Role of Human FKBP51 in P-TEFb Mediated Transcriptional Regulation


A THESIS

SUBMITTED FOR THE PARTIAL

FULFILLMENT OF THE

REQUIREMENTS FOR THE DOCTOR

OF PHILOSOPHY DEGREE (SCIENCE)

OF

JADAVPUR UNIVERSITY

2022

BY
DHEERENDRA PRATAP MALL

MOLECULAR GENETICS DIVISION
CSIR- INDIAN INSTITUTE OF CHEMICAL BIOLOGY
KOLKATA-700032
INDIA

सी.एस.आई.आर–भारतीय रासायनिक जीवविज्ञान संस्थान
वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद की एक इकाई
विज्ञान एवं प्रौद्योगिकी मंत्रालय के अधीन, एक स्वायत्त निकाय, भारत सरकार
4, राजा एस. सी. मल्लिक रोड, यादवपुर, कोलकाता - 700 032
CSIR - INDIAN INSTITUTE OF CHEMICAL BIOLOGY
A Unit of Council of Scientific & Industrial Research
An Autonomous Body, under Ministry of Science & Technology, Government of India
4, Raja S. C. Mullick Road, Jadavpur, Kolkata-700 032

CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled "The Role of FKBP51 in P-TEFb mediated Transcriptional Regulation in Eukaryotes" Submitted by Sri Dheerendra Pratap Mall who got his name registered on 11th May 2017 for the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of Dr. Debabrata Biswas and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

09/04/22

Dr. Debabrata Biswas
Senior Principal Scientist,
CSIR-IICB, Kolkata-700032

Debabrata Biswas, Ph.D.
Senior Principal Scientist,
CSIR - Indian Institute of Chemical Biology
Professor, Academy of Scientific and Innovative Research (AcSIR)
4, Raja S. C. Mullick Road, Kolkata-700032, India

Phone : 2499-5700 (Pilot No. / EPABX No.) ● Website : www.iicb.res.in ● Telegram : LIVINGCELL
Fax : 91-33-2473-5197 (Dir) ● 91-33-2473-5368 (Dir) ● Fax : 91-33-2414-9475 (A.O.) ● 91-33-2429-8490 (A.O.)
91-33-2483-1983 (F&AO) ● 91-33-2483-1982 (SPO)

Preface

The study and findings presented in this thesis entitled ***"The role of human FKBP5 in P-TEFb mediated transcriptional regulation"*** *was started in July 2014. This study adds another level of information for the regulation of P-TEFb inside the cell.*

This thesis has been divided into six major chapters. ***Chapter I*** *contains the basic introduction of eukaryotic transcription system and its regulation.* ***Chapter II****contains the review of literature about P-TEFb mediated transcriptional regulation and nature of eukaryotic FKBP5 protein as well as its involvement in regulation of different cellular phenomenon.* ***Chapter III*** *describes detailed material and methods used to carry out this thesis work.* ***Chapter IV*** *contains the results and data of this study.* ***Chapter V*** *contains the Summery of the whole work.And final* ***Chapter VI*** *contains discussion of the present study.*

Due acknowledgment has been made for reporting the scientific observations and findings made by other scientists. I take all the responsibility for any kind of unintentional error or misinformation in this thesis.

(Dheerendra Pratap Mall)

Molecular GeneticsDivision

CSIR-IICB, Kolkata-700032

DEDICATED TO MY

BELOVED

"Family"

Acknowledgement:

First of all, I want to thank my mentor, ***Dr. Debabrata Biswas,*** *who gave me the opportunity to work under his guidance, without which it was an impossible task to pursue my doctoral studies. I am very much thankful for his continuous effort to make my path easier to walk on. His dedication and untiring efforts for science always amazed and inspired me to work more with honesty. His vibrant presence always gave positive energy and a positive environment to work in. I would like to take this opportunity to express my sincere gratitude for him for his all-time presence with his valuable advises to help me whenever I needed. If I have to say something about him, he is* ***"The Most Passionate and Hard-Working Researcher".***

As a biologist we know the importance of good environment for expression of the good genes and our lab environment is wonderful because of these wonderful people around. So, I must have to thank all the present and past members of the Biswas-lab for making this lab such a nice place to work. ***Our lab seems to me a small India*** *with all its diversities and beauty. I thank each and every present and past member of Biswas-lab, starting from* ***Dipika Di, Mahesh Da, Nidhi, Koushik, Sunny, Subham, Sundaram, Arijit, Sujoy, Prathama, Sushrita****,* ***Abhik and Poushali*** *for creating such a nice environment to work in and being such a nice lab member. I would like to thank* ***Mehuli, Shraddha, Nivedita Anchal and Rupsha*** *for their support and their contribution during their short time stay in our lab.*

Personally, I want to thank ***Dipika di*** *forher help and support like an elder sister. I want to thank* ***Mahesh da*** *for all his help and support as my senior. I am really grateful to* ***Koushik*** *for being such a good friend of mine. I am very much thankful for his valuable suggestions as and when required. I want to thank* ***Nidhi, Sunny, Subham, Arijit, Sujoy, Prathama, Sushrita, Abhik and Poushali*** *for all their direct and indirect support.*

I want to convey my sincere regards to ***Dr. Arun Bandopadhyay (Director CSIR- IICB)*** *and* ***all the past directors of CSIR-IICB*** *for giving me the opportunity to work at IICB.*

*I express my sincere regards to **all the scientists of CSIR-IICB** fortheir continuous help and valuable suggestion as and when required.*

*I am grateful to the **technical staff members** of the central instrumentation facility (CIF) who always extended their helping hand during using the central instrumental facility. Special mention should be made in this regard of **Tapas Da** in Chemidoc section.*

*I cannot imagine myself at this stage without the help of my childhood teachers, just as a building cannot be imagined without its foundation and the foundation makers. I would take this opportunity to express my sincere gratitude and respect to all my teachers who have taught me since my childhood, particularly **Sahab dayal acharya ji, Thakur acharya ji, Bimlesh acharya ji, Virendra acharya ji, Omprakash acharya ji, Sitaram acharya ji, Gorakhnath acharya ji, kharbhan acharya ji, Ashwani acharya ji.. Prakash Narayan sir, PN mall sir, Mangru gupta sir……** Without them, I may never have gotten to where I am today.*

*I would like to give special thanks to **Dr. A K Raghuvanshi sir** forhis extraordinary, confidence building teaching during my college days.*

*I am very much thankful to all teachers of my M.Sc. days, Particularly, I am thankful to **Prof. SP Adhikari, Prof. Amit Roy, Dr. Samiran Saha, Dr, Jolly Basak, Dr.Narattam day, Dr. Neelanjana Das and Prof. Tathagata choudhuri,** who played a very crucial role at the time of transition from M.Sc. to PhD.*

*I would like to thank my college friends, **Ravi, Amit, Atul, Aashish, Munna, shatish,** and the university friends **Rohit, Ravi, Tejveer, Alfred, Anjali, Kasturee, Archana, Sayani, Chandan** and my beloved seniors **Sanjay bhaiya, Ashok bhaiya Manish Bhaiya, Tushar bhaiya, Dhanesh bhaiya, Nitin Bhaiya, Vinod Bhaiya, Prabuddha bhaiya, Aritra bhaiya, Madhu Bhaiya, Nisha di, Nivedita di and Sonali di…….** for being always there for me and making my days memorable.*

*I would like to acknowledge my school friends **Sudhakar, Ankit, Janardan, Kanhiya, kesh, Sahab, Ajay, Manoj, Jayprakash, Mahaver, Avinash, Shariq, Mnjoor, Aabid,***

Dinkar, Diwakar, Surendra, Sunil, Santosh, Pradeep, ……. *For making my school days wonderful.*

I would like to thank my childhood friends ***Vindhyachal, Upendra, Amit, Ajay, Mithun, Sahab, Chandan, Ashok, Vivekanand, Monu, Digvijay, Golu, Deepak, Rinku, Bhola, Sanjay, Krishnakant, Washim ……*** *for making my childhood awesome and being always there for me.I am really blessed to have friends like them.*

I am grateful to ***Shyamsundar mall*** *for listening me, always offering me advice, and supporting me like my elder brother throughout this entire process, especially during my initial days.*

Research is not complete without food. I would like to thank all the canteen-staff for their generous behavior during my stays at IICB. In this regard, ***Kashi Da, Avishek Da, and Mithu Da****'s name will come first.*

Besides doing science I consider myself a sports person. So, I would like to acknowledge all my colleagues for finding time out of their busy lab schedule to play ***Cricket, Table tennis, Badminton and carrom****. I enjoyed most and these memories will be close to my heart throughout my life.*

Acknowledgement page is incomplete without a special acknowledgement to my favourite sport ***Cricket*** *which played a role of guru and taught me so many valuable lessons of my life, specially whenever I got depressed for any reason, and it worked as a stressbuster whenever I was stressed-out in my life.*

I would like to offer my acknowledgements to ***Dushyant, Avijeet, Sambit, Anirban, Purna, Anoy, Chandrasobha, Rimita, Aymen, Achyut, Pallavi, Ayan, Sumit, Bheem, Ajay Da, Saurabh, Biswajit, Pinaki Da, Nirmal, Dipayan Sarkar, Prasanta Da, Mainak Da, Titli di, Moumita di, Sougata, Pratiti, Saheli, Ankita, Deepesh, Subarno, Suman da, Avishek (Mandal), Basuraj, Animesh, Koushik, Sumanta da, Achinta, Nayan Da, Pulak, Subhankar Da, Chinmoy Da, Shiladitya, Abhishek Da, Subhadeep Da, Arijit Da, Samrat, Piyush Da, Madhu Di, Diya Di, Deeksha Di, Arijeet Da, Nicki, Sonali, Sneha, Ramkrishna,***

Sugreev Da, Parash, Eshani, Dwiprohi, RaghavendraDa, Sandip Da, *for their helping hand wheneverneeded.*

It would be rather incomplete if I miss acknowledging ***3 no. Gate of Jadavpur University****. That is the place which worked as a stressbusting yard and I spend a lot of time besides my lab during these years. In this regard I would like to thank* ***Koushik, Sunny, Anand, Rajeev, Saroj, Ritesh, Kamran, Snehasish, Jeet, Saif, Chittron, Debojyoti, Subrata, Kajal, Peter, Mithun Da, Som Da, Shameel Da, Tridib Da, Sayanika Di, Sarfaraj Da, Abdus Da, and Dipayan Da.*** *The gathering and debate were never complete without a continuous supply of tea. So, thank you* ***didi and kakima****for non-stop supplying of tea during that time.*

I would like to express my gratitude to my ***Bade MAMA, Chote MAMA, MAMI, Chunnu Bhaiya, Bablu Bhaiya, Alok, Nivedita Rahul and Dali*** *for their love and support.*

I would like to thank all my sisters and sister in-laws starting from ***Guddi didi, Dayasankarjijaji, Sunita didi, Chandrashekhar jijaji, Sita didi, Late Nandkumar jijaji, Babli didi, Dinesh jijaji, Sadhna didi and Rajkumar jijaji*** *unconditional love and care. It is all your sacrifices and unconditional love that makes me what I am today.*

I would like to express my heartfelt thanks to all my ***nephews, nieces*** *starting from* ***Pushkal, Pushpendra, Madhvi, Shimpi, Roshni, Pankaj, Sudhir, Khushbu, Puja, Vikash, Deepu, Suchitra, Deepanshu, Suhani, Anjali, Saumya and Adarsh*** *for all their well wishes and love.*

I want to express my thanks from core of my heart to ***Sampita****for your continuous support and understanding. Day by day you r becoming the prime reason for my all the successes. Thank you for being always with me in my ups and downs.*

I want to express my heartfelt thanks to ***Bhaiya*** *for his unconditional love and support throughout my life. Without you I couldn't have even dreamed about fulfilling my dreams. You have always been an inspirational character for me. Thanks a lot for being always with me and for being such a nice* ***elder brother*** *of mine.*

*I would like to express my heartfelt gratitude to my **Bhabhi** for her continuous support and love. She taught me so many lessons of my life which really helped me to stand firmly in any situation.*

***Pita Ji,** I know you are looking at me from heaven and I hope, I have partially fulfilled your dreams. I will always be indebted of your thousands of sacrifices for me and my happiness. You taught me the most important lessons of my life. Countless words to say…. But I can just express my gratitude with tearful eyes. I miss you every moment and I'll miss you forever.*

***Maa**, in words it's impossible to express my gratitude for you. Though, I am thanking you for giving me this life, for your truly truly unconditional love, support and caring for me. It is your hard work and sacrifices that taught me how to chase my dream. You taught me, how to be **patient,** the best lesson of my life. It was your face which always stopped me whenever I was down and about to quit. Thank you **Maa**.*

(Dheerendra Pratap Mall)

Table of content:

List of figures:

List of tables:

List of Abbreviations

Pol II	-	RNA polymerase II
P-TEFb	-	Positive transcription elongation factor b
CDK9	-	Cyclin dependent kinase
FKBP5	-	FK506 Binding Protein 51
HSP90	-	Heat shock Protein 90
NELF	-	Negative elongation factor
DSIF	-	DRB sensitivity inducing factor
GTF	-	General Transcription factor
7SK snRNP	-	7SK small nuclear ribonucleoprotein
MePCE	-	Methyl phosphate capping enzyme
PIP3	-	Phosphoinositide 3-kinase
Cyp40	-	Cyclophilin 40
TPR	-	Tetratricopeptide repeat
GR	-	Glucocorticoid receptor
PIC	-	Preinitiation complex
CTD	-	Carboxy terminal domain
TSS	-	Transcription start site
TF	-	Transcription factor
eRNA	-	Enhancer RNA
Inr	-	Initiation element
HAT	-	Histone acetyltransferase
HDAC	-	Histone deacetylase
Brd4	-	Bromodomain containing protein 4
SEC	-	Super elongation complex
ELL	-	Eleven- Nineteen lysine- rich Leukemia
EAF	-	ELL associated factor
DPE	-	Downstream promoter element

BRE	-	TFIIB recognition element
TBP	-	TATA binding protein
TAF	-	TBP associated protein
CPSF	-	Cleavage and polyadenylation specificity factor
CSTF	-	Cleavage stimulation factor
DBD	-	DNA binding domain
KMT	-	Lysine methyl transferase
SAM	-	S-adenosyl methionine
DNMT	-	DNA methyl transferase
DMSO	-	Dimethyl sulfoxide
β-me	-	beta-mercaptoethanol
TRM	-	TAR recognition motif
LARP7	-	La related protein7
HEXIM	-	Hexamethylene bisacetamide inducible
UV	-	Ultra violate
AR	-	Androgen receptor
LPS	-	Lipopolysaccharide
DMEM	-	Dulbecco's Modified Eagle Medium
PCV	-	Packed cell volume
NPV	-	Nuclear pellet volume
IPTG	-	Isopropyl β-D-1- thiogalactopyranoside
BSA	-	Bovine serum albumin
RT-qPCR	-	Realtime quantitative polymerase chain reaction
ChIP	-	Chromatin immunoprecipitation
GO	-	Gene ontology
DAVID	-	Database for annotation, visualization, and integrated discover

CHAPTER I

General introduction of transcription regulation

1. General introduction of eukaryotic transcription and transcription regulation

1.1 Transcription overview:

If we look at the central dogma of molecular biology, starting from prokaryotes to eukaryotes, the very first step of it is transcription. Through transcriptional process, information(s) coded in the form of DNA inside the cell, copied in the form of RNA, which ultimately can be translated into a protein/peptide molecule (Figure 1.1). Eukaryotic transcription process and its regulation is quite different from prokaryotes. Different sets of genes are expressedat different times in multicellular organisms, including humans. Gene expression pattern changes depending on the cell type of an organism. Eukaryotic transcription, unlike prokaryotic gene-transcription, is a much more organized event which happens inside the membrane enclosed nucleoplasm. Regulation at the level of transcription is particularly significant during whole event of a gene expression, since it comes before most other regulatory stages, including as chromatin accessibility, RNA processing, RNA stability, translation, post-translation modification, and so on. In contrast to bacteria which use a single polymerase for transcription, eukaryotic cells use three different RNA-polymerases to transcribe their DNA, depending on the type of gene. RNA polymerase I catalyses rRNA synthesis (Jiang and Peterlin 2008; L. Li, Lou, and Wang 2011; Binder et al. 2004), whereas RNA polymerase III catalyses tRNA biogenesis as well as several other non-coding short RNAs (Dieci et al. 2007; Weinmann and Roeder 1974). RNA polymerase II (pol II) is responsible for the transcription of protein-coding genes as well as short RNAs such as siRNA and miRNA (Kornberg 2007). The information held in DNA is carried to the protein synthesis machinery by messenger RNA (mRNA), which is transcribed from protein coding genes.

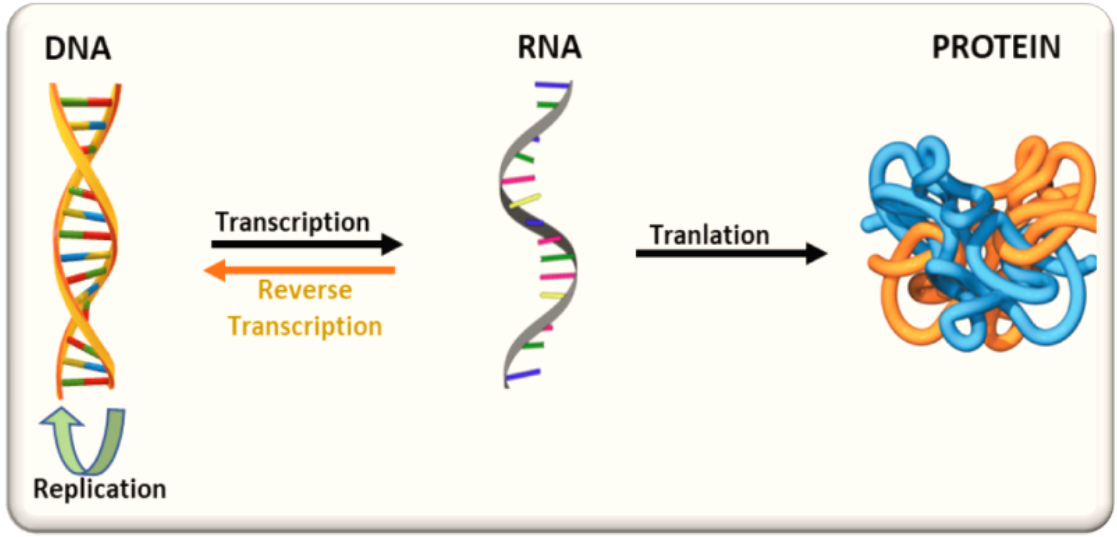


Figure 1.1 Schematic representation of Central dogma of molecular biology (Adapted from Central dogma, translation, transcription | Dai Liang Group (cityu.edu.hk**).**

Figure shows basic steps of molecular biology where, Replication indicates the production of DNA from DNA, Transcription indicates the production of RNA from DNA, Reverse transcription indicates the production of DNA from RNA and lastly Translation indicates the production of Protein from RNA.

Overall transcriptional outcome is regulated by the factors which regulate chromatin organization and accessibility for other transcription factors. These processes are regulated by several epigenetic modifiers which decide chromatin compactness and accessibility.

If we look at a finer level of transcription regulation (especially Pol II mediated transcription), it is directly controlled by several transcription factors. Transcription mediated by Pol II is a complex process with three broad stages: initiation, elongation, and termination. With the aid of numerous general transcription factors, Pol II is recruited to the promoter and catalyzes mRNA synthesis in a template-dependent manner. The basal level of transcription is regulated by the combinatorial activity of two transcription components, technically known as the trans-acting element and cis-acting element, (Buratowski 1994; Metzger et al. 2016).

Here we have discussed the major ways of eukaryotic transcription regulation.

1.1.1 Chromatin organization anld transcription:

DNA inside the eukaryotic cells remains in the form of chromatin which is a DNA-Histone protein complex. Chromatin fluidity and compaction is one of the very initial steps of transcription regulation, which is maintained by many other factors called chromatin modifiers and remodelers. In order to form a chromatin structure, DNA is tightly wrapped around histone octamer forming a nucleosome which, with the help of other nucleoproteins, gets more compacted to form chromatin. On the basis of compactness, chromatin is catcacategorized into two forms i.e., euchromatin and heterochromatin. Euchromatin region of chromatin is generally a less condensed state of chromatin which is known to be an active chromatin region where availability of other nucleoproteins is very high. While heterochromatin region chromatin is a highly condensed state of chromatin which is known to be silenced chromatin region and availability of other nucleoproteins is relatively very low in this region (Swigut and Wysocka 2007). Euchromatin region is known to be transcriptionally active region and, as this region is less compact, it flexibly allows an access to chromatin modifiers and remodelers. As and when required, several chromatin modifiers and remodelers work in coordinated manner and helps in nucleosome dispersal and making nucleosome free DNA region where different transcription factors and RNA polymerase can easily bind and transcription process can be started (Bai et al. 2010; C. K. Lee et al. 2004). Again, when transcription silencing is needed, another set of chromatin remodelers, histone chaperones (e.g., nucleosome assembly protein 1 i.e., NAP1) work for nucleosome assembly and chromatin compaction. Here we have provided a simple diagrammatic representation of Chromatin organization and its effect on transcription.

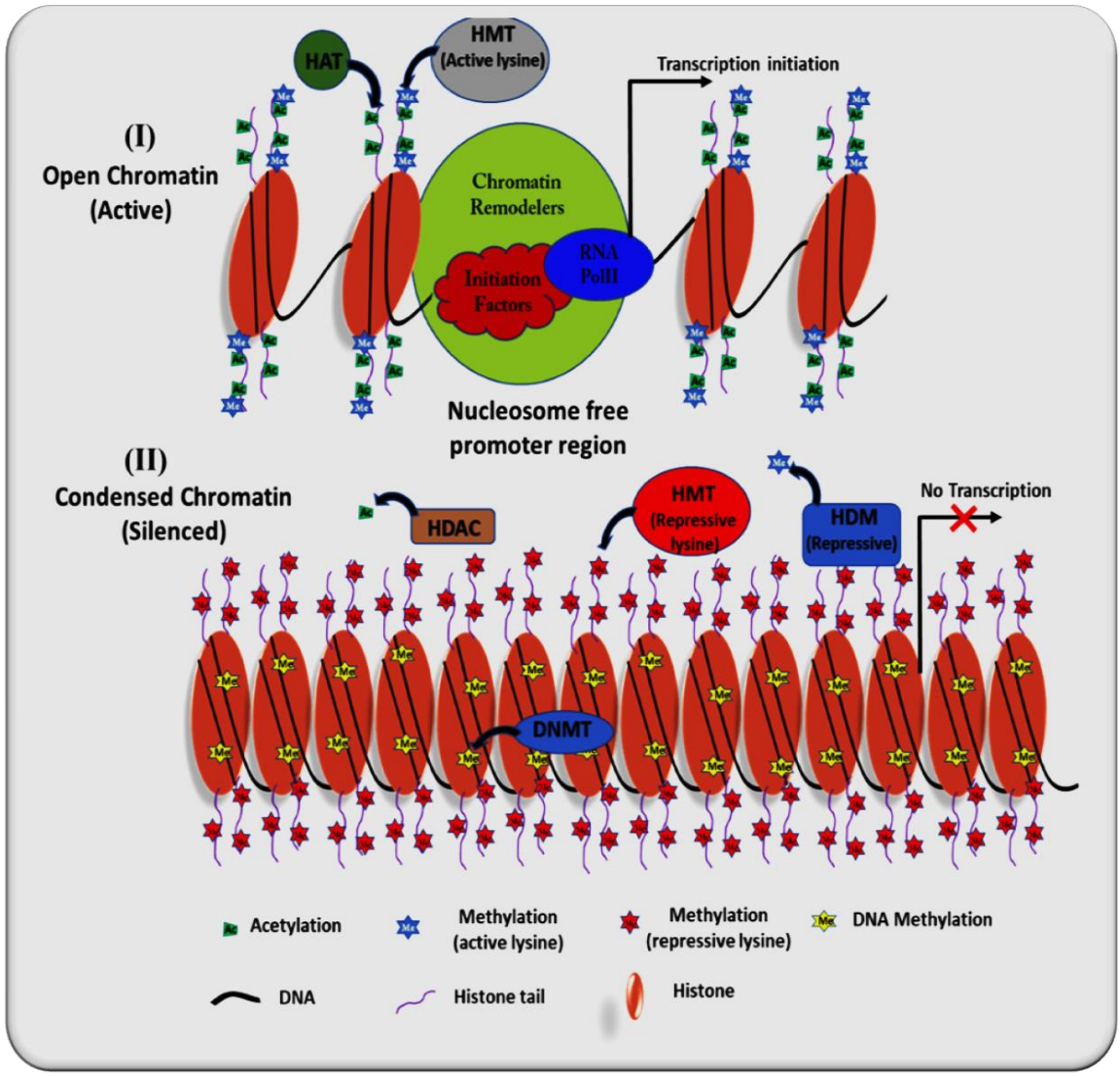


Figure 1.2 Pictorial representations of Chromatin organization, its modification, modifiers and its effect on transcription. (Adapted from https://doi.org/10.3389/fncel.2014.00446).

Upper panel **(I)** represents the open chromatin structure where different chromatin modifiers are shown which are involved in establishing and maintaining the open chromatin (Nucleosome free region) state, where polII and other initiation factors gets easy access to promoter and transcription happens. Lower panel **(II)** represents the Closed chromatin structure where different chromatin modifiers are shown which are involved in establishing and maintaining the closed chromatin state, where polII and other initiation factors doesn't get access to promoter and transcription doesn't happen.

1.1.2 Epigenetic regulation of transcription:

Expression of different sets of genes in a particular pattern is required for normal functioning of a cell, and it is accomplished by the epigenetic regulation of gene expression. Epigenome varies with cell type, and for normal cellular differentiation, stable pattern of gene expression is essential, and this is achieved by cell type specific epigenome. Unlike the genome, which is essentially identical in all cells of a vertebrate and stable throughout the life-time of an individual, the epigenome differs from cell to cell and is plastic, changing with time and with exposure to the environment (Jirtle and Skinner 2007). Epigenetic regulation basically regulates the chromatin conformation, and it explains the questions related to plasticity of chromatin. Epigenetic changes encompass different type of molecular components like acetylation, phosphorylation, ubiquitination, crotonylation etc. Here, epigenetic players are divided and explained in following categories.

- **Writer:**

As we discussed earlier, eukaryotic DNA remains in condensed form and is tightly bound with histone proteins that is called chromatin. Chromatin decondensation is prerequisite for any gene to be expressed. Some epigenetic modifications, which help in chromatin decondensation, play major decisive role in gene expression. Epigenetic players (Proteins) which covalently modify histone or DNA are termed as writers in the field of epigenetics, and these players directly or indirectly help in chromatin decondensation. There are many types of epigenetic writers, some of which are discussed below.

Among important epigenetic writers' histone methyltransferases, histone acetyltransferases, kinases and ubiquitin ligases are well studied. Histone methyltransferases include lysine methyltransferase (KMT) and arginine methyltransferases (RMT) which methylates lysine and arginine residues of histone respectively. All the histone methyltransferases use cofactor S-adenosyl methionine (SAM) as a source of methyl group. Degree of methylation varies for both the residues as lysine undergoes mono, di, and trimethylation and arginine shows only mono and di-methylation (McBride and Silver 2001; Whetstine et al. 2006). Degree of methylation significantly affects the gene activation and deactivation as epigenetic readers, that

recognise methylation marks, can distinguish between different levels of methylation. Again, methylation of arginine residue can be symmetric as well as asymmetric. Symmetric dimethylation of arginine residue is associated with the gene repression whereas asymmetric dimethylation is associated with the gene activation(Bauer et al. 2002). There are methyltransferases, which can methylate DNA itself, called DNA methyltransferases (DNMTs)(Fujita et al. 1999). DNA gets methylated on major grove by DNMTs which are majorly associated with gene silencing becausemethylated DNA may recruit multiple protein factors which prevent transcription by inhibiting pol II recruitment on the chromatin.

Histone acetylation is also another major epigenetic mark that plays a decisive role in gene activation. Histone acetyltransferases (HATs) transfer acetyl group on lysine residue on histone tails. Here, cofactor acetyl-coA works as acetyl group donor. Addition ofacetyl group on lysine residues on histone tails neutralize the positive charge of lysine, which results in weakening the histone-DNA interaction, and open chromatin state is achieved because of loosely wrapped DNA around the histones. Research employing chicken erythrocytes by Dr. Crane Robinson's group was the first to establish a direct relationship between histone acetylation and transcriptionally active chromatin(Hebbes, Thorne, and Crane-Robinson 1988).

Histone phosphorylation is another epigenetic modification that plays a major role in chromatin de-condensation and modulates both gene expression as well as DNA damage repair. One of the well-known kinases, JAK, phosphorylates histone H3 at lysine 41 (H3Y41). This phosphorylation leads to disruption of the binding of protein HP1-α on heterochromatin which causes chromatin de-condensation and it leads to the activation of target oncogene Lmo2 (Dawson et al. 2009). Another well-studied phosphorylation of histone H2AX at S139 (aka γH2AX) in surrounding regions of double-stand breaks and is a marker of the DNA repair region. MDC1 binds to the γH2AX and in turn, recruits DNA repair factors (Stewart et al. 2003).

➢ Eraser:

A group of enzymes is called erasers or eraser enzymes that delete the epigenetic markers. These enzymes reverse the effect of writers. Histone deacetylases, demethylases, and phosphatases are among the most frequent eraser enzymes that have been widely studied.

➢ Reader:

Reader domain-containing proteins are divided into four categories: chromatin architectural proteins, chromatin remodelling enzymes, chromatin modifiers, and adaptor proteins that recruit other geneexpression machinery. The first category, chromatin architectural proteins, attaches to nucleosomes and can either directly cause chromatin compaction or act as a shield to inhibit transcription-related proteins from binding to them. Unlike chromatin architectural proteins, chromatin remodelling enzymes provide a more open chromatin architecture, which makes it easier for DNA transcription to take place. The energy of ATP hydrolysis drives this structural alteration in chromatin architecture. Many other proteins with reader domains, aside from chromatin remodeling enzymes and architectural proteins, do not directly alter chromatin architecture, but rather recruit secondary chromatin modifiers to further modify chromatin or undo an existing chromatin modification.Adaptor proteins are the last kind of reader domain-containing proteins, and their major role is to recruit components involved in DNA metabolism activities including transcription, DNA damage repair, DNA recombination, DNA replication, and RNA processing. MDC1's BRCT domain interacts with a phosphorylated serine residue on histone H2AX, acting as an adapter to recruit the histone ubiquitin ligase RNF8 to double-strand break-flanking chromatin(Stucki et al. 2005). Following histone ubiquitination, repair machinery is recruited, including the tumour protein p53 binding protein 1 (TP53BP1).

