Most of the tumor suppressor proteins that regulate Pol III transcription also target Brf1 for their action (White et al., 2005; Felton-Edkins et al., 2003a; 2003b; Gomez-Roman et al., 2006).

For *in vitro* transcription of a gene which can direct TFIIIB binding through its TATA box, no other factors are required for transcription initiation. But for TATA less genes (which constitutes most of the Pol III transcribed genes in budding yeast) and for transcription of chromatin templates of certain TATA containing genes like SNR6, TFIIIC is required to recruit and position TFIIIB. TFIIIC achieves this through the interaction between its TFC4 subunit and Brf1 (Geiduschek and Kassavetis, 2001).

2.2 Transcription factor IIIC

For *in vitro* transcription of a gene which can direct TFIIIB binding through its TATA box, no other factors are required for transcription initiation. But for TATA less genes (which constitutes most of the Pol III transcribed genes in budding yeast) and for transcription of chromatin templates of certain TATA containing genes like SNR6, TFIIIC is required to recruit and position TFIIIB. TFIIIC achieves this through the interaction between its TFC4 subunit and Brf1 (Geiduschek and Kassavetis, 2001).

TFIIIC is the most complex transcription factor for pol III in budding yeast as it is composed of 6 subunits and recognizes two sequence elements simultaneously. These 6 subunits are coded by the genes Tfc1, Tfc3, Tfc4, Tfc6, Tfc7 and Tfc8 making an aggregate molecular weight of 520 kD. These subunits are arranged in two different domains τA and τB, connected by a flexible and proteolysis sensitive linker (Marzouki et al., 1986). τA is composed of Tfc1, Tfc4 and Tfc7 and binds to A box. Tfc4 subunit plays the key role in recruitment of TFIIIB by interacting with Brf1 and Bdp1. τB is composed of Tfc3, Tfc6 and

Tfc8. Tfc8 also acts as the linker between τA and τB. Tfc3 binds to the B box while Tfc6 occupies the most downstream position of TFIIIC complex and binds to the terminator region (Fig. 2.2). Yeast TFIIIC does not show a HAT activity. Recently *S. pombe* TFIIIC was found to act as a chromatin organizer independent of Pol III transcription function (Noma et al., 2006). Similar role for TFIIIC in *S. cerevisiae* has also been reported (Simms et al., 2008).

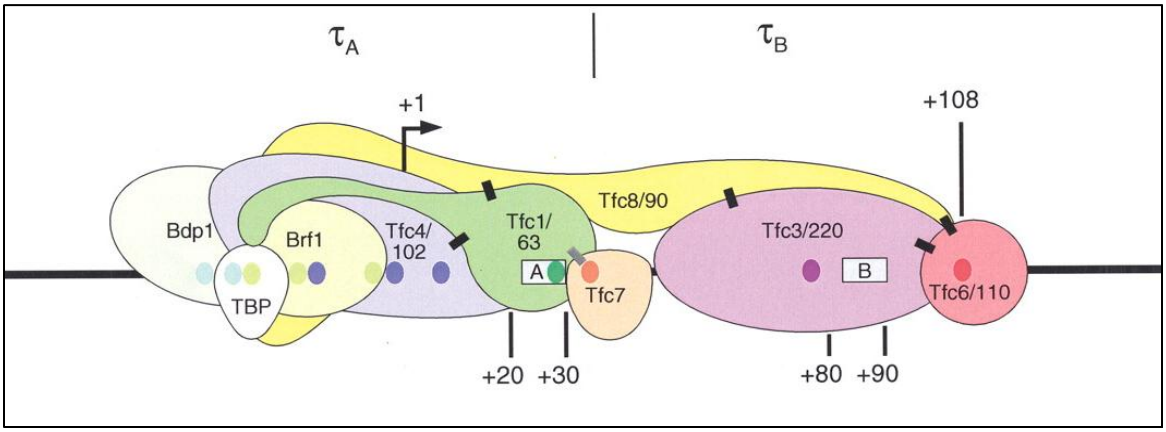

Figure 2.2 Assembly of the six subunits of TFIIIC to form τA and τB domains. (Schramm et al., 2002)

2.3 Proteins of Interest

The selected proteins were identified in the lab data and vigorous studies are being held on them. Some of them are known to be chromatin remodelers like SPT16, SWC5 and some are RNAP II associated factors, like MAF1, PAF1,TAF12 etc. Interestingly, these proteins are found in TFIIIC as well as TFIIIB interactome, this area has not been studied earlier. Description about few of these proteins has been highlighted below.

SPT16: A Component of the FACT (Facilitates Chromatin Transcription) complex, a general chromatin factor that acts to reorganize nucleosomes. The FACT complex is involved in multiple processes that require DNA as a template such as mRNA elongation, DNA replication and DNA repair (Belotserkovskaya et al., 2003; Formosa et al., 2002). During transcription elongation, the FACT complex acts as a histone chaperone that both destabilizes and restores nucleosomal structure. It facilitates the passage of RNA polymerase II and transcription by promoting the dissociation of one histone H2A-H2B dimer from the nucleosome, then subsequently promotes the reestablishment of the nucleosome following the passage of RNA polymerase II. It has a molecular weight of 118,629 Da (Hainer et al., 2012)

SWC5: It is SWR1-complex protein 5; Component of the SWR1 complex which mediates the ATP- dependent exchange of histone H2A for the H2A variant HZT1 leading to transcriptional regulation of selected genes by chromatin remodeling. It has a molecular weight of 34,343 Da (Krogan et al., 2003; Mizuguchi et al., 2004)

PAF1: A Component of the PAF1 complex (PAF1C) which has multiple functions during transcription by RNA polymerase II and is implicated in regulation of development and maintenance of embryonic stem cell pluripotency (Betz et al., 2002). PAF1C associates with RNA polymerase II and is involved in transcriptional elongation. It is also involved in SER3 repression by helping to maintain SRG1 transcription-dependent nucleosome occupancy; It is a homolog of human PD2/hPAF1. It has a molecular weight of 51,800 Da (Tous et al., 2011; Pruneski et al., 2011)

MAF1: It is a *Saccharomyces cerevisiae* protein which is highly conserved in eukaryotic cells. *S. cerevisiae* MAF1 is a negative effector of RNA polymerase III (Pol III). It responds to changes in the cellular environment and represses Pol III transcription (Boguta et al., 1997; Upadhya et al., 2002) . Biochemical studies identified the initiation factor TFIIIB as a target for Maf1-dependent repression. It is involved in tRNA processing and stability; inhibits tRNA degradation via rapid tRNA decay (RTD) pathway; binds N-terminal domain of Rpc160p subunit of Pol III to prevent closed-complex formation; localization and activity are regulated by phosphorylation, mediated by TORC1, protein kinase A, and Sch9p; localizes to cytoplasm during vegetative growth and translocates to nucleus and nucleolus under stress conditions. It has a molecular weight of 44,732 Da (Pluta et al., 2001; Desai et al., 2005).

TAF12: Control of transcription by RNA polymerase II involves the basal transcription machinery which is a collection of proteins. These proteins with RNA polymerase II, assemble into complexes which are modulated by transactivator proteins that bind to cis-regulatory elements located adjacent to the transcription start site (Tora, 2002). Some modulators interact directly with the basal complex, whereas others may act as bridging proteins linking transactivators to the basal transcription factors. Some of these associated factors are weakly attached while others are tightly associated with TBP in the TFIID complex. Among the latter are the TAF proteins (TBP associated factors). Different TAFs are predicted to mediate the function of distinct transcriptional activators for a variety of gene promoters and RNA polymerases (Grant et al., 1998). TAF12 interacts directly with TBP as well as with TAF2I. Subunit (61/68 kD) of TFIID and SAGA complexes; involved in RNA polymerase II transcription initiation and in chromatin modification, similar to histone H2A. Diseases associated with TAF12 include *epidermodysplasia verruciformis*, and *progeria*, and among its related super-pathways are RNA Polymerase II Pre-transcription Events and Assembly of RNA Polymerase-II Initiation Complex. TAF12 has a molecular weight of 61,072 Da (Lee et al., 2000).

TAF14 : In *S. cerevisiae*, TAF14 is a protein physically associated with many critical multisubunit complexes including the general transcription factors TFIID and TFIIF, the chromatin remodeling complexes SWI/SNF, Ino80 and RSC, Mediator and the histone modification enzyme NuA3 (Peterson et al., 1998). TAF14 is a member of the YEATS superfamily, conserved from bacteria to eukaryotes and thought to have a transcription stimulatory activity (Myer and Young, 1998). However, besides its ubiquitous presence and its links with transcription, little is known about Taf14's role in the nucleus. TAF14 has a molecular weight of 27,440 Da (Hampsey , 1998).

3. MATERIALS AND METHODS

Most of the fine biochemicals used were from Sigma (USA) and Merck (USA). The protein marker and 1kB DNA ladder used was from New England Biolabs. Antibodies were purchased from Millipore (Germany) or Abcam (USA) FLAG M2 agarose was from Sigma, US while all other resins used were from GE Healthcare (India) . PCR components were procured from Invitrogen (US) and the reaction mixture was prepared according to the instructions given on the kit.

3.1 Genetic manipulation of yeast

We have used polymerase chain reaction (PCR)-based strategy for the targeted introduction of heterologous DNA to genomic location. Tagging of genes by chromosomal integration of PCR amplified cassettes is a widely used and fast method to label proteins *in vivo* in the yeast *Saccharomyces cerevisiae*. The strategy requires:

(a) a pair of primers that contain within their 5 prime region sequences of homology to the genomic target location;

(b) PCR-cassettes (also term 'modules') that can be amplified using these primers. An additional primer is needed for gene deletion .

This amplified PCR product was then transformed in desired yeast strain so that it integrates in the genome by homologous recombination, leading to C-terminal tagging of protein (Fig. 3.1).

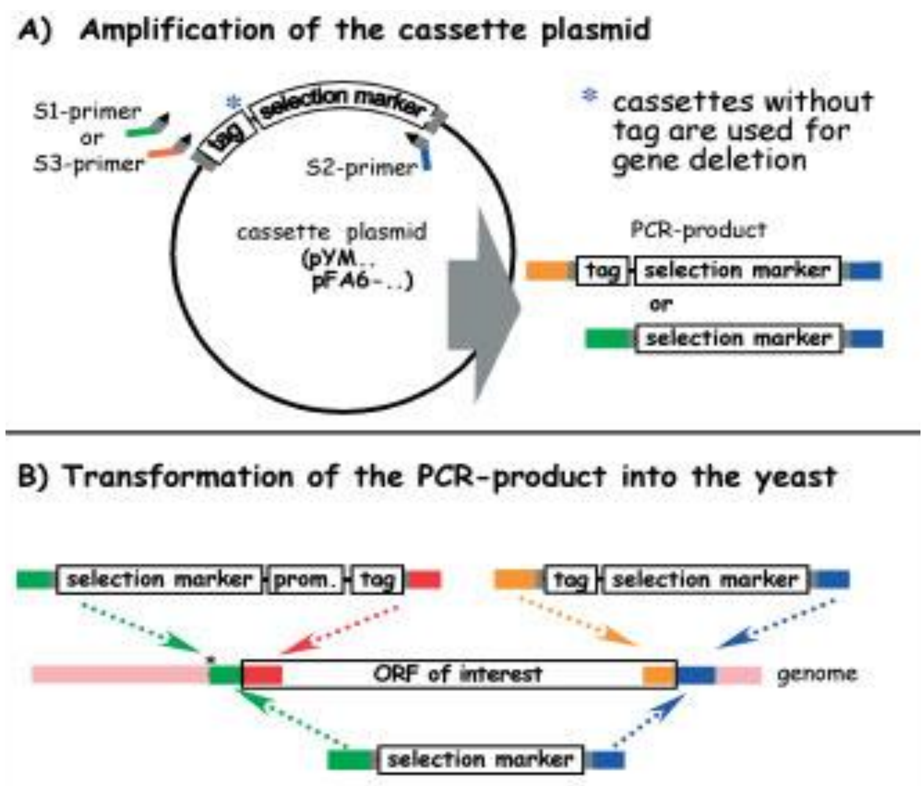

Figure 3.1 PCR based tagging followed by transformation in *S.Cerevisiae.* (Janke et al., 2004)

All the primers used in this study were custom synthesized by Bioserve, Hyderabad. The primers sequences of foward(S2) and reverse(S3) primers to tag the target proteins are mentioned below:

TAF12

S2:CTATAACTTTAGGAATTTTTTCTGTGGAAGAGTGGAGAATGTGGGTAACATAATTAATCGATGA
ATTCGAGCTCG
S3: GCAAAAAACAATGGAAACAATGTTGCAAGCTTGAATACAAAAAAACGTACGCTGCAGGTCGA

TAF14

S2:CGTGTTTTATTTATACAAACATAAAAGCGCGCATTTAACGCCCTTTTACCTTTTATTAATCGATGA
ATTCGAGCTCG
S3: GAGGGATTATTGAAAAGTCTATGGGACTACGTTAAGAAAAATACCGAGCGTACGCTGCAGGT
CGAC

SPT16

S2: TTCTGTCAGATCAAGGTCTTGCTGGTGAAACCCAGTAAGTGTTATAAAGTCTAATCGATGAAT TCGAGCTCG
S3: GAGAAAAAGGCTGCTAGGGCTGATAGGGGTGCAAACTTAGAGATCGTACGCTGCAGGTCGAC

SWC5

S2: CCTCGGCGGTTAAAAAAATTTGAGCAGAAAAGCATGTTATTTAATACATGTAATATTTGTCTAATC GATGAATTCGAGCTCG
S3: ACAACTTGCTCAGCAGTTGCAGCAGGATAGCGAAGCTTCACGTACGCTGCAGGTCGAC

PAF1

S2: TCTGGTCTAATCGATGAATTGCGCATTTAACGCCCTTTTAACTACGTGCAGAAATAATCGATGAAT TCGAGCTCG
S3: CGTACGCTCGTACGCTGCAGTTATAATGTAATATTTAAGTCTAATGGTCGAC

MAF1

S2:TGTTGCAAGCTTGAAATGTGATGCCGGTAAATTAAGCGCATTTAACGATGCCCTTTCGATGAATTC GAGCTCG
S3: ATGTTGCAAGCTTGAATACGTTGGAGAATGTGGGTAACATAACGCTGCAGGTCGAC

3.1.1 Plasmid isolation

Genetic manipulation of yeast viz., C-terminus tagging of the gene with an epitope, were carried out using plasmid-cassettes synthesised from DH5α ultra competent *E. coli* cells. 1 µl PYM16 plasmid was added to 50 µl competent cells (carried out in ice) followed by a heat shock at 42 ^{o}C for 90 seconds. 700 µl LB (Luria broth) media is added to the above mixture and incubated at 37 ^{o}C for approx 45 min. The media is removed by centrifugation and the cells are resuspended in fresh LB media and spread on LB pates with ampicillin antibiotic as marker. The plates were kept at 37 ^{o}C overnight.

Further, as colonies appeared on the plate, single colony was picked and inoculated in 20 ml LB media and kept for overnight at 37^{o}C. Cells were pelleted at 10000 xg for 5 min and supernatant was discarded. Pellet was resuspended in Solution I and vortexed for 2 min then Solution II was added and mixed gently, lastly Solution 3 was added. The mixture was then centrifuged for 15500 xg for 30 min at 4^oC. Supernatant was recovered and 2.5X volume of Isopropanol was added then centrifuged for 30 min at max speed. The pellet recovered was washed with 70% ethanol and dried. Pellet was resuspended in 100 µl TE RNase and stored at -20^oC.

3.1.2 Polymerase Chain Reaction

PCR modules for C-terminal tagging were amplified using foward (S2) and reverse (S3) primers with PCR tool box plasmids as template. Plasmids were selected depending upon the tag required as HA or myc and selection marker present in the plasmid. The plasmid used for this work was PYM16, which contains Hygromycin B as the selection marker. Table 3.1 elaborates the constituents of PCR mix .

Table 3.1 Contents of PCR mix

Reagents required	**Volume(µl)**
5X buffer	17
S2 (10pm/µl)	6.4
S3 (10pm/µl)	6.4
dNTP mix (1mM)	3
$MgCl_2$ (10mM)	3
Phusion taq polymerase (2 units)	1
Plasmid (1mg/ml)	13
Milli-Q	50.2
Total	100

For tagging PCR, initial denaturation was done at 95°C for 10mins followed by 15 cycles of annealing at 50°C for 30 sec and extension at 72°C for 2.40 min. Again denaturation was done at 95°C for 30 sec followed by 20 cycles of annealing at 65°C for 30 sec and extension at 72°C for 2.40 mins. Final extension was done at 72°C for 10mins followed by hold at 4°C for atleast 10mins.

The PCR product was run on 1% agarose gel.

3.1.3 Yeast Transformation

Yeast transformation was done by Lithium Acetate-Poly Ethyl Glycol (LiAc-PEG) method (Gietz et al., 2007). Cells from 5 ml log phase culture were harvested and washed with Milli-Q (MQ). The pellet was resuspended in 1 ml MQ and 500 µl was transferred to a fresh tube. Water was removed after spinning and the cells were resuspended in 70 µl Lithium acetate, 50 µl of PCR product and 10 µl of single strand denatured salmon sperm DNA, 600 µl of PEG solution and incubated at 30^0C for 30 minutes before giving heat shock at 42^oC for 30 minutes. After the heat shock, cells were pelleted, resuspended in 200 µl MQ and allowed to grow for 4-5 hours before plating on to the respective antibiotic selection YPD agar plates.

Transformed colonies appeared on plate after 3-4 days. Colonies were screened for the insertion of PCR cassette. Replica plating was done for the selection of positive transformants. The success of integration was tested by immunoblotting and sequencing.

Protein was precipitated by TCA (TriChloroacetic Acid) method followed by detection by Western blotting.

Single colony was inoculated in 5 ml media for overnight. Cells were pelleted and washed with water. To the cell pellet 20% TCA solution was added according to the bed volume along with glass beads. This was vortexed for 20 min and the supernatant was transferred to a fresh vial. Equal amount of 5% TCA solution was added and kept at 4^oC for 30 min. Protein pellet was recovered by spinning at 3000 rpm for 10 min. Supernatant was discarded and to this protein pellet 200 µl 1x sample loading buffer and 50 µl tris of pH 8.8 was added. Samples were then boiled and loaded on 8% SDS-PAGE and run for 1 hour 30 min at constant current (25 mA).

3.1.4 Western Transfer

SDS-Polyacylamide gel was then transferred on PVDF (Polyvinyline Fluoride membrane). Wet Transfer was used for this, PVDF membrane and 2 filter papers were cut according to gel size. PVDF membrane being hydrophilic is dipped in methanol for 5 min for enhancing the binding properties. The transfer was carried out at 200 mA for 3 hours at 4^{o}C.

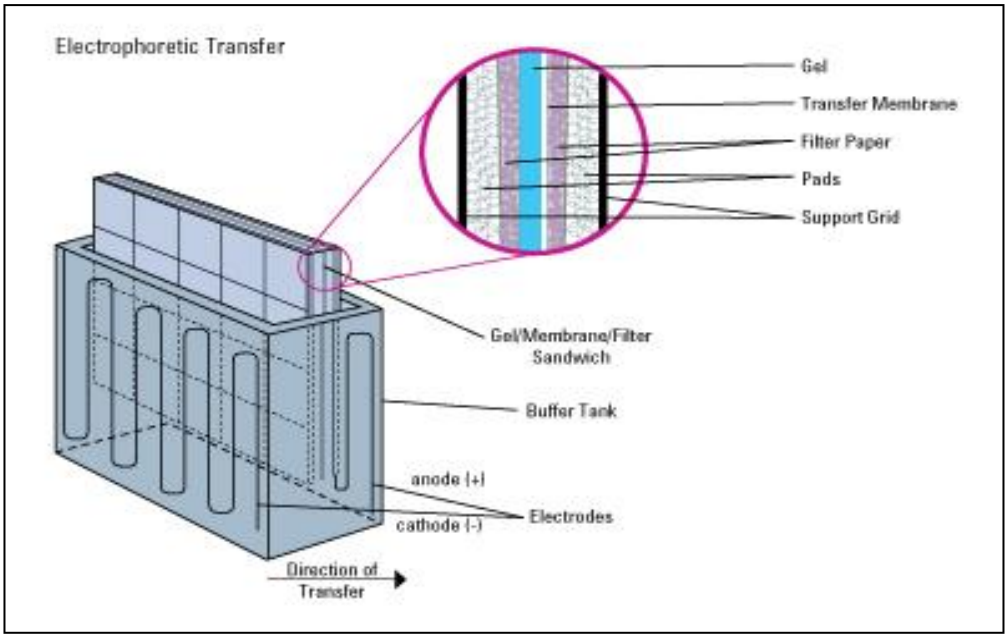

Figure 3.2 Tank transfer apparatus for Western blotting. Schematic showing the assembly of a typical Western blot apparatus with the position of the position of the gel, transfer membrane and direction of protein in relation to the electrode position. (Towbin, et al., 1979)

3.1.5 Detection by Western blotting

After the transfer, blots were incubated in 5% milk powder in 1X TBST for at least 1 hour, blots were then washed with 1X TBST and incubated with fresh primary antibody (anti HA or anti FLAG, dilution ranges from 1:1000-1:15000) for 2 hours at room temperature. Then blots were washed thrice with 1X TBST 10 min each. Blots were incubated with secondary antibody conjugated with Horseradish Peroxidase for 40 min. Secondary antibody can be either anti-mouse or anti-rabbit and its dilution ranges from 1:10000-1:15000. Blots were again washed thrice with 1X TBST 10 min each. These blots were then viewed under chemiluminiscence imaging instrument(chemi-doc). ECL(Enhanced Chemi Luminescence) substrate which binds to secondary antibody and imparts chemiluminiscence was evenly added, blots were then viewed under chemi- doc instrument and image was captured.

3.2 Media and growth condition

Yeast extract and peptone used for preparing the media were purchased from Difco. Rich media used were YEPD (Yeast Extract, Peptone, dextrose) composed of 1% Yeast extract, 2% peptone and 2% glucose. Cells were grown in an incubator shaker at 30^0C for respective time intervals. Cells for protein interaction studies were usually harvested at A_{600} =1.00 in Active sets. In Repressed sets cultures attaining the absorbance 1.00 at A_{600} were further kept for 2 hours in nutrient starvation condition i.e pelleting the cells from rich media and resuspending them in 0.15X YEP (Yeast Extract, Peptone) without glucose.

3.3 Immunoprecipitation and Co- Immunoprecipitation

Immunoprecipitation technique is widely used to study protein-protein interactions *in vivo*. In technique where an antibody against a specific target protein forms an immune complex with that target in a sample, such as a cell lysate. The immune complex is then captured, or precipitated, on a beaded support to which an antibody-binding protein is immobilized (such as Protein A or G), and any proteins not precipitated on the beads are washed away. Finally, the antigen (and antibody, if it is not covalently attached to the beads and/or when using denaturing buffers) is eluted from the support and analyzed by SDS-PAGE, often followed by Western blot detection to verify the identity of the antigen.

Doubly tagged strains (e.g. Protein A- flag tagged Protein B HA-tagged) were inoculated in YEPD media and grown upto A_{600} =1.00 and harvested immediately by centrifuging at 4000 rpm for 1 min at 4 °C . This has been followed for all active Set IPs. In the Repression Sets, 2 hour repression, as described above was given for all the test proteins Cells of both the sets were stored -80°C.

3.3.1 Preparation of cell lysates

Cells stored in -80°C were thawed, resuspended in 900 µl of modified lysis buffer containing protease inhibitors and phosphatase inhibitors. Resuspended samples and glass beads were added to 2 ml screw cap tubes and put in bead beater at 4°C for a cycle of 30 seconds ON and 90 seconds OFF. 21-25 of these cycles were performed to obtained lysed cells (80-90% lysis). Cell lysates recovered after 21 cycles was checked under microscope (40X) to check the lysis and then centrifuged at 10000 rpm for 10 min. Supernatant was transferred to fresh tube.

3.3.2 Pulldown of tagged proteins with Antibody or Antibody coupled beads

FLAG-M2 agarose (FLAG Antibody coupled with agarose beads) was used for IP and CL4B beads was used as mock. Lysates were divided into 3 tubes 50 µl was taken as Input, 400 µl as IP and 400 µl as Mock. This is done for each protein and it's both Active and Repressed Sets. 40 µl of 50% slurry of FLAG-M2 agarose was added in IP set and similarly 40 µl of 50% slurry of CL4B was added in Mock set, and left for binding at 4^0C for 3-8 hours.

Similar strategy was followed in case of HA pulldown except lysates were pre-cleared with 50% slurry of 50 µl protein A/G sepharose prior the addition of antibody. This was done to reduce the non specific signals in control lanes. Preclearing was done for 1 hour at 4^0C, and then lysates were centrifuged for at 4000 rpm for 1 min. Beads were discarded and supernatant was divided as above. 50 µl was taken as Input and to the 400 µl IP set Anti HA (Millipore) was added and NO antibody was added to 400 µl Mock set, and kept for antibody binding at 4^0C for overnight. then next day 30 µl of 50% slurry protein A/G sepharose beads was added to both IP and Mock set. Beads were left for binding to antibody at 4^0C for 3 hours.