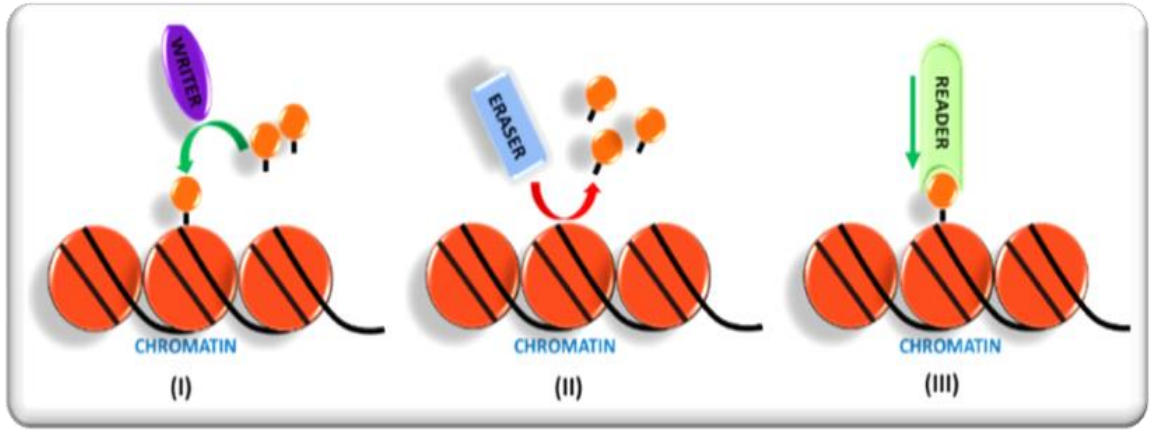


Figure 1.3: Pictorial representation of epigenetic modifiers (Adapted from https://www.med.unc.edu/~bstrahl/research.html).

(I) Represents Writer which is shown to add some kind of entity to modify the chromatin structure. **(II)** Represents Eraser which works in opposite way of writers and it modifies the chromatin structure in opposite way of writer. **(III)** Represents the Readers which recognize the entities added by the writer.

1.1.3 Transcription regulation by trans-acting elements:

There are multiple DNA sequences, termed as trans-acting elements, which regulate transcription in an indirect manner where they synthesize different factors which regulate the transcription. Factors synthesized from trans acting elements, generally work on cis-acting elements to regulate transcription. Depending on their role, trans-acting factors might be of several types, some of which are discussed below.

1.1.3.1 General transcription factors (GTFs):

There are many factors which are necessary for the onset of transcription. These factors are termed as general transcription factors (GTFs). GTFs are also known as basal transcription factors. General transcription factors mostly have DNA binding domain and they bind to the specific sites of DNA (Mostly on cis-regulatory elements) to help transcription initiation. There are 6 well known General transcription factors (GTFs)

namely TFIID, TFIIA, TFIIB, TFIIF, TFIIE, and TFIIH. On the promoter region of a gene, these components create a megadalton complex with Pol II, which is known as the pre-initiation complex (PIC) assembly. Basic information regarding these GTFs and their functioning has been provided in brief in this section.

- **TFIID:** TFIID is a complex comprised of several factors, helping in the placement of pol II on the promoter in order to transmit the regulatory signal. TATA-binding protein (TBP) is the main constituent of TFIID complex. There are several other TBP-associated factors (TAFs) which together constitute TFIID complex. (Sanders, Garbett, and Weil 2002). TBP identifies the TATA box on the promoter, whereas TAFs increase promoter selectivity, notably in TATA-less promoters (Martinez et al. 1994; Pugh and Tjian 1991). Its components, including TAF1, TAF7, and others, operate as transcriptional co-activators in addition to their involvement in PIC assembly (Devaiah et al. 2010; Kloet et al. 2012; Maile et al. 2004). TFIID functions as a scaffold for the assembly of rest of the factors required for the pre-initiation complex (PIC) formation.

- **TFIIA:** TFIIA is heterodimer of two subunits. TFIIA interacts directly with TBP and stabilizes the TFIID association with TATA box on promoter (DeJong and Roeder 1993; Yokomori et al. 1994).

- **TFIIB:** TFIIB is a single 33 KDa peptide, which binds with the B recognition element (BRE) of core promoter. Its binding with the promoter stabilizes the TFIID complex association with DNA and it helps in Pol II recruitment on the promoter (Fairley et al. 2002; Hawkes and Roberts 1999)

- **TFIIE:** TFIIE is a tetrameric complex that recruits TFIIH and helps in the formation of pre-initiation complex with other GTFs (H. T. Chen, Warfield, and Hahn 2007; Langelier et al. 2001). It promotes TFIIH's DNA-dependent ATPase and Pol II C-terminal domain kinase activity. TFIIH and TFIIE are both necessary for RNA polymerase to clear the promoter.

- **TFIIF:** TFIIF is one of the GTFs which binds with free Pol II and helps in the recruitment of Pol II on the promoter by binding to TBP and TFIIB. Thus, it ensures the specific recruitment Pol II on promoter (Khaperskyy et al. 2008; Purrello et al. 1995).

- **TFIIH:** It is a multi-subunit complex, which comprises of 10 subunits. Two of its subunits XPB and XPD have ATPase and helicase activity respectively which helps in transcription-bubble formation (Fuss & Tainer, 2011). Two other subunits of TFIIH, Cyclin H and associated CDK7,

have kinase activity towards Pol II CTD (C-Terminal Domain of RNAPol II binding protein1) and this activity plays a crucial rol in Pol II promoter escape (Shiekhattar et al. 1995).

1.1.4 Transcription regulation by cis-acting elements:

Cis-acting elements are regulatory DNA sequences, located in vicinity of gene sequences. Mostly trans-acting factors bind to their cis-regulatory sequences to control gene expression. Cis-acting elements are DNA sequences that function as regulators in the proximity of gene sequences. To influence gene expression, trans-acting factors bind to the cis-regulatory regions.

We've provided a brief overview of the many types of Cis-acting components here.

1.1.4.1 Core regulatoryelement:

Core regulatory mainly comprises of promoter region which contains several different regulatory sequences.

1.1.4.1.1 Promoter: Promoter is the DNA sequence which provides the idea about the transcription start site. It recruits the factors which are responsible for starting the transcription. A core promoter is often made up of some conserved sequences. A detailed description of a typical promoter has been provided here (Figure 1.4).

- **TATA BOX (TATA sequence)**: TATA box is a consensus sequence comprised of "T" and "A" base pairs. This sequence was first identified by David Hogness in 1978.It serves as recognition site for TBP subunit of TFIID and that's how it helps in pre-initiation complex (PIC) formation. TATA box is located ~25 base pairs upstream of TSS (Transcription Start Site). TATA box was assumed to be present in every promoter at first. However, with advances in understanding, it is currently thought that there are thousands of TATA-independent promoters (Pugh and Tjian 1991; Tokusumi et al. 2007). There are several examples of TATA-independent promoters recruiting Pol II via the SAGA complex (Baptista et al. 2017; Bonnet et al. 2014).

- **B recognition element (BRE)**: The BRE element is a TFIIB recognition sequence found in the core promoter. Depending on the relative position to the TATA box, it can be positioned as BREu (BRE upstream) or BREd (BRE downstream). The BREu element has a consensus sequence of SSRCGCC, while the BREd element has a sequence of RTDKKKK.

- **Initiator sequence (Inr)**: Initiator sequence facilitates binding of general transcription factor TFIID. This sequence or motif has a consensus sequence of YYANWYY. Inr element generally strengthens the binding of general transcription factors to promoter (O'Shea-Greenfield and Smale 1992a).

- **Downstream promoter element (DPE)**: In *Drosophila melanogaster*, T.W Burke and James T. Kadonaga were the first to characterize the downstream promoter element (Burke and Kadonaga 1997). It's about 28 to 33 nucleotides after the transcription start site (TSS). The proper spacing between the DPE and the Inr element is required for DPE-dependent basal transcription (O'Shea-Greenfield and Smale 1992b).

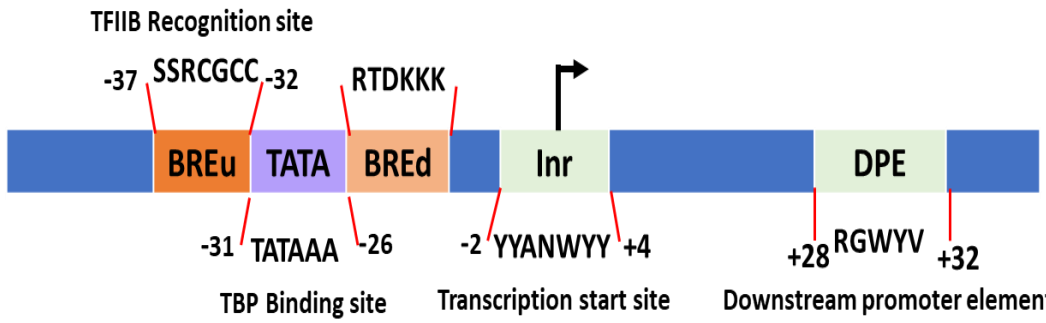


Figure 1.4 Pictorial representation of a core-promoter and its elements with their sequences.

1.1.4.2 Distal regulatory element:

1.1.4.2.1 Enhancer:

A short stretch of DNA sequence that regulates the gene expression, can be found both upstream and downstream of the transcription start site (TSS), is known as a enhancer (Bulger and Groudine 2011). The mechanism of its action remained unknown for a long time, despite the fact that the enhancer feature was found in the early 1980s. The cis-acting role of enhancer has been addressed with the advent of microscopy and other biochemical experiments. It can influence a single or numerous promoters and are likely to trigger transcription. For powerful transcriptional activation, many enhancers can exist as a unit through chromatin looping with the support of transcription factor and Mediator complex, and are referred to as super enhancers(Whyte et al. 2013). Enhancers which are linked with the H3K27ac histone mark are active in nature, whereas enhancers which are linked with the H3K27me3 is poised in nature (Creyghton et al. 2010). There are two hypotheses which can explain enhancer-mediated spatiotemporal control of eukaryotic transcription.

1.1.4.2.2 Enhancer-promoter looping mediated transcription regulation:

The promoter-enhancer looping hypothesis is widely accepted and often used to describe the function of enhancers. The promoter-enhancer looping can be observed by proximity ligation test which is used to evaluate the co-localization of various DNA fragments in close proximity at a certain time. Dynamic looping between promoter and enhancer was discovered in a 3D genomic structure based on “chromosome conformation capture” technique (Rao et al. 2014). To influence the transcriptional output from a homologous promoter, the enhancer can interact with different transcription factors. High-resolution imaging and high-throughput sequencing data have conclusively confirmed the transfer of transcription factors and other regulatory signals from cognate enhancers to corresponding promoters through chromatin looping, (Gurumurthy et al. 2019; Z. Liu and Tjian 2018).

1.1.4.2.2.1 eRNA mediated transcription regulation:

Using high-throughput DNA and RNA sequencing data, researchers discovered a new type of non-coding RNA molecule known as enhancer RNA or eRNA that controls transcription from the respective promoter. eRNAs are non-coding RNAs that are 50-2000 bp long and are transcribed from enhancer DNA using Pol II (T. Kim et al., 2010). It may be classified into two groups based on their sizeand directionality of synthesis, and polyadenylation status, namely 1D and 2D eRNA (Natoli and Andrau 2012). Out of the two above mentioned eRNAs, 1D eRNA is produced unidirectionally as polyadenylated RNA, whereas the latter is tiny and non-polyadenylated. 2D eRNAs get their name from the fact that they are generated *via* bidirectional transcription from enhancer DNA (Mikhaylichenko et al. 2018). Recently, it has been observed that eRNA can have both cis- as well as trans-acting function (Melo, Léveillé, and Agami 2013). eRNA increases the recruitment of regulatory proteins at the site of its own production in a cis-acting pathway. For example, Enhancer of Cyclin D1 produces eRNA, which serves as an adaptor for acetyltransferases to be recruited to the eRNA synthesis site. Cyclin D1 transcription is suppressed when this eRNA is genetically depleted (Wang and Fischer 2008).Evf2, which promotes transcription of the DLX gene distal to the eRNA synthesis site, is a classic example of trans-acting eRNA.

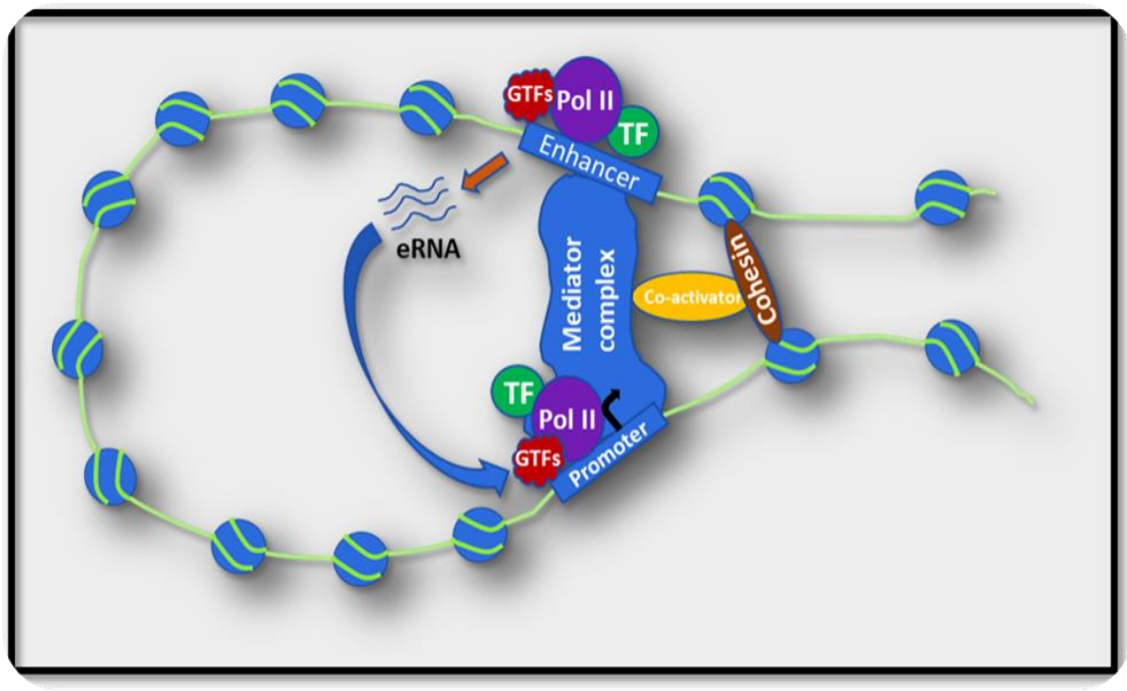


Figure 1.5 Promoter-enhancer looping and regulation of transcription (Adapted from https://doi.org/10.3390/genes10010043).

- Cartoon diagram representing coordinated action of mediator complex, cohesin, co-activators bringing enhanser and cognate promotor close through loop formation to convey the transcriptional signal. Additionally, promoter function is also odulated by eRNAs synthesized from enhancer
-

1.1.4.2.3 ***Silencer:***

Silencer, unlike enhancer, represses transcription from a homologous promoter. Silencer elements, like enhancers, use a looping mechanism to transport transcriptional repressors to the target promoter. Silencer elements interact directly with Pol II and either promote formation of more condensed chromatin or block it. Some silencer components are always active, whereas others are activated just in selected cells. For example, the silencer for the CD4 expressing gene is inactive in the majority of T cells that display the CD4 marker, but it is active in a fraction of CD4 negative T cells (Jiang and Peterlin 2008; Sawada et al. 1994).

1.1.4.2.4 Insulator:

As its name suggests, insulator creates a physical barrier between an enhancer and promoter to inhibit the enhancers activity onto the cognate promoter. The insulator interacts with a number of components that aid in DNA looping and connects two insulators while separating the enhancer and promoter. Barrier insulators have also been linked to nucleosome removal during heterochromatin spreading, in addition to enhancer blockage (Gaszner and Felsenfeld 2006). It can also change the nucleosome by recruiting histone modifiers, which prevent heterochromatin signals from spreading. In humans, the HS5 insulator disrupts the looping of the LCR (locus control region) and the downstream promoter of the β-globin gene. Instead, it generates a new CTCF-dependent loop that prevents β-globin genes from being expressed (Splinter et al., 2006.). Intrachromosomal and interchromosomal interactions mediated by insulators may play a role in cell-specific gene expression. As a result, insulator dysfunction is detrimental to healthy cellular growth, potentially leading to cancer.

1.2 Major stages of Pol II-mediated transcription:

Mammalian transcription process involves many steps and all the steps are regulated by many different sets of transcription factors. Here, in this part, major stages of transcription have been discussed in detailed and sequential manner.

1.2.1 Transcription initiation:

The first and obviously the most important step of the transcription process is its initiation. Transcription initiation is a major rate-limiting step of the whole transcription process. During this process, Pol II gets recruited on to the promoter with the help of many other factors and get itself ready to enter in elongation phase. The whole event of transcription initiation has been discussed below in step wise manner.

1.2.1.1 *Preinitiation complex formation:*

TAcomplex formationTA box recognition by TBP of the TFIID complex initiates the development of the preinitiation complex. TFIIA and TFIIB then assist in the stabilisation of the TFIID-DNA complex and the recruitment of the Pol II and TFIIF

complexes. TFIIF acts as a connector between TATA-bound TBP and Pol II. Finally, TFIIE recruits the TFIIH complex to complete the construction of the preinitiation complex (PIC) (Louder et al. 2016; Tang et al. 1996). Sequential assembly of PIC has been provided here (Figure 1.5).

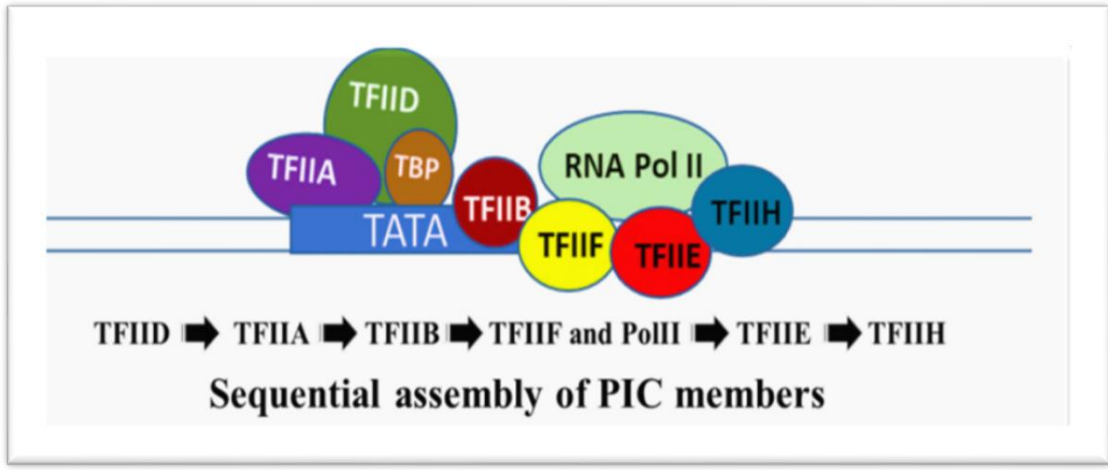


Figure 1.6 Pre initiation complex (PIC) formation.

Picture representing sequential assembly of pre-initiation complex (PIC) members which starts with TFIID and ends with TFIIH recruitment on the promoter.

1.2.1.2 Promoter melting and open complex formation:

TFIIH draws double-stranded DNA into the cleft of polymerase and aids in strand separation to produce transcription bubbles via its ATPase and kinase activities (T. K. Kim, Ebright, and Reinberg 2000; Pal, Ponticelli, and Luse 2005). Following the transition from closed to open complex, Pol II adds complementary ribonucleotides to produce a hetero-duplex of nascent RNA and template DNA strands.

1.2.1.3 Promoter escape:

The polymerase may halt the transcription and release shortened RNA called abortive transcription until the nascent RNA reaches a threshold length (Goldman, Ebright, and Nickels 2009; Revyakin et al. 2006). When nascent RNA hits the RNA exit channel after

multiple rounds of unsuccessful transcription, polymerase breaks all contacts with other initiation complex regulatory proteins and escapes the promoter. The phosphorylation of the carboxy-terminal domain (CTD) of Rpb1, the major subunit of Pol II, by TFIIH at Serine 5 locations of its heptad repeat is one of the essential processes of promoter escape (Akhtar et al. 2009; Ramanathan et al. 2001).

1.2.2 Transcription elongation:

After successful promoter escape, Pol II enters into the elongation phase of transcription with several other transcription factors. Elongating Pol II halts more often during the elongation process due to several reasons like mRNA capping, DNA damage, etc. After receiving an appropriate signal, the halted Pol II either falls off the template DNA or restarts transcription. Here, some stages of elongation are discussed below.

1.2.2.1 Promoter proximal pausing:

Two pause-inducing factors, DRB-sensitivity-inducing factor (DSIF) and negative elongation factor (NELF), get recruited with the early elongation complex and the RNA transcript when the nascent RNA reaches ~20 nucleotides (nt) in length, limiting further synthesis. DSIF and NELF have been found all throughout the genomic locations. Pol II is found at promoters in virtually all genes, implying that it transitions through a paused phase. The length of pause, on the other hand, varies greatly from gene to gene. (From a few seconds to many minutes) and is determined by the pace at which pause-release factors are recruited. The CDK7 component of TFIIH phosphorylates the Pol II C-terminal domain (CTD) in the early elongation complex. Both Serine 5 (S5) and Serine 7 (S7) residues on the CTD are targeted by CDK7, and both modifications are enriched promoter proximally. Because the capping enzyme complex (CE) interacts with DSIF as well as the S5-phosphorylated CTD, both Pol II pausing and CTD phosphorylation are expected to increase the efficacy of capping the 5′ end of the RNA.

1.2.2.2 Pause release:

Through phosphorylation of the pause-inducing factors by the kinase P-TEFb, the transcriptional barrier produced by DSIF and NELF is removed. Phosphorylation is considered to separate NELF from Pol II, transforming DSIF into a positive elongation factor that promotes productive RNA synthesis. TFs, chromatin changes, and coregulatory

complexes are among the processes that cause P-TEFb to be recruited and the stalled elongation complex to be released. Importantly, TFs that promote P-TEFb recruitment are frequently different from those that regulate PIC assembly, allowing TFs that control the initiation and elongation phases to collaborate to decide transcription output.

1.2.2.3 Productive elongation:

From 3' to 5', Pol II travels along with antisense DNA, producing complementary RNA strands in the 5' to 3' direction. Pol II, being a multi-subunit protein, has its own helicase function, which allows it to unwind DNA ahead of polymerase while also allowing it to rewind the unwound DNA behind it (Gnatt et al. 2001; Westover, Bushnell, and Kornberg 2004). A number of elements bind Pol II as it transforms to a completely elongation-competent form, assisting it in its downstream travel. Some of these factors may operate directly on Pol II to speed up or improve the processivity of transcription elongation, while others aid in the disassembly and reassembly of nucleosomes in the route of Pol II transcription. Phosphorylation of the CTD at S2 is a characteristic of the fully elongation-competent Pol II complex. As Pol II travels downstream into the gene, P-TEFb performs this change, which is likely boosted by the kinase Cdk12/CyclinK. The S2-phosphorylated CTD offers a wide binding surface for the elongation complex's entourage of components, which includes chromatin-modifying enzymes and RNA-processing machinery that carry out splicing and 3′ end creation. Here, we have provided the pictorial representation of different stages of pol II-mediated transcription.

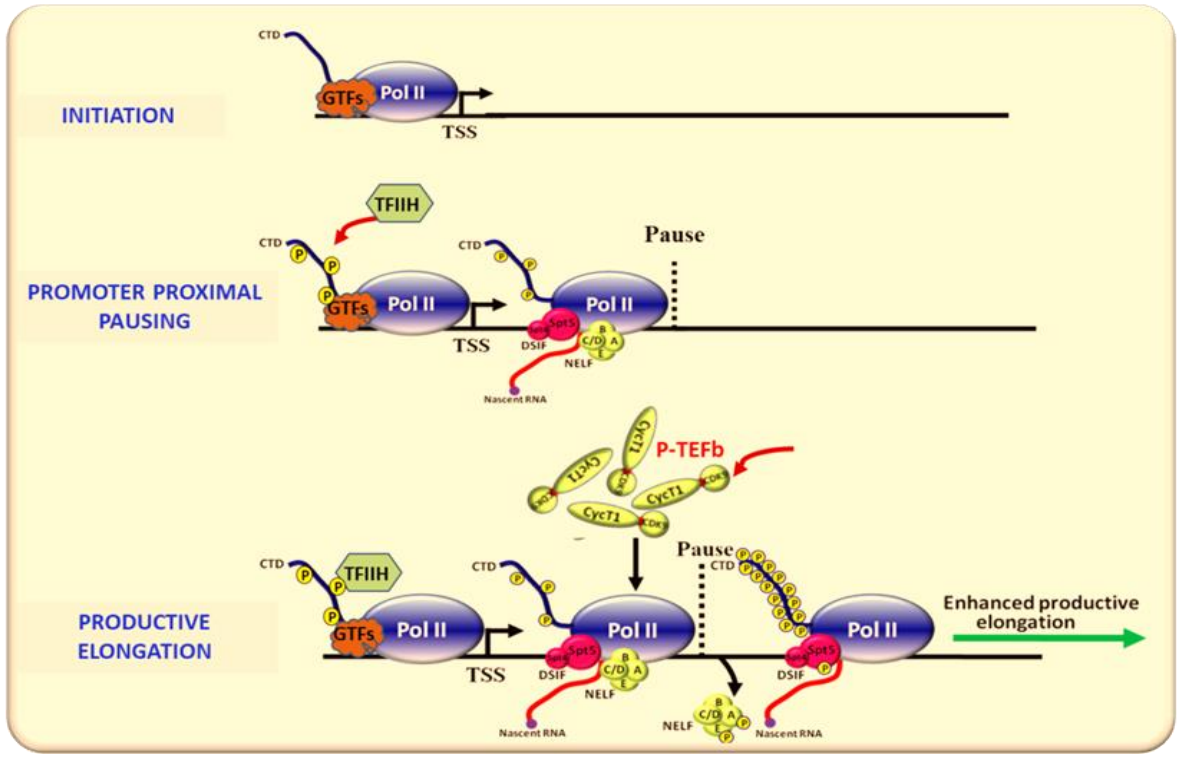


Figure 1.7 Cartoon diagram representing three major stages of eukaryotic transcription.

Upper panel shows transcription initiation where recruitment of polII and GTFs on promoter and start of the transcription is happening. Middle panel shows, Promoter proximal pausing caused by pause inducing factors. Lower panel shows pause release with the help of P-TEFb triggering productive elongation.

1.2.3 Transcription termination:

Transcription termination is a necessary step for a gene to be expressed properly, as delayed stopping can lead to a defective protein. When Pol II reaches the end of a gene, two CTD-associated protein complexes called CPSF (cleavage and polyadenylation specificity factor) and CSTF (cleavage stimulation factor) terminate the gene (Richard and Manley 2009). These proteins recruit numerous additional protein complexes to carry out cleavage and polyadenylation of freshly transcribed RNA after identifying the poly (A) tail signal (Barilla, Lee, and Proudfoot 2001). In a template-independent way, Poly-A polymerase

adds roughly 200 adenines to the cleaved 3' end of RNA. There are two commonly recognized models which explain how transcription termination of a gene takes place.

1.2.3.1 Allosteric model:

According to the allosteric model, when an elongating polymerase reaches the termination sequence, many termination factors are recruited, creating a conformational shift in the elongation complex, which causes dissociation of the elongation complex and, ultimately, transcription termination (Kuehner, Pearson, and Moore 2011).

1.2.3.2 Torpedo model:

This model of transcription termination is a “Run and Chase” mechanism. As we know majority of genes does not have discreate transcription termination site, so the allosteric model cannot explain the transcription termination of these genes. It can be explained by the torpedo model, where highly processive exonuclease chase down Pol II and when exonuclease catches Pol II, it chews up free end of RNA coming out of pol II (Porrua and Libri 2015).

1.3 Transcription factor (TF):

Total transcription output, apart from basal level, varies from a cell type to other according to the cellular requirement. This is achieved by a set of factors which can directly bind to the DNA and can control the transcriptional output. Presence of DNA binding domain (DBD) is one of the definitive features of a transcription factor (Mitchell and Tjian 1989). Functionally, these factors can be divided in two categories i.e., Activator and Repressor.

1.3.1 Transcription Activator:

Transcription activators have DNA Binding Domain (DBD) which helps an activator to boost a gene's transcriptional output through direct DNA binding. It may control transcription *via* altering the accessibility of Pol II to the DNA template or directly altering DNA bases or histones (Ptashne and Gann 1997). An activator can also serve as a scaffold for the recruitment of other transcription-related proteins.

1.3.2 Transcription Repressor:

Transcriptional repressors, unlike a transcriptional activators, are proteins that bind to particular locations on DNA and stop neighboring genes from being transcribed. A repressor molecule reduces the transcriptional outcome of a gene by blocking Pol II on template DNA or modifying chromatin, making them transcriptionally repressive.

1.3.3 Coactivator and Corepressor:

Several protein factors or substances, in addition to transcription factors, may fine-tune the transcriptional result by altering the action of an activator or repressor. These factors are termed as coactivator or corepressors depending on their action of target factors on transcription. These factors, unlike activators and repressors, do not contain a DNA binding domain, but they can be recruited to transcription machinery *via* protein-protein interactions.

CHAPTER II

Review of literature

2. Review of Literature

2.1 FKBP5, a multidimensional protein

2.1.1 Introduction:

Different cellular pathways are regulated by the coordinated action of different regulatory proteins, which decide the ultimate cell fate. Transcription is one of the most basic and vulnerable cellular processes which needs to be regulated with utmost precision. There are a whole lot of literature talking about transcriptional regulation and the regulators involved, still there is huge scope to find out new regulators of transcription. Here in this review section, I will discuss the available literature based on which one can hypothesize the possible role of FKBP5 in transcriptional regulation.

FKBP5 (FK506 binding protein 5) is a member of the immunophilin family and drugs like FK506, rapamycin and cyclosporine are known to target members of this family. FKBP5 was first cloned from a HeLa cell cDNA library and identified as a 51 KDa protein in murine T cells (that is why sometimes referred to as FKBP5)(Baughman et al. 1995). It is encoded by the gene *FKBP5* which is located on chromosome no. 6 (p21.31). This encoded protein is a cis-trans prolyl isomerase. Both FKBP domains and tetratricopeptide repeat (TPR) domains are found in members of this family (Kang et al. 2008). Peptidylprolyl isomerase (PPIase) activity is found in the FKBP domain, which catalyzes the cis-trans conversion of peptidyl-prolyl bonds, which is critical for protein folding (Fruman, Burakoff, and Bierer 1994). Protein-protein interactions are mostly mediated by the TPR domains at the C terminus (Scheufler et al. 2000a).FKBP5 is involved in modulating the action of steroid receptors, such as progesterone, androgen, and glucocorticoid receptors (GR), by creating a complex with the heat shock proteins Hsp90/Hsp70. Recent research has suggested that FKBP5 might be a biomarker for tumorigenesis and chemoresistance, which is of equal or more importance than these effects on steroid hormone signaling. FKBP5 has some similarities to other FKBPs, but it also has some distinguishing traits, such as its function in the regulation of numerous signalling pathways, as well as tumorigenesis and chemoresistance. Beyond its roles as a co-chaperone and a PPIase, it plays a role in those processes. In addition to its

involvement in tumorigenesis, various studies have suggested that FKBP5 may have a role in mental illnesses such as depression and in response to depression therapy via hormone receptor regulation (Schosser and Kasper 2009). FKBP5 and Hsp90 are also important for the clearance of tau, a microtubule-associated protein that accumulates in a group of neurodegenerative disorders such as Alzheimer's disease (Jinwal et al. 2010).

Here, in the following lines structural and functional details (according to the available literature) of FKBP5 have been provided, which is important to understand the importance of FKBP5 and its diverse working/regulatory mechanisms.

2.1.1.1 Domains of FKBP5 and their Functional importance:

FKBP5 structurally consists of three domains i.e., an N-terminal FK1 domain, a PPIase-like FK2 domain in the middle, and a C-terminal TPR domain. Details of these domains and their functions are discussed below.