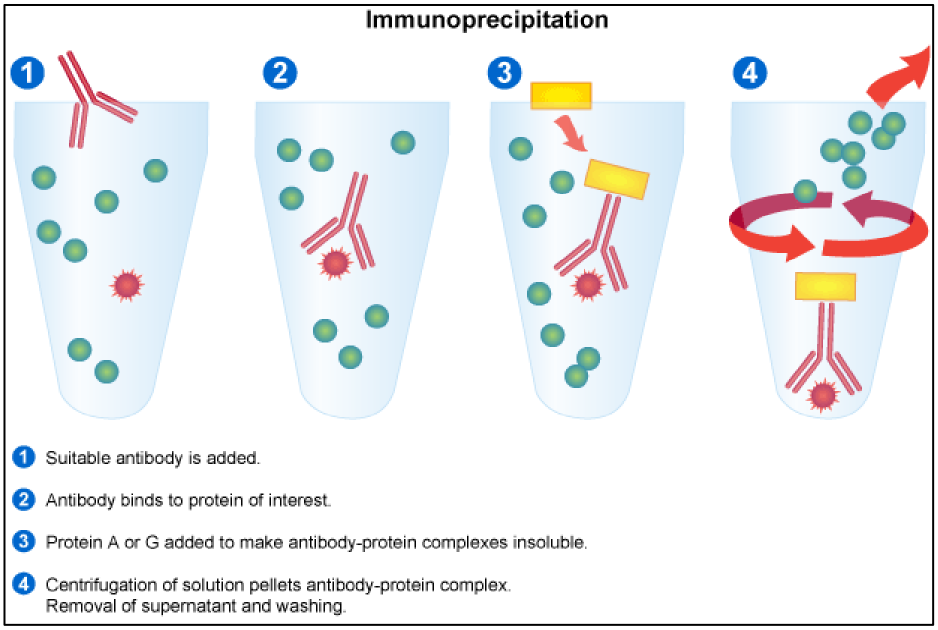

Figure 3.3 Diagram illustrating Immunoprecipitation (Bonifacino et al., 2001)

In both cases, FLAG pulldown and Anti HA pulldown, supernatant was discarded, and beads bound with our protein of interest, were washed 3-5 times with MLB. Each wash was of 5 min and last wash was done with 10 mM Tris to minimize detergent and salt concentration.

30 µl 2X Laemmli Buffer was added to these beads, beads were boiled at 95^{0}C for 5 min, so that bound proteins can come out in the solution. This was spun at high speed to settle down the beads and sup was loaded onto 8% SDS-PAGE. Gel was transferred to PVDF membrane and this PVDF membrane was probed with anti-HA and anti-FLAG antibodies to check the interactions.

Band appearances in IP and mock lanes depicted their interactions.

3.4 Preservation of strain

For the preservation of confirmed doubly tagged strains, glycerol stock was prepared for each of them. 800 µl of freshly grown cells were mixed with 200 µl of 100% glycerol and were quickly frozen in liquid nitrogen for a few minutes. This glycerol stock was stored at -80^{0}C.

4. RESULTS

4.1 STRAIN CONSTRUCTIONS

PCR based tagging approach was used for epitope tagging of the target proteins. Plasmid pYM16 from PCR tool box was used for the amplification of the module which contains 6X HA tag and Hygromycin B as selection marker. Chimeric primers containing target gene sequences and plasmid sequences were already available in the lab. 2 ul of PCR product was run on 1% agarose gel and stained with ethidium bromide. An expected size band of 2 kb was observed which was further used in transformation.

Fig. 4.1 depicts the successful amplification of PCR product of each gene at 2kB. These were transformed in Tfc1 flag tagged strain and Brf1 flag tagged strain creating twelve doubly tagged strains .

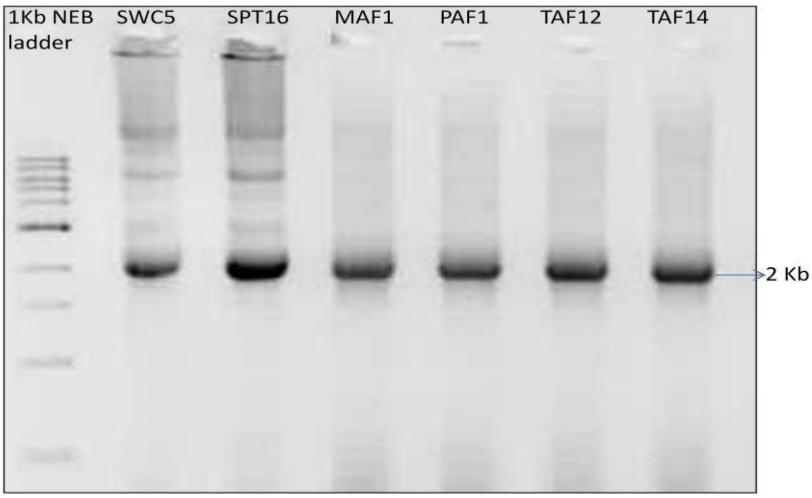

Figure 4.1. Agarose gel electrophoresis showing PCR product of target proteins

The tagging was confirmed at both genetic levels by sequencing and at protein level by western analysis.

Confirmation by Western

Yeast total protein extractions for all the twelve strains were done by TCA precipitation method. 20 µl of from each sample was resolved on 8% SDS-PAGE, transferred on PVDF membrane and probed with anti HA antibody.

Appearance of band at correct size i.e. protein MW + 6XHA epitope MW confirmed tagging in both Tfc1 and Brf1 sets (Fig.4.2).

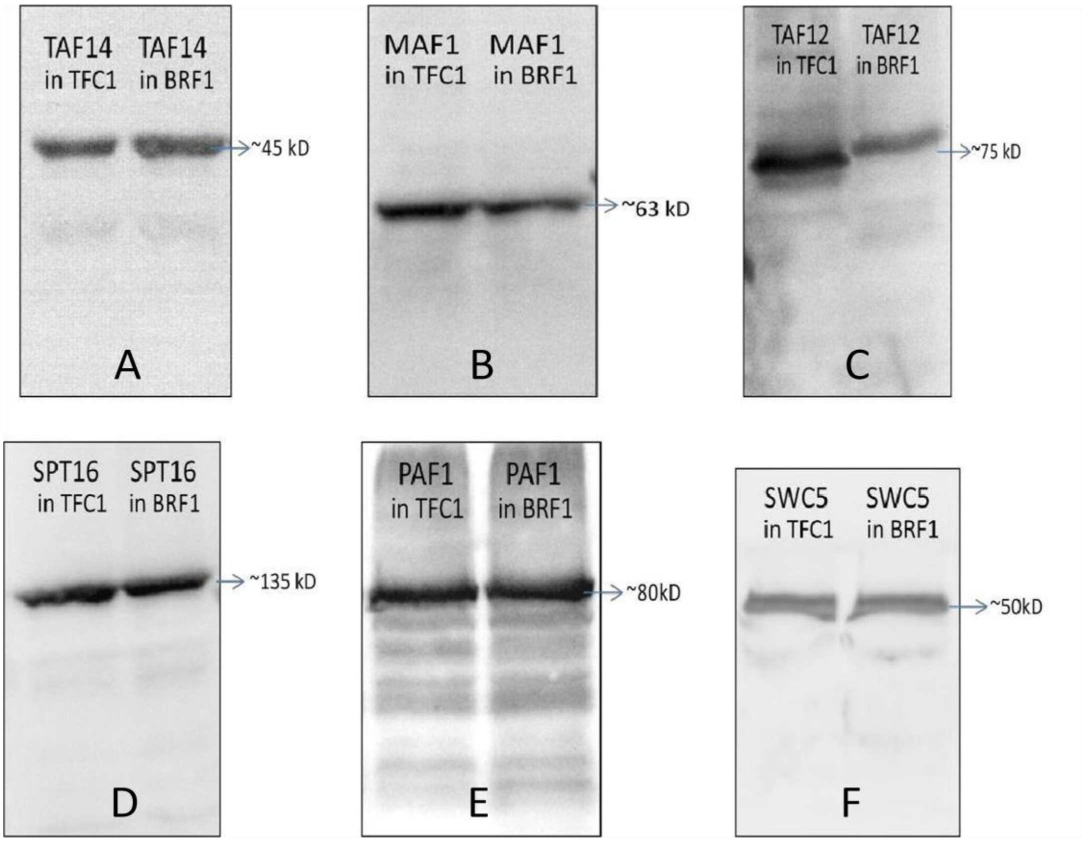

Figure 4.2 Western blots showing bands for all six genes in both TFC1 flag and BRF1 flag strains. (A) confirms the tagging of TAF14 with 6X HA epitope in both in both TFC1 and BRF1 backgrounds as the band appeared on an expected size of ~45 kD. (B) also confirms the tagging of MAF1 as a band appeared on an expected size of ~63 kD.(C) shows the tagging of TAF12 as a band appeared on an expected size of ~75 kD. (D) confirms the tagging of SPT16 with the same epitope as the band appeared on an expected size of ~135 kD. (E) illustrates the confirmed tagging of PAF1 with the appearance of band at ~80 kD. (F) confirms the tagging of SWC5 as the band appeared on an expected size of ~50kD.

These doubly tagged strains were further confirmed by sequencing with specific primer for each gene.

Hence a total of twelve strains were generated, as mentioned in table 4.1:

Table 4.1 Twelve doubly tagged strains constructed

TFC1 FLAG-SPT16 HA	BRF1 FLAG-SPT16 HA
TFC1 FLAG-SWC5 HA	BRF1 FLAG-SWC5 HA
TFC1 FLAG-MAF1 HA	BRF1 FLAG-MAF1 HA
TFC1 FLAG-PAF1 HA	BRF1 FLAG-PAF1 HA
TFC1 FLAG-TAF12 HA	BRF1 FLAG-TAF12 HA
TFC1 FLAG-TAF14 HA	BRF1 FLAG-TAF14 HA

4.2 PROTEIN –PROTEIN INTERACTION STUDIES

Strains constructed in section 4.1, were used to check the interactions individually. Immunoprecipitation was performed for all the twelve strains in two conditions Normal (Active) and Nutrient Starved (Repressed). One protein at a time was pulldown and presence of target protein was checked in the immunocomplex.

In doubly tagged strains, flag pulldown was done and presence of HA tagged protein was checked using anti HA specific antibody. Similarly in HA pulldown presence of flag tagged protein was checked with specific anti flag antibody.

Interaction results of each protein are described below in detail.

Note: In- Input, IP- Test, Mock-control

4.2.1 INTERACTION OF PAF1 WITH BRF1 AND TFC1

(A) PAF1 with BRF1

Fig. 4.3.1 represents the flag pulldown for doubly tagged strain BRF1- FLAG#PAF1 HA and blot probed with anti HA antibody which detected PAF1 at MW of 80 KDa in the IP lanes in both active and repressed conditions. BRF1 pull down was checked with anti FLAG antibody, a band was detected at MW 85 KDa on the membrane. PAF1 and BRF1 interact in both active and repressed conditions.

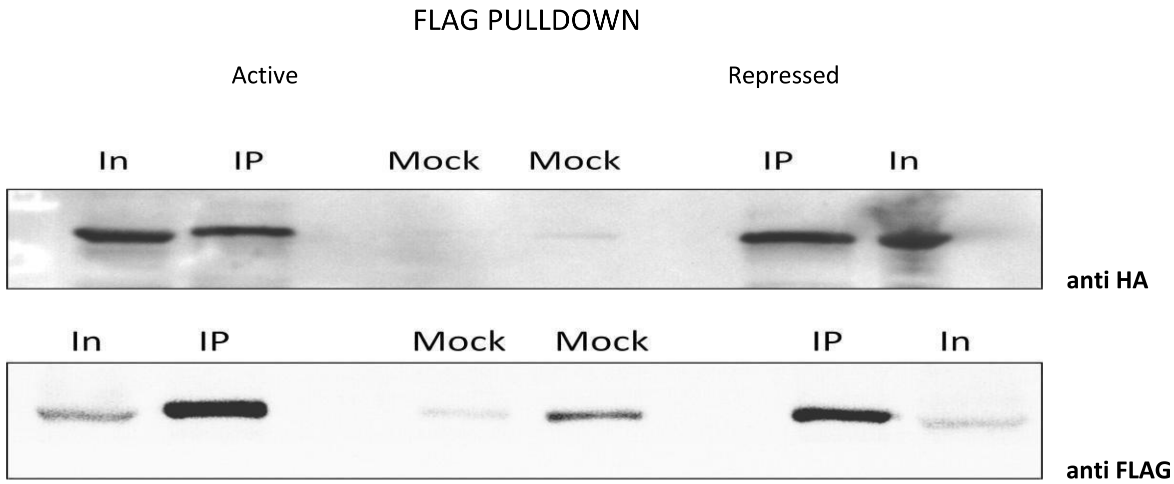

Figure 4.3.1 Western Blots showing Paf1 presence in Brf1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred.(In- Input, IP- Test, Mock-control)

HA pulldown for doubly tagged strain BRF1- FLAG#PAF1 HA was done and blot was probed with anti FLAG antibody which detected BRF1 at molecular size of 85 KDa in the IP lanes in both active and repressed conditions. PAF1 pull down was checked with anti HA antibody, a band was detected at MW 80 KDa on the membrane. Again PAF1-BRF1 interacts in both active and repressed conditions (Figure 4.3.2).

Figure 4.3.2 Western Blots showing Brf1 presence in Paf1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

(B) PAF1 with TFC1

Fig. 4.4.1 depicts flag pulldown for doubly tagged strain TFC1- FLAG#PAF1 HA and blot probed with anti HA antibody which detected PAF1 at MW of 80 KDa in the IP lanes in both active and repressed conditions. TFC1 pull down was checked with anti FLAG antibody, a band was detected at MW ~100 KDa on the membrane. PAF1 and TFC1 interact in both active and repressed conditions.

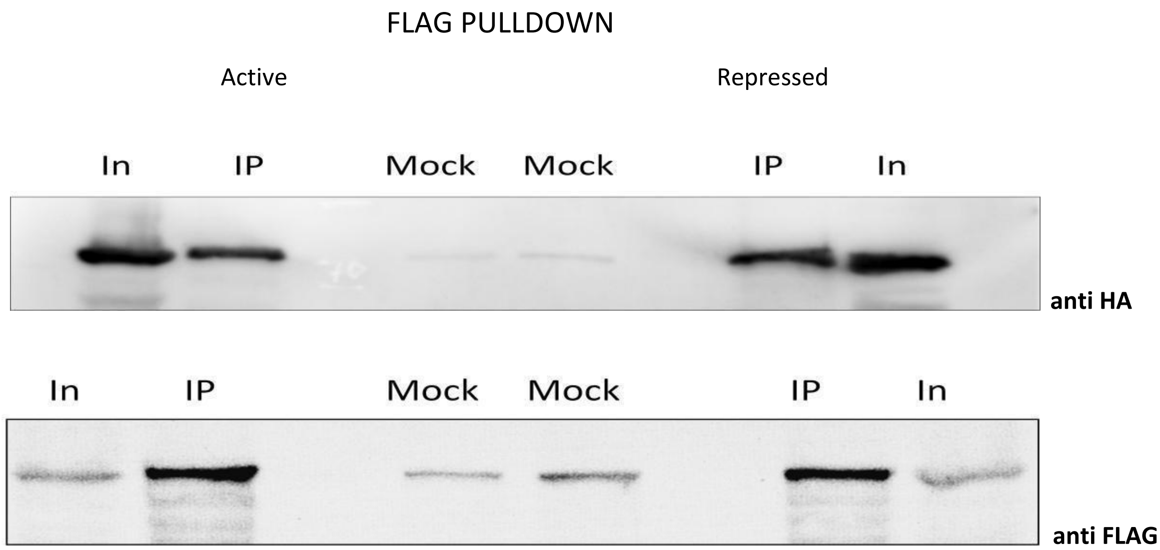

Figure 4.4.1 Western Blots showing Paf1 presence in Tfc1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

HA pulldown for doubly tagged strain TFC1- FLAG#PAF1 HA was done and blot was probed with anti FLAG antibody which detected TFC1 at molecular size of ~100 KDa in the IP lanes in both active and repressed conditions. However a band in the mock of Repressed lane is seen which may be because of weak interaction of PAF1-TFC1 in Repressed condition**.** PAF1 pull down was checked with anti HA antibody, a band was detected at MW 80KDa on the membrane. PAF1-TFC1 does interact in both active and repressed conditions(Fig.4.4.2).

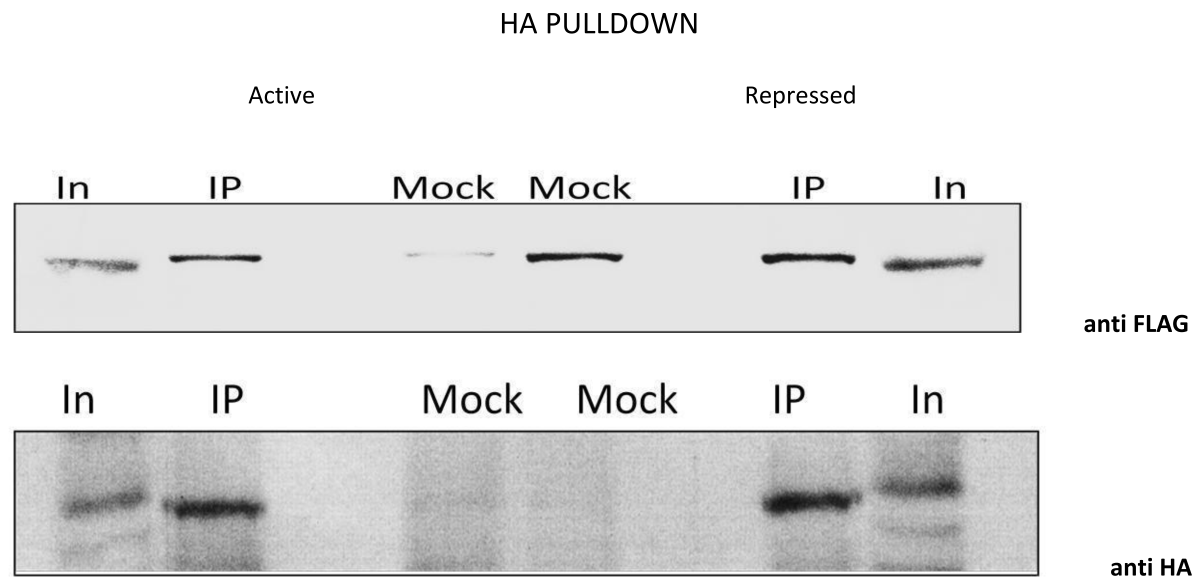

Figure 4.4.2 Western Blots showing Tfcf1 presence in Paf1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

4.2.2 INTERACTION OF TAF12 WITH BRF1 AND TFC1

(A) TAF12 with BRF1

Fig. 4.5.1 illustrates Flag pulldown for doubly tagged strain BRF1- FLAG#TAF12 HA and blot probed with anti HA antibody which detected TAF12 at MW of ~ 75 KDa in the IP lanes in both active and repressed conditions. BRF1 pull down was checked with anti FLAG antibody, a band was detected at MW ~85 KDa on the membrane. TAF12 and BRF1 interact in both active and repressed conditions.

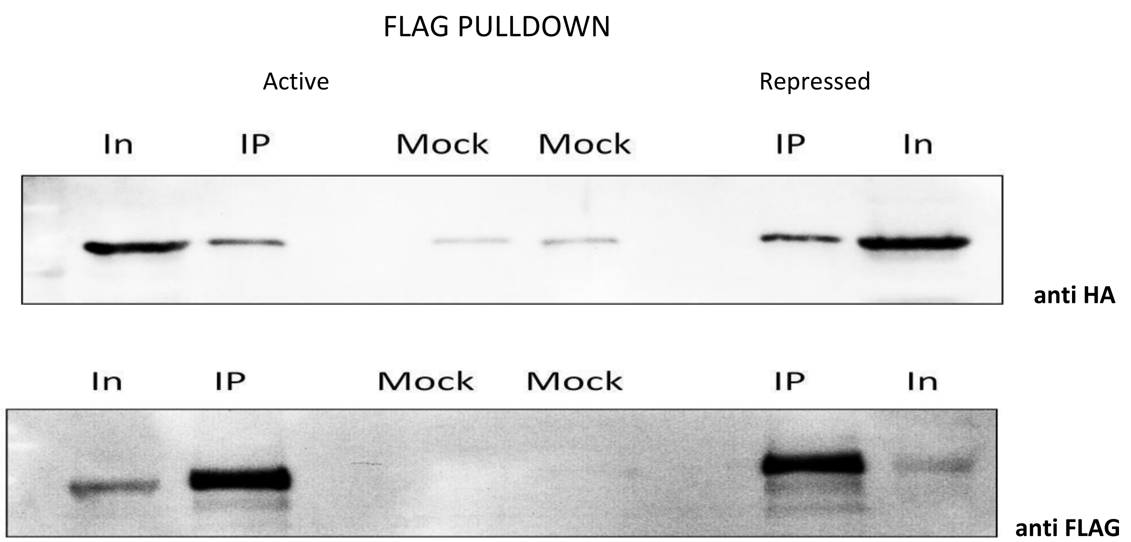

Figure 4.5.1 Western Blots showing Taf12 presence in Brf1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

Fig. 4.5.2 given below shows HA pulldown for doubly tagged strain BRF1- FLAG#TAF12 HA and blot probed with anti FLAG antibody which detected BRF1 at molecular size of ~85 KDa in the IP lanes in both active and repressed conditions. TAF12 pull down was checked with anti HA antibody, a band was detected at MW ~ 75 KDa on the membrane. TAF12 and BRF1 interact in both active and repressed conditions.

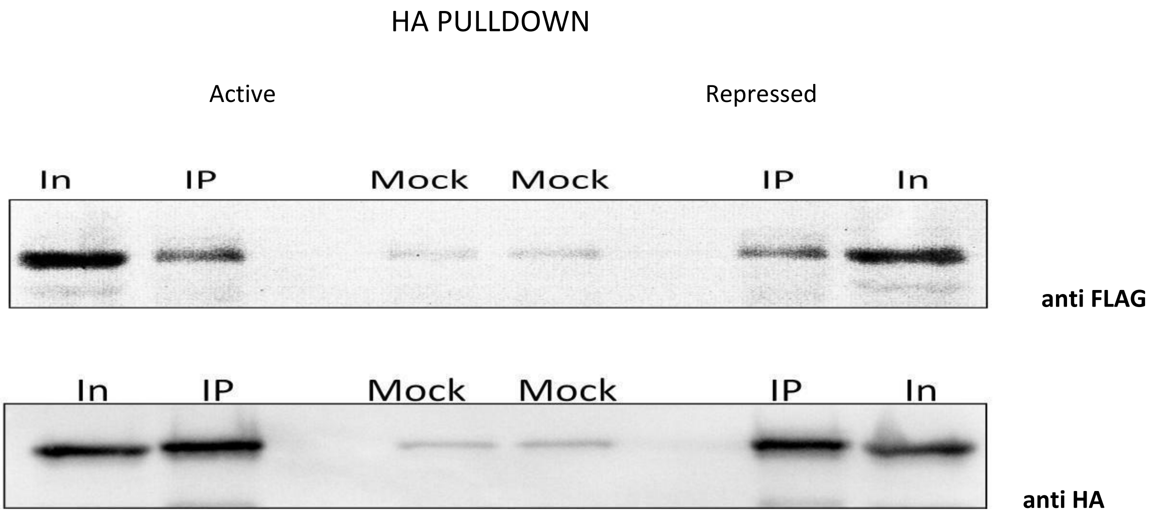

Figure 4.5.2 Western Blots showing Brf1 presence in Taf12 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

<u>(B) TAF12 with TFC1</u>

Flag pulldown for doubly tagged strain TFC1- FLAG#TAF12 HA was done and blot was probed with anti HA antibody which detected TAF12 at MW of ~ 75 KDa in the IP lanes in both active and repressed conditions. TFC1 pull down was checked with anti FLAG antibody, a band was detected at MW ~100 KDa on the membrane. TAF12 and TFC1 interact in both active and repressed conditions Fig. (4.6.1).

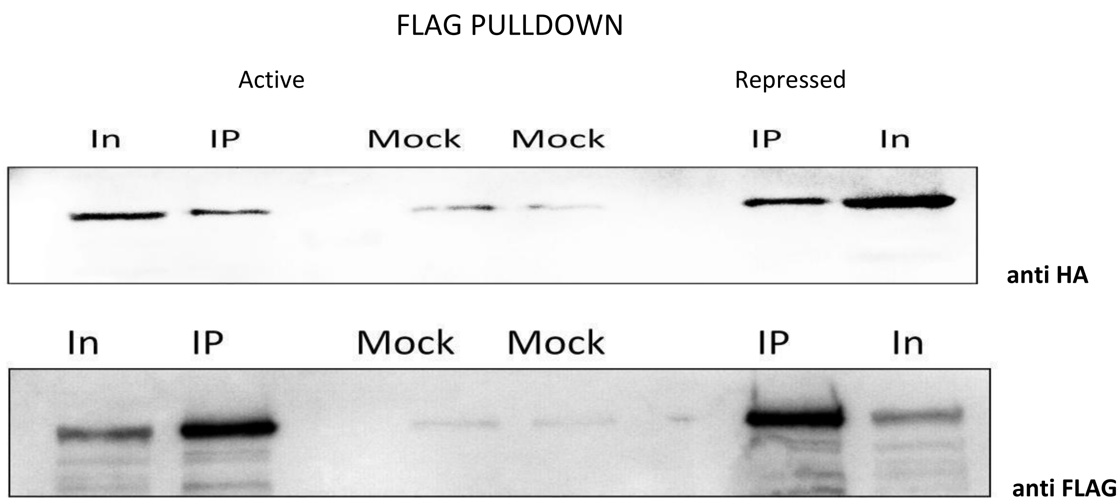

Figure 4.6.1 Western Blots showing Taf12 presence in Tfc1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

Fig. 4.6.2 suggests HA pulldown for doubly tagged strain BRF1- FLAG#TAF12 HA and blot probed with anti FLAG antibody which detected TFC1 at molecular size of ~100 KDa in the IP lanes in both active and repressed conditions. TAF12 pull down was checked with anti HA antibody, a band was detected at MW ~ 75 KDa on the membrane. TAF12 and TFC1 interact in both active and repressed conditions.