- **FK1 domain:**

This is the first FKBP domain of FKBP5 at its N-terminal end. The first FKBP domain has five antiparallel β-strands curled around a core α-helix, which is the usual FK fold. FKBP5 has a PPIase activity of 0.48 X 10^6 $M^{-1}s^{-1}$(Yeh, Bierer, and McKnight 1995), which is comparable to FKBP12's 0.64 X 10^6 $M^{-1}s^{-1}$(Harrison and Stein 1990). A double point mutation in FK1 reduces human FKBP5's enzymatic activity by >90% (Barent et al. 1998), suggesting that this domain is the demonstrably active PPIase domain in FKBP5. So, this domain is the most important region of FKBP5 for its enzymatic activity. When compared to FKBP12, the FK1 domain binds the immunosuppressive drugs Rapamycin and FK506 with somewhat lower affinities (Rapamycin: Ki = 3.7 0.9 nM, FK506: Ki = 104 14 nM) (Kozany et al. 2009). FK1 domain also interacts with some other regulatory factors like AKT.

- **FK2 domain:**

After FK1 domain there is one more FKBP like domain present in the FKBP5 protein, which is called FK2 domain. Despite having just 26% sequence identity and 44%

structural similarity, FK2 is structurally similar to FKBP12. PR and AKT interact with FK2 domain of FKBP5. The FK2 domain has a structure similar to that of FKBP, however, it has no isomerase activity *in vitro* and does not bind FK506 or Rapamycin (Storer et al. 2011; Dunyak and Gestwicki 2016).

➢ TPR1, TPR2 and TPR3 domains:

FKBP5 is molecular Cochaperone protein which works with HSP90. Usually, most of the molecular chaperone machinery contains multiple protein components that have one or more structural domains composed of tetratricopeptide repeat (TPR) motifs. As the name suggests, the TPR is composed of a, 34–amino acid consensus motif that is found in tandem repeats of varying number (Lamb et al. n.d.). In case of FKBP5 there are 3 tetratricopeptide repeat (TPR) motifs namely TPR1, TPR 2 and TPR 3. These three TPR repetitions are structurally similar to the Hsp90-binding domains of PP5 (Das, Cohen, and Barford 1998), Hop (Scheufler et al. 2000b), and Cyp40 (Taylor et al. 2001). α1 and α3 are longer than the normal 12–15 residues, with 21 and 25 residues, respectively, whereas α2 and α4–α6 are within the average size for TPR helices. FKBP5 has a seventh helix that continues beyond the last TPR motif, similar to the structures of PP5 and Cyp40, where the domains were not reduced by digestion. Mostly TPR domains are dedicated to protein-protein interaction.

The domain structure of FKBP5 is shown below.

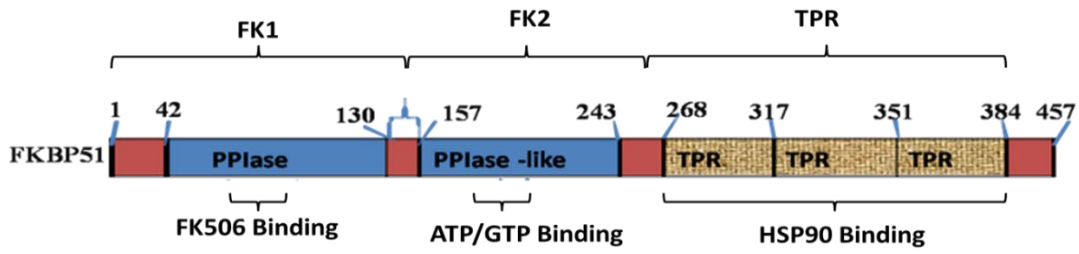


Figure 2.1 Schematic diagram of the FKBP5 domains (adapted from Li et al., 2011)

Schematic diagram showing important domains of FKBP5. N-terminal FK1 is a peptide prolyl isomerase domain and the FK2 domain is a peptide prolyl isomerase like domain. C-terminal TPR1, 2, and 3 are domains responsible for protein-protein interaction.

2.1.2 Crystal structure of FKBP5:

The Crystal structures of human FKBP5 were first published in 2003 by Sinars CR group(Sinars et al. 2003). The human FKBP5 crystal structure (2.7A°), adapted from the above-mentioned paper, has been provided here (Figure. 2.2). Final refined models comprise residues 33–412 of 457, with a few tiny gaps (38–45, 62–66, 70–75, and 382–385). The n-terminal two domains, residues 33–138 and 147–251, are typical FKBP domains, with an antiparallel five-stranded β-sheet wrapped around a core α-helix (van Duyne et al. 1991). The third domain, which contains residues 261 to 400, is similar to the HSP90-binding TPR domains of PP5 (protein phosphatase 5) (Das, Cohen, and Barford 1998), Hop (Hsp70/90 organizing protein)(Scheufler et al. 2000), and Cyp40 (cyclophilin 40)(Taylor et al. 2001). TPR domains are all-helical structures made up of 2–16 units of a 34-aa consensus motif (Das, Cohen, and Barford 1998). A single unit is made up of two 12 to 15 aa residue long α-helices that cross at a 20° angle to each other. Three of these units, plus an extra helix, are found in the FKBP5 TPR domain. The alignment of the FKBP domains' binding pockets and the TPR domain's binding groove reveals a plausible domain orientation for FKBP5 in multiprotein complexes. The first FKBP domain, FK1, has a binding pocket that is positioned 180° from the putative binding pocket of the second FKBP domain, FK2. The link between FK1 and FK2 is only eight residues long, but it contains a lot of interactions that seem to limit its flexibility. The connection begins with a modest three-amino acid (residues 140–42) antiparallel strand contact with FK2's β1. The following four amino acids (residues 143–146) create a tight loop that is maintained by hydrogen bonding between the loop's backbone nitrogens and the side-chain carboxylates of Asp-141 and Asp-145.

The TPR domain's concave face faces the same direction as the FK2 domain's binding pocket, although it's 26 Å distant. The connecting region, residues 252–260, extends 16.7 Å from Cα to Cα with a single helical turn (residues 256–258), and α1 of the TPR domain is 21 Å from the last residue of FK2's β3. The first two α-helices then come back toward the FK2 domain, forming two direct hydrogen bonds between FK2 and TPR α1, and a salt bridge involving Asp-195, Lys-274, and Tyr-278, and two water-mediated hydrogen bonds. Here, we have provided crystal structures of human FKBP5.

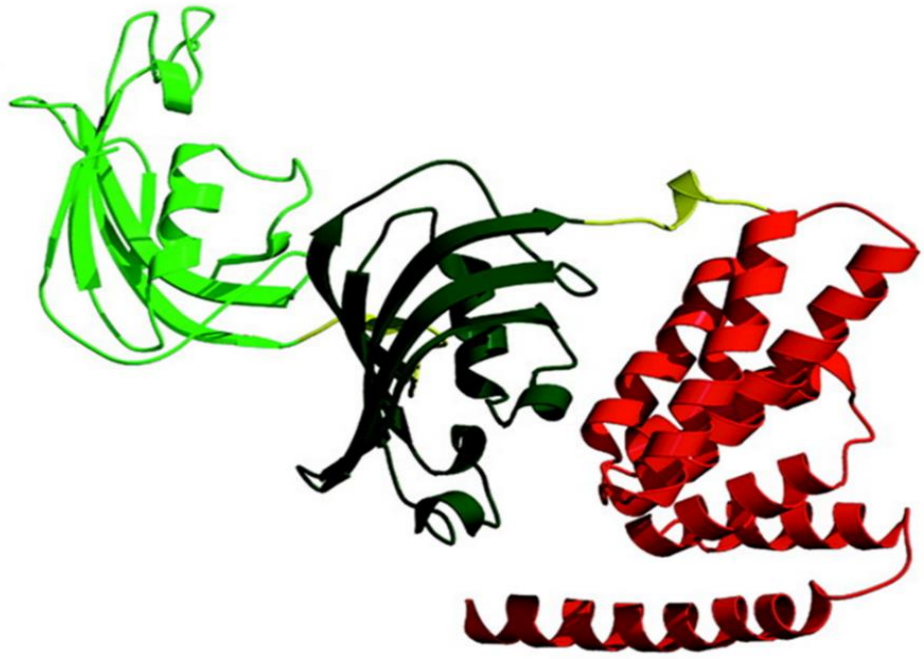


Figure 2.2 Crystal structure of FKBP5.

The Crystal structures of human FKBP5 to 2.7 Å. Picture has been adapted from Sinars CR et al. 2003.

2.1.3 Role of FKBP5 in different cellular signaling pathways:

FKBP5 is involved in a variety of biological processes and it regulates a variety of signaling pathways. It affects protein folding by acting as a peptidyl-prolyl isomerase (PPIase) (GALAT 1993; Fruman, Burakoff, and Bierer 1994b). FKBP5 can also act as cochaperones with chaperones and hence play a role in cell trafficking (Schiene-Fischer, letters, and 2001 n.d.). FKBP5 affects many signaling pathways in different ways. It has an effect on steroid receptor signaling (Denny et al. 2000; Barent et al. 1998), the NFκB pathway (Bouwmeester et al. 2004), and the Akt pathway (Denny et al. 2000; Barent et al. 1998; Pei et al. 2009). FKBP5 is also involved in the regulation of drug responses (Jiang and Peterlin 2008; L. Li, Lou, and Wang 2011; Binder et al. 2004).

2.1.4 FKBP5 in cancer pathogenesis andresponse to chemotherapy:

As mentioned above, FKBP5 is a protein that regulates a number of essential signaling pathways in the cell, including the Akt (Pei et al. 2009), NFκB(Bouwmeester et al. 2004), and androgen receptor pathways (L. Ni et al. 2010). All of these signaling pathways have been linked to tumorigenesis and drug response. It has been proposed that FKBP5 has a tissue-specific role in tumorigenesis and antineoplastic treatment. FKBP5 can either promote or inhibit tumor growth and chemoresistance depending on the cellular environment. Here, the role of FKBP5 has been discussed in one of the important cases of tumorigenesis and the involved pathways (Akt pathway regulation).

- **Role of FKBP5 in pancreatic cancer pathogenesis (through Akt pathway regulation):**

The Akt signalling pathway is one of the most significant signalling pathways, regulating a variety of cellular functions such as cell proliferation, growth, and other critical processes for cell survival (Manning and Cantley 2007). Akt is a serine/threonine kinase that requires the phosphorylation of its Ser473 and Thr308 residues in order to completely activate this process and is facilitated by different factors like, Phosphoinositide 3-kinase (PIP3), PDK1, and the mTOR complex 2 (Alessi et al. 1996; Engelman, Luo, and Cantley 2006; Sarbassov et al. 2005). Phosphatases, such as PP2 holoenzymes and PHLPP, dephosphorylate Akt and stop it from working (Padmanabhan et al. 2009). The activity of the Akt pathway is determined by the balance of phosphorylation levels, which affects all downstream cellular activities. If the Akt pathway is over-activated, it can contribute to tumor formation, progression, and eventually treatment resistance. FKBP5 low expression levels have been linked to resistance to numerous chemotherapy treatments in genome-wide association studies of cytidine analogues (Pei et al. 2009; L. Li, Lou, and Wang 2011). Functional investigations revealed that FKBP5 works as a scaffolding protein, strengthening contact between Akt's phosphatase - PHLPP and Akt, and therefore lowering Akt-Ser473 phosphorylation, (Pei et al. 2009). FKBP5 expression is downregulated in pancreatic and breast cancer cells, but Akt-Ser473 phosphorylation is upregulated, perhaps leading to chemoresistance. It was also proposed that FKBP5 may operate as a tumor suppressor gene by inhibiting Akt activity (Pei et al. 2009).

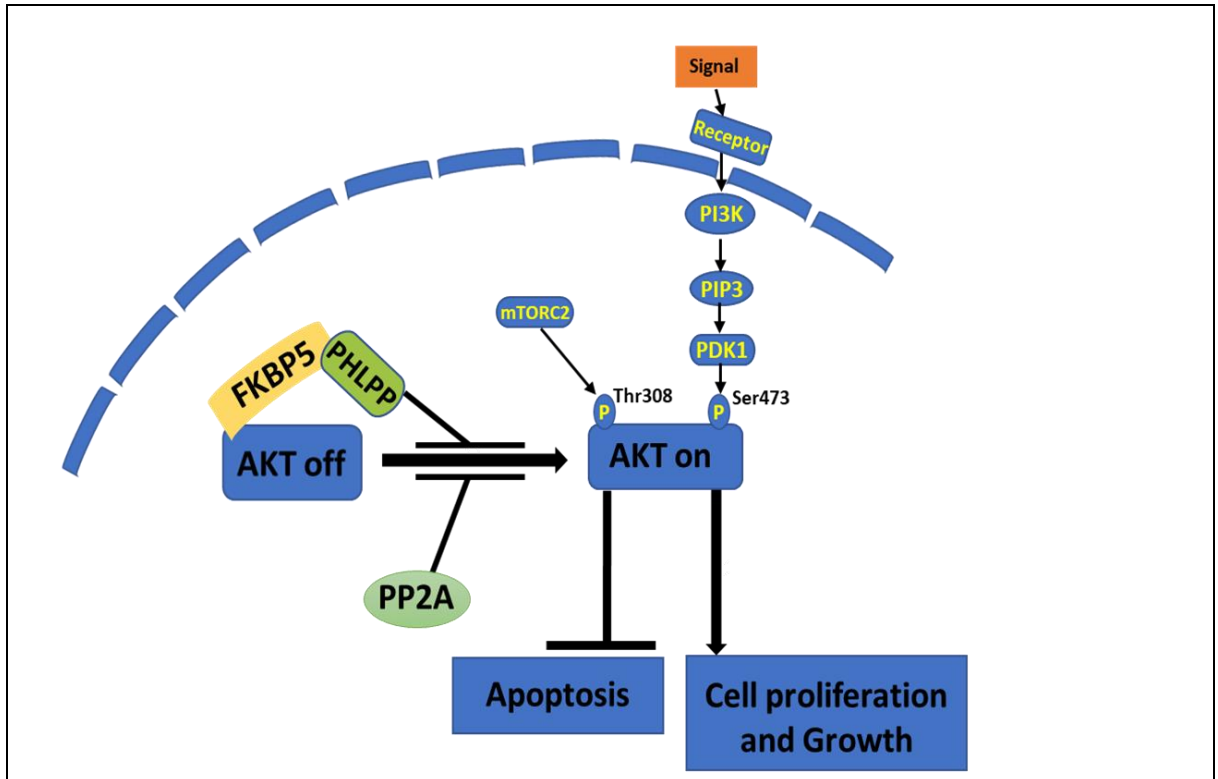


Figure 2.3 schematic representation of FKBP5 regulatory activity of Akt pathway (adapted from Li et al. 2011).

FKBP5 interact with PHLPP and AKT acting as a scaffold and promoting interaction between AKT and PHLPP, thereby enhancing the AKT-dephosphorylation and inactivating it, which results in blockde of AKT signalling for cell survival, growth and leads to apoptosis.

2.2 P-TEFb, one of the most crucial factors of transcriptional regulation:

2.2.1 Introduction:

RNA polymerase II (Pol II) regulates numerous processes of eukaryotic transcription, including initiation, promoter clearance, elongation, co-transcriptional processing of nascent transcripts, termination, mRNA cleavage, and polyadenylation (Cramer 2019; Kornberg 2007; Lis 2019; Roeder 2019). Each of the transcriptional regulatory process involve a variety of negative and/or positive regulators.Elongation is a vital regulatory step of transcription, as evidenced by the rapid development of genome-wide transcriptome

study (Jonkers and Lis 2015; Rahl and Young 2014a; Saldi et al. 2016; Nechaev and Adelman 2011; Zhou, Li, and Price 2012; Gilchrist, Fargo, and Adelman 2009).P-TEFb complex, among all the regulators of transcriptional elongation, plays a major role during activation of majority of developmental and early response gene expression.

In 1992, the existence of positive and negative transcription elongation factors came into consideration. Marshall and Price discovered in 1992 that partly purified nuclear fractions induce Pol II elongation in a way that is reliant on the kinase inhibitor 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) (N. F. Marshall and Price 1992).They termed this protein P-TEFb (positive transcription elongation factor b) and isolated it as a heterodimer with 124 and 43 kDa(N. F. Marshall and Price 1995). Understanding the molecular roles of P-TEFb has progressed significantly during last three decades of study.

P-TEFb is a heterodimer made up by CDK9 and one of the C-type cyclins T1, T2a, T2b, or K (Kohoutek 2009a). The CDK9 component has kinase activity, which is the P-TEFb complex's main function (Anshabo et al. 2021). CDK9 exists into two isoforms with molecular weights of 42 and 55 kDa, respectively. Both the shorter and longer versions of CDK9 may form a heterodimer with CyclinT1 (Shore et al. 2003). CyclinT1 has a TAR recognition motif (TRM) that distinguishes it from other Cyclin isoforms (CycT2a/CycT2b) (Wei et al. 1998). TRM is involved in the development of the Tat/TAR/P-TEFb complex within the host cells during HIV transcription (Wimmer et al. 1999). Following discussion will be focused on P-TEFb composed of CDK9 and CylinT1 as it is the most abundant among all species of P-TEFb inside the cell.

➢ P-TEFb regulates promoter proximal pause release:

It has already been discussed earlier that, after initiation Pol II gets stalled after synthesizing just a small stretch of mRNA. This stalling of PolII involves two important complexes namely DSIF and NELF complex. Here after, as and when there is a proper signal for a productive transcription of that particular gene, P-TEFb comes into the picture and helps Pol IIin getting released from this pausing site. The P-TEFb complex phosphorylates the conserved serine residues serine-2 and serine-5 in the C-terminal domain (CTD) of the Pol II, DSIF, and NELF complexes as and when transcriptional activation is required(Zhou and Yik 2006a). This phosphorylation helps Pol II in the

overriding of the negative effects of the DSIF and NELF complexes, allowing Pol IIto enter productive elongation and, eventually, gene expression. Inside the cell, the P-TEFb complex exists as both active and inactive complexes. In combination with Hexim1/2, LARP7, MePC2, and 7SK RNA, the majority of the cellular P-TEFb complex is sequestered in an inactive complex (Zhou and Yik 2006b). P-TEFb is released from the inactive complex for transcriptional activation by many positive regulators. The P-TEFb complex interacts with various transcriptional activator complexes when it is liberated from its inactive state, including the Brd4 complex, ZMYND8 complex, TFIID and Super Elongation Complex (SEC)(Biswas et al. 2011; Basu et al. 2020; Ghosh et al. 2018; Yadav et al. 2019).

➢ Protein structure of P-TEFb subunits:

Asmentioned above, P-TEFb is a heterodimer which is composed of one of the two isoforms (42 KDa and 55Kda) of CDK9 and one of the C-type Cyclin. CDK9-42 and CDK9-55 are two isoforms of the CDK9 gene that are expressed from two different transcription start sites in the CDK9 gene (Shore et al. 2005; Giacinti et al. 2008; Morales and Giordano 2016; H. Liu and Herrmann 2005). The catalytic site of CDK9 is situated between N- and C-lobes in the kinase domain of CDK9 (Wang and Fischer 2008; Sonawane et al. 2016; Franco et al. 2018a; Paparidis, Durvale, and Canduri 2017). The N-lobe operates as an inhibitory domain, with a helix specific to each CDK (PITALRE for CDK9), while the C-lobe serves as an activation domain, with a regulatory T-loop structure (Figure 2.4). The phosphorylation of threonine residue (T186) in the T-loop activates CDK9 (Renee M. Marshall and Grana 2006; Q. Li et al. 2005; R. Chen, Yang, and Zhou 2004; Ramakrishnan, Dow, and Rice 2009; Ramakrishnan and Rice 2012).

CycT1, T2a, and T2b (collectively, CycT) have two very conserved cyclin box structures in their N-terminus, that interact with CDK9. Following the C-terminal cyclin box there is a small stretch of basic amino acids that comprises the nuclear localization signal. The Tat-TAR recognition motif (TRM) is a region in CycT1 that interacts with HIV Tat and TAR via a crucial C261 residue (Wei et al. 1998; Garber et al. 1998)(Figure 2.4). The 278 amino acids in CycT1's N-terminal region are adequate for Tat transactivation (Garber et al. 1998; Bieniasz et al. 1998; Fujinaga et al. 1999). Amino acid substitutions in this area result in

CycT1 mutants that are dominant negative and inhibit endogenous P-TEFb activity (Kuzmina et al. 2014; Verstraete et al. 2014; Jadlowsky, Nojima, Okamoto, et al. 2008; Jadlowsky, Nojima, Schulte, et al. 2008).The C-terminal region of CycT proteins differs (Peng et al. 1998). CycT1 has a region (aa 480–550) rich in histidine residues that directly interact with Pol IICTD(Taube et al. 2002). This area was later discovered to help the P-TEFb complex develop a phase-separated condensate formation(Lu et al. 2018). CycT1's C-terminal end (aa 706–726) forms a PEST motif, which controls the protein's stability in cells (Wei et al. 1998) (Figure 2.4).

The most abundant species of P-TEFb is composed of smaller isoform of CDK9 and CyclinT1. Schematic presentation of CDK9 isoforms (42 KDa and 55KDa) and different CycTs (CyclinT1, CyclinT2a, and CyclinT2b) protein structure has been given bellow.

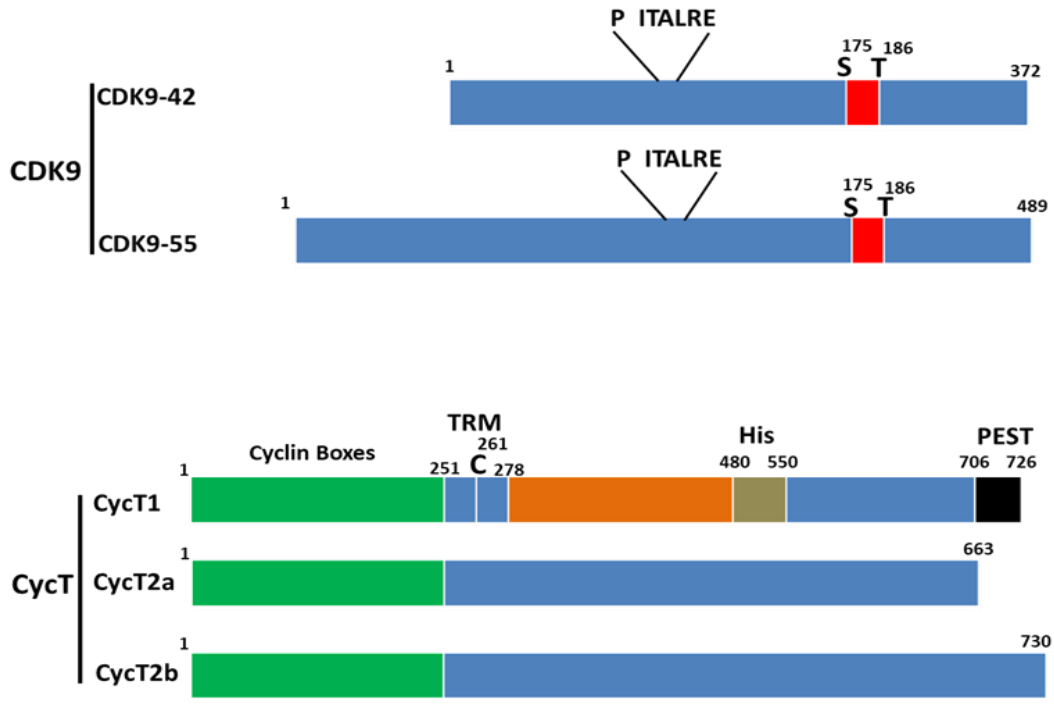


Figure 2.4 Schematic representations of CDK9 Isoforms and CycT varients.

- Upper panel represents two isoforms of CDK9 i.e., CDK9-42 and CDK9-55.
- Lower panel represents three varients of CycT i.e, CycT1, CycT2a and CycT2b.

➢ Functional regulation of P-TEFb:

P-TEFb resides in two different forms inside the cell. One is active form and other is inactive form. Active form of P-TEFb remains either alone or associated with the different factors which recruits P-TEFb to its target gene. An inactive P-TEFb complex remains associated with 7SK snRNA (7SK small nuclear RNA), HEXIM1/2 (hexamethylene bisacetamide-inducible mRNAs 1 and 2) proteins, LARP7 (La-related protein 7) and MePCE (methyl phosphate capping enzyme), also known as the 7SK small nuclear ribonucleoprotein (7SK snRNP)(Yang et al. 2001; Nguyen et al. 2001; Diribarne and Bensaude 2009). Depending on the cellular requirement P-TEFb shuttles between active and inactive forms. There are several ways to release P-TEFb from inactive complex such as exposure to UV light, heat, inhibition of transcription etc. There are some small compounds such as histone deacetylase inhibitors (HDACis), PKC agonists, DNA damage-inducing agents, a strong cell-differentiation inducer like hexamethylene bisacetamide (HMBA)also release P-TEFb from 7SK snRNP*via* various known and unknown mechanisms and activate transcription elongation mediated by P-TEFb(Peterlin and Price 2006; Zhou and Yik 2006c; Kohoutek 2009b; Bartholomeeusen et al. 2013; 2012; Contreras et al. 2007; Y. K. Kim et al. 2011). The *HEXIM1* gene, on the other hand, is one of the first genes to respond to P-TEFb release, and freshly generated HEXIM1 proteins promptly re-incorporate P-TEFb into 7SK snRNP, causing cell growth halt (P. Liu et al. 2014; Tan et al. 2016; Bandukwala et al. 2012).Many anti-cancer drugs have been discovered to be strong P-TEFb-releasers/activators due to this negative feedback mechanism.

➢ Recruitment of P-TEFb on its target genes:

Transcription of most of the genes is regulated at the early elongation step. PolII stalls just after synthesizing a small stretch of mRNA. This stalling is termed as promoter proximal pausing and it is achieved with the help of two major factors namely NELF complex and DSIF complex (Hargreaves, Horng, and Medzhitov 2009; Rahl et al. 2010; Z. Ni et al. 2008; Mayer et al. 2010). Whenever a gene in paused state needs to be expressed P-TEFb gets recruited to the pause site with the help of different recruiters of it and it phosphorylates NELF complex, DSIF complex and Pol II CTD at Ser2p and Ser5p

positions. These phosphorylations helps in pause release and ultimately causes gene expression.

P-TEFb is recruited to its target genes by a variety of gene-specific and non-gene-specific binding partners, some of which are listed below.

(a) Chromatin-binding proteins like Bromodomain-containing protein 4 (Brd4) (Yang et al. 2005; Moon et al. 2005).

(b) Pol II-associated factors such as PolII-Associated Factor 1c (PAF1c) (Y. Chen et al. 2009; Yu et al. 2015),

(c) Mediator complex components such as Med26 (Takahashi et al. 2011).

(d) Super Elongation Complex components such as AFF4(Lu et al. 2014; Luo, Lin, and Shilatifard 2012a; Smith, Lin, and Shilatifard 2011).

(e) DNA-bound transactivators such as NFκB(Barboric et al. 2001), cMyc(Eberhardy and Farnham 2002; Kanazawa et al. 2003; Gargano et al. 2007), STAT3 (Brasier 2008; Giraud et al. 2004; Hou, Ray, and Brasier 2007), MyoD (Giacinti et al. 2006; Simone et al. 2002), and (MEF2) (Nojima et al. 2008), as well as hormone receptors such as ER and AR (D. K. Lee, Duan, and Chang 2001; Wittmann et al. 2005).

(f) Transactivators that do not bind to DNA, such as the Autoimmune Regulator (AIRE) and the Class II Transactivator (CIITA)(Kanazawa, Okamoto, and Peterlin 2000; Oven et al. 2007; Žumer et al. 2011).

(g) HIV Tat and other RNA-bound transactivators(Asamitsu, Fujinaga, and Okamoto 2018a; Jeang 1998; Jones 1997; Karn 2011; Ott, Geyer, and Zhou 2011a; A. P. Rice 2019).

➢ P-TEFb alteration and associated human diseases:

P-TEFb, being one of the most important regulators of transcription, its own regulation becomes very crucial. Any alteration in P-TEFb function leads to serious abnormalities through various different mechanisms. However, due to the complexity of the above-mentioned P-TEFb regulatory mechanisms and the cellular transcriptional network, developing efficient treatment techniques that may possibly target P-TEFb necessitates a

greater knowledge of P-TEFb-dependent transcriptional regulation. The following are some examples of human disorders in which P-TEFb plays a crucial role.

- **AIDS:** P-TEFb is an important cellular cofactor for HIV transcription sinceCyclinT1 interacts with the viral Tat protein and the viral promoter *via* TAR RNA, (Ott, Geyer, and Zhou 2011b; A. P. Rice 2017; Asamitsu, Fujinaga, and Okamoto 2018b; Karn 1999).The HIV long-terminal repeat (LTR) DNA sequence,which acts as a promoter and enhancer for HIVtranscription, also contains NFκB binding sites, and NFκB also requires P-TEFb for its activity *via* directinteraction (Jones 1997; Mbonye and Karn 2017; Cary, Fujinaga, and Peterlin 2016). Therefore, P-TEFb is required for both Tat-dependent and Tat-independentHIV transcription. Latent infection of HIV is another major hurdle in treating HIV/AIDS patients. HIV latency is established shortly after a fresh infection by a rapid reduction of viral transcription via multiple mechanisms such as epigenetic gene silencing, a lack of cellular co-factors, transcription interference, and so on (Dahabieh, Battivelli, and Verdin 2015; Darcis, van Driessche, and van Lint 2017; Margolis 2010; Rasmussen and Lewin 2016; Siliciano and Greene 2011; Xing and Siliciano 2013; Kimata, Rice, and Wang 2016). Because latently infected cells do not express HIV proteins, they avoid detection and clearance by the host's immune system. Resting CD4+ T cells and memory subsets containing silent proviruses are thought to be long-lived viral reservoirs (Chomonta et al. 2011; Eisele and Siliciano 2012; Murray et al. 2016; Renée M. Marshall et al. 2005). Interestingly, CycT1 protein expression is preserved at a vanishingly low level in resting CD4+ T cells by post-transcriptional processes (Liou et al. 2006; Liou, Herrmann, and Rice 2002; A. Rice and Herrmann 2005). T cell signalling activation *via* TCR engagement and downstream PKC activation raises CycT1 protein levels, which is required for HIV reactivation from latently infected cells (A. Rice and Herrmann 2005; Garriga et al. 1998; Liou et al. 2006; Renée M. Marshall et al. 2005). As a result, P-TEFb is also required for HIV latency maintenance.
- **Cardiac Hypertrophy:** Other thanHIV/AIDS, cardiac hypertrophy is one of the first example of diseases where P-TEFb activity has been shown to play a critical role. Studies shows that treatment of cardiomyocytes with hypertrophic stimulations or mechanical stress releases P-TEFb from 7SK snRNP and ultimately activates P-TEFb. In contrast, antisense oligonucleotide disruption of 7SK snRNP resulted in P-TEFb activation and

hypertrophic responses (Sano et al. 2002). Finally, animals that overexpress CycT1 develop cardiac hypertrophy (Sano et al. 2002). Siddiqui and colleagues demonstrated that HEXIM1 knockout mice are lethal in late fetal stages due to heart failure, and that bigenic mice overexpressing CycT1 in a HEXIM (+/-) heterozygote background exhibit increased susceptibility to cardiac hypertrophy with elevated Cdk9 activity (Espinoza-Derout et al. 2009; 2007). The connection between CDK9 and HEXIM1 in cardomyocytes is reduced in a hypertrophic mouse model with calcineurin overexpression, indicating continual up-regulation of P-TEFb activity (Garriga et al. 1998).