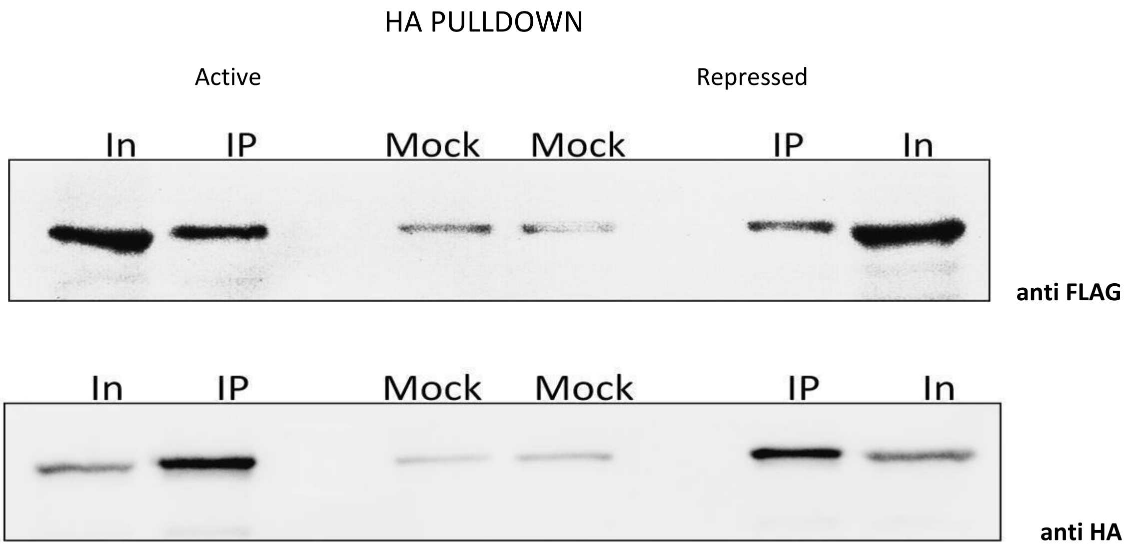

Figure 4.6.2 Western Blots showing Tfc1 presence in Taf12 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

4.2.3 INTERACTION OF TAF14 WITH BRF1 AND TFC1

(A) TAF14 with BRF1

Fig. 4.7.1 shows Flag pulldown for doubly tagged strain BRF1- FLAG#TAF14 HA and blot probed with anti HA antibody which detected TAF14 at MW of ~ 45 KDa in the IP lanes in both active and repressed conditions. BRF1 pull down was checked with anti FLAG antibody, a band was detected at MW ~85 KDa on the membrane. TAF14 and BRF1 interact in both active and repressed condition

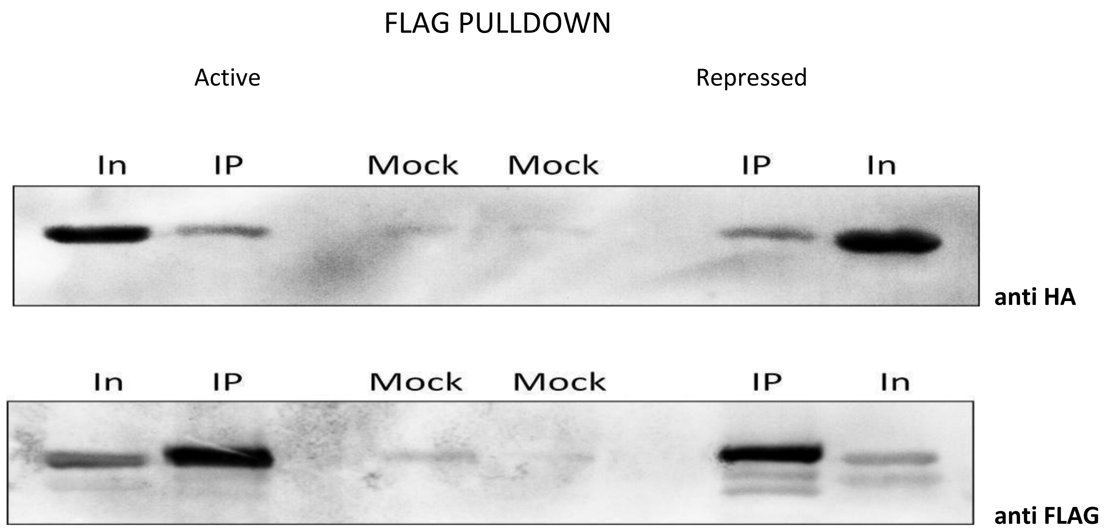

Figure 4.7.1 Western Blots showing Taf14 presence in Brf1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

HA pulldown for doubly tagged strain BRF1- FLAG#TAF14 HA was done and blot was probed with anti FLAG antibody which detected BRF1 at molecular size of ~85 KDa in the IP lanes in both active and repressed conditions. TAF14 pull down was checked with anti HA antibody, a band was detected at MW ~ 45 KDa on the membrane. TAF14 and BRF1 interact in both active and repressed conditions (Fig. 4.7.2).

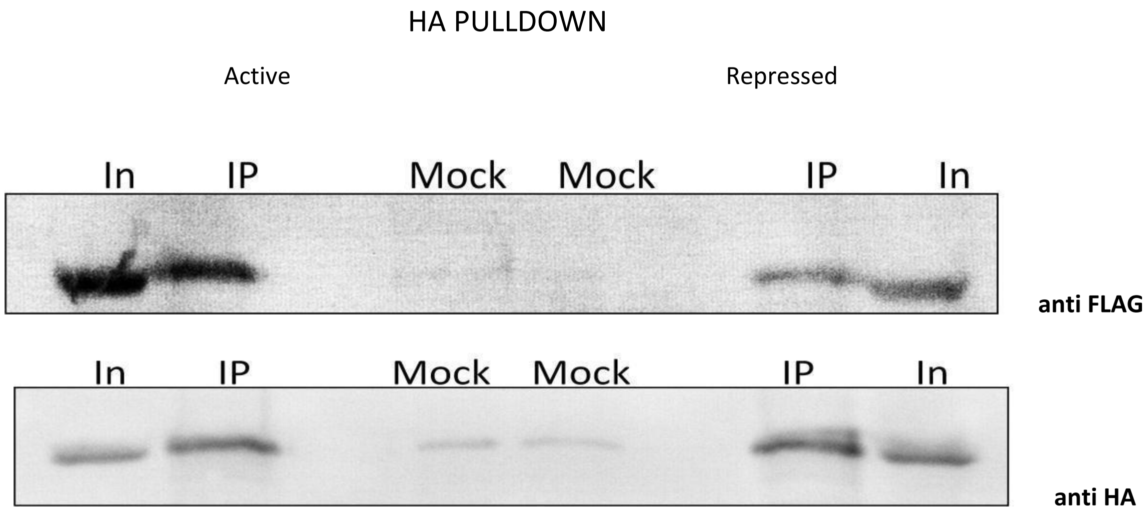

Figure 4.7.2 Western Blots showing Brf1 presence in Taf14 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

(B) TAF14 with TFC1

Fig. 4.8.1 represents Flag pulldown for doubly tagged strain TFC1- FLAG#TAF14 HA and blot probed with anti HA antibody which detected TAF14 at MW of ~ 45 KDa in the IP lanes in both active and repressed conditions. TFC1 pull down was checked with anti FLAG antibody, a band was detected at MW ~100 KDa on the membrane. TAF14 and TFC1 interact in both active and repressed conditions.

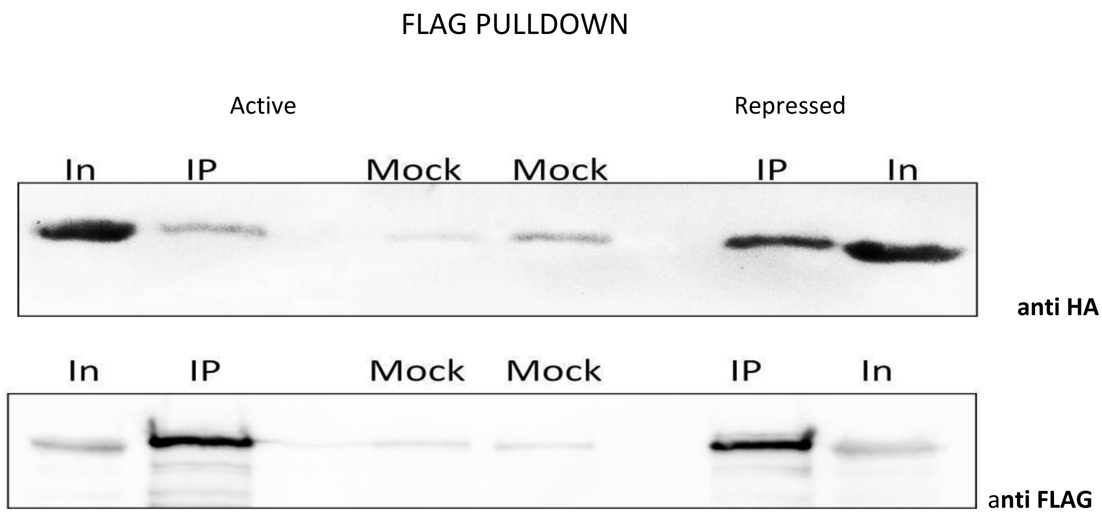

Figure 4.8.1 Western Blots showing Taf14 presence in Tfc1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

HA pulldown for doubly tagged strain TFC1- FLAG#TAF14-HA was done and blot was probed with anti FLAG antibody which detected TFC1 at molecular size of ~100 KDa in the IP lanes in both active and repressed conditions. However bands appeared in Mock lanes, this may be because of either handling errors or there is weak interaction between TFC1 and TAF14. This need to done again to get confirmative results (Fig. 4.8.2).

TAF14 pull down was checked with anti HA antibody, a band was detected at MW ~ 45KDa on the membrane. TAF14 and TFC1 have weak interaction.

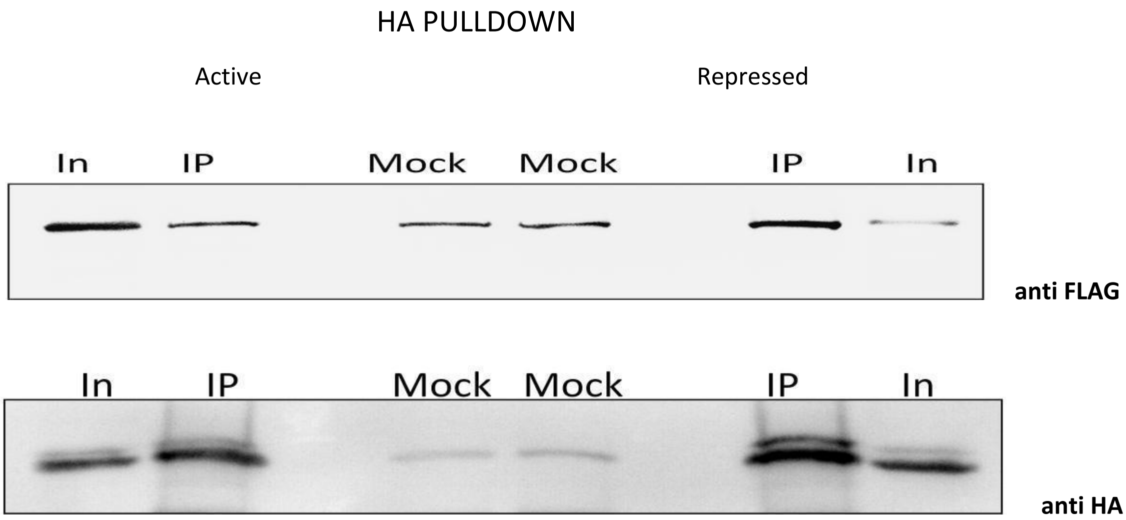

Figure 4.8.2 Western Blots showing presence of Tfc1 in Taf14 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

4.2.4 INTERACTION OF SPT16 WITH BRF1 AND TFC1

(A) SPT16 with BRF1

Fig. 4.9.1 shows that Flag pulldown for doubly tagged strain BRF1- FLAG# SPT16-HA was done and blot was probed with anti HA antibody which detected SPT16 at MW of ~ 135 KDa in the IP lanes in both active and repressed conditions. BRF1 pull down was checked with anti FLAG antibody, a band was detected at MW ~85 KDa on the membrane. SPT16 and BRF1 interact in both active and repressed conditions.