- **Cancer:** It is not unexpected that P-TEFb is associated in numerous forms of cancer because it induces transcription elongation of manycellular genes(Romano 2013; Navone 2010; Franco et al. 2018). Mixed-lineage leukaemia (MLL) is an example of P-TEFb's direct participation in cancer, with frequent translocation and genomic rearrangement between the MLL gene and components of SEC, which is a strong recruiter of P-TEFb(Boffo et al. 2018; Smith, Lin, and Shilatifard 2011). The bulk of MLL rearrangements (50 percent of infant cases and 75 percent of adult cases of acute lymphoblastic leukaemia with MLL rearrangement) occur in in-frame fusion proteins between MLL and SEC's AFF1 subunit (Lin et al. 2010). Other SEC subunits, such as ENL and AF9, also undergo MLL rearrangement (Luo, Lin, and Shilatifard 2012b). These MLL rearrangements dysregulates transcription elongation by P-TEFb.The production and function of c-Myc, which is increased in many forms of cancer, is another example of P-TEFb's involvement in cancer (Rahl and Young 2014b). P-TEFb recruited by Brd4 regulates c-Myc gene expression, and BETi mediated removal of Brd4 from the c-Myc locus produces a potent anti-tumor impact (Mertz et al. 2011; Delmore et al. 2011).Therefore, P-TEFb plays a central role also in c-Myc-dependent tumorigenesis.In addition, P-TEFb activity is abnormally increased in a variety of malignancies, possibly due to a change in the P-TEFb equilibrium between free P-TEFb and P-TEFb sequestered in 7SK snRNP complex.LARP7 mutations, in particular, seen in breast cancer patients, disrupt 7SK snRNP and result in constitutive activation of P-TEFb-dependent transcription (Zhou and Yik 2006a; Cheng et al. 2012; Dey, Chao, and Lane 2007; Ji et al. 2014).

  There is a long list of P-TEFb involvement in different tumorigenesis. Importantly, whereas widespread activation of transcription elongation by P-TEFb has been linked to

cancer, it is unclear which specific sets of P-TEFb-dependent genes are responsible for tumorigenesis. Depending on how P-TEFb function is disrupted, distinct groups of P-TEFb-target genes may be upregulated in different forms of cancer (Garriga and Graña 2014; Garriga et al. 2010; Gomes et al. 2006).

2.3 Rationale behind the present study:

Human FKBP5 is a peptidyl-prolyl cis-trans isomerase and co-chaperone protein. It mostly functions in conjunction with the Hsp90 chaperone protein but there are several functions of FKBP5 which are HSP90 independent. A recent study shows that FKBP5 inhibits CDK5 mediated DNMT1 phosphorylation by competing with FKBP52 (the activator of CDK5) to bind with CDK5 (Gassen et al. 2015). Few AP-MS studies show the interaction between FKBP5 and CDK9 (Uniprot ID- EBI-1383449; EBI-306914), (Taipale et al. 2014). Altered level of FKBP5 inside the cell has been shown to be associated with several abnormalities. FKBP5 has been shown to be overexpressed and downregulated in several human malignancies. FKBP5 is overexpressed in brain tumors, prostate cancer, lymphoma, head and neck cancer, and melanoma, according to the oncomine. FKBP5 has also been found to be downregulated in pancreatic cancer, melanoma, colon cancer, and testicular cancer (Pei et al. 2009).FKBP5 has been shown to regulate some important signaling pathways like NF-κB pathway, AKT pathway, GR pathway, AR pathway, PR pathway etc.(L. Li, Lou, and Wang 2011), which can explain the role of FKBP5 in different abnormalities to a certain level but proper mechanistic understandings of role of FKBP5 in these abnormalities needs detailed study. Till date most of the mechanistic studies has been done to explain the tumorigenic potential of overexpressed FKBP5, but as mentioned above, there are several malignancies where FKBP5 has been shown to be downregulated but the mechanistic understanding of these cases is least explored. These questions drive us to study mechanism of tumorigenesis in low FKBP5 level.

Present study gives a clear explanation of the tumor suppressor role of FKBP5. Next chapter discusses the detailed results of this study.

CHAPTER III

Materials and methods

3. Materials andmethods:

3.1 Cellculture :

3.1.1 Animal cellculture :

HEK293T, a human embryonic kidney cell line, was used in the majority of the research in this study. This study also employed cancer cell lines such as PC3 (Human Prostate Cancer) and HCT116 (Human Colorectal Cancer Cell Line). Cells-lines were maintained in DMEM-pH-7.4 supplemented with 10% FBS (Fetal Bovine Serum) and 1% Penicillin-Streptomycin solution for all animal cell cultures. The cells were kept in a 37°C CO2-incubator with a humidity of ≥90% and a CO_2 concentration of 5%. Table no. 1 summarizes all of the cell lines employed in this research.

Table 1: Cell lines used in this study

Name of the cell line	Purposes of the cell line
HEK 293T	The human embryonic kidney cell line used for transient expression for majority of the experiments and for making stable knock-down cell lines
Flp-In 293	Used for the purpose of making stable cell line of targetgene with constitutive expression of protein of interest.
Flp-in T-Rex	Used for making stable cell lines of target genes with doxycycline inducible system for expression of the protein of interest.
PC3	The prostate cancer cell line (PC3) used for cell-proliferation related experiments and as a different cell system for testing the cell type specificity of effects seen in HEK293T.
HCT116	Used as a different cell system for testing the cell type specificity of effects seen in HEK293T.

3.1.2 Insect cellculture :

Insect cell, Sf9 (*Spodoptera frugiperda*), system was used as a heterologous system for direct protein-protein interaction studies, for protein purification and protein complex reconstitution and purification. Sf9 cells were maintained in Grace's insect media supplemented with 10% FBS and gentamycin (7µg/ml) in adhered condition at 26˚C in

BOD incubator. Large scale culture was also done as cell-suspension culture in double side arm spinner flasks. In case of suspension culture above mentioned insect culture, Grace's insect media was also supplemented with 0.1% Pluronic acid (v/v).

Name of the cell line	Purposes of the cell line
Sf9 (*Spodoptera frugiperda*)	This insect cell line was used as a heterologous system for protein-protein interaction, for protein/ protein complex purification.

3.1.3 Bacterial culture

This study employs a variety of bacterial strains for various experimental goals. *E. coli* strains DH5α and DH10-Bac were utilised for vector transformation and subsequent amplification in this investigation. DH10-Bac was utilised to make recombinant bacmids, which were then used to make recombinant baculovirus particles in Sf9 cells. In many cloning experiments, DH5α was utilised to amplify plasmids. The BL21(DE3) and Rosetta (DE3) strains were employed for recombinant protein expression and purification.

❖ **Antibiotics used in this study**

Several antibiotics were utilized in the preparation of the selection media in this whole study. Table 2 lists the antibiotics as well as their working concentrations, utilized in this study.

Table 2: List of Antibiotics used in this study

Name of the antibiotic	Working concentration
Ampicillin	100 µg/ml
Penicillin + Streptomycin solution	1%
Hygromycin	200 µg/ml
Puromycin	10 µg/ml
Gentamycin	7µg/ml
Tetracyclin	20 µg/ml

Kanamycin	20 µg/ml
Chloramphenicol	50 µg/ml
X-Gal	40 µg/ml
IPTG	100µM

3.2 Transformation:

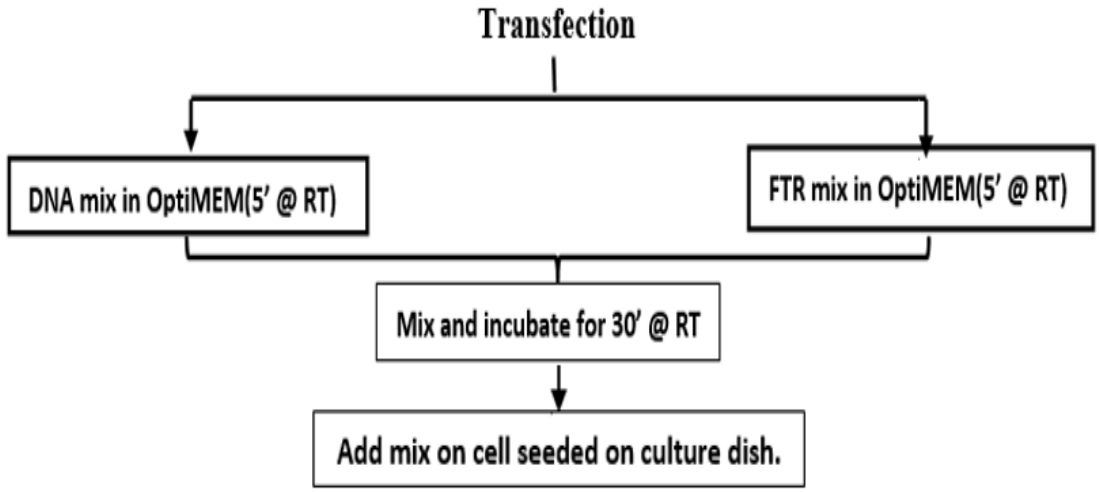
All the bacterial transformation in *E coli.* (DH5α, BL-21(DE3) etc.) was performed through conventional heat shock method of transformation (i.e., Mix Competent cell + DNA –Incubate on ice for 30' – Heat shock @ 42ºC for 90" – Incubate on ice for 10'- revival in LB media for 1 hr @ 37 ºC Shaker (at 180rpm) – Plating on antibiotic (For selection) containing LB-agar plate). Further, pFastBac™transformation in DH10-Bac was performed using the electroporation method (i.e., Cell+DNA - Incubate on Ice for 30' – Electric pulse in Bio-Rad Gene Pulser electroporator @2.1kV,100 Ω, and 25 µF, for 2.6 milliseconds). All the bacterial strains used in this study has been discussed in Table no3.

Table 3: Bacterial strain used in this study

Name of Strain	Strain used for
DH5α	This *E.coli* strain was used for regular transformation during cloning, and also for plasmid amplification.
DH10-Bac	This *E.coli* strain was used for making recombinant bacmid which was subsequently used for the production of baculoviruses.
BL21(DE3)	This *E.coli* strain was mostly used for recombinant protein purification
Rosetta (DE3) pLysS	This *E.coli* strain was also used for recombinant protein purification

3.3 Transfection

We utilised Fugene Transfection Reagent (FTR) for all transfections in mammalian cell lines, as directed by the manufacturer. I've included a flow chart with a full procedure for transfection in mammalian cell lines that we followed.



3.4 Cloning

Human FKBP5 construct (cloned in pCMVSPORT6 vector) was purchased from "Open Biosystem". FKBP5 was PCR-amplified using this construct as a templet and subcloning were done in different expression vectors. For making stable cell line, FKBP5 and CDK9 were cloned in FLAG-HA tagged pcDNA-FRT-TO vector. Genes/fragments were PCR-amplified using specific cloning primer subsequently restriction digestion was done using suitable restriction enzymes, and finally T4-DNA ligase was used to ligate digested-vector and insert fragments. Using this ligated product, we transformed DH5α, and selection of positive clone was done by growing these transformed cells on selection media. Positive clones were amplified and stored in -20ºC for further use. FKBP5, its fragments and majority of factors required in this study were cloned in 1X- epitope-tagged pcDNA-FRT-TO vector. For heterologous expression of proteins in Sf9 cells, Genes/Fragments were cloned in pFastBac^{TM} and subsequently transformed into DH10-Bac for the generation of recombinant bacmid. Helper plasmid helps in integration of pFastBac^{TM} with the bacmid.

Recombinant bacmid containing colonies were screened through blue-white selection method. Recombinant bacmids were isolated using manual plasmid isolation procedure. All the primer details and restriction enzyme used in cloning related work has been discussed in Table no 4.

Table 4: Cloning details of factors cloned in different vectors:

Cloning details	Primer details	Primer Sequence (5'-3')
FKBP5 cloned into FLAG-HA-pcDNA5-FRT-TO and FLAG-pFASTBAC vectors	5'NheI primer	GGAGAAGCTAGCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCC TCAGGTTTCTCTTCTTCC
Human FKBP5 gene cloned in 6-His-pFASTBAC vector	5'BamHI primer	GGAGAAGGATCCATGACTACTGATGAAGGT GCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCC TCAGGTTTCTCTTCTTCC
Human FKBP5 gene cloned in GST-pFASTBAC vector	5' BamHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5 cloned in BamHI-XhoI sites of GST-pGEX vector.	5' BamHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5 cloned in XhoI-	5' XhoI primer	GGAGAACTCGAGATGACTACTGATGAAG

BamHI sites of 6-His pET-11d vector.		GTGCCAAGAACAATGAAG
	3' BamHI primer	GGAGAAGGATCCTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5 cloned in BamHI-XhoI sites of NT- pFASTBAC vector	5' BamHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5 cloned in BamHI-XhoI sites of Flag- pcDNA5-FRT-TO	5' BamHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5 cloned in BamHI-XhoI sites of HA- pcDNA5-FRT-TO	5' BamHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3'XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5 cloned in BamHI-XhoI sites of 6-His- pcDNA5-FRT-TO	5' BamHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5Δ1 fragment (from 1-384 aa) cloned into BamHI-XhoI sites of Flag- pcDNA5-FRT-TO.	5' BamHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCACTCATTGGCAC TGTCCAGTCCAAGGG
FKBP5Δ2 fragment (from 1-351 aa)cloned into BamHI-XhoI sites of Flag- pcDNA5-FRT-TO.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCACTCATTGGCAC TGTCCAGTCCAAGGG
FKBP5Δ3 fragment (from 1-317 aa) cloned into BamHI-XhoI sites of Flag- pcDNA5-FRT-TO.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCAAAGGAGAAAT GATTCAGAAGCTTTCGATTCC

FKBP5Δ4 fragment (from 1-268 aa) cloned into BamHI-XhoI sites of Flag- pcDNA5-FRT-TO.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCAAGCCTGCTCCA ATTTTTCTTTGGTATCC
FKBP5Δ5 fragment (from 1-157 aa) cloned into BamHI-XhoI sites of Flag- pcDNA5-FRT-TO.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCACTCTCCTTTCC GTTTGGTTCTCCGG
FKBP5Δ6 fragment (from 130-rest aa) cloned into BamHI-XhoI sites of Flag-pcDNA5-FRT-TO.	5' BmHI primer	GGAGAAGGATCCTTTGAGATTGAGCTCCT TGATTTCAAAGG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5Δ7 fragment (from 243-rest aa) cloned into BamHI-XhoI sites of Flag- pcDNA5-FRT-TO.	5' BmHI primer	GGAGAAGGATCCTATGAAGTTACACTTAA GAGCTTCG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
Full-length FKBP5 cloned into pM vector	5' EcoR1 primer	GGAGAAGAATTCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XbaI primer	GGAGAATCTAGATTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5Δ1 fragment (from 1-384 aa) cloned into BamHI-XhoI sites of Flag-pFASTBAC vector.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCAATTCTGGGGGT TTACTTCCAGCACTTTC
FKBP5Δ2 fragment (from 1-351 aa) cloned into BamHI-XhoI sites of Flag-pFASTBAC vector.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCACTCATTGGCAC TGTCCAGTCCAAGGG
FKBP5Δ4 fragment (from 1-268 aa) cloned into BamHI-XhoI sites of Flag-pFASTBAC vector.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI	GGAGAACTCGAGTTATCAAGCCTGCTCCA

	primer	ATTTTTCTTTGGTATCC
FKBP5Δ5 fragment (from 1-157 aa) cloned into BamHI-XhoI sites of Flag-pFASTBAC vector.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCACTCTCCTTTCC GTTTGGTTCTCCGG
FKBP5Δ6 fragment (from 130-rest aa) cloned into BamHI-XhoI sites of Flag-pFASTBAC vector.	5' BmHI primer	GGAGAAGGATCCTTTGAGATTGAGCTCCT TGATTTCAAAGG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5Δ7 fragment (from 243-rest aa) cloned into BamHI-XhoI sites of Flag-pFASTBAC vector.	5' BmHI primer	GGAGAAGGATCCTATGAAGTTACACTTAA GAGCTTCG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5Δ1 fragment (from 1-384 aa) cloned into BamHI-XhoI sites of GST-pGEX vector.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCAATTCTGGGGGT TTACTTCCAGCACTTTC
FKBP5Δ2 fragment (from 1-351 aa) cloned into BamHI-XhoI sites of GST-pGEX vector.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCACTCATTGGCAC TGTCCAGTCCAAGGG
FKBP5Δ3 fragment (from 1-317 aa) cloned into BamHI-XhoI sites of GST-pGEX vector.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCAAAGGAGAAAT GATTCAGAAGCTTTCGATTCC
FKBP5Δ4 fragment (from 1-268 aa) cloned into BamHI-XhoI sites of GST-pGEX vector.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCAAGCCTGCTCCA ATTTTTCTTTGGTATCC
FKBP5Δ5 fragment (from 1-157 aa) cloned into BamHI-XhoI sites of GST-pGEX vector.	5' BmHI primer	GGAGAAGGATCCATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' XhoI primer	GGAGAACTCGAGTTATCACTCTCCTTTCC GTTTGGTTCTCCGG

FKBP5Δ6 fragment (from 130-rest aa) cloned into BamHI-XhoI sites of GST-pGEX vector.	5' BamHI primer	GGAGAAGGATCCTTTGAGATTGAGCTCCT TGATTTCAAAGG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5Δ7 fragment (from 243-rest aa) cloned into BamHI-XhoI sites of GST-pGEX vector.	5' BmHI primer	GGAGAAGGATCCTATGAAGTTACACTTAA GAGCTTCG
	3' XhoI primer	GGAGAACTCGAGTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
Human CDK7 gene cloned into Flag-pFASTBAC vector.	5' SacI primer	GGAGAAGAGCTCTGATGGCTCTGGACGTG AAGTCTCGGGCAAAGCG
	3' XhoI primer	GGAGAACTCGAGCTATTAAAAAATTAGTT TCTTGGGCAATCCTCCTTGTTCTAAGGCC
Human CDK7 gene cloned into FLAG-HA pcDNA5-FRT-TO vector.	5' SacI primer	GGAGAAGAGCTCTGATGGCTCTGGACGTG AAGTCTCGGGCAAAGCG
	3' XhoI primer	GGAGAACTCGAGCTATTAAAAAATTAGTT TCTTGGGCAATCCTCCTTGTTCTAAGGCC
Human CDK8 gene cloned into FLAG-HA pcDNA5-FRT-TO vector.	5' NheI primer	GGAGAAGAGCTCTGATGGACTATGACTTT AAAGTGAAGCTGAGCAGCGAGCGGG
	3' XhoI primer	GGAGAACTCGAGCTATCAGTACCGATGTG TCTGATGTGAGTACTGTGGAGGCTGC
FKBP5Δ1 fragment (from 1-384 aa) cloned into BamHI-XhoI sites of 6-His pET-11d vector.	5' XhoI primer	GGAGAACTCGAGATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' BamHI primer	GGAGAAGGATCCTTATCAATTCTGGGGGT TTACTTCCAGCACTTTC
FKBP5Δ2 fragment (from 1-351 aa) cloned into BamHI-XhoI sites of 6-His pET-11d vector.	5' XhoIprimer	GGAGAACTCGAGATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' BamHI primer	GGAGAAGGATCCTTATCACTCATTGGCAC TGTCCAGTCCAAGGG
FKBP5Δ3 fragment	5' XhoI primer	GGAGAACTCGAGATGACTACTGATGAAG

(from 1-317 aa) cloned into BamHI-XhoI sites of 6-His pET-11d vector.		GTGCCAAGAACAATGAAG
	3' BamHI primer	GGAGAAGGATCCTTATCAAAGGAGAAAT GATTCAGAAGCTTTCGATTCC
FKBP5Δ4 fragment (from 1-268 aa) cloned into BamHI-XhoI sites of 6-His pET-11d vector.	5' XhoI primer	GGAGAACTCGAGATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' BamHI primer	GGAGAAGGATCCTTATCAAGCCTGCTCCA ATTTTTCTTTGGTATCC
FKBP5Δ5 fragment (from 1-157 aa) cloned into BamHI-XhoI sites of 6-His pET-11d vector.	5' XhoI primer	GGAGAACTCGAGATGACTACTGATGAAG GTGCCAAGAACAATGAAG
	3' BamHI primer	GGAGAAGGATCCTTATCACTCTCCTTTCC GTTTGGTTCTCCGG
FKBP5Δ6 fragment (from 130-rest aa) cloned into BamHI-XhoI sites of 6-His pET-11d vector.	5' XhoI primer	GGAGAACTCGAGTTTGAGATTGAGCTCCT TGATTTCAAAGG
	3' BamHI primer	GGAGAAGGATCCTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
FKBP5Δ7 fragment (from 243-rest aa) cloned into BamHI-XhoI sites of 6-His pET-11d vector.	5' XhoI primer	GGAGAACTCGAGTATGAAGTTACACTTAA GAGCTTCG
	3' BamHI primer	GGAGAAGGATCCTTATCATACGTGGCCCT CAGGTTTCTCTTCTTCC
CDK9Δ1 (1-276) cloned into FLAG-pcDNA5-FRT-TO vector.	5’ BamHI primer	GGAGAAGGATCCATGGCAAAGCAGTACG ACTCGGTGG
	3’ XhoI primer	GGAGAACTCGAGTTATCACTTCACCTTCC GCTTCTGGCCCTTGACC
CDK9Δ2 (32-276) cloned into FLAG-pcDNA5-FRT-TO vector.	5’ BamHI primer	GGAGAAGGATCCATGGCAAAGCAGTACG ACTCGGTGG
	3’ XhoI primer	GGAGAACTCGAGTTATCACTTCACCTTCC GCTTCTGGCCCTTGACC
CDK9Δ3 (67-276) cloned into FLAG-	5’ BamHI primer	GGAGAAGGATCCATGGCAAAGCAGTACG ACTCGGTGG

pcDNA5-FRT-TO vector.	3’ XhoI primer	GGAGAACTCGAGTTATCACTTCACCTTCC GCTTCTGGCCCTTGACC
CDK9Δ4 (100-276) cloned into M447	5’ BamHI primer	GGAGAAGGATCCATGGCAAAGCAGTACG ACTCGGTGG
	3’ XhoI primer	GGAGAACTCGAGTTATCACTTCACCTTCC GCTTCTGGCCCTTGACC
CDK9Δ5 (150-276) cloned into FLAG-pcDNA5-FRT-TO vector.	5’ BamHI primer	GGAGAAGGATCCATGGCAAAGCAGTACG ACTCGGTGG
	3’ XhoI primer	GGAGAACTCGAGTTATCACTTCACCTTCC GCTTCTGGCCCTTGACC
CDK9Δ1 (1-276) cloned into Flag-pFASTBAC vector.	5’ BamHI primer	GGAGAAGGATCCATGGCAAAGCAGTACG ACTCGGTGG
	3’ XhoI primer	GGAGAACTCGAGTTATCACTTCACCTTCC GCTTCTGGCCCTTGACC
CDK9Δ2 (32-276) cloned into Flag-pFASTBAC vector.	5’ BamHI primer	GGAGAAGGATCCATGGCAAAGCAGTACG ACTCGGTGG
	3’ XhoI primer	GGAGAACTCGAGTTATCACTTCACCTTCC GCTTCTGGCCCTTGACC
CDK9Δ3 (67-276) cloned into Flag-pFASTBAC vector.	5’ BamHI primer	GGAGAAGGATCCATGGCAAAGCAGTACG ACTCGGTGG
	3’ XhoI primer	GGAGAACTCGAGTTATCACTTCACCTTCC GCTTCTGGCCCTTGACC
CDK9Δ4 (100-276) cloned into Flag-pFASTBAC vector.	5’ BamHI primer	GGAGAAGGATCCATGGCAAAGCAGTACG ACTCGGTGG
	3’ XhoI primer	GGAGAACTCGAGTTATCACTTCACCTTCC GCTTCTGGCCCTTGACC
CDK9Δ5 (150-276) cloned into Flag-pFASTBAC vector.	5’ BamHI primer	GGAGAAGGATCCATGGCAAAGCAGTACG ACTCGGTGG
	3’ XhoI primer	GGAGAACTCGAGTTATCACTTCACCTTCC GCTTCTGGCCCTTGACC
CDK9 gene cloned into Flag-pcDNA5-FRT-TO vector.	5’ BamHI primer	GGAGAAGAATTCTGATGGCAAAGCAGTA CGACTCGGTGGAGTGC
	3’ XhoI primer	GGAGAACTCGAGTTATCAGAAGACGCGCT CAAACTCCGTCTGGTTGG

CDK9 gene cloned into HA-pcDNA5-FRT-TO vector.	5' BamHI primer	GGAGAAGAATTCTGATGGCAAAGCAGTA CGACTCGGTGGAGTGC
	3' XhoI primer	GGAGAACTCGAGTTATCAGAAGACGCGCT CAAACTCCGTCTGGTTGG
CDK9 gene cloned into His-pcDNA5-FRT-TO vector.	5' BamHI primer	GGAGAAGAATTCTGATGGCAAAGCAGTAC GACTCGGTGGAGTGC
	3' XhoI primer	GGAGAACTCGAGTTATCAGAAGACGCGCT CAAACTCCGTCTGGTTGG
CDK9 cloned into GST-pGEX vector.	5' BamHI primer	GGAGAAGAATTCTGATGGCAAAGCAGTAC GACTCGGTGGAGTGC
	3' XhoI primer	GGAGAACTCGAGTTATCAGAAGACGCGCT CAAACTCCGTCTGGTTGG
Human CDK8 gene cloned into 6-His-pFASTBAC vector.	5' SacI primer	GGAGAAGAGCTCTGATGGACTATGACTTTA AAGTGAAGCTGAGCAGCGAGCGGG
	3' XhoI primer	GGAGAACTCGAGCTATCAGTACCGATGTGT CTGATGTGAGTACTGTGGAGGCTGC

3.5 Generation of stable cell line:

Flp-in 293 cells were used to create a stable FKBP5 cell line that constitutively produces ectopic FLAG-HA epitope-tagged FKBP5 protein under the control of the CMV promoter. The Flp-in T-Rex cell line, which possesses a doxycycline-inducible system and expresses FLAG-HA-tagged CDK9 during doxycycline induction, was used to create a stable CDK9 cell line. Doxycycline at a concentration of 5µg/ml was employed to stimulate FLAG-HA CDK9 expression. In a nutshell, the following things are described.

• Day 1: 5µg of the corresponding mammalian expression construct was transfected into Flp-in 293/ Flp-in T-Rex cells in a 10 cm plate.

• Day 2: Fresh FBS-containing media was added to the transfected medium.

• Day 4: Cells were selected with hygromycin (200 μg/ml) after 72 hours of transfection.

• Day 7: After 72 hours of hygromycin selection, the media was replaced daily for 15 days with new hygromycin-containing medium.

• Individual colonies were selected and planted in a 24-well plate after being screened with hygromycin.

• It was put onto a 6-well plate once it reached confluency.

• After achieving confluency, several cells from the 6-well plate were split and utilised for western blotting to confirm the expression of the desired protein using tag-specific antibody.

• For long-term preservation, produced stable cells were preserved in liquid nitrogen with 10% DMSO containing FBS after establishing the stable expression of individual proteins.

3.6 Generation of stable knockdown of FKPP5 and CDK9 in different mammalian cell lines.

For this study, we created a stable knockdown cell line of FKBP5 and CDK9. Using publiclyavailable tools, oligos for generating shRNA targeting FKBP5 and CDK9 were developed. The sequence of oligos is given in detail in Table 5. We employed the Lentiviral delivery technique to ensure steady integration of shRNA-producing constructs. The designed oligos were initially cloned in PLKO.1 to make lentiviruses. We purchased PLKO.1-puro vector from Addgene. For transfection, 3 x 10^5 cells were plated in a 6-well plate. We transfected cells with 500 ng sh-PLKO.1 (containing the target shRNA) after 18-20 hours of cell seeding along with psPAX2 (packaging plasmid): 125 ng, pMD2.G vector: 375 ng (envelop plasmid). The medium containing transfection reagents was replaced with new DMEM media after 12-15 hours of transfection. The supernatant (virus particle) was collected after 72 hours and kept at -80°C for long-term storage. 3x10^5 HEK293T or cells were transduced with viral particles (300μl sup) in the presence of polybrene (8mg/ml) for transduction purposes. After24 hours, transduced cells were exposed to puromycin (3mg/ml) selection for 2-3 passages. Puromycin-positive cells were used for checking the expression of target genes by qRT-PCR and immunoblotting analyses.

We made a shRNA mediated stable knockdown cell line of FKBP5 and CDK9 for the present

study. Oligos for producing shRNA, targeting FKBP5 and CDK9, were designed using publicly available software. A detailed sequence of oligos has been provided in Table no 5. For stable integration of shRNA producing oligos we used lentiviral delivery system. To produce lentivirus, the designed oligos were first cloned in PLKO.1 puro vector purchased from Addgene. 3 x 10^5 cells were seeded in 6-well plate for transfection. After 18-20hr of cell seeding, we transfected cells using 500 ng sh-PLKO.1, 125 ng psPAX2 (packaging plasmid) and 375 ng of pMD2.G vector (envelop plasmid). After 12-15hr, media containing transfection reagents was changed with fresh DMEM media. After 72-hours, supernatant (virus particle) was collected and stored at -80°C for long term storage. To check the efficiency of knockdown, again 3x10^5 HEK293T or cells were transduced with virus particle (300µl sup) in presence of polybrene (8 µg/ml). After 24-hours, transduced cells were subjected to puromycin (3µg/ml) selection for 2-3 passages. Puromycin-positivels were checked for target gene expression through mRNA (RT-PCR) along with immunoblotting analyses.

Table 5: Sequence of oligos, used in the generation of FKBP5 and CDK9 knockdown.

Name of the oligo	Sequence of oligo
shRNA-CDK9#1	**Upper oligo-** CCGGCCGCTGCAAGGGTAGTATATACTCGAGTATATAC TACCCTTGCAGCGGTTTTTG
	Lower oligo- AATTCAAAAACCGCTGCAAGGGTAGTATATACTCGAGT ATATACTACCCTTGCAGCGG
shRNA-FKBP5#1	**Upper oligo-** CCGGACCTAATGCTGAGCTTATATACTCGAGTATATAAGC TCAGCATTAGGTTTTTTG
	Lower oligo- AATTCAAAAAACCTAATGCTGAGCTTATATACTCGAGTAT ATAAGCTCAGCATTAGGT

shRNA-FKBP5#2	**Upper oligo-** CCGGCGAAGGAGCAACAGTAGAAATCTCGAGATTTCTAC TGTTGCTCCTTCGTTTTTG
	Lower oligo- AATTCAAAAACGAAGGAGCAACAGTAGAAATCTCGAGAT TTCTACTGTTGCTCCTTCG

3.7 Nuclear extract preparation

To make nuclear extract, cells were collected (scraped) in 1xPBS, centrifuged for 5 minutes at 4°C at 3000 rpm, and the supernatant was discarded. After measuring the packed cell volume (PCV), the cells were resuspended in 2X-PCV of NE1 buffer, a hypotonic solution, and incubated for 15 minutes on ice. The cells were then homogenized using a 23-gauge needle (10 pulses). The nuclear pellet was obtained by centrifuging the cell lysate at 6000 rpm for 5 minutes in cold (4°C) conditions. The nuclear pellet was quantified as NPV and the supernatant was maintained as a cytosolic fraction (S100). The nuclear pellet was then resuspended in pre-chilled NE2 buffer at 0.5X NPV. The resuspended nuclear pellet was then mixed with an equivalent volume (0.5X NPV) of cooled NE3 buffer and vortexed intermittently by maintaining in a cold condition while stirring for 45 minutes. Finally, separate the nuclear fraction from the chromatin fraction by spinning at 14000 for 20 minutes at 4°C. The salt-extracted nuclear protein is kept in the supernatant at -80°C until needed. Table no. 6 describes the buffer composition used in nuclear-extract processing.