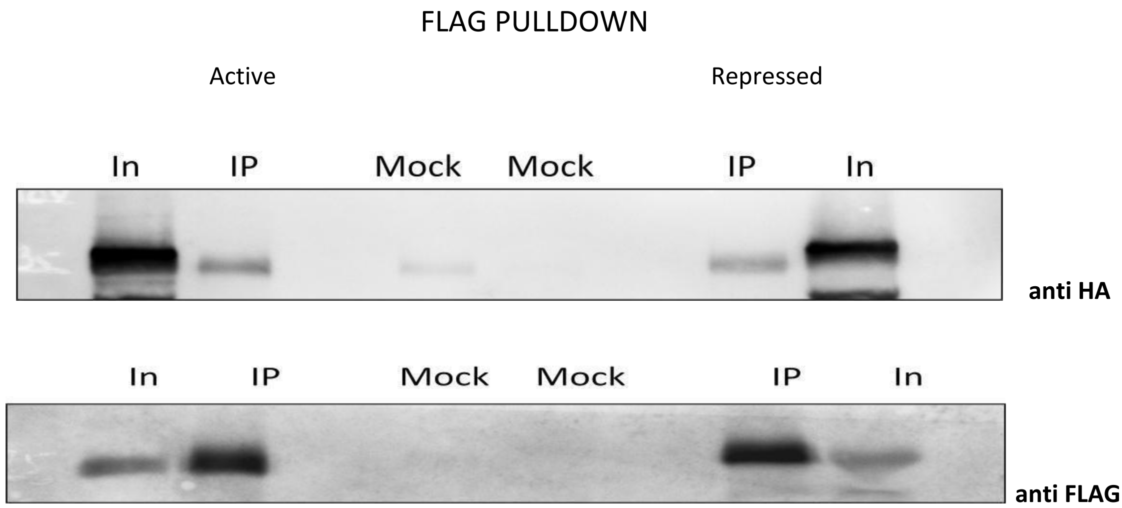

Figure 4.9.1 Western Blots showing presence of Spt16 in Brf1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

HA pulldown for doubly tagged strain BRF1- FLAG#SPT16- HA was done and blot was probed with anti FLAG antibody which detected BRF1 at molecular size of ~85 KDa in the IP lanes in both active and repressed conditions. SPT16 pull down was checked with anti HA antibody, a band was detected at MW ~ 135 KDa on the membrane. SPT16 and BRF1 interact in both active and repressed conditions (Fig. 4.9.2).

Figure 4.9.2 Western Blots showing presence of Brf1 in Spt16 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

<u>(B) SPT16 with TFC1</u>

Flag pulldown for doubly tagged strain TFC1- FLAG# SPT16-HA was done and blot was probed with anti HA antibody which detected SPT16 at MW of ~ 135 KDa in the IP lanes in both active and repressed conditions. TFC1 pull down was checked with anti FLAG antibody, a band was detected at MW ~100 KDa on the membrane. SPT16 and TFC1 interact in both active and repressed conditions (Fig. 4.10.1).

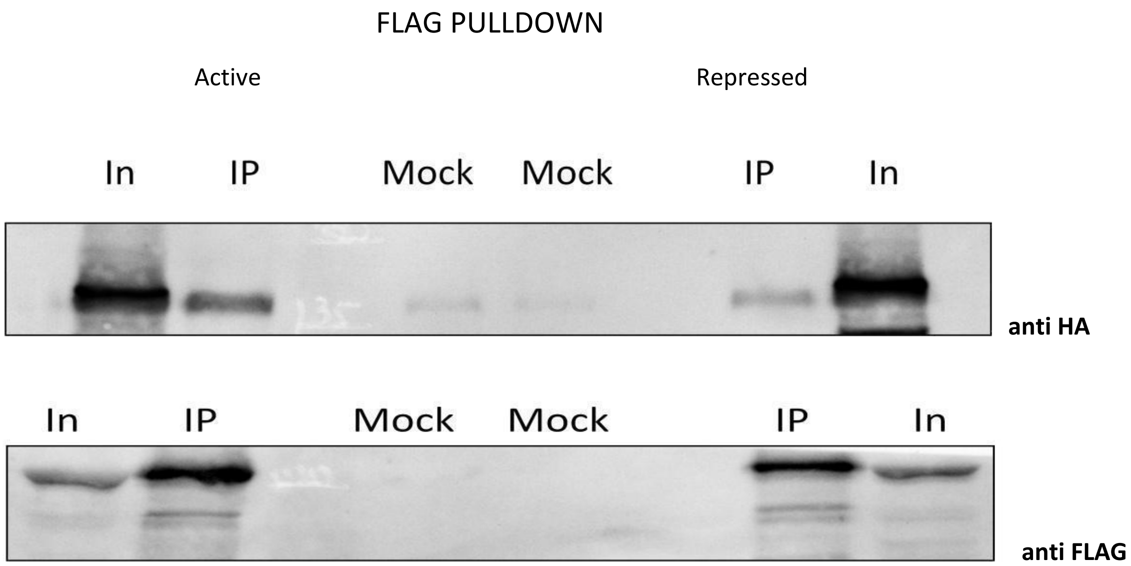

Figure 4.10.1 Western Blots showing presence of Spt16 in Tfc1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

Fig. 4.10.2 conveys that HA pulldown for doubly tagged strain TFC1- FLAG#SPT16- HA was done and blot was probed with anti FLAG antibody which detected TFC1 at molecular size of ~100 KDa in the IP lanes in both active and repressed conditions. SPT16 pull down was checked with anti HA antibody, a band was detected at MW ~ 135 KDa on the membrane. SPT16 and TFC1 interact in both active and repressed conditions.

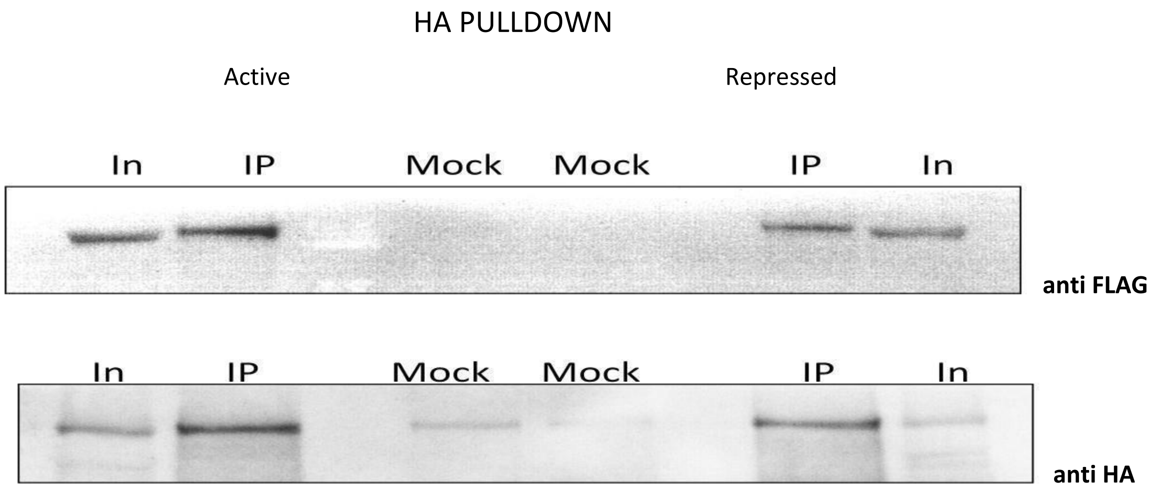

Figure 4.10.2 Western Blots showing presence of Tfc1 in Spt16 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

4.2.5 INTERACTION OF MAF1 WITH BRF1 AND TFC1

(A) MAF1 with BRF1

Fig 4.11.1 describes that Flag pulldown for doubly tagged strain BRF1- FLAG# MAF1-HA was done and blot was probed with anti HA antibody which detected MAF1 at MW of ~ 63 KDa in the IP lanes in both active and repressed conditions. BRF1 pull down was checked with anti FLAG antibody, a band was detected at MW ~85 KDa on the membrane. MAF1 and BRF1 interact in more in repressed conditions than active.

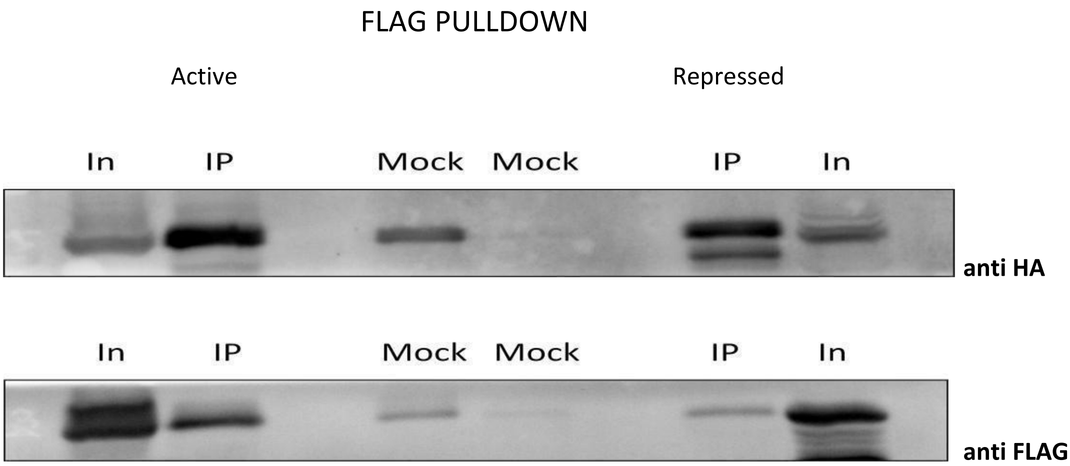

Figure 4.11.1 Western Blots showing presence of Maf1 in Brf1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

Fig. 4.11.2 mentioned next illustrates HA pulldown for doubly tagged strain BRF1-FLAG#MAF1- HA and blot probed with anti FLAG antibody which detected BRF1 at molecular size of ~85 KDa in the IP lanes in both active and repressed conditions. MAF1 pull down was checked with anti HA antibody, a band was detected at MW ~ 63 KDa on the membrane. MAF1 and BRF1 interact more in repressed conditions than active.

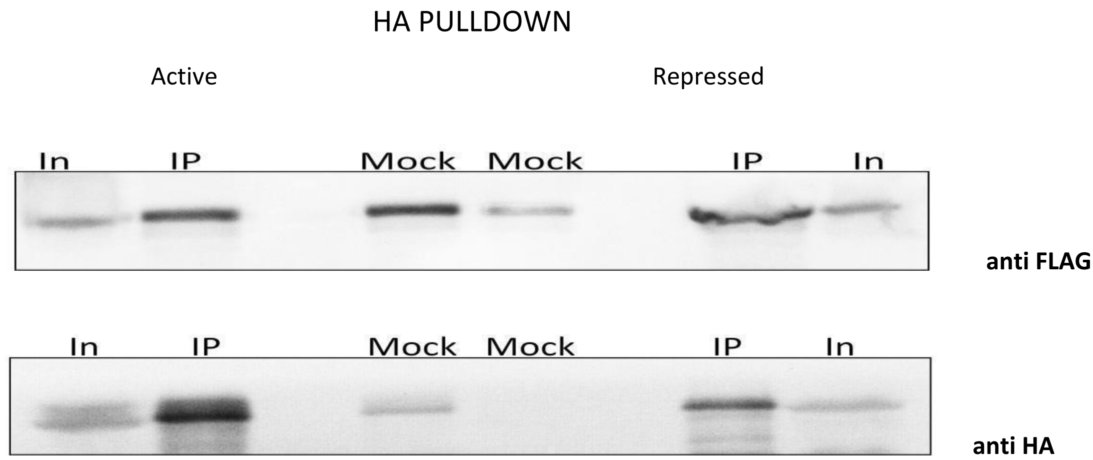

Figure 4.11.2 Western Blots showing presence Brf1of in Maf1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

<u>(B) MAF1 with TFC1</u>

Fig. 4.12.1 describes that Flag pulldown for doubly tagged strain TFC1- FLAG# MAF1-HA was done and blot was probed with anti HA antibody which detected MAF1 at MW of ~ 63 KDa in the IP lanes in both active and repressed conditions. TFC1 pull down was checked with anti FLAG antibody, a band was detected at MW ~100 KDa on the membrane. MAF1 and TFC1 interact more in repressed conditions than active.