3.8 Protein complex purification from a stable cell line.

CDK9 and FKBP5 complexes were isolated from stable cell lines in this study. Both stable cell lines were cultured at a high scale to purify these complexes, which was followed by nuclear extract preparation, immunoprecipitation, and affinity elution. The above-mentioned methodology was used to prepare the nuclear extract. For immunoprecipitation, nuclear extract was first pre-cleared for 1 hour at 4°C with protein A-agarose. The FLAG antibody-coated (M2) magnetic beads were then added overnight with the precleared extract. After an overnight incubation period, beads and sup were separated on a magnetic stand and washed three times in wash buffer. Finally, the bead-bound protein complex was eluted using 3X

FLAG peptide (250ng/µl) for 30 minutes at 4°C. To detect the protein complex purification, it was separated on a 4-12% SDS-PAGE gradient gel and then stained with silver stain. The silver staining was done according to the manufacturer's instructions.

3.9 Immunoprecipitation of Ectopically-expressed proteins

To confirm the probable interactors of a protein, firstly it was cloned in mammalian expression vector which expresses epitope-tagged protein which can be easily immuno-precipitated. Subsequently, this construct was transfected in 6-well dish (1µg/well). After 48hrs of transfection cells were harvested with 1ml of PBS at 3000rpm for 5' at 4°C. Then, cells were lysed in lysis buffer for 2hrs at 4°C, 16rpm. The lysed cells were centrifuged at 12000rpm for 20' at 4°C and supernatant was taken out (Whole cell extract). This WCE was incubated with epitope tag-specific beads for over-night at 4°C, and rotataed at 16 rpm. After over-night incubation, bead-bound proteins were washed rigorously with wash buffer (3x). Finally, bead-bound protein complex was eluted by heating at 95°C. Finally, western blotting was done to identify the interactors of the target protein.

3.10 Immunoprecipitation of endogenous proteins

Endogenous immunoprecipitation was used to evaluate the protein-protein interaction in normal cellular circumstances utilizing a target antibody immobilized on a protein-G magnetic bead. First, a whole-cell extract was produced in BC150 buffer, and then the cell lysate was pre-cleared for 1 hour at 4°C using protein A-agarose beads. Protein-G beads were blocked for 1 hour at 4°C with 1% BSA in BC150 + 0.1 percent NP40. Pre-blocked protein-G beads were now treated for 4 hours at 4°C with 2µg of target antibody. After pre-clearing the extract, it was immunoprecipitated using antibody-bound protein-G beads overnight at 4°C and rotating at 16 rpm. After overnight incubation at 4°C, the protein-G magnetic beads-associated protein complex was rigorously washed (3x) with the same buffer (BC150 + 0.1 percent NP40) used for immunoprecipitation. Finally, the bead-bound protein complex was heated to 95°C to elute it. Now, western blotting was used to find the target protein's likely interactors.

Table no. 6 summarizes the composition of all immunoprecipitation buffers.

Table 6: Buffers used for nuclear extract preparation, protein purification and immunoprecipitation analysis

Name of the buffers	Composition
RIPA lysis buffer	20 mM Tris-Cl pH8, 150 mM NaCl, 1% Triton-X100, 0.1% SDS
BC 100 elution buffer	100 mM KCl, 20mM Tris-Cl pH8, 20% glycerol, 0.1% NP40 + 150mM 3x flag-peptide.
BC 300 lysis buffer	300 mM KCl, 20mM Tris-Cl pH8, 20% glycerol, 0.1% NP40 + freshly added PIC and 0.7µl/ml β-mercaptoethanol (β-me).
Wash buffer	Same as lysis buffer but without PIC and β-me.
NE1 buffer	10 mM Tris-Cl pH-7.3, 1.5mM MgCl2, 10mM NaCl + 0.7µl/ml of β-me
NE2 buffer	25% Glycerol, 20 mM Tris-Cl pH-7.3, 1.5 mM $MgCl_2$, 20 mM NaCl + 0.2 mM EDTA pH-8 + 0.7µl/ml of β-me + 0.2 mM PMSF
NE3 buffer	25% Glycerol, 20 mM Tris-Cl pH-7.3, 1.5 mM $MgCl_2$, 1200 mM NaCl + 0.7µl/ml of β-me + 0.2 mM PMSF

3.11 *In vitro* kinase assay:

Several *in vitro* kinase assays were carried out in this study to determine the influence of FKBP5 on P-TEFb kinase activity, i.e., the phosphorylation level of different P-TEFb substrates such as Pol II-CTD, NELF complex, and DSIF complex. The Sf9 system was used to reassemble and purify P-TEFb and NELF complexes, whereas the DSIF complex and Pol II-CTD were purified using the bacterial expression system. All kinase assay procedures were performed at 30°C for 30'- 2 hours in kinase assay buffer (50mM Tris-Cl pH 8.0, 2mM $MgCl_2$, and 500µM ATP) (As per experimental set-up). Following the reactions, the amount of phosphorylation of the substrates was determined using either western blotting with phosphorylation specific antibody (for GST-CTD phosphorylation) or autoradiography (For NELF complex and DSIF complex phosphorylation). Along with the 500µM ATP, an additional 0.2µCi ^{32}P-ATP was employed in autoradiography-based experiments.

3.12 Purification of recombinant proteins using a baculoviral system.

Many of the proteins like FLAG-CDK9, FLAG-FKBP5etc, and protein complexes like P-TEFb, NELF complex etc, used in this study were reconstituted and purified using insect cell (Sf9) system. We employed a baculoviral expression method to reconstitute and purify proteins or protein complexes from insect cells. Baculovirus particles were applied directly to 90% confluent Sf9 cells for the purification of expressed proteins/complexes, either alone or in combination, depending on the experimental requirements. After 48 hours of infection, the cells were collected. For 2 hours at 4°C, cell lysates were prepared in BC300 lysis buffer (20mM Tris-Cl, 200mM KCl, 2mM EDTA, and 20% Glycerol) with 0.1 % NP-40, 0.7µl/ml β-mercaptoethanol, and 1X protease inhibitor cocktail (PIC). Cell lysate was centrifuged at 12000rpm for 20 minutes at 4°C to remove cell debris. After that, immunoprecipitation with epitope-tag specific agarose beads was used to bring down the protein/protein complex. Immunoprecipitation was done for overnight at 4°C, by rotating at 16rpm. Beads were washed three times (10min each at 4°C) with washing buffer containing BC300 + 0.1% NP40 after overnight binding. This bead-bound protein/protein combination has been used directly or eluted utilising epitope-based competitive elution. FLAG-tagged proteins were eluted with 3X FLAG peptide (250ng/µl) in BC100 buffer after vigorous washing, whereas GST-tagged proteins were eluted with GST peptide solution (9µg/ml) in BC100 buffer.

3.13 Purification of recombinant proteins using a bacterial system

Several proteins, used in this study, were purified from the BL21 or Rosetta (Both *E.coli* strain) which was used to produce recombinant proteins. Mainly GST-tagged and HIS-tagged proteins were purified using a bacterial expression system. Genes were cloned as GST-tag and HIS-tag in PET11-d vectors. These constructs were used for transformation in BL-21/Rosetta cells. A single colony was selected after regular transformation and grown in ampicillin-containing LB for initial culture at 37°C for O/N. After that, a secondary culture was inoculated at 1:100 dilutions, and when the OD reached 0.4, IPTG (100mM final concentration) was added for induction, and the protein expression was carried out for 3-4 hours in a 37°C shaker.

Hereafter, for purification of GST-Tagged proteins, the cells were harvested and washed in

PBS before resuspending in BC300 buffer with freshly added β-mercaptoethanol and a protease inhibitor cocktail (PIC). Sonication was done with a 100-watt input and a 30-second on/off cycle for 10 minutes to prepare the cell lysate. The supernatant was collected when the lysate was spun down at 12000 rpm for 20min. GST-tagged protein was purified from the lysate using anti-GST beads. Beads bound GST- tagged protein was eluted for 1hr at 4°C in an elution buffer (9µg/ml reduced GST peptide in BC100 + 0.1% NP40). Eluted proteins were flash-frozen with liquid-N2 and stored at -80°C for subsequent usage.

For purification of His-tagged protein, lysate was prepared as above. His-tagged proteins were purified from the lysate using Ni-NTA beads. Bead-bound His-tagged protein was washed vigorously with wash buffer for 3 times for 30 mins. Beads bound His-tagged protein was eluted for 1hr at 4°C, with His-elution buffer. Eluted proteins were flash-frozen with liquid-N2 and stored at -80°C for subsequent usage. Buffers used in His-tagged protein purification are discussed in Table no. 7.

Table 7: Buffers used for HIS-tagged protein purification.

Buffer	**Buffer composition**
Lysis Buffer	Na2HPO4 (50mM) + NaCl (300mM) + Imidazole (10mM) +Glycerol (20%)
Wash Buffer	Na2HPO4 (50mM) + NaCl (300mM) + Imidazole (20mM) + Glycerol (20%)
Elution Buffer	Na2HPO4 (50mM) + NaCl (300mM) + Imidazole (250mM) + Glycerol (20%)

3.14 *In vitro* direct interaction analysis

We performed direct *in vitro* protein-protein interaction assays to test interaction between different proteins like FLAG-CDK9 and HIS-FKBP5. We employed pure recombinant target factors, either immobilized to agarose beads or introduced exogenously for direct *in*

vitro protein-protein interaction assays. Purified proteins were incubated overnight at 4°C in a binding buffer comprising 20mM Tris pH 8.0, 20% glycerol, 2mM EDTA, 150mM KCl, 0.1 % NP-40, and 20-80ng/ml BSA. After that, protein-bound beads were washed three times with binding buffer (10 minutes each at 4°C). The bound proteins were eluted by incubation in 1xSDS loading dye for 10 minutes at 95°C. Western blots using particular antibodies were used to identify the interactors.

3.15 *In vitro* competition assays

We employed purified bead-immobilized FLAG-CDK9, as well as exogenously added GST-CyclinT1 and His-FKBP5, in an *in vitro* competitive binding assay. We employed a constant quantity (0.5µg) of bait protein (immobilized FLAG-CDK9), as well as a constant amount of one of the proteins (GST-CyclinT1/His-FKBP5) and a gradient amount of the other competitor (His-FKBP5/GST-CyclinT1). In a binding buffer comprising 20mM Tris pH 8.0, 20% glycerol, 2mM EDTA, 100mM KCl, 0.1% NP-40, and 80ng/ml BSA, the binding process was carried out overnight at 4°C. The beads were thoroughly washed in binding buffer after an overnight incubation. Bead-bound proteins were eluted by incubating them in 1xSDS loading dye for 10 minutes at 95°C. Western blots with particular antibodies were used to identify the interactors.

3.16 *In vitro* dissociation assay:

We employed a purified bead-immobilized P-TEFb complex consisting of FLAG-CDK9+GST-CyclinT1 for the *in vitro* dissociation experiment. Both proteins were purified separately and assembled into the P-TEFb complex by combining them in an assembly buffer (20mM Tris pH 8.0, 20% glycerol, 2mM EDTA, 300mM KCl, 0.1%NP-40) and incubating for O/N at 4°C. To completely saturate the FLAG-CDK9 binding to GST-CyclinT1, bead-bound FLAG-CDK9 was treated with an excess quantity of purified GST-CyclinT1. Extensive washing was used to eliminate any remaining unbound GST-CyclinT1. This immobilized P-TEFb complex was then employed in the reaction setup with increasing concentrations of purified FKBP5 protein in reaction buffer (20mM Tris pH 8.0, 20% glycerol, 2mM EDTA, 100mM KCl, 0.1 percent NP-40, 80ng/ml BSA) and incubated at 4°C for O/N. The reactions were centrifuged briefly, and the supernatant fraction was collected. The bead-bound proteins were washed twice with reaction buffer

before eluting in 1X SDS loading dye and being treated as bead-bound fractions by incubating them at 95°C for 10 minutes. Each supernatant and bead-bound fractions were loaded in parallel for western blots using particular antibodies to compare the quantity of target proteins present in both fractions. A similar experimental technique was employed with bead-bound P-TEFb complex immunoprecipitated from whole cell extract from mammalian cells coupled with purified FKBP5 protein for immunoprecipitated P-TEFb complex.

3.17 Western blot analysis:

Western blot analysis was used for majority of the biochemical experiments. Samples were prepared in lammeli buffer for western blotting. To prepare the sample, 5x lammeli buffer was added to the sample (according to the sample volume) and it was boiled for 10 minutes at 95°C to denature the protein. The SDS-PAGE gel was loaded with a denatured protein sample (as per required percentage of gel). The proteins were resolved using gel electrophoresis at a constant voltage of 100 volts, combined with a molecular weight marker. Proteins were separated using gel electrophoresis and then transferred to the nitrocellulose/PVDF membrane using the wet-transfer technique. To prevent non-specific antibody binding, the membrane was blocked for 1 hour at room temperature in a 5% skimmedmilk solution in TBST/PBST. After blocking, the milk was removed and a new primary antibody was applied to the membrane, which was then incubated overnight at 4°C on a rocker. It was washed three times with 1X TBST/PBST after an overnight incubation in primary antibody (10 minutes each at RT). Following washing, an HRP conjugated secondary antibody in 5% milk in TBST/PBST was added for 1 hour at room temperature. After 1hr incubation with the secondary antibody, the membrane was washed three times with 1X TBST/PBST (10 minutes each at RT). Finally, either in the darkroom on X-ray film or in the chemidoc system, commercially available ECL solution (Bio-Rad) was used to develop the blot.

Table no. 8 lists all of the antibodies utilized in this investigation for western blotting.

Table 8: List of antibodies used in this study.

Name of the antibody	Source	Identifier
FKBP5	Bethyl Laboratories	Cat# A302-090A
CyclinT1	Santa Cruz Biotechnology	H-245
CDK9	Santa Cruz Biotechnology and Bethyl Laboratories	Cat# SC-8338 and Cat# A303-492A
FLAG epitope	Sigma	Cat# F7425
GST	Santa Cruz Biotechnology	Cat# sc-53909
His epitope	Santa Cruz Biotechnology	Cat# sc-8036
AFF1	Abcam	Cat# ab31812
AF9	Bethyl Laboratories	Cat# A300-596
ELL	Bethyl Laboratories	
NELF A	Bethyl Lab	Cat# A301-531
NELF E	Bethyl Lab	Cat# A303-279
Rpb1 (4H8 clone)	Cell Signaling Technology	Cat# 2629
Phospho Rpb1 CTD (Ser2)	Cell Signaling Technology	Cat# 13499
Phospho Rpb1 CTD (Ser5)	Cell Signaling Technology	Cat# 13523
Beta Actin	Santa Cruz Biotechnology	Cat# sc47778
Normal Rabbit IgG	Cell Signaling Technology	Cat# 9064
Normal Mouse IgG	Cell Signaling Techn ology	Cat# 11912
HRP conjugated secondary antibody (Rabbit)	Bio-Rad	Cat# 1706515
HRP conjugated secondary antibody (Rabbit)	Bethyl	A120-101P
HSP90β	Bio-Rad	VPA0080
Histone H3	Cell Signaling Technology	2650S

3.18 Luciferase reporter assay

Dual luciferase reporterassay was performed using HIV1-LTR-luciferase (firefly) construct. We seeded 50000 cells in each well of 24-well dish in 2 sets, one set of scramble cells and one set of FKBP5-KD cell line. Next day we transfected 3 different concentrations (in 8 different sets) of HIV1-LTR-luciferase with equal concentration ofrenilla luciferase construct (as internal control for transfection) in both the cell-lines. We lysed cells after 40hrs of transfection in 50µl of PLB buffer and we proceeded for luciferase assay. We used Dual-glow Luciferase assay kit from Promega and followed manufacturer's protocol for obtaining expression data. Here in this experiment renilla luciferase construct was used as internal control and we normalized data with renilla luciferase expression data.

3.19 Plasmid DNA and bacmid DNAisolation

For different purposes like cloning, Plasmid DNA was isolated from DH5α and for the purpose of baculovirus production recombinant bacmid DNA was isolated from DH10Bac. For both these DNA isolations, we followed manual alkaline lysis method. Plasmid DNAs isolated for the transfection purpose, were isolated, using Qiagen miniprep Kit following the manufacturer protocol.

3.20 RNA isolation and subsequent assays:

For different experimental purposes like RNA-seq, qRT-PCR etc, total cellular RNA was isolated. RNA isolation was done using TRIzol following the conventional method. After harvesting cells from a confluent well of 6-well dish (with 1ml of PBS at 3000rpm for 5 min), cells were lysed using 400µl of TRIZOL, and vortexing for 30 seconds and incubating at room temperature for 5 minutes which dissociates nucleoprotein complexes in of the cell. Then, 200µl of chloroform was added to the suspension and vortexed vigorously for 15-20'' and incubated at room temperature for 2-3 minute. Subsequently, the whole suspension was centrifuged at 12000rpm for 15 minutes at 4°C. This high-speed centrifugation leads to the separation of an upper transparent, RNA containing aqueous phase, middle interphase containing degraded proteins etc. and a lower organic phase. Top layer, containing total cellular RNA, was carefully taken out in a separate tube by pipetting

without mixing with the middle and lower phase. Subsequently, to precipitate RNA in aqueous phase, we added 500µl of molecular biology grade isopropanol, mixed and incubated for 10 minutes at room temperature. Then, centrifuging the solution at 12000 rpm for 10 minutes at 4°C causes RNA precipitation. The supernatant was discarded, leaving the RNA pellet intact. RNA pallet was resuspended in 500µl of 75% ethanol through vertexing briefly. Again, centrifugation at 12000rpm for 5 minutes at 4°C was done to pellet down the RNA and the supernatant was discarded. After air-drying the pellet for 15min at RT, it was resuspended in nuclease-free water and incubated in a water bath maintaining 45°C for 15 minute to dissolve the RNAcompletely. Finally, the isolated RNA concentration was measured in nanodrop for further use, and remaining RNA sample was stored in -80°C fridge for future use.

3.20.1 cDNA synthesis from mRNA

cDNA was synthesized for the purpose of qRT-PCR analysis. For cDNA synthesis, we used 1µg of isolated RNA. We used Verso cDNA synthesis kit (Thermo Scientific), following the manufacturer protocol for cDNA synthesis.

3.20.2 qRT-PCR analysis

Total cDNA synthesized from 1µg RNA, was diluted 50 times before using in qRT-PCR analyses. The qRT-PCR analysis was performed using iTaq universal SYBR Green supermix from BioRad laboratories and target gene-specific primer (See Table no 9). qRT-PCR was performed using BIORAD CFX 96^{TM}Real-Time PCR system software. For target gene expression analysis, expression of GAPDH and 18S was used as internal control as per the requirement.

Table 9: List of primers for RT-qPCR

GeneName	5' forward primer	3' reverse primer
FKBP5	TCCTTGCTGCCTTTCTG	CTTTCTCAAAGTCACCCTTG
CDK9	GCATCATGGCAGAGATGTG	GTTGTCCACGTTTGGCC
CCND1	CAAACACGCGCAGACCTTC	GATCACTCTGGAGAGGAAGCG
MYC	GCTTGTACCTGCAGGATC	GACTCCGTCGAGGAGAG
CDKN1A	GGACAGCAGAGGAAGACCATG	CTGTCATGCTGGTCTGCC
CDKN1B	CGACGATTCTTCTACTCAA	TTACGTTTGACGTCTTCTG
CDKN1C	GCTGCACTCGGGGATTTC	GGACATCGCCCGACGACT
PTPRS	AACACAGAAGTGCCCGCAC	GTGACGTGTGGGCCTTGGAG
TM2D2	GGACACTACTTCATAACCAC	AATAAGGTCAACAAACCACC
E2F2	GGAGCCGGACAGTCCTTC	GCTGTCAGTAGCCTCCAAG
CDC25B	GGCACATCAAGACTGCGG	GGTAGTCGTTGACAGCACG
CDK6	GGAGTGTTGGCTGCATATTTG	CGATATCTGTTACAAACTTC
TAF4	GACGACAGATATGAGCAGG	GTTGCTGCATCTCCTTTG
ATF2	GTACCAGGCCCATTTCCTCTTC	GAACGAGTGGGACTGCAGCTG
MDM2	TTGGATCAGGATTCAGTTTC	GAGAGTTCTTGTCCTTCTTC
TEF	GCTCTTCCACAGCATCC	GCACACTGGAGAGCAC
CRABP2	CCCTACACCAACAAAGAGG	CCCTCAAGTCCCCTTTAG
GLUT1	GAAGTGTCACCCACAGCCCTTC	GGGCCACAGGTCCTTGTTGC
STIP1	GAGAAAATCCTGAAGGAGCAAG	ATGCTTCATGGCCTGGGGATA
RGS2	AAGATTGGAAGACCCGTTTGAG	GCAAGACCATATTTGCTGGCT
MAP2	AATAGACCTAAGCCATGTGACATCC	AGAACCAACTTTAGCTTGGGCC
LDLR	CTGGAAATTGCGCTGGAC	GTCTTGGCACTGGAACTCGT
GAPDH	GACAACTTTGGTATCGTGG	GTTCAGCTCAGGGATGAC

3.21 Chromatin immunoprecipitation (ChIP) analysis.

Cells were cross-linked for 10 minutes at room temperature with 1 percent formaldehyde (Sigma) and then incubated with 125 mM glycine for 5 minutes to terminate the cross-linking process. The cross-linked cells were rinsed three times with ice-cold PBS. Resuspending the cells in ChIP lysis buffer (0.5% NP40, 1% Triton X-100, 300mM NaCl, 20mM Tris (pH 7.5), 2mM EDTA, and protease inhibitor cocktail) and incubation on ice for 30 minutes yielded nuclear extract for downstream immunoprecipitation analysis. The lysate was spun down at 5000 rpm for 10 minutes at 4°C after being syringe passaged eight times via a 23-gauge needle. ChIP sonication buffer (1% SDS, 50mM Tris (pH 8.0), 10mM EDTA, and 1X protease inhibitor cocktail) was used to resuspended the nuclear pellet. After that, the resuspended nuclear pellet was sonicated for 20 minutes at high efficiency with the Bioruptor TM UCD-200. The sonicated samples were spun at 15K for 20 min. The generated supernatant was pre-cleared by using65µg sonicated DNA sample for 30 minutes at 4°C using 20µl Protein-A agarose beads. ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.1mM EDTA, 20mM Tris-Cl (pH 8.0), and 167mM NaCl) were used to dilute the pre-cleared lysate ten times. IgG was utilized as a negative control and each IP sample was immunoprecipitated overnight at 4°C with 2µg of target antibody. Protein G-magnetic beads were blocked overnight in a separate process with 50µg of salmon sperm DNA. The next day, 25 µl of pre-blocked Protein G-magnetic beads were added to the immunoprecipitated sample and incubated at 4°C for 1 hour. After a brief spin at 3000 rpm, the antibody-protein-DNA complex was washed twice with low salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Cl (pH 8.0), 150mM NaCl, and protease inhibitor cocktail), high salt buffer (500mm NaCl, 0.1 percent SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Cl pH8 and PIC). The immunoprecipitated protein-DNA complexes were eluted by incubating them at room temperature for 30 minutes in elution buffer (1% SDS, 0.1M $NaHCO_3$). For de-crosslinking, the eluted DNA-protein complex was incubated with 190 mM NaCl at 65°C. Proteinase K was used to digest the remaining proteins for 45 minutes at 45°C. By following the manufacturer's instructions, the eluted DNA was purified using the Qiagen PCR purification kit. The purified eluted DNA was then subjected to qRT-PCR testing with primers specific to the target gene locus.

Primer against intergenic locus was used to determine non-specific recruitment of over-expressed factor whenever required (See Table no11).

Table 10: Buffers used in ChIP-qPCR analysis.

Description of buffers	Composition
Cross-linking reagent	1% formaldehyde in minimal DMEM
Quenching solution	125 mm glycine in PBS
ChIP lysis buffer	20 mm Tris pH7.5, 300 mm NaCl, 2 mm EDTA, 0.5% NP-40, 1% Triton X-100 +PIC
ChIP sonication buffer	50 mm Tris pH8, 10 mm EDTA, 1% SDS and PIC
ChIP dilution buffer	20 mm Tris-Cl pH8, 167 mm NaCl, 1.1 mm EDTA, 0.01% SDS, 1.1% Triton X-100
Low salt buffer	20 mm Tris-Cl pH8, 150 mm NaCl, 2 mm EDTA, 0.1% SDS, 1% Triton X-100 + PIC
High salt buffer	20 mm Tris-Cl pH8, 500 mm NaCl, 2 mm EDTA, 0.1% SDS, 1% Triton X-100 + PIC
Lithium chloride buffer	10 mm Tris-Cl pH8, 250 mm LiCl, 1 mm EDTA, 1% NP-40, 1% deoxycholic acid (sodium salt)
Elution buffer	1% SDS, 0.1M $NaHCO_3$

Table 11: Primers used for ChIP-qPCR analysis

Gene name	5' forward primer	3' reverse primer
CCND1gene promoterregion	CTTATGGTACTGTAACTGAGCTAAC	GCGGGACTATGGTTGCTGAC
CCND1 gene coding region	CAATCAAATCATTCCGGATACTGCG	CAAATCTATACATTAAGACGACTCG
***C-MYC* genepromoter Region**	CATACACGAGCCCTGAGCAT	GATAGCCTTCTCCTGCGCTAG
***C-MYC* genecoding Region**	CCCTACACCAACAAAGAGG	CCCTCAAGTCCCCTTTAG
***RGS2* genepromoter Region**	GGAAATCTGAGGGAACGAGG	CTCCAGATGCCAGGGAAG

3.22 Gene ontology (GO) analysis:

Gene ontology (GO) analysis was performed, using RNA-seq data, to identify and classify the gene sets according to their functional characteristics. Database for annotation, visualization, and integrated discovery (DAVID) software, an online bioinformatics tool, was used to provide a functional interpretation of gene sets which were upregulated or downregulated upon FKBP5 knockdown (Huang, Sherman, and Lempicki 2009). GO terms with *p*-values>0.05 as determined by the EASE score, a modified Fisher Exact P-value test, integrated into the DAVID workflow, were considered as significant. The entire pathway described in GO analysis had at least four genes with a *p*-value of < 0.05.

3.23 Cell proliferation assay:

To test the effect of FKBP5 knockdown on proliferation rate of HEK293T and PC3 cell lines, we simply counted cells at different intervals, after knocking down FKBP5, using hemocytometer under microscope. $6x10^4$ cell from scramble and FKBP5-KD were seeded (in triplicate) in-6-well dish. On 3^{rd}, 4^{th}, and 5^{th} day after seeding, cells were resuspended with trypsin and DMEM and diluted 10 times with fresh DMEM. 10µl cell suspension was applied to the hemocytometer counting chamber to count the cell number under

microscope. Finally, average count of FKBP5-KD cells was plotted with the average count of scramble cells on excel-sheet.

3.24 Statistical analysis ofdata:

To get the statistical significance of our data sets, statistical analyses and quantification of different experiments were performed using one-tailed student's t test. Data represents a minimum of two biological repeats and three technical replicates. wherein * denotes $p \leq 0.05$, ** denotes $p \leq 0.01$, *** denotes $p \leq 0.001$, and ns denotes not significant.

3.25 RNA-Seq analysis:

To analyze the effect of FKBP5 on differential expression of target genes we performed RNA-Seq analysis in normal (Scramble control) and FKBP5 knockdown conditions. For this purpose, we isolated RNA from both scramble and FKBP5-KD cell lines. Equal amount of RNA was sent for sequencing where RNA-seq analysis was performed following steps mentioned here.

A. RNA QC:

First of all, RNA quantity and quality was checked. For checking RNA quantity Qubit RNA HS assay kit (Thermo Fisher Scientific) was used and RNA quality was checked using Agilent Bioanalyzer RNA Nano 6000 Kit (Agilent Technologies). Subsequently library preparation was done with the samples having RNA integrity number (RIN) >7.

B. Library Preparation:

NEB NEXT RNA Ultra II Library preparation kit was used for NGS library preparation.For this, mRNA enrichment was needed which was achieved by using 800 ng of total RNA as input for poly (A) enrichment. Subsequently, it was fragmented which was followed by reverse transcription to generate cDNA. Adaptor index primers with unique barcodes for each sample was ligated to fragmented double strand cDNA. Now, adaptor ligated fragments were purified using ampure beads. Finally, the purified product was amplified using Illumina primers to generate sequencing library.

C. Library QC:

Quality of the library was checked with Agilent Bioanalyzer DNA 1000 kit (Agilent Technologies) and quantity was checked using Qubit DNA HS assay kit (Thermo Fisher Scientific). Libraries were diluted to 2 nM and pooled to make equimolar sequencing library

pool.

D.Sequencing

The pooled libraries were sequenced using Illumina HiseqX to generate 150 bp paired end reads. The library pool was diluted to 250 pM before loading it into the flow cell.Illumina bcl2fasta v 2.19 was used to generate fasta files.

E. Raw sequencing data analysis

For analyzing differential gene expression, the raw fastq sequences of all the samples were mapped to *Homo sapiens* GRCh38 genome using STAR (v2.27.2b) in order to create bam files (Dobin et al. 2013; Yates et al. 2020). These bam files were then processed using Rsamtools, Rsubread and Genomic Alignments R packages and the abundance table was created from the alignments (Lawrence et al. 2013; Liao, Smyth, and Shi 2019). The abundance tables were further normalized and differentially abundant genes were identified using the DESeq2 R package(Love, Huber, and Anders 2014. Genes with Log2 fold change (Log2FC) of 1.5-fold or more, having p-value <0.05 and adjusted p-value <0.05 were considered significantly differentiated gene expression. The heatmap of the selected genes was generated using counts per millionnormalizations from raw counts by implementing edgeR package and subsequent visualization was done with heatmap R function (McCarthy, Chen, and Smyth 2012).

3.26 RNA expression analysis in different cancer cohorts:

To analyze the expression of FKBP5, CDK9 and some of the target genes like CCND1 and cMyc in different cancer cohorts, we obtained gene expression data from a publicly available database i.e., The Cancer Genome Atlas (TCGA), stored as the part of the Broad Institute of MIT and Harvard TCGA Government Data Analytics Center (GDAC). Expression data of above indicated genes in 11 different types of cancers were obtained using above-mentioned TCGA database of 38 different cancer cohorts which contains over 14000 sequencing samples. The firehouse analysis pipeline, which was developed by the Broad Institute itself, was utilized through its Java-dependent web application Fire Browse, for easy data retrieval and analysis. For greater accuracy in quantification the cohort specific mRNA expression was represented as log2RSEM. One-tailed student t-test was performed to get statistical significance considering $p<0.05$ as significant.

CHAPTER IV

Results:

4. RESULTS:

4.1 A new role of Human FKBP5 in controlling cell proliferation rate by controlling P-TEFb activity.

❖ Introduction:

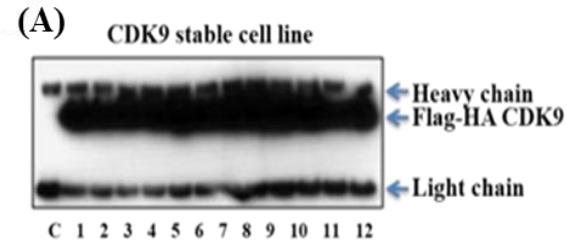
Human FKBP5 is a well-known co-chaperone that performs its function in association with HSP90. FKBP5 has also been shown to control several cellular signaling pathways without any association with HSP90. In our present study, we examined and established another extremely important role of FKBP5, where the cellular pool of FKBP5 has been shown to control the active P-TEFb pool inside the cell that in turn regulates the expression of target genes.

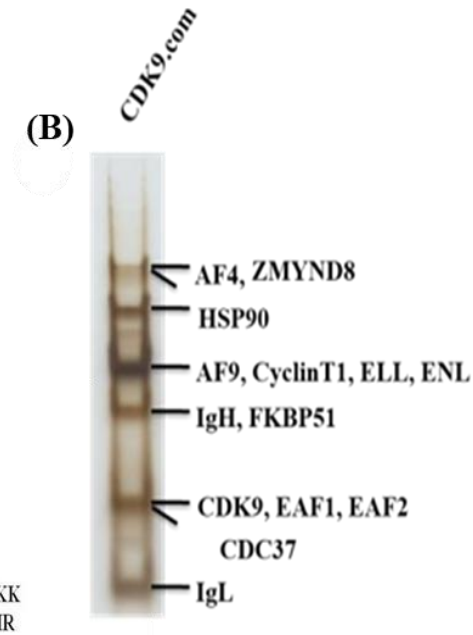
4.1.1 FKBP5 is a novel CDK9 associated protein in mammalian cells.

Present study has been started with the identification of novel interactors of CDK9 to find out other P-TEFb interactors. That is why, we started our study with identifying novel P-TEFb interactors within mammalian cells. Here, we have explained every experimental finding in detail.

4.1.1.1 Mass-spectrometry of CDK9 revealed FKBP5 as a very strong CDK9 interactor.

In search of other P-TEFb regulators (Interactors), we did mass spectrometric analysis of CDK9-associated proteins. For mass spectrometric analysis, we generated stable cell line which expresses FLAG-HA epitope-tagged CDK9 upon doxycycline induction. We purified FLAG-HA-CDK9.com through FLAG-affinity purification which we used for subsequent mass-spectrometric analysis (Figure 4.1A, B (Ghosh et.al 2018)). In this mass-spectrometry data, along with other known and unknown CDK9-interactors, we identified FKBP5 as a very strong interactor of CDK9 (Figure 4.1C, D).





(C)

Name of protein	Percentage coverage	Total no. of peptides	Total no. of unique peptides	Representative peptide
FKBP5	78.56	38	38	KGEGYSNPNEGATVEIHLEGR
FKBP5	78.56	38	38	NNEESPTATVAEQGEDITSKK
FKBP5	78.56	38	38	KGEICHLLCKPEYAYGSAGSLPK
FKBP5	78.56	38	38	TTDEGAKNNEESPTATVAEQGEDITSKK
FKBP5	78.56	38	38	IPSNATLFFEIELLDFKGEDLFEDGGIIR
FKBP5	78.56	38	38	ASESFLLAAFLNLAMCYLK
FKBP5	78.56	38	38	FDSSHDRNEPFVFSLGK
FKBP5	78.56	38	38	FAEQDAKEEANKAMGK

(D)

Description	Coverage	Proteins	Unique Peptides	Sum	Score
Peptidyl-prolyl cis-trans isomerase FKBP5 OS=Homo sapiens GN=FKBP5 PE=1 SV=2 - [FKBP5_HUMAN]	**78.56**	1	38	38	**340**

Figure 4.1 Association of FKBP5 with CDK9.

(A) Immunoblot analysis using anti-FLAG antibody, demonstrating the production of stable cell lines expressing FLAG-HA CDK9. Stable cell lines were generated in Flp-In-TREx 293 cells using the methods outlined in the Invitrogen manual.

(B) Purification of a CDK9-associated protein complex using one-step FLAG-affinity purification using nuclear extract from a stable cell line expressing FLAG-HA tagged CDK9. Eluted proteins were run on a 4%–12% gradient gel and silver stained to visualize interactors before being identified by mass spectrometry.

(C) Representative FKBP5 peptides identified by Mass Spectrometry in a CDK9 pull-down sample**.**

(D) Representation of mass spectrometry data showing an association of FKBP5 with CDK9.

4.1.1.2 FKBP5 and CDK9 preferentially localizes in nuclear region of mammalian cells.

After getting the clue from mass spectrometry data, that FKBP5 and CDK9 interacts with each other in HEK293T cell line, we suspected that FKBP5 might have some effect on P-TEFb-mediated transcriptional regulatory activities on target genes. Inorder to study that, at first, we addressed the localization of FKBP5 and CDK9 inside the cell. Using an online program, The Human Protein Atlas, we found out that FKBP5 preferentially localizes in nuclear region of the cell (Figure 4.2A). Subsequently, we tried to validate it by western blotting. To test the localization of FKBP5 and CDK9 inside the cell through western blot analysis, we separated the nuclear fraction and cytoplasmic fraction following the nuclear extraction protocol (Described in material methods section) and prepared the sample of both the fractions with 5X protein loading dye. Both the samples were analyzed through western blotting using the FKBP5 and CDK9 antibodies with Actin as cytoplasmic marker and HistoneH3 as nuclear marker. Data obtained from western blotting also supported that both FKBP5 and CDK9 proteins preferentially localizes inside the nucleus (Figure 4.2B).

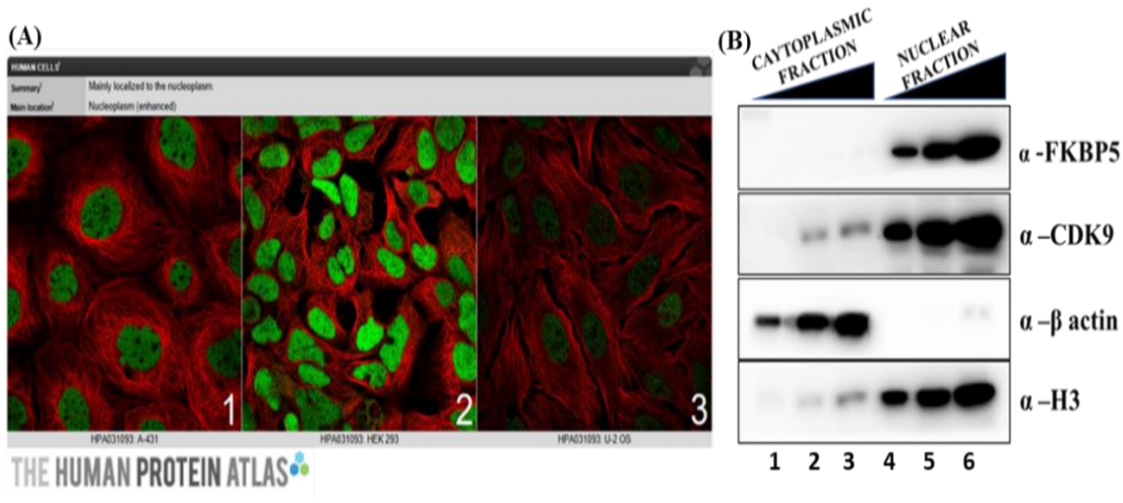


Figure 4.2 Localization of FKBP5 and CDK9 inside the cell.

(A) Confocal microscopy picture demonstrating that FKBP5 preferentially localizes to the nucleus in several mammalian cell types (picture adapted from "The Human Protein Atlas").

(B) FKBP5 and CDK9 co-presence in the nuclear compartment of mammalian 293T cells as revealed by Western blotting. The histone H3 protein is employed as a nuclear localization marker, whereas the actin protein is used as a cytoplasmic localization marker.

4.1.1.3 CDK9-FKBP5 Interaction analysis in ectopically-expressed conditions in mammalian cells.

To validate our mass spectrometry data, we tested the interaction between FKBP5 and CDK9 through their ectopic expression followed by immunoprecipitation and western blotting. First of all, we did immunoprecipitation using nuclear extract obtained from stable line of FLAG-CDK9. We prepared nuclear extract by following the nuclear extraction protocol described in material methods section, and subsequently immunoprecipitation using Flag-agarose beads (following immunoprecipitation protocol described in material methods section). Finally, we analyzed the interaction through western blotting (Figure 4.3A). Again, for reciprocal analysis, we made the stable line constitutively expressing FLAG-HA-FKBP5 in Flp-293 cells and checked the expression of FLAG-HA-FKBP5 through western blot analysis using FLAG-antibody (Figure 4.3B). We also purified the FKBP5-associated protein complex, (following purification protocol described in the material methods section), performed silver-staining and observed the presence of CDK9 band along with other known interactors of FKBP5 like HSP90(Figure 4.3C) which can be further validated by western blotting. To further validate this interaction, we prepared whole cell extract and subsequently immunoprecipitation using FLAG-agarose beads. Finally, we analyzed the interaction through western blotting and found out that FKBP5 strongly interacts with CDK9 along with a known FKBP5 interactor i.e., HSP90 but it failed to show any interaction with Cyclin T1 or other SEC members (Figure 4.3D). We also performed co-immunoprecipitation assay to confirm and substantiate our earlier observation of interaction between FKBP5 and CDK9. To perform co-immunoprecipitation, we ectopically expressed both FKBP5 and CDK9, with different epitope tag, i.e., FLAG and HA, in HEK293T cells. Subsequent immunoprecipitation one of the factors (In this case FLAG-FKBP5 was immunoprecipitated) and finally western blot analyses using antibody for other factor's epitope (HA) was done to see the interaction between the two proetins. By this co-immunoprecipitation assay also, we observed a very strong interaction between FKBP5 and CDK9 (Figure 4.3E).

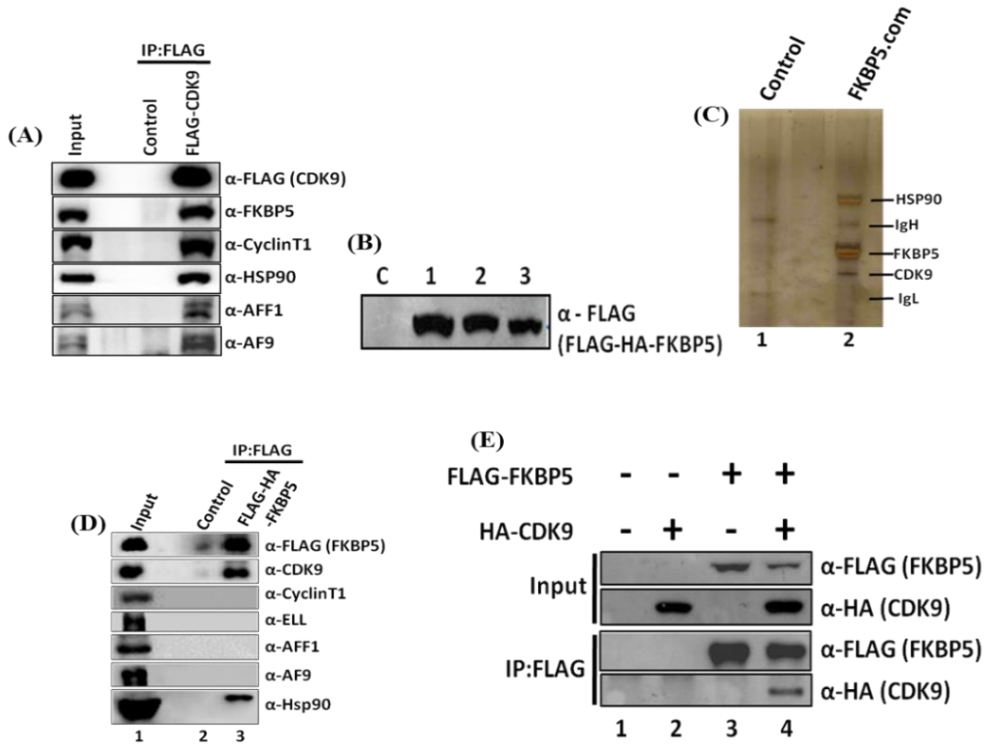


Figure 4.3 Interaction analyses between FKBP5 and CDK9 inside the cell.

(A) Identification of CDK9 interactors through immunoprecipitation and subsequent western blot analysis, showing FKBP5 as a novel CDK9 interactor with other known interactors (SEC members).

(B) Immunoblot analysis demonstrates the establishment of a stable cell line expressing the FLAG-HA-FKBP5 protein. Here, cells that did not express the FLAG-HA-FKBP5 protein were used as a control (marked as C).

(C) Purification of FKBP5 associated protein complex using one-step FLAG-affinity purification using nuclear extract from a stable cell line expressing FLAG-HA tagged FKBP5. Eluted proteins were run on a 4%–12% gradient gel and silver-stained to visualize interactors before being identified by mass spectrometry.

(D) Identification of FKBP5 interactors through immunoprecipitation and subsequent western blot analysis, showing CDK9 as a novel FKBP5 interactor with another known interactor of FKBP5 i.e., HSP90.

(E) Coimmunoprecipitation followed by western blotting reveals ectopically expressed FLAG-FKBP5, which pulls down ectopically expressed HA-CDK9 in mammalian 293T cells.

4.1.1.4 Immunoprecipitation of endogenous proteins to test interaction between CDK9 and FKBP5 in endogenous context.

Till now all the interaction studies are done in ectopically over-expressed condition of one or more factors which might be different from the normal cellular conditions. So, before concluding anything or proceeding our study further, we wanted to test the interaction between FKBP5 and CDK9 in normal cellular condition. To achieve that, we performed immunoprecipitation of endogenous proteins (protocol described in material methods section) followed by western blotting analyses. At first, we performed immunoprecipitation of endogenous FKBP5 using specific antibody, and subsequent western blotting analysis confirmed the association of CDK9 along with another known interactor of FKBP5 i.e., HSP90 (Figure 4.4A). Here also, FKBP5 failed to pull-down components of SEC. We tried to do the vice-versa experiment using CDK9 antibody for endogenous-immunoprecipitation assay. In this assay we found similar result as of overexpressed condition and we could clearly see that CDK9 is pulling down FKBP5 along with its other interacting SEC partners (Figure 4.4B).

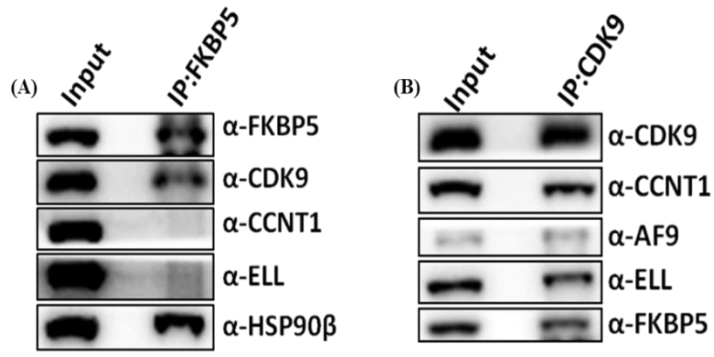


Figure 4.4 Endogenous-Immunoprecipitation to see interaction between FKBP5 and CDK9 in normal cellular conditions.

A. Immunoprecipitation of endogenous FKBP5 using specific α-FKBP5 antibody and subsequent western blotting analysis showing FKBP5 interaction with CDK9 along with HSP90 in normal cellular condition. FKBP5 failed to pull-down other SEC members.
B. Immunoprecipitation of endogenous CDK9 using specific α-CDK9 antibody and subsequent western blotting analysis showing CDK9 interaction with FKBP5 along with SEC components in normal cellular condition. CDK9 could successfully pull-down other SEC members (Known CDK9 interactors).

4.1.2 The interaction of FKBP5 with CDK9 is direct and specific.

Till now all the interaction studies are done in mammalian cell lines. Where, we have shown that FKBP5 and CDK9 interacts with each other. But, up until now, it is not clear whether CDK9 and FKBP5 interact directly with each other and there is a possibility of some other intermediate player which may have a role in making this interaction possible. In order to address this issue, we choose a heterologous system (i.e., insect cell (Sf9)) system for interaction analysis.

4.1.2.1 FKBP5 interacts with CDK9 but not with another subunit (i.e., CyclinT1) of the P-TEFb complex.

To clarify whether interaction between FKBP5 and CDK9 is direct or mediated by some other factor(s), we used a heterologous system i.e., insect (Sf9) cell system for understanding the protein-protein interaction. In this heterologous system, the interefernce from the expressed proteins are minimal and thus the observed interactions imply direct in nature between the interacting proteins. At first, we made different high-titer (P2) baculoviruses, (detailed protocol is provided in material and method section), which infects Sf9 cells and produces the specific proteins which they are made for. Subsequent co-infection with the viruses followed by immunoprecipitation and western blotting confirms the type (Direct or indirect) of interaction between the two proteins. Here, at first, we co-infected Sf9 cells with baculoviruses expressing FLAG-FKBP5 and NT-CDK9 (non-tag CDK9). Subsequent immunoprecipitation using FLAG-beads and western blotting confirms that there is direct interaction between FKBP5 and CDK9 proteins (Figure 4.5A). Similar experiment was done to see the interaction between FKBP5 and CyclinT1. Here we found that FKBP5 does not interact with CyclinT1(Figure 4.5B). Again, we tried to test if there is a tri-partite complex formation that could possibly take place. For this assay, we co-infected Sf9 cells with baculoviruses expressing FLAG-FKBP5, NT-CDK9 and NT-CyclinT1. Subsequent immunoprecipitation using FLAG-beads and western blotting confirms that there is direct interaction between FKBP5 and CDK9 proteins but there is no tripartite complex formation (Figure 4.5C).

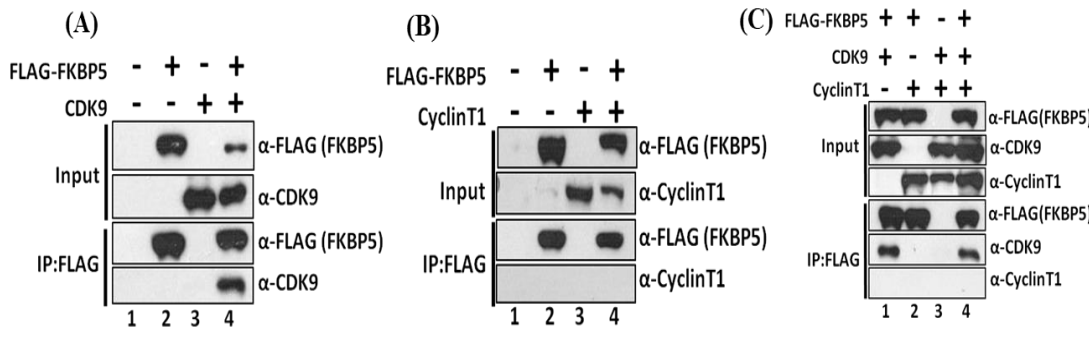


Figure 4.5 Direct-Interaction analysis between FKBP5 and CDK9/CyclinT1 using insect cell system.

(A) Immunoprecipitation followed by western blot analysis showing the direct interaction between FKBP5 and CDK9 through their coexpressing in heterologous insect cell system.

(B) Immunoprecipitation followed by western blot analysis showing no interaction between FKBP5 and CyclinT1 through their coexpressing in heterologous insect cell system.

(C) Immunoprecipitation followed by western blot analysis showing specific interaction of FKBP5 with CDK9 through their coexpression in heterologous Sf9 cells. Even though CyclinT1 expression was as high as CDK9, FKBP5 failed to interact with it in this system.

4.1.2.2 Interaction of FKBP5 is very specific to CDK9 only and not with other CDKs.

To rule out the possibility of generalized type of interaction of FKBP5 with CDKs, we wanted to check the FKBP5 interaction with other kinases which are similar to CDK9. For this, we chose CDK7 and CDK8 and made baculoviruses expressing FLAG-CDK8 and FLAG-CDK7. Subsequently, in first set we co-infected P2-viruses of FLAG-CDK8 and NT-FKBP5 and in other set we co-infected P2-viruse of FLAG-CDK7 and NT-FKBP5. Subsequent immunoprecipitation using FLAG-beads and western blotting confirms that there is no-direct interaction between FKBP5 and CDK8 (Figure 4.6A) or CDK7 proteins (Figure 4.6B).

So, until now, we could conclude that FKBP5 interacts directly with CDK9 and not with the whole P-TEFb or SEC. FKBP5 specifically interacts with CDK9 and it fails to

show any interaction with other kinases which are similar to CDK9 (e.g., CDK7, CDK8).

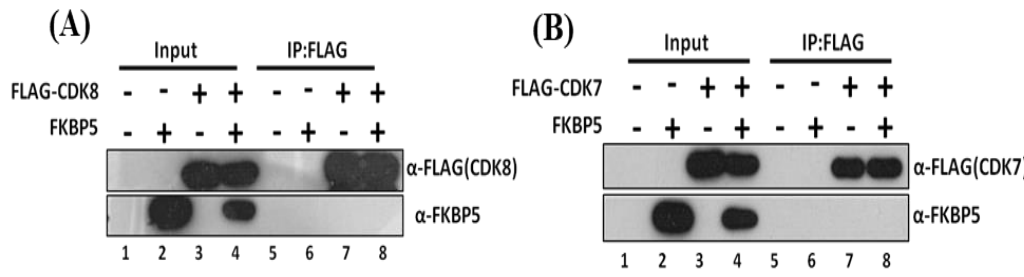


Figure 4.6 Direct-Interaction analysis between FKBP5 and CDK7/CDK8 using a heterologous insect cell system.

(A) Immunoprecipitation and subsequent western blot analysis demonstrated that when CDK7 and FKBP5 are co-expressed in heterologous Sf9 cells, they do not interact. Sf9 cells were co-infected with baculoviruses producing the relevant proteins, and protein lysates were used for immunoprecipitation and subsequent western blotting with particular antibodies to determine their interaction.

(B) Immunoprecipitation and subsequent western blot analysis demonstrated that when CDK8 and FKBP5 are co-expressed in heterologous Sf9 cells, they do not interact. Sf9 cells were co-infected with baculoviruses producing the relevant proteins, and protein lysates were used for immunoprecipitation and subsequent western blotting with particular antibodies to determine their interaction.

4.1.2.3 The TPR1 domain of FKBP5 plays the most important role for its interaction with CDK9.

We wanted to identify the domain of FKBP5 which is involved in interaction with CDK9. In order to do that, we cloned different deletion fragments of FKBP5 in different expression vectors (Mammalian, Baculoviral and bacterial) with different epitope tags (Cloning details is provided in material and method section). Detailed domain structure has been shown here (Figure 4.7A). At first, we tried to do domain analysis through co-immunoprecipitation assay in mammalian system (HEK293T). We co-transfected FLAG-tagged wild-type and different fragments cloned in mammalian expression vector with HA-tagged CDK9. Subsequent immunoprecipitation using FLAG-agarose beads followed by western blot clearly shows that the FKBP5-fragment

devoid of TPR1 domain (Deletion fragment 4, 5) cannot interact with CDK9 (Figure 4.7B).

Further, we also addressed this domain analysis in heterologous system (Sf9 system). We made baculoviruses expressing FLAG-tagged FKBP5 and all the domains. Subsequently, we co-transfected these viruses with baculovirus expressing NT-CDK9, and immunoprecipitation using FLAG-agarose beads followed by western blot shows the similar result as obtained in mammalian system i.e., FKBP5-fragment devoid of TPR1 domain (Deletion fragment 4, 5) cannot interact with CDK9 (Figure 4.7C).

The strength of interaction of different fragments of FKBP5 with CDK9 varies between two systems, which can be justified because of difefrent nature of these two systems for protein-protein interactions.

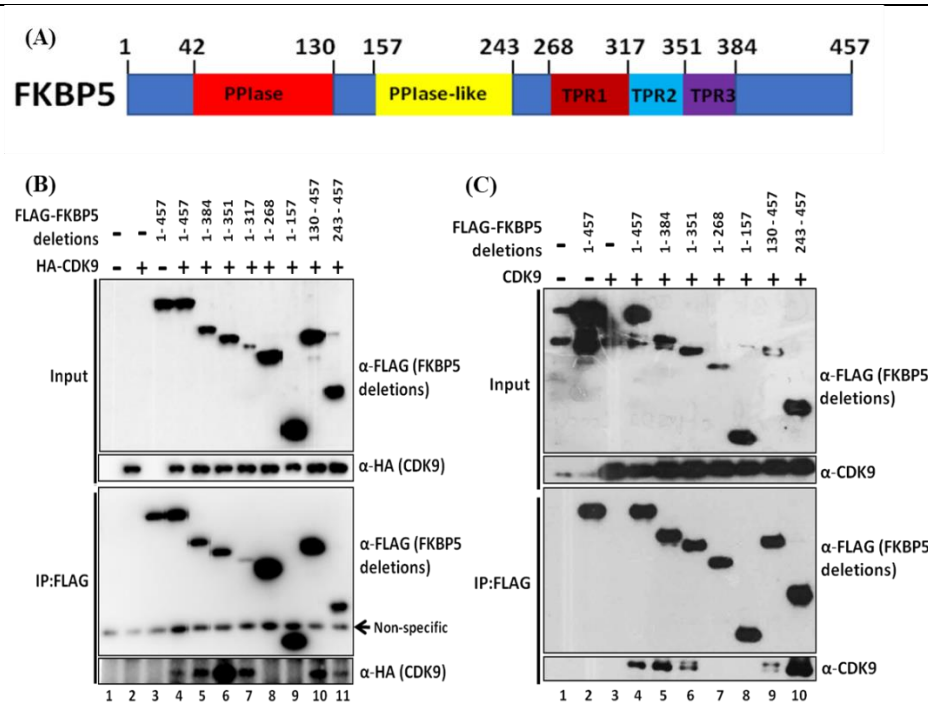


Figure 4.7 Domain analysis of FKBP5 for its interaction with CDK9.

(A) Cartoon diagram representing the important domains of FKBP5.

(B) The immunoblots representing FKBP5 domains (as indicated) and their interaction with CDK9 through their coexpression in heterologous Sf9 cells.

(C) The immunoblots representing FKBP5 domains (as specified) and their interaction with CDK9 through their coexpression in mammalian 293T cells and subsequent immunoprecipitation and western blotting analysis.

4.1.3 Functional importance of FKBP5 and CDK9 interaction.

Here, in this part of our study, after stablishing the interaction between FKBP5 and CDK9, we wanted check the functional importance of this interaction. To explore this arena, we did different *invitro* and cell-based assays which finally established FKBP5 as a negative regulator of transcription. Here, we have successfully shown that, FKBP5 competes with CyclinT1 to bind with CDK9 and ultimately makes the P-TEFb complex nonfunctional by dissociating it into CDK9 and CyclinT1. Detailed experimental design and results are provided here.

4.1.3.1 Effect of FKBP5 on P-TEFb-mediated phosphorylation activity *in vitro*.

First of all, we tried to see if FKBP5 have any effect on P-TEFb kinase activity *in vitro.* To test this, we designed different type of *in vitro* kinase assays using different P-TEFb substrates like Pol II CTD, NELF complex, and DSIF complex and tried to test the FKBP5 on phopshorylation activity of P-TEFb on these substrates. Our results in this part of study shows that FKBP5 have negative impact on P-TEFb kinase activity *in vitro*irrespective of the type of substrates being used in the assay. Effect of FKBP5 on P-TEFb-mediated phosphorylation of various substrates are discussed in this section in details.

4.1.3.1.1 Standardization of P-TEFb-mediated kinase assays*in vitro*.

On the basis of detection method, we performed two types of *in vitro* kinase assays, one is based on kinase reaction followed by western blotting and other is based on kinase reaction followed by autoradiographic detection. To perform all these assays, we purified all the required proteins and protein complexes like His-FKBP5, P-TEFb complex, GST-CTD, NELF complex, and DSIF complex. His-FKBP5, GST-CTD and DSIF complex were purified using bacterial expression system (detailed purification protocol is provided in material and method section) (Figure 4.8 A-C). P-TEFb and NELF complexes were purified using baculoviral expression system (detailed purification protocol is provided in material and method section) (Figure 4.8 D, E).

First of all, we standardized the phosphorylation assays for estimating proper enzyme substrate ratio. For that, we fixed the concentration of substrates with varying amount of P-TEFb to estimating the best possible ratio that could be used for subsequent assays (Figure 4.8 F, G).

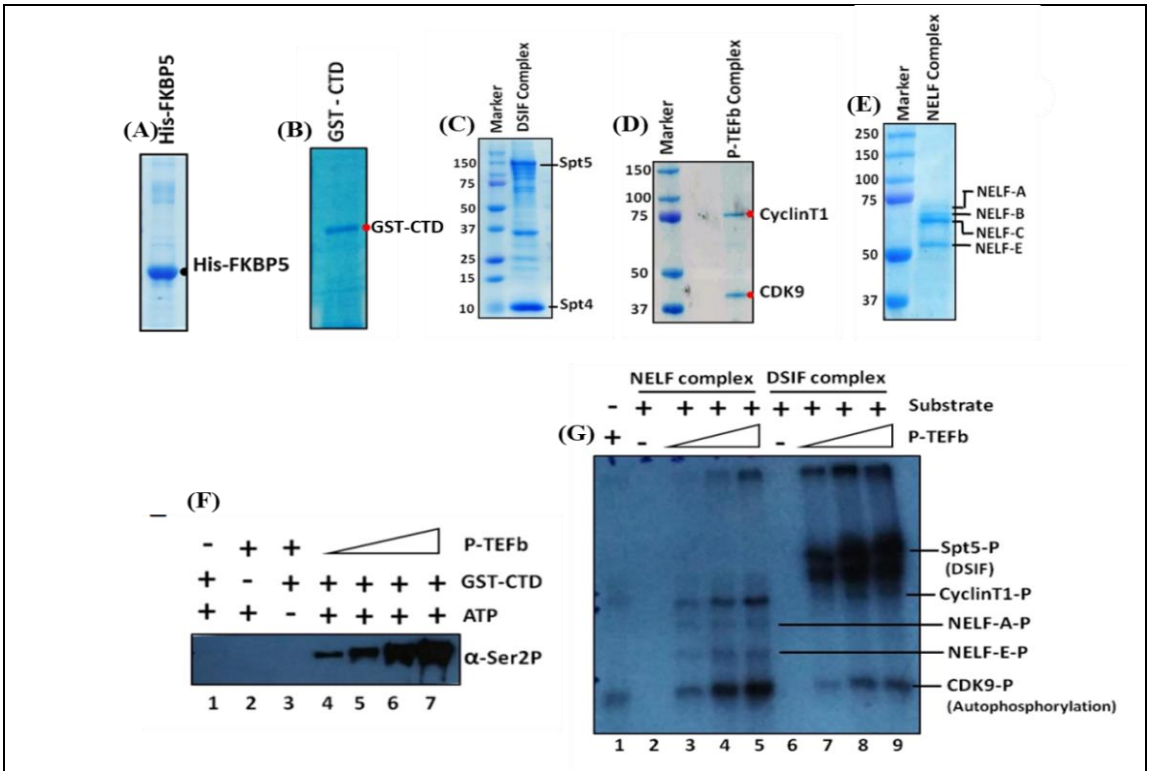


Figure 4.8 Standardization of *in vitro* kinase assays.

(A) SDS-PAGE Coomassie staining demonstrating the purification of recombinant His-FKBP5 through its expression in a bacterial expression system.

(B) SDS-PAGE Coomassie staining demonstrating the purification of recombinant GST-CTD through its expression in a bacterial expression system.

(C) SDS-PAGE, Coomassie staining demonstrating the purification of HIS-DSIF complex. The complex was purified using a bacterial expression system.

(D) SDS-PAGE coomassie staining demonstrating the purification of the P-TEFb complex. The P-TEFb complex was purified through the baculovirus-mediated expression of its subunits in Sf9 cells.

(E) SDS-PAGE coomassie staining demonstrating the purification of the NELF complex. The NELF complex was purified through the baculovirus-mediated expression of its subunits in Sf9 cells.

(F) *In vitro* kinase assay followed by immunoblotting analysis to test the enzymatic activity of purified P-TEFb complex. GST-CTD was used as a substrate and antibodies specific to phospho-Ser2 and -Ser5 were used for detecting phosphorylated GST-CTD protein.