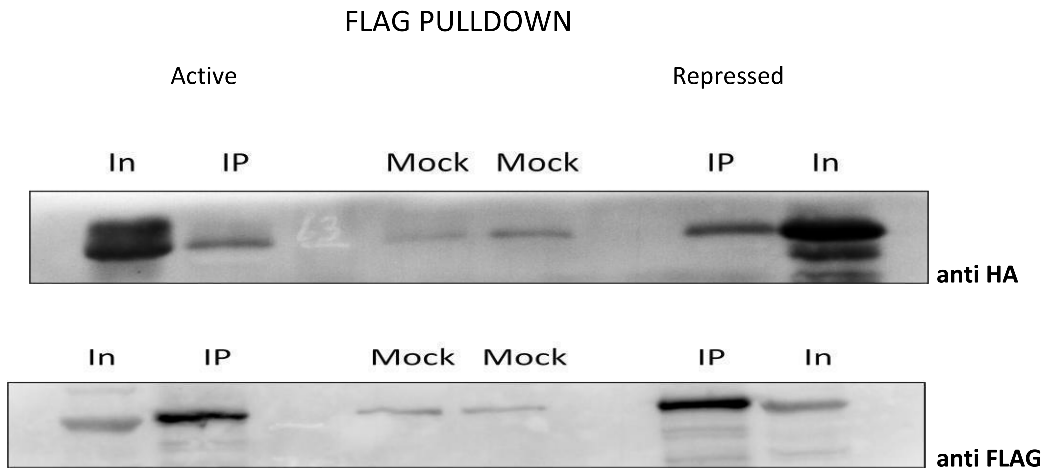

Figure 4.12.1 Western Blots showing presence of Maf1 in Tfc1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

HA pulldown for doubly tagged strain TFC1- FLAG#SPMAF1- HA was done and blot was probed with anti FLAG antibody which detected TFC1 at molecular size of ~100 KDa in the IP lanes in both active and repressed conditions. MAF1 pull down was checked with anti HA antibody, a band was detected at MW ~ 63 KDa on the membrane (Fig. 4.12.2). MAF1 and TFC1 interact more in repressed conditions than active.

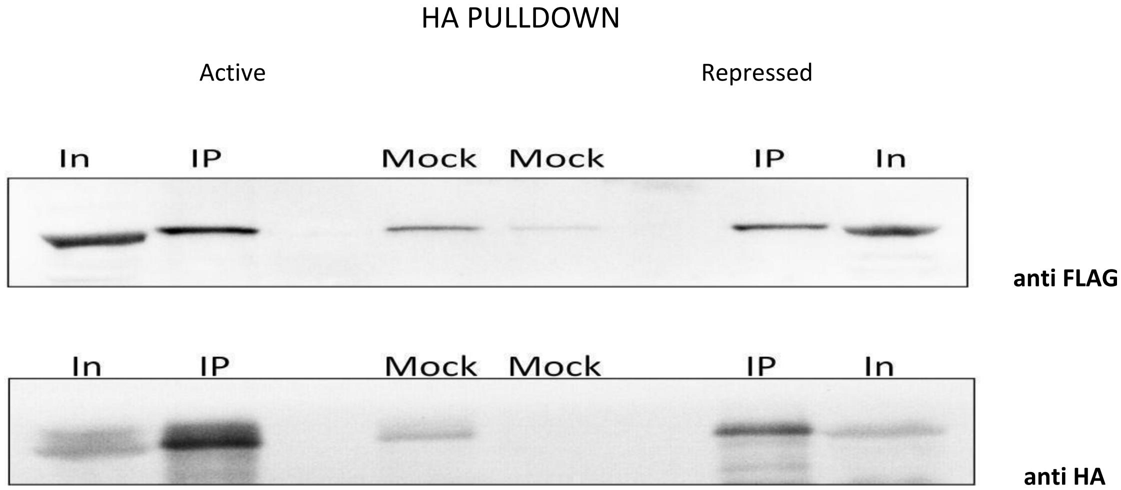

Figure 4.12.2 Western Blots showing presence of Maf1 in Tfc1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

4.2.6 INTERACTION OF SWC5 WITH BRF1 AND TFC1

(A) SWC5 with BRF1

Fig. 4.13.1 demonstrates that Flag pulldown for doubly tagged strain BRF1- FLAG# SWC5-HA was done and blot was probed with anti HA antibody which detected SWC5 at MW of ~ 50 KDa in the IP lanes in both active and repressed conditions. However bands were observed in mock lanes also in both the conditions. This may indicate a poor or transient interaction. We couldn't capture the interaction hence nothing can be said about their interaction from these blots. Though IP has worked as BRF1 pull down was checked with anti FLAG antibody, and a band was detected at MW ~85 KDa on the membrane.

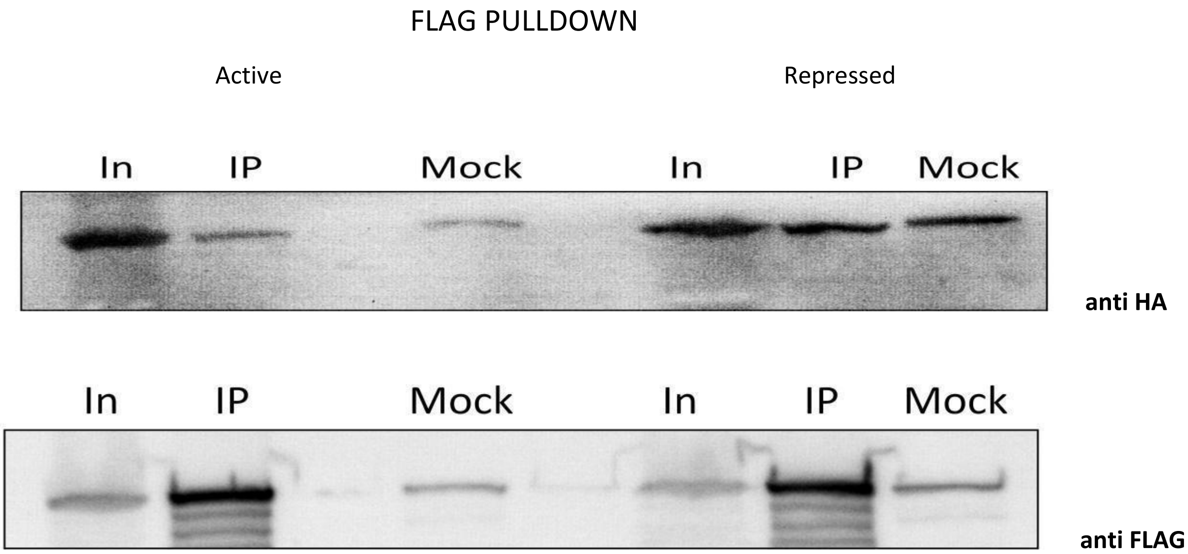

Figure 4.13.1 Western Blots showing presence of Swc5 in Brf1 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

HA pulldown for doubly tagged strain BRF1- FLAG#SWC5- HA was done and blot was probed with anti FLAG antibody which detected TFC1 at molecular size of ~100 KDa in the IP lanes in both active and repressed conditions. However in this blot band appeared in mock lane of Active set. Interaction might be too transient to be captured. But there is visible difference in Mock and IP lane of repressed set. We can say there is interaction particularly in repressed condition. IP has worked as SWC5 pull down was checked with anti HA antibody, and a band was detected at MW ~50 KDa on the membrane (Fig. 4.13.2).

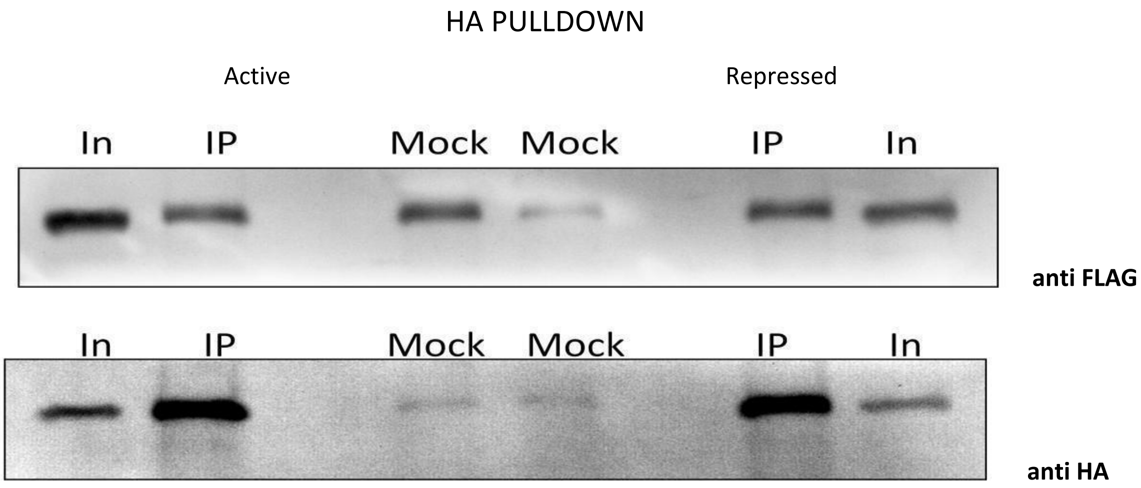

Figure 4.13.2. Western Blots showing presence of Brf1 in Swc5 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

<u>(B) SWC5 with TFC1</u>

Fig. 4.14.1 mentions Flag pulldown for doubly tagged strain TFC1- FLAG# SWC5-HA and blot probed with anti HA antibody which detected SWC5 at MW of ~ 50 KDa in the IP lanes in both active and repressed conditions. TFC1 pull down was checked with anti FLAG antibody, a band was detected at MW ~100 KDa on the membrane. SWC5 and TFC1 interact with each other in both active and repressed conditions.

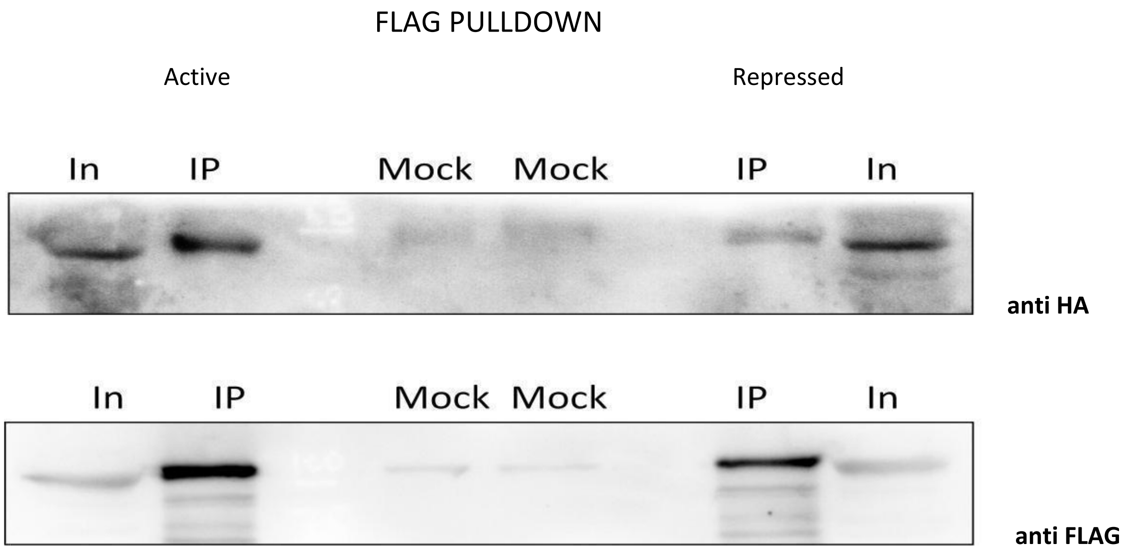

Figure 4.14.1. Western Blots showing presence of Brf1 in Swc5 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

Fig. 4.14.2 displays that HA pulldown for doubly tagged strain BRF1- FLAG#SWC5 HA was done and blot was probed with anti FLAG antibody which detected TFC1 at molecular size of ~100 KDa in the IP lanes in both active and repressed conditions. SWC5 pull down was checked with anti HA antibody, a band was detected at MW ~ 50 KDa on the membrane. SWC5 and TFC1 interact in both active and repressed conditions.