(G) *In vitro* phosphorylation and subsequent autoradiography analysis showing P-TEFb-mediated phosphorylation of Spt5 subunit of DSIF complex, NELF-A and NELF-E subunits of NELF complex. γ32P-ATP was used for labelling the P-TEFb-mediated phosphorylation and subsequently autoradiographed for detecting phosphorylation events.

4.1.3.1.2 FKBP5 negatively regulates the phosphorylation activity of P-TEFb *in vitro.*

After standardizing *in vitro* kinases assays, we addressed the effect of FKBP5 on P-TEFb kinase activity. To perform these assays, we chose standardized concentration of enzyme and substrate.

First of all, we tested the effect of FKBP5 on P-TEFb-mediated phosphorylation of Pol II GST-CTD substrate. We tested this in two different ways, (i) FKBP5-concentration dependent and (ii) time dependent (0hrs-4hrs) in presence of constant FKBP5. In concentration dependent assay, followed by western blotting with α-Ser2P and α-Ser5P antibodies, we observed that the level of Ser2P and Ser5P significantly goes down when upon addition of increased concentraton of FKBP5 (Figure 4.9A). Again, in time-kinetic assay, it was followed by western blotting with α-GST antibody, where in absence of FKBP5 we found a significant up-shift with the time but there was no shift observed with the time in presence of FKBP5 (Figure 4.9B). From both of these experiments, we could clearly state that there is a strong negative effect of FKBP5 on P-TEFb-mediated kinase activity of GST-CTD substrate *in vitro*.

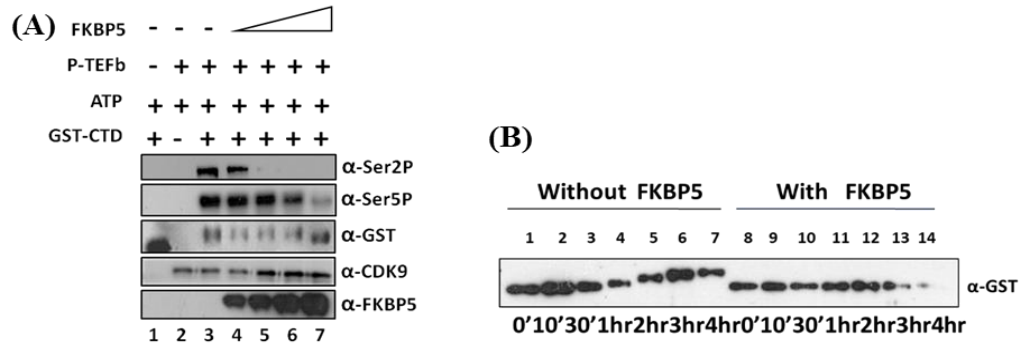


Figure 4.9 Effect of FKBP5 on P-TEFb kinase activity towards PolII-CTD *in vitro*

(A) *In vitro* kinase assay using purified P-TEFb complex, and GST-CTD as substrate and in presence/absence of purified HIS-FKBP5. Antibodies specific to phospho-Ser2 and phospho-Ser5 were used for detecting phosphorylated GST-CTD proteins.

(B) *In vitro* kinase assay using purified P-TEFb complex, and GST-CTD as substrate and in presence/absence of purified HIS-FKBP5. Antibody specific to GST was used for detecting shift of phosphorylated GST-CTD proteins.

4.1.3.1.3 There is no substrate specificity for FKBP5-mediated inhibition of P-TEFb kinase activity.

After observing strong negative effect of FKBP5 on P-TEFb kinase activity towards Pol II CTD phosphorylation, we addressed the substrate specificity of this inhibition. In order to study that, we chose other two well-known P-TEFb substrates i.e., DSIF complex and NELF complex. Again, two different kinase assays wereperformed with these two substrates in presence of FKBP5 and assay was followed by auto-radiography. Here also, not to our surprise, a very strong negative effect of FKBP5 was observed on P-TEFb kinase activity towards phosphorylation of Spt5 subunit of DSIF complex (Figure 4.10A) and NELF-A and NELF-E subunits of NELF complex (Figure 4.10B).

With these observations, it was concluded that FKBP5 have strong negative effect on P-TEFb kinase activity without any substrate specificity. Model depiting the inhibition of P-TEFb kinase activity by FKBP5 on diverse substrates is presented in Figure. 4.10(C).

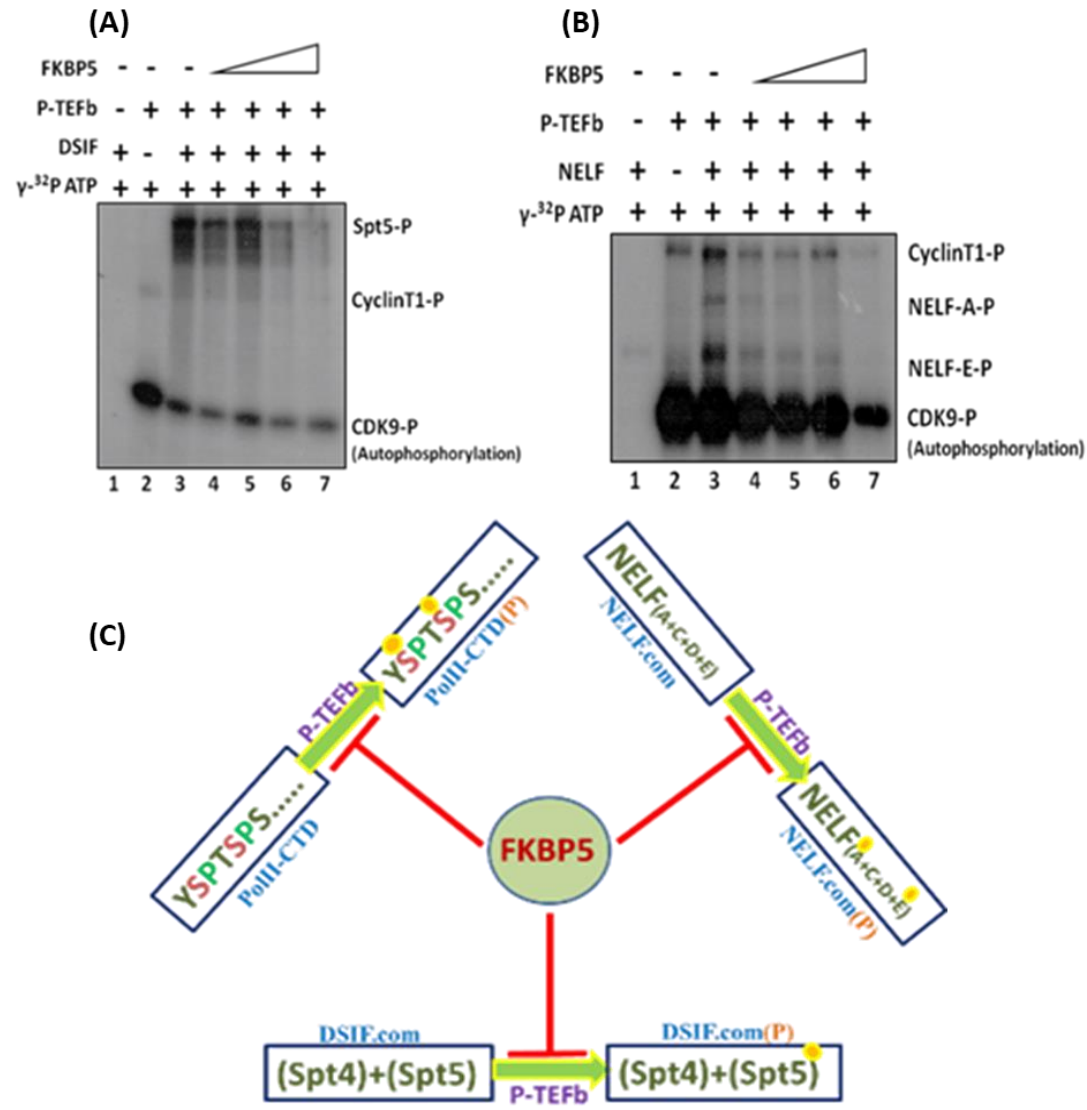


Figure 4.10 Effect of FKBP51 on P-TEFb kinase activity towards different substrates of P-TEFb.

(A) *In vitro* phosphorylation and subsequent autoradiography analysis showing inhibition of P-TEFb-mediated phosphorylation of Spt5 subunit of DSIF complex by FKBP5. γ32P-ATP was used for labelling the P-TEFb-mediated phosphorylation and subsequently autoradiographed for detecting phosphorylation events.

(B) *In vitro* phosphorylation and subsequent autoradiography analysis showing inhibition of P-TEFb-mediated phosphorylation of NELF-A and NELF-E subunits of NELF complex by FKBP5. γ32P-ATP was used for labelling the P-TEFb-mediated phosphorylation and subsequently autoradiographed for detecting phosphorylation events.

(C) Cartoon diagram representing substrate independent inhibition of P-TEFb kinase activity by FKBP5.

4.1.3.1.4 TPR1 domain of FKBP5 also plays the most important role for its inhibitory action towards kinase activity of P-TEFb *in vitro*.

After establishing FKBP5 as a negative regulator of P-TEFb kinase activity *invitro*, domain analysis of FKBP5 was performed to find out the specific domain, which is responsible for this inhibition. In order to do that, we purified all the deletion fragments of FKBP5 using bacterial expression system (Figure 4.11A, B). Subsequently, we used equal molar amount of all the fragments in kinase assay using GST-CTD substrate and P-TEFb, which was followed by western blotting to address inhibitory capacity of different deletion fragments on phosphorylation level of Pol II CTD. In this assay, we found out that the fragments devoid of TPR1 domain have least inhibitory capacity (Figure 4.11C).

Here, the point to be noticed is that the same domain of FKBP5 which is responsible for interaction with CDK9 is also responsible for inhibitory action of FKBP5 on P-TEFb.

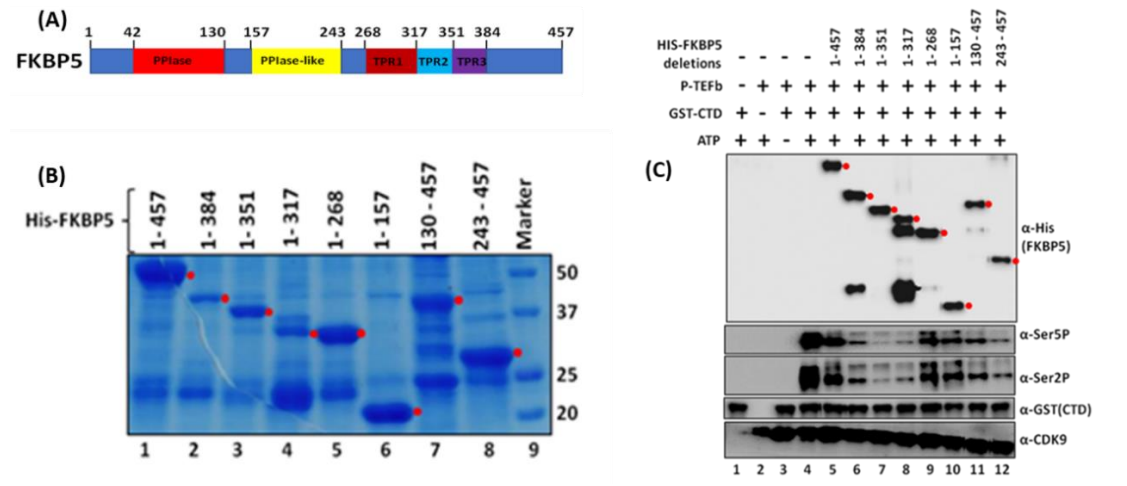


Figure 4.11 Identification of FKBP5 domain responsible for P-TEFb activity inhibition

(A) Cartoon diagram representing the important domains of FKBP5.

(B) SDS-PAGE Coomassie staining demonstrating the purification of recombinant His-FKBP5 domains through their expression in a bacterial expression system.

(C) *In vitro* phosphorylation and subsequent Western blot analysis demonstrate the influence of distinct FKBP5 domains on the suppression of P-TEFb-mediated phosphorylation of Pol II CTD at Ser2 and Ser5 residues. Each reaction mixture included 30 mM GST-CTD, 15 mM P-TEFb in each lane, and 250 ng of purified FKBP5 (full-length and deletion segments).

4.1.4 Global effect of FKBP5 on P-TEFb activity within mammalian cells.

Since we could successfully show that FKBP5 interacts with CDK9 and that this interaction is critical for its inhibitory effect on P-TEFb-mediated phosphorylation activity of diverse substrates, we also wanted to address whether this phenomenon would also be observed within mammalian cells. In this section, we discuss several experimental results showing role of FKBP5 in regulation of P-TEFb-mediated functions within mammalian cells and its implications in target gene expression.

4.1.4.1 Knockdown of FKBP5 in different cell lines

We generated sh-RNA-mediated stable FKBP5 knockdown in different cell lines to study the global effect on P-TEFb-mediated functions within mammalian cells (detailed protocol for making stable knockdown cell line is discussed in material method section). Knockdown efficiency was very high and protein level was checked through western blotting (Figure 4.12A-C).

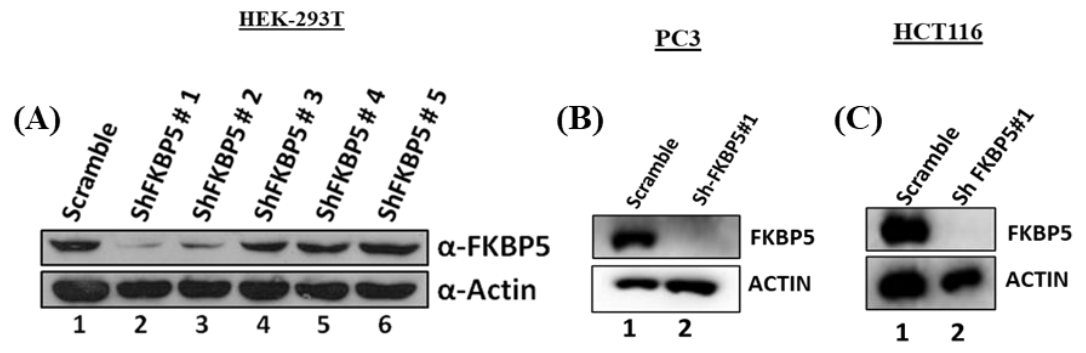


Figure 4.12 knocking down FKBP5 in different cell lines using a lentiviral system.

(A) Immunoblot assay demonstrating the stable knockdown of FKBP5 within mammalian 293T cells using multiple using short hairpin RNA (shRNA).
(B) Immunoblot assay demonstrating the stable knockdown of FKBP5 within mammalian PC3 cells using multiple using short hairpin RNA (shRNA).
(C) Immunoblot assay demonstrating the stable knockdown of FKBP5 within mammalian HCT116 cells using multiple using short hairpin RNA (shRNA).

4.1.4.2 Effect of FKBP5 knockdown on phosphorylation of P-TEFb targets inside the mammalian cell.

P-TEFb is known to regulate target gene expression by phosphorylating Pol II CTD at Ser2 and Ser5 position of heptad repeat (YSPTSP), Spt5 subunit of DSIF complex and NELF-A, NELF-E subunits of NELF complex. As it has already been shown earlier through *in vitro* analysis in this study that FKBP5 negatively regulates P-TEFb-mediated phosphorylation of GST-CTD substrate, we addressed the effect of FKBP5 knockdown on P-TEFb activity towards phosphorylation of Pol II CTD substrate inside the mammalian cells. Through western blotting we checked the change in level of phosphorylation of Ser2 ad Ser5 of Pol II CTD in FKBP5 knockdown condition. Immunoblotting analyses using whole cell extract clearly showed that upon FKBP5 knockdown there was a substantial increase in global Pol IICTD phosphorylation at both Ser2 and Ser5 residues when it was compared to control knockdown cells (Figure 4.13 A and B for quantitation). We also observed a significant increase in global PolII level, which may result in increased Ser2P and Ser5P levels. The other possibility is that, increased Ser2P and Ser5P level has earlier been shown to protect Pol II from proteasomal degradation by E3 ubiquitin ligase Def1 (Somesh et al. 2005). Normaization of increase in total Ser2P and Ser5P with that of total Pol II nonetheless showed modest increase in Ser2P and Ser5Pwithin mammalian HEK293T cells (Figure 4.13C).

Further we also addressed the cell-type specificity of the global changes observed upon FKBP5 knockdown in HEK293T. Similar results were also observed when we performed knockdown of FKBP5 in colon carcinoma cell line (HCT116) cell line (Figure 4.13 D and E, F for quantitation) as well as prostate cancer (PC3) cell line (Figure 4.13 G and H, I for quantitation) and thus rules out the observed global effect observed on FKBP5 to be limited to the only cell type i.e., mammalian 293T cells.

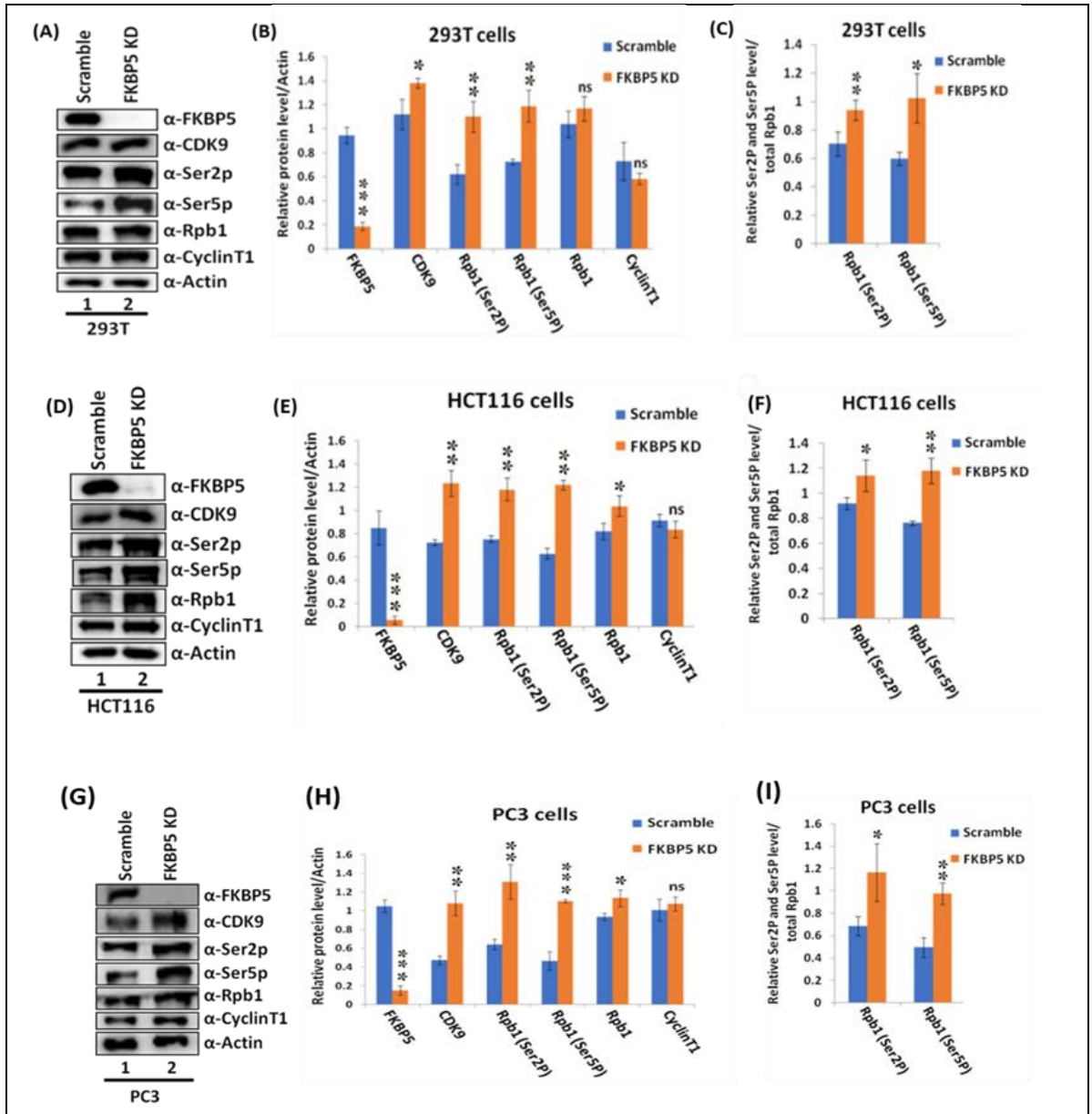


Figure 4.13 Global negative effect of FKBP51 on P-TEFb mediated phosphorylation in different cell lines.

(A) The impact of stable FKBP5 knockdown on global levels of several variables (as stated) within mammalian 293T cells is shown by immunoblot analysis.

(B) Densitometric quantification of various protein levels (as indicated) in control (scramble) and FKBP5 knockdown in mammalian 293T cells (as shown in Figure. 4.13 A). In our experiments, actin levels were employed as an internal control and for standardization.

(C) Quantification of the relative amount of increase in the phosphorylated Ser2 and Ser5 forms of Pol II in mammalian 293T cells, normalized to the total quantity of Pol II (as shown in Figure. 4.13 A).

(D) The impact of stable FKBP5 knockdown on global levels of several variables (as stated) within mammalian HCT116 cells is shown by immunoblot analysis.
(E) Densitometric quantification of various protein levels (as indicated) in control (scramble) and FKBP5 knockdown in mammalian HCT116 cells (as shown in Figure. 4.13 D). In our experiments, actin levels were employed as an internal control and for standardization.
(F) Quantification of the relative amount of increase in the phosphorylated Ser2 and Ser5 forms of Pol II in mammalian HCT116 cells, normalized to the total quantity of Pol II (as shown in Figure. 4.13 D).
(G) The impact of stable FKBP5 knockdown on global levels of several variables (as stated) within mammalian PC3 cells is shown by immunoblot analysis.
(H) Densitometric quantification of various protein levels (as indicated) in control (scramble) and FKBP5 knockdown in mammalian PC3 cells (as shown in Figure. 4.13 G). In our experiments, actin levels were employed as an internal control and for standardization.
(I) Quantification of the relative amount of increase in the phosphorylated Ser2 and Ser5 forms of Pol II in mammalian PC3 cells, normalized to the total quantity of Pol II (as shown in Figure. 4.13 G).

4.1.4.3 Global changes observed upon FKBP5 knockdown are dependent on its interaction with CDK9.

Next, we addressed whether the effects observed upon FKBP5 knockdown on global increase in Pol II CTD Ser2 and Ser5 phosphorylation were specific to FKBP5 effect on P-TEFb activity or represents an indirect effect. In order to address that, we re-expressed wild type (WT) FKBP5 in stable FKBP5 knockdown cells. Re-expressing WT FKBP5 reverses the effects observed upon FKBP5 knockdown on global increase in Pol II CTD Ser2 and Ser5 phosphorylation. More importantly, when we re-expressed the FKBP5 fragment (1-157), which does not interact with CDK9 (shown earlier), it could not reverse the effect observed upon FKBP5 knockdown, even when it is expressed higher than wild type (Figure 4.14 A and B for quantification).

So, these results also indicate that there is an important role of interaction between FKBP5 and CDK9 in the overall inhibitory effect of FKBP5 on P-TEFb kinase activity on Pol II GST-CTD substrate that we observed *in vitro* and *in vivo* within mammalian cells.

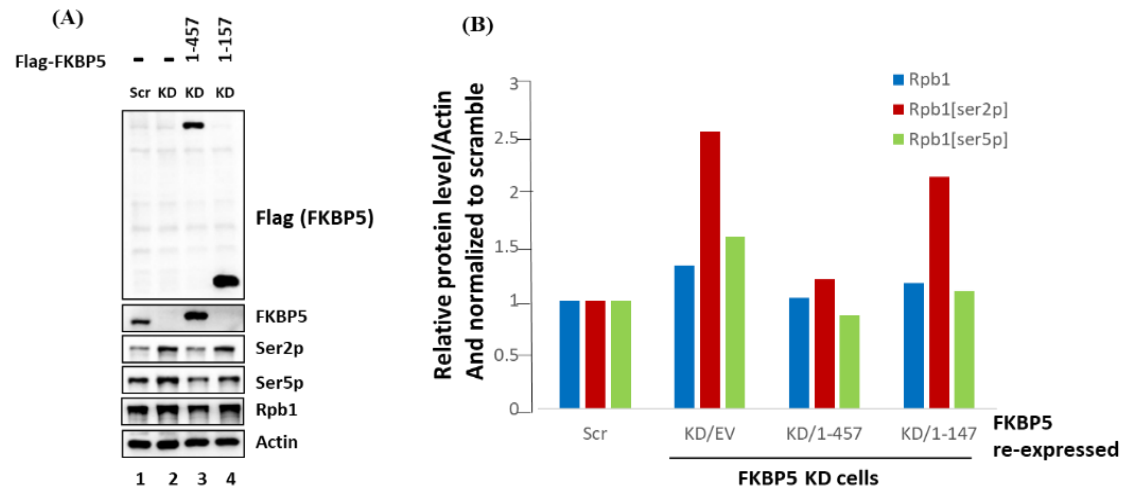


Figure 4.14 Reexpression of FKBP5 reverses the effects of its knockdown in mammalian 293T cells and this reversal effect is dependent on the CDK9 interacting domain of FKBP5.

(A) Immunoblot examination of the effect of reexpression of full-length and CDK9 interaction-deficient FKBP5 proteins on the global levels of different factors in FKBP5 knockdown 293T cells, as indicated.

(B) Quantification of the relative amount of changes in the phosphorylated Ser2 and Ser5 forms of Pol II in mammalian 293T cells. In this experiment, actin levels were employed as an internal control and for standardization.

4.1.4.4 FKBP5 regulates global Pol II CTD phosphorylation within mammalian cells in HSP90-independent manner.

The function of FKPP5 is well characterized as a co-chaperone of HSP90 (Fries, Gassen, and Rein 2017), which assists protein folding, trafficking and proteasomal degradation. Further, HSP90 has also been shown to assist CDK9 folding and stabilizing as client protein (Bacon and D'Orso 2018). Therefore, there is a possibility that, in FKBP5 knockdown condition there is enhanced activity of HSP90 towards folding of its client protein CDK9, resulting in production of higher level of active pool of P-TEFb complex which ultimately results in increased Pol II Ser2 and Ser5 phosphorylation as observed in different cell lines.

To test this HSP90-dependent effect upon FKBP5 knockdown, we designed two different experiments. In first approach, we used a chemical inhibitor of HSP90 i.e.,

Geldanamycin. We treated stable FKBP5 knockdown cells with geldanamycin and, through subsequent western blotting, we tested the effect on global changes of Pol II Ser2 and Ser5 phosphorylation which we observed in FKBP5 knockdown condition. We observed that the treatment with geldanamycin reduces the level of CDK9, a known effect as reported earlier (Citri et al. 2006), however, it fails to show any effect on global increase in Pol II Ser2 and Ser5 phosphorylation (Figure 4.15 A). In our second approach, we tried to see the change in association between HSP90 and CDK9 in FKBP5 knockdown condition. For this, we overexpressed FLAG-CDK9 ectopically in FKBP5 knockdown cell line and in control scramble knockdown cell line. Subsequent immunoprecipitation using FLAG beads followed by western blotting shows no difference in HSP90-CDK9 association upon FKBP5 knockdown when compared to scramble control (Figure 4.15 B).

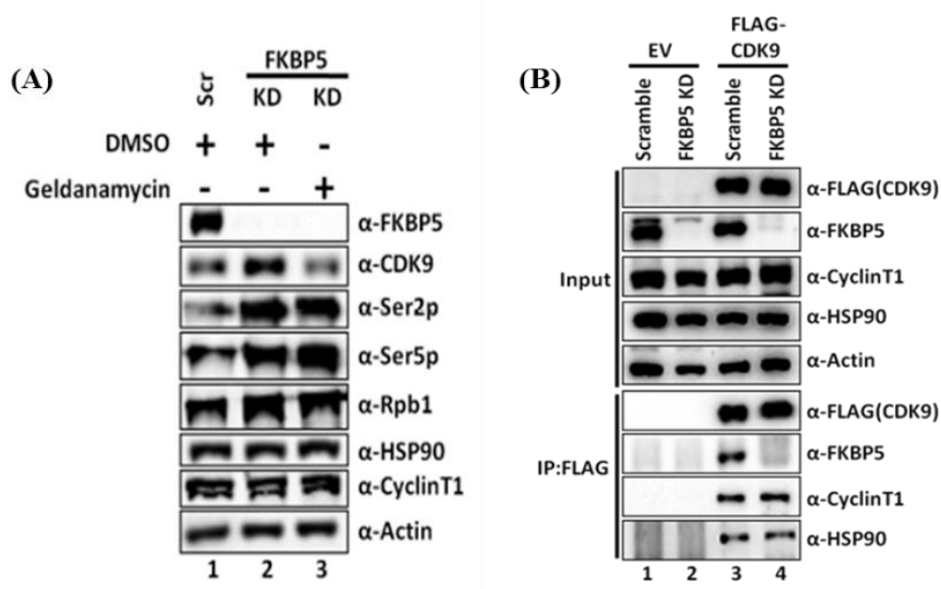


Figure 4.15 FKBP5 mediated P-TEFb inhibition is HSP90 independent.

(A) Immunoblot analysis of the effect of geldanamycin treatment (5 mM final concentration) on the global level of Pol II CTD phosphorylation in 293T cells in FKBP5 knockdown cells. The CDK9 level was employed as a control for the geldanamycin treatment.

(B) Immunoblot analysis showing the effect of FKBP5 knockdown on the interaction between ectopically expressed FLAG-CDK9 and endogenous HSP90 compared to that in control scramble cells.

4.1.5 Mechanism of FKBP5 mediated P-TEFb inhibition:

Our earlier results have shown that FKBP5 has an inhibitory role in regulation of P-TEFb function *in vitro* as well as within mammalian cells. We have also shown that interaction between FKBP5 and CDK9 plays an important role in this inhibitory effect of FKBP5. In HEK293T cells, it has also been shown that the global changes observed in level of Pol II Ser2 and Ser5 phosphorylation upon FKBP5 knockdown, is independent of HSP90. Still the mechanism behind this inhibitory effect of FKBP5 on P-TEFb need to be explored. In this section, we describe the essential mechanism of functions of FKBP5 towards negative regualtion of functions of P-TEFb complex.

4.1.5.1 FKBP5 does not interact with P-TEFb substrates:

It's a very common phenomena and there are several studies which show the site inhibitors binds to the substrates and thus block the access to the effector proteins for performing their site-specific modifications. In order to explore the working mechanism behind FKBP5 mediated P-TEFb inhibition, we initially addressed whether FKBP5 interacts with the substrates of P-TEFb and thus blocks the site of phosphorylation. At first, we did interaction analysis between FKBP5 and Pol II CTD *in vitro*. For this interaction analysis we purified His-FKBP5 and GST-CTD. Subsequently, we performed *in vitro* interaction analysis (detailed protocol has been described in material method section) followed by western blotting. In this assay, we haven't observed any interaction between FKBP5 and Pol II CTD (Figure 4.16 A, B, E)

Again, we tested interaction of FKBP5 with other substrates of P-TEFb i.e., DSIF complex and NELF complex. For these assays we purified GST-FKBP5, DSIF complex and NELF complex (detailed purification protocol has been described in material method section). Subsequently, we performed *in vitro* interaction analysis separately for both the substrates (detailed protocol has been described in material method section) followed by western blotting. In both of these assays we neither found any interaction between FKBP5 and Spt5 subunit of DSIF complex nor with NELF-A or NELF-E subunits of NELF complex which are known to get phosphorylated by P-TEFb (Figure 4.16 A, C, D, F and G). Thus, we can conclude from our results that the substrate blocking mechanism does not play any role in this case of inhibition of P-TEFb by FKBP5.