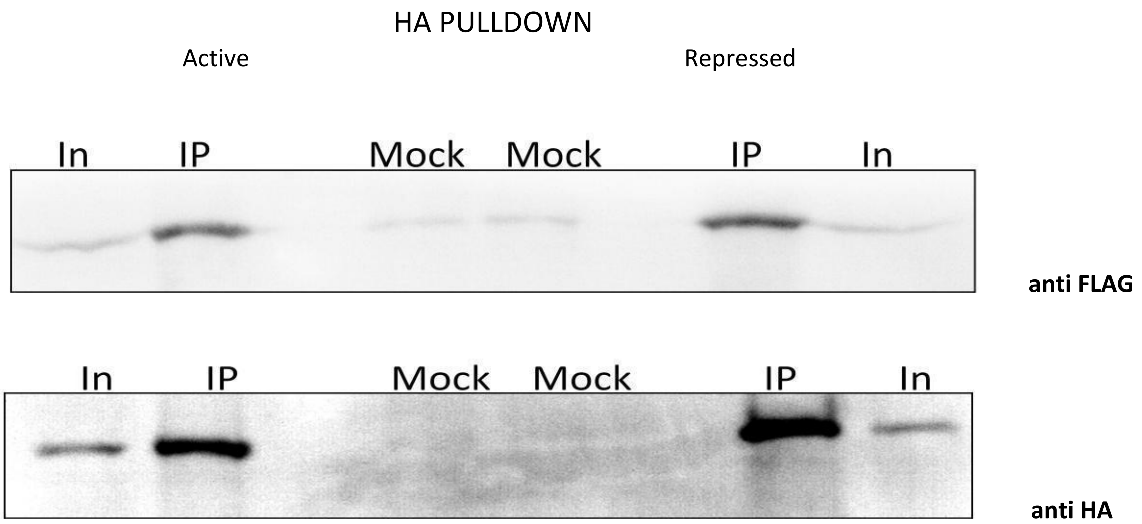

Figure 4.14.2. Western Blots showing presence of Brf1 in Swc5 immunocomplex. Presence of band in IP lane represents the pulldown has occurred. (In- Input, IP- Test, Mock-control)

5. DISCUSSIONS

RNA polymerase III is the most complex among the three polymerases as it contains highest number of subunits, that is, 17 subunits (Ducrot et al., 2006). The main transcription factors associated with RNA pol III are TFIIIB and TFIIIC. TFIIIB complex is composed of TATA binding protein (TBP), TFIIB related factor 1 (BRF1) and BDP1, whereas TFIIIC is composed of 6 subunits coded by the genes Tfc1, Tfc3, Tfc4, Tfc6, Tfc7 and Tfc8 (Schramm et al., 2002).
To study the interactions of target proteins, PAF1, TAF12, TAF14, SPT16, MAF1 and SWC5 with RNA Pol III, their interactions with BRF1 and TFC1, subunits of TFIIIB and TFIIIC respectively were checked.

In the present study, PAF1 is seen to interact with both TFC1 and BRF1 which indicates a positive interaction with TFIIIC and TFIIIB respectively. Though its interaction with TFIIIC in nutrient starved condition seems less, PAF1 is interacting with TFIIIB in both conditions which demonstrates that PAF1 may play a role with TFIIIC in active state only. In case of RNA Polymerase II, PAF1p is a nuclear RNA polymerase II-associated factor important for cell growth and required for full expression of a subset of cell cycle-regulated genes (Shi X, et al., 1996).It also includes general initiation factors TFIIB and TFIIF. Role of PAF1 complex is also elucidated in transcription elongation and modulates the activity of both RNA polymerase I and II (Tous et al.,2011).
Involvement of PAF1 in RNA Pol II is evident, but, this study reports for the first time interaction of PAF1 with TFIIIB and TFIIIC subunits of RNA Pol III. This interaction with RNA Pol III transcription factors confirms its involvement somewhere in RNA pol III transcription.

The results show that the interactions of TAF 12 with TFIIIB and TFIIIC are positive in both active and repressed conditions. Studies carried out by Grant et al. (1998) report that TAF12 is a subunit of RNA pol II transcription factor TFIID and a chromatin remodeler SAGA complexes which functions in nucleosomal histone acetylation and chromatin associated transcriptional activation or repression. It is also known to involve in RNA polymerase II transcription initiation and in chromatin modification. These evidences display a clear role of TAF12 in RNA pol II transcription. Interaction of TAF12 with TFIIIB and TFIIIC suggests that TAF12 may have a role in RNA Pol III transcription. This interaction along with PAF1 strengthens the Pol II and RNA pol III crosstalk.

TAF14 is seen to interact fairly good with TFIIIB in both active and repressed conditions but not with TFIIIC. TAF14 is a subunit of TFIID and TFIIF which are RNA pol II transcription factors and chromatin remodelers such as INO80, SWI/SNF, and NuA3 complexes (Peterson et al., 1998). All of them are known to be involved in RNA polymerase II transcription initiation and in chromatin modification. TFIIIB interacting with TAF14 could be very interesting as TAF14 is the subunit RNA polymerase II transcription factors, detailed studies are required to elucidate the exact role of TAF14 in RNA pol III transcription.
TFIIIB recruits RNA pol III on tRNA genes while TFIIIC is an assembly factor for RNA Pol III genes. Hence TAF14 might be having role direct in RNA Pol III transcription .

In the current studies, SPT16 has a clear interaction with both TFIIIB and TFIIIC. SPT16 is a subunit of the heterodimeric FACT complex (Spt16p-Pob3p); FACT associates with chromatin via interaction with Nhp6Ap and Nhp6Bp, and reorganizes nucleosomes to facilitate access to DNA by RNA and DNA polymerases (Belotserkovskaya et al., 2003). Studies by Ransom et al. (2009) suggest that in RNA polymerase II (RNAP II) transcription initiation, the FACT complex increases chromatin accessibility to transcription factors within promoter regions . Both genetic and physical evidence for the involvement of the FACT complex in DNA replication in *S. cerevisiae* has been reported by Schlesinger and Formosa (2000). Though SPT16 is seen to play an evident role in RNA Pol II transcription, the interactions illustrate that SPT16 plays a role in RNA Pol III transcription also. Hence SPT16 has a direct or an indirect role in RNA pol III regulation.

MAF1 interacts with both TFC1 and BRF1, subunits of TFIIIC and TFIIIB respectively, but in nutrient starved condition only. Previous studies identified MAF1 as a highly conserved negative regulator of RNA polymerase III. It is involved in tRNA processing and stability (Upadhya et al., 2002). It affects tRNA suppressor efficiency and interacts genetically with

Pol III. This interaction supports the previous known role of MAF1 as suggested through studies by Pluta et al. (2001).This interaction evident in nutrient starved state RNA pol III transcription abolishes. Studies by Rollins et al. (2007) also reveal human MAF1 negatively regulates RNA Polymerase III transcription via the TFIIB family members Brf1 and Brf2. As mentioned above, MAF1 is also a negative regulator of RNA Pol III in yeast , indicative that MAF1 is a common repressor in eukaryotes in both RNA Pol II and III.

In the present study, SWC5 is seen to interact with TFIIIC but a weak interaction is observed in case of TFIIIB. SWC5 a component of the SWR1 complex. SWR1 complex exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A (Mizuguchi G et al., 2004). Its interaction with TFIIIC could be very interesting as histone variant H2AZ is known to come on RNA pol III genes. Transient or weak interaction seen with TFIIIB may be a result of less number of molecules in the cell, ie. 1680 molecules/cell (Ghaemmaghami et al., 2003) Though, According to Tkach et al. (2012) protein abundance increases in response to DNA replication stress. This can be related to the faint interaction that is observed with TFIIIB, giving an insight to some mechanism connecting SWC5 and TFIIIB under stressed conditions.

Salient findings

The prominent findings of the present work is summarised below:

1.TAF14, PAF1 and TAF12 interactions with RNA Pol III share common link with RNA pol II transcription. This strengthens the Pol II and RNA pol III crosstalk. This has not been reported before.

2. MAF1 has already been identified as a negative regulator of RNA Pol III . The results aligned with the existing reports of MAf1, highlighting its repressive function.

3. Though not much work has been reported about SPT16 in RNA pol III, the results conveyed that SPT16 plays a role in both RNA pol II and III.

4. SWC5 did not show an evident interaction with TFIIIB, though it clearly plays a role with TFIIIC subunit of RNA Pol III.

5. CONCLUSIONS

We have successfully generated twelve doubly tagged *S.cerevisiae* strains. These are as follows :

TFC1::*3XFLAG*::*KanMX PAF1::6XHA::HygB*	*BRF1*::*3XFLAG*::*KanMX PAF1::6XHA::HygB*
TFC1::*3XFLAG*::*KanMX MAF1::6XHA::HygB*	*BRF1*::*3XFLAG*::*KanMX MAF1::6XHA::HygB*
TFC1::*3XFLAG*::*KanMX TAF12::6XHA::HygB*	*BRF1*::*3XFLAG*::*KanMX TAF12::6XHA::HygB*
TFC1::*3XFLAG*::*KanMX TAF14::6XHA::HygB*	*BRF1*::*3XFLAG*::*KanMX TAF14::6XHA::HygB*
TFC1::*3XFLAG*::*KanMX SPT16::6XHA::HygB*	*BRF1*::*3XFLAG*::*KanMX SPT16::6XHA::HygB*
TFC1::*3XFLAG*::*KanMX SWC5::6XHA::HygB*	*BRF1*::*3XFLAG*::*KanMX SWC5::6XHA::HygB*

All strains are isogenic to W303-1a (*MAT*a {*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15*})

These doubly tagged strains were successfully used for the respective interactions. Individual interaction results are below :

- Both TFIIIC and TFIIIB interact with TAF12 in active and nutrient starved conditions. TAF12 is a subunit of TFIID (Transcription Factor for RNA pol II) and SAGA complex (Chromatin remodeler).

- Both TFIIIC and TFIIIB interact with SPT16 in active and nutrient starved conditions. SPT16 is subunit of FACT complex (Chromatin remodeler).

- PAF1 interacts with TFIIIB in both active and nutrient starved conditions. while TFIIIC seems to interact less in nutrient starved set. PAF1 complex is involved in transcription elongation of RNA pol II.

- TFIIIB interacts fairly good with TAF14 in both active and nutrient starved conditions while TFIIIC has weak/transient interaction with TAF14. TAF14 is component of many RNA Pol II transcription factors such as TFIID, TFIIF and several chromatin remodelers INO80, SWI/SNF, and NuA3 complexes involved in chromatin modification.

- SWC5 a component another chromatin remodeler SWR1 complex interacts fairly good with TFIIIC in both active and nutrient starved conditions while TFIIIB doesn't seem to interact with SWC5 in either of the condition.

- MAF1, which is negative regulator of RNA pol III transcription, interacts with both TFIIIC and TFIIIB in nutrient starved only.

Hence in the present work, some very interesting interactions of RNA pol III transcription factors TFIIIC and TFIIIB are validated. These interactions can put further insights into RNA pol III regulation.

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ANNEXURE A

Commonly used Buffers

1.TGS running buffer (per litre) Tris 15.14g Glycine 93.83g SDS 5g	**2. 10x TBE Buffer (per litre)** Tris 108g Boric acid 55g 0.5M EDTA 40ml
3. Western transfer Buffer (per litre) Tris 5.8g Glycine 14.4g SDS 0.4g Methanol 200ml	**4. 10x TBST buffer (per litre)** Tris 12.11g Tween20 5ml NaCl 87.66g
5. Modified Lysis buffer (for 50ml) 1M HEPES(pH7.5) 2.5ml 5M NaCl 1.5ml NP40(10%) 100µl Glycerol(100%) 5ml 0.5M EDTA 50 µl	**6. Laemmli sample loading buffer(for 20ml)** 1.5M Tris-Cl pH 6.8 4ml Glycerol 10ml B-mercaptoethanol 5ml SDS 2g 1%Bromophenol blue 1ml
7. Alkali lysis buffer 1 (Solution I) 500mM Glucose 10ml 500mM EDTA pH 8.0 2ml 1M Tris pH 8.0 2.5ml Distilled water 85.5ml	**8. Alkali lysis buffer 2 (Solution II)** 20% SDS 50 µl 10 N NaOH 20 µl dH2O 930 µl
9. Alkali lysis buffer 3 (Solution III) 5 M KOAc 60 ml glacial acetic acid 11.5 ml dH2O 28.5 ml	**10. Mild stripping buffer (for 200ml)** Glycine 1.5g Tween 20 1ml 20% SDS 0.5ml

11. Protease cocktail inhibitor 1M NaF 140ul 0.5M Na_2VO_3 56ul 0.2M PMSF 140ul 1M BGP 840ul MLB 14ml	**12. 10X DNA gel-loading dye (stock solution 10 mL)** Glycerol 3.9 ml 10% SDS 500 µl 0.5 M EDTA 200 µl bromophenol blue 0.025g xylene cyanol 0.02 g
13. TE buffer 10 mM Tris-Cl pH 8.0 1 mM EDTA pH 8.0	

ANNEXURE B

1. SDS gel composition

Separating Gel (20 ml)	10%	8%
Miili-Q	7.9 ml	9.3 ml
30% Acrylamide	6.7 ml	5.3 ml
1.5M Tris pH 8.8	5 ml	5 ml
10% SDS	200 µl	200 µl
10% APS	200 µl	200 µl
TEMED	8 µl	12 µl

Stacking Gel (6 ml)	5%
Miili-Q	4.1 ml
30% Acrylamide	1 ml
1M Tris pH 6.8	0.75 ml
10% SDS	60 µl
10% APS	60 µl
TEMED	6 µl

2. Agarose gel

Agarose gel (50 ml)	1%
Seakem agarose	0.5g
Miili-Q	50 ml