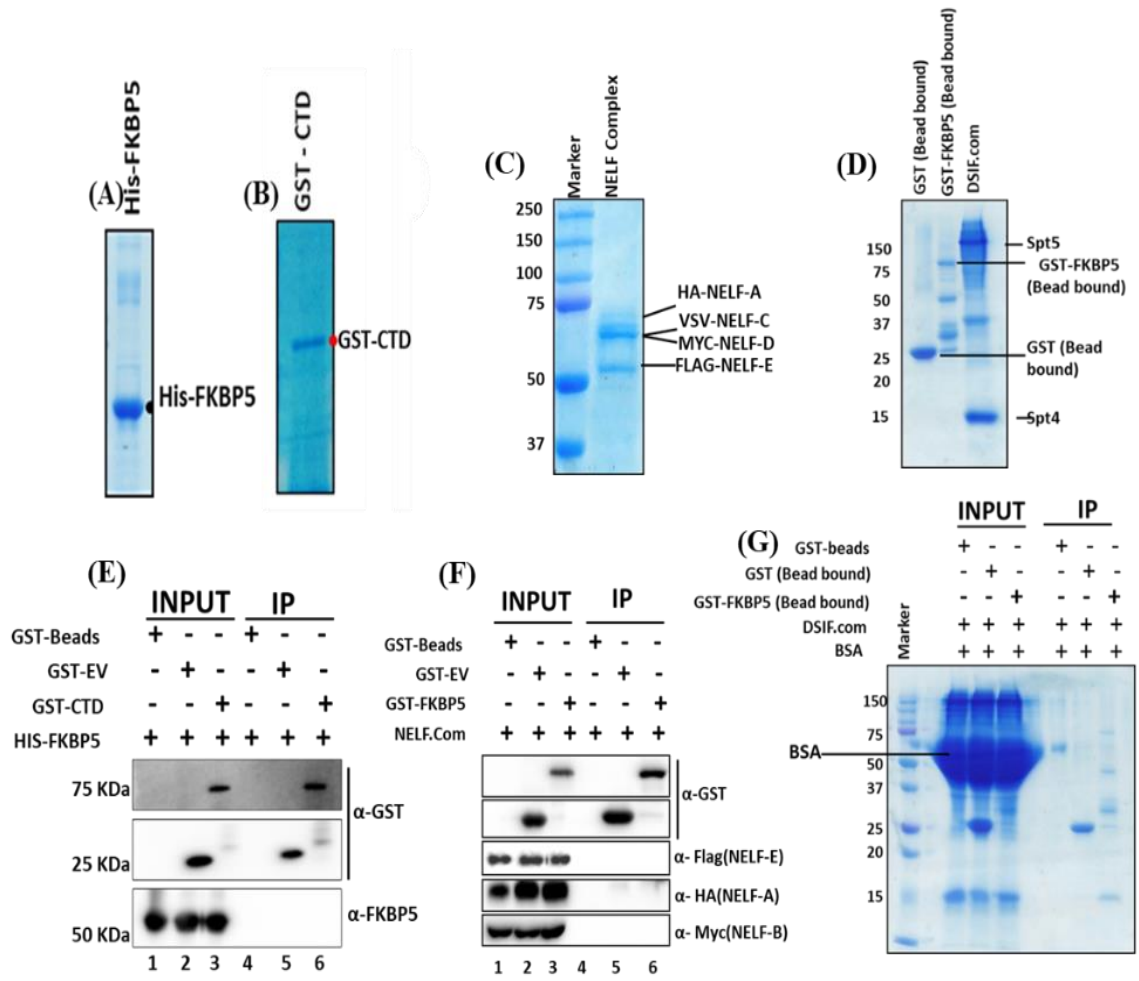


Figure 4.16 Interaction analysis of FKBP5 with different P-TEFb substrates.

(A) SDS-PAGE Coomassie staining demonstrating the purification of recombinant His-FKBP5 through its expression in a bacterial expression system.

(B) SDS-PAGE Coomassie staining demonstrating the purification of recombinant GST-CTD through its expression in a bacterial expression system.

(C) SDS-PAGE coomassie staining demonstrating the purification of the NELF complex. The NELF complex was purified through the baculovirus-mediated expression of its subunits in Sf9 cells.

(D) SDS-PAGE, Coomassie staining of purified HIS-DSIF complex, GST, and GST-FKBP5. These proteins were purified using a bacterial expression system.

(E) Immunoblotting analysis of *in vitro* interaction assay showing failure of GST-CTD to interact with FKBP5.

(F) Immunoblotting analysis of *in vitro* interaction assay showing failure of NELF complex to interact with FKBP5.

(G) SDS-PAGE Coomassie staining of *in vitro* interaction assay showing failure of DSIF complex to interact with FKBP5.

4.1.5.2 FKBP5 does not have phosphatase activity.

As we discussed earlier that P-TEFb is a kinase which phosphorylates its substrates, which have their different downstream effects. In this study until now, through many *in vitro* assays, we have already shown that in presence of FKBP5 reduces kinase activity of P-TEFb. As we already ruled out the possibility of substrate blocking mechanism of inhibition, there is strong possibility for a P-TEFb inhibitor (FKBP5) to have phosphatase activity and thus resulting in reduced level of substrate phosphorylation as observed. We applied two different approaches to examine the phosphatase activity of FKBP5 *in vitro*. In first assay, we applied autoradiography-based assay, where P-TEFb was incubated with radioactive ATP (P^{32}) to get auto-phosphorylated at CDK9. Subsequently, FKBP5 was added to the reaction mix and incubated for 2hrs. Autoradiography was done to visualize the changes in CDK9 phosphorylation level. But we could not observe any decrease in intensity of CDK9 phosphorylation signal with increasing FKBP5 (Figure 4.17A). So, based on this assay, we can presume that FKBP5 does not have any phosphatase activity (at least towards auto-phosphorylated CDK9). Further, another different type of colorimetric assay was done to test the phosphatase activity of FKBP5, where PNPP (Para Nitro Phenyl Phosphate) was used as substrate and calf intestinal phosphatase (CIP), bovine serum albumin (BSA) was used as positive and negative control respectively. Here also, consistent with our earlier observation, we couldn't see any phosphatase activity of FKBP5 (Figure 4.17B, C).

Thus, we conclude that FKBP5 doesn't have any phosphatase activity and it has already been shown that it does not function as substrate blocking mechanism of P-TEFb inhibition. So, there could possibly be some other mechanism by which FKBP5 negatively regultes kinase activity of P-TEFb. These aspects are explored in our subsequent assays.

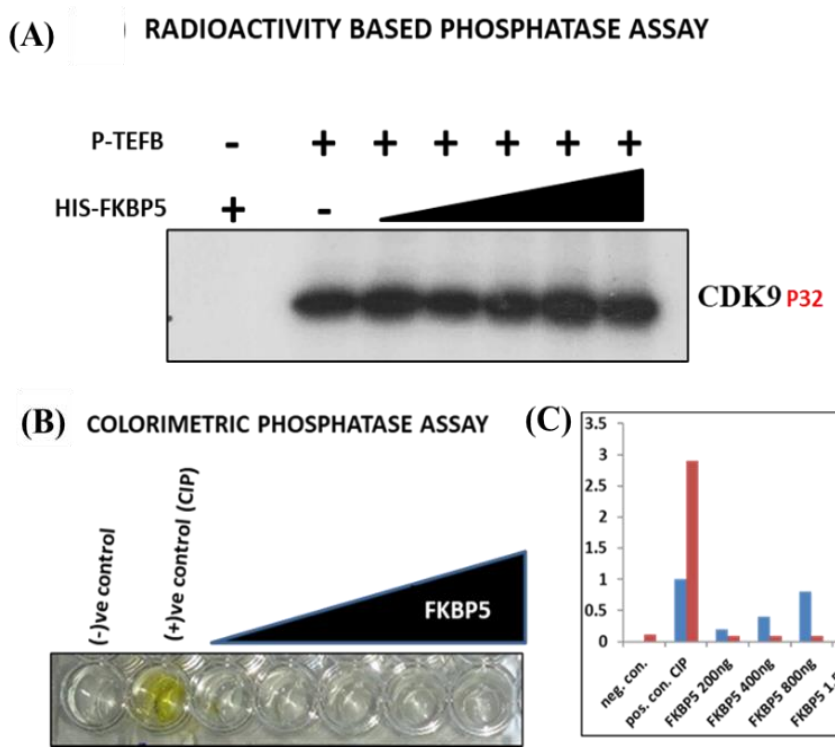


Figure 4.17 *In vitro* phosphatase assays to test phosphatase activity of FKBP5.

(A) Autoradiograph showing failure of FKBP5 possessing any phosphatase activity. Pre-phosphorylated (using ATP^{32}) CDK9 was used as a substrate in an i*n vitro* phosphatase assay.

(B) *In vitro* Colorimetric Phosphatase assay showing failure of FKBP5 possessing any phosphatase activity.

(C) Quantification of colorimetric phosphatase assay.

4.1.5.3 FKBP5 and CyclinT1 interact with the same domain of CDK9.

There was an indication in interaction analysis of FKBP5 with CDK9 and CyclinT1 in Sf9 system that FKBP5 and CDK9 probably interacts with the same domain of FKBP5 (as there was no interaction between CyclinT1 and CDK9 when FKBP5 was present). Later on, through different assays, we observed a strong negative effect of FKBP5 on P-TEFb kinase activity. Taking these two observations together, we can presume that there might be competition between FKBP5 and Cyclin T1 to interact with CDK9, if both the proteins interact with the same domain of FKBP5. For addressing this, we performed domain analysis of CDK9 for its interaction with FKBP5 and CyclinT1. In

order to do the domain analysis, we cloned different fragments (Figure 4.18 A) of CDK9 to express in different systems.

Subsequently, for domain analysis of CDK9 for its interaction with FKBP5 in Sf9 system, we generated P2-baculoviruses expressing CDK9 fragments as FLAG-tag and co-infected with P2-bacculoviruses of NT-FKBP5 (non-tag FKBP5). Subsequent immunoprecipitation followed by western blotting showed that extreme N-terminus (1-31 aa) of CDK9 interacts with FKBP5 (Figure 4.18 B). Again, in similar set of experiment in Sf9, P2-baculoviruses expressing CDK9 fragments as FLAG-tag were co-infected with P2-bacculoviruses of NT-CyclinT1 (non-tag Cyclin T1). Subsequent immunoprecipitation followed by western blotting showed that the same extreme N-terminus (1-31 aa) of CDK9 interacts with CyclinT1 too (Figure 4.18 C).

As both of the above-mentioned assays were done in a heterologous system i.e., Sf9 system, we wanted to confirm these results in mammalian system (in HEK293T cells). In order to address this, we transfected CDK9 fragments expressing as FLAG-tag in HEK293T. Subsequent immunoprecipitation using FLAG beads followed by western blotting with FKBP5 antibody again showed the similar result as obtained from Sf9 system, i.e., N-terminal 1-31 aa of CDK9 interacts with FKBP5 (Figure 4.18 D). In the same assay western blotting with CyclinT1 antibody also showed the similar result as obtained in Sf9 system i.e., N-terminal 1-31aa of CDK9 interacts with CyclinT1 (Figure 4.18 E). These analyses strongly indicate presence of competition between FKBP5 and Cyclin T1 to interact with CDK9 possibly leading to disruption of P-TEFb complex and thus ultimately negatively regulating its functions.

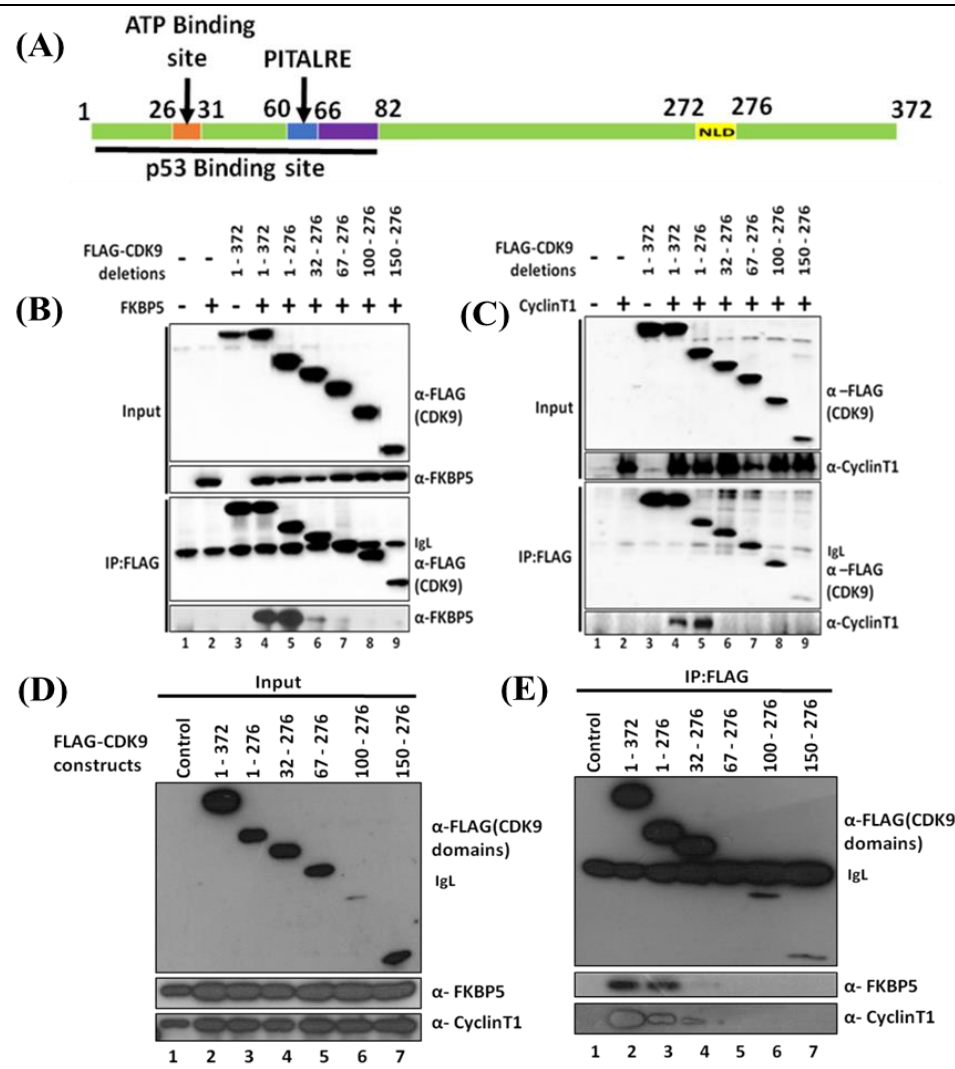


Figure 4.18 Domain analysis of CDK9 for its interaction with FKBP5 and CyclinT1.

(A) Cartoon graphic depicting the several functional domains of CDK9 that are critical for its activities. NLD stands for nuclear localization domain. ATP-binding site (amino acids 26–31); PITALRE (amino acids 60–66); p53-binding site (amino acids 1–82).

(B) Domain analysis of CDK9 for its interaction with FKBP5 in Sf9 cells using baculovirus-mediated expression (using several domains as indicated). Sf9 cells were co-infected with the baculoviruses as specified followed by immunoprecipitation and subsequent Western blotting for identifying their interaction within this heterologous system.

(C) Domain analysis of CDK9 for its interaction with CyclinT1 in Sf9 cells using baculovirus-mediated expression (using several domains as indicated). Sf9 cells were co-infected with the baculoviruses as specified, followed by immunoprecipitation and subsequent Western blotting for identifying their interaction within this heterologous system.

(D) , **(E)** Domain analysis of CDK9 for its interaction with FKBP5 and CyclinT1 in mammalian 293T cells. Cells were transfected with plasmids expressing different CDK9 domains with FLAG tag, and cell lysates were immunoprecipitated and analyzed using factor-specific antibodies for determining their interaction inside 293T cells.

4.1.5.4 FKBP5 and CyclinT1 compete with each other for their binding to CDK9 *in vitro* and *in vivo*.

As of now, in several assays, there is an indication of competition between FKBP5 and CyclinT1 for interaction with CDK9. Further, we designed experiments to explore this possibility which can explain the FKBP5 mediated inhibition of P-TEFb functions.

In the initial analysis in HEK293T cells, we co-transfected equal amount of FLAG-CDK9 construct and increasing amount of GAL-FKBP5 construct to test whether increased expression and subsequent binding of FKBP5 with CDK9 would reduce its binding with endogenous CyclinT1. Subsequent, immunoprecipitation using FLAG beads followed by western blotting showed decreased association between CDK9-CyclinT1 on increased expression and binding of FKBP5 with CDK9 and thus indicating presence of competition between FKBP5 and CyclinT1 for interaction with CDK9 (Figure 4.19A compare lane 3 vs 5). In *vice versa* experiment, where increasing expression of HA-CyclinT1 was transfected with fixed expression of FLAG-CDK9 was used to test whether increased expression and subsequent binding of CyclinT1 with CDK9 CDK9 would reduce its binding with endogenous FKBP5. FLAG-CDK9 immunoprecipitation followed by western blotting showed decreased association between CDK9-FKBP5 on increased expression of CyclinT1 and its binding to CDK9. Here also, clear competition can be seen between FKBP5 and CyclinT1 for interaction with CDK9, as increased expression and thus binding of CyclinT1 concomitantly reduced CDK9 interaction with endogenous FKBP5 (Figure 4.19 B compare lane 3 vs 5).

Above-mentioned competition assays have been done through ectopically expressed factors, which can differ from normal cellular conditions. Therefore, to explore the scenario in normal cellular condition, immunoprecipitation of endogenous CDK9 was done using stable FKBP5 knockdown cells followed by western blotting. In this analysis, when compared with control scramble, we observed significantly increased CDK9 associated CyclinT1 in FKBP5 knockdown cells (Figure 4.19C compare lane 2 vs 3). To confirm that these effects are because of FKBP5-CDK9 interaction, we co-transfected HA-CDK9 and full-length FKBP5 (FL-FKBP5) and CDK9-interaction

deficient domain in equal amount followed by HA-immunoprecipitation to test endogenous CyclinT1 interaction with CDK9. Here also we found that CDK9 interaction deficient domain of FKBP5 did not have any effect on CDK9-CyclinT1 interaction (Figure 4.19D), whereas, the full-length FKBP5 showed reduced interaction between CDK9 and endogenous CyclinT1.

Inside the cell there are multiple other factors which can have their direct or indirect effect on interaction between two proteins. To rule out the possibility of unknown factors affecting the overall interaction and thus our interpretation, we performed competition assay with purified proteins *in vitro*. For this we purified FLAG-CDK9 from HEK293T, His-FKBP5 and GST-CyclinT1 using bacterial system (purification protocols are described in material method section) (Figure 4.20A, B, C). Before performing *in vitro* competition assays we wanted to check the strength of *in vitro* interaction between CDK9-FKBP5 as well as CDK9-CyclinT1. At first, *in vitro* interaction analysis was performed using bead-bound FLAG-CDK9 and purified His-FKBP5 in presence of BSA. We observed a very strong interaction between CDK9 and FKBP5 (Figure 4.20 D). Similar *in vitro* interaction analysis using bead-bound FLAG-CDK9 and purified GST-CyclinT1 in presence of BSA also showed a very strong interaction between CDK9 and CyclinT1 (Figure 4.20E). Next, we designed different *in vitro* competition assays using purified proteins, to show the competition between FKBP5 and CyclinT1. In our in vitro assay, we have used bead-bound FLAG-CDK9 and added equal amount of GST-CyclinT1 with increasing amount of His-FKBP5. After incubation for binding, western blot analysis was peerformed that showed decreased CDK9 binding with CyclinT1 with increasing binding of FKBP5 (Figure 4,20F). Similar experiment was performed with bead-bound FLAG-CDK9, equal amount of His-FKBP5 and increasing amount of GST-CyclinT1. After incubation for binding western blot analysis was performed that showed decreased CDK9 binding with FKBP5 upon increasing CyclinT1 (Figure 4.20G).

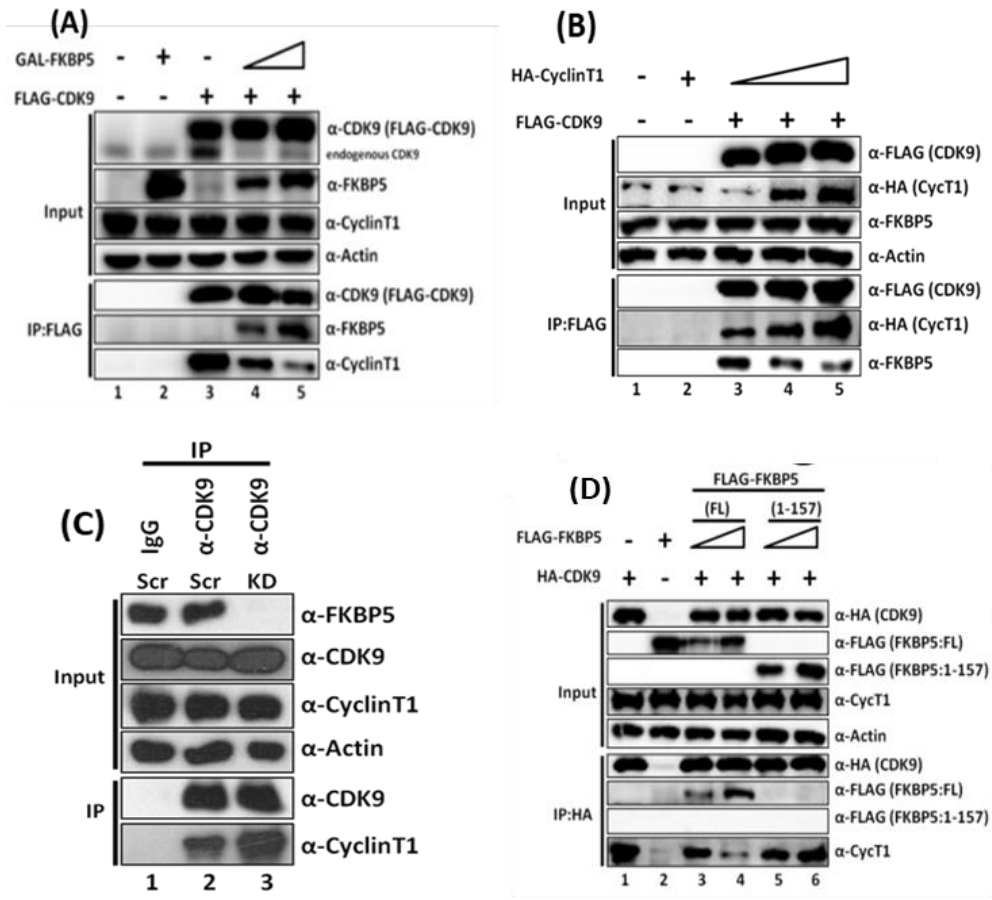


Figure 4.19 FKBP5 and CyclinT1 compete with each other for their binding to CDK9 in vivo within mammalian cells.

(A) Immunoblotting analysis showing the effect of FKBP5 overexpression on association of CDK9 and CyclinT1 in mammalian 293T cells.293T cells were co-transfected with the indicated plasmids, and cell lysates were immunoprecipitated using epitope tag-specific agarose beads, followed by western blotting analysis using factor-specific antibodies to identify their interaction.

(B) Immunoblotting analysis showing the effect of CyclinT1 overexpression on association of CDK9 and FKBP5 in mammalian 293T cells.293T cells were co-transfected with the indicated plasmids, subsequent immunoprecipitation using epitope tag-specific agarose beads, followed by western blotting analysis using factor-specific antibodies to identify their interaction.

(C) Immunoblotting assay demonstrating the effect of FKBP5 knockdown on endogenous CDK9-CyclinT1 interaction. Endogenous CDK9 was immunoprecipitated with a CDK9 antibody, and its interaction with endogenous CyclinT1 was determined using western blotting.

(D) Immunoblotting analysis of the effect of FL-FKBP5 and CDK9 interaction-defective (1-157 amino acids) FKBP5 proteins on ectopically expressed CDK9 and endogenous CyclinT1 association in 293T cells. 293T cells were co-transfected with the indicated plasmids, subsequent immunoprecipitation using epitope tag-specific agarose beads, followed by western blotting analysis using factor-specific antibodies to identify their interaction.

4.1.5.5 FKBP5 can dissociate preformed P-TEFb complex into CDK9 and CyclinT1.

Until now, FKBP5 has been shown to compete with CyclinT1 to bind with CDK9. Now we wanted to examine the potential of FKBP5 to dissociate preformed P-TEFb complex, which will provide more realistic explanation of FKBP5-mediated inhibition of P-TEFb complex function. In order to do that we applied two approaches to get preformed, bead-bound P-TEFb which we used in subsequent dissociation assays. In first approach, we reconstituted P-TEFb through *in vitro* binding assay using purified FLAG-CDK9 and GST-CyclinT1. Subsequent incubation of this preformed complex with increasing amount of purified FKBP5 and downstream western blotting analyese showed that FKBP5 have potential to dissociate CyclinT1 from the preformed P-TEFb (Figure 4.20 H, I). In second approach, we purified preformed P-TEFb from whole cell lysate. Again, similar experiment was done where addition of increasing amount of FKBP5, and subsequent analysis by western blotting showed same result that FKBP5 have potential to dissociate CyclinT1 from preformed P-TEFb complex from mammalain cells (Figure 4.20J).

Thus, with all these results mentioned until now, we have established the mechanism of regulation of P-TEFb complex functions by FKBP5 through a competition model that shows presence of competition between FKBP5 and CyclinT1 for their binding to CDK9 resulting in FKBP5 dissociating CyclinT1 fron available P-TEFb complex *in vitro* as well as *in vivo* within mammalian cells. This mechanism clearly explain the negative role of FKBP5 in regulation of P-TEFb functions as observed in multiple of our studies presented earlier (Figure 4.20K).

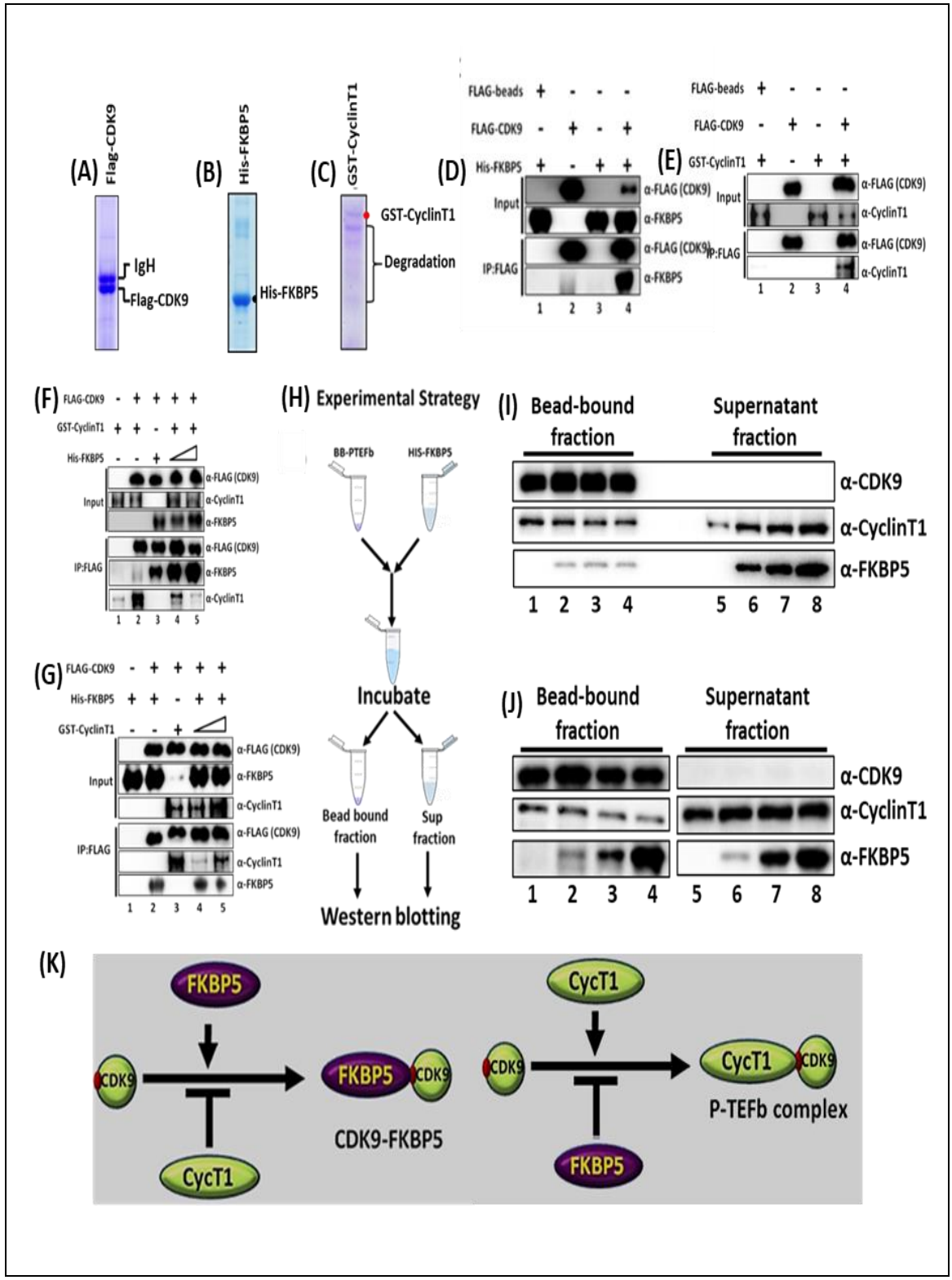

(A)
Flag-CDK9
IgH
Flag-CDK9
(B)
His-FKBP5
His-FKBP5
(C)
GST-CyclinT1
GST-CyclinT1
Degradation
(D)
FLAG-beads
FLAG-CDK9
His-FKBP5
Input
IP:FLAG
α-FLAG (CDK9)
α-FKBP5
(E)
GST-CyclinT1
α-CyclinT1
(F)
(G)
(H) Experimental Strategy
BB-PTEFb
HIS-FKBP5
Incubate
Bead bound fraction
Sup fraction
Western blotting
(I) Bead-bound fraction
Supernatant fraction
α-CDK9
α-CyclinT1
α-FKBP5
(J)
(K)
FKBP5
CDK9
CycT1
CDK9-FKBP5
P-TEFb complex

Figure 4.20 *In vitro* competition assays showing competition between FKBP5 and CyclinT1 .

(A) SDS-PAGE Coomassie staining demonstrating the purification of FLAG-CDK9 through overexpression in mammalian 293T cells and subsequent purification in high salt buffer conditions.

(B) SDS-PAGE Coomassie staining demonstrating the purification of recombinant His-FKBP5 through its expression in a bacterial expression system.

(C) SDS-PAGE Coomassie staining demonstrating the purification of recombinant GST-CyclinT1 through its expression in a bacterial expression system.

(D) Immunoblotting analysis showing a direct interaction between CDK9 and FKBP5. In this *in vitro* interaction analysis, purified recombinant CDK9 and FKBP5 proteins were utilized and added as stated.

(E) Immunoblotting analysis showing a direct interaction between CDK9 and cyclin T1. In this *in vitro* interaction analysis, purified recombinant CDK9 and cyclin T1 proteins were utilized and added as stated.

(F) An immunoblotting analysis demonstrating the effect of increasing FKBP5 on CDK9 and CyclinT1 interaction *in vitro*. Purified recombinant FKBP5, CDK9 and cyclin T1 proteins were utilized and added as stated.

(G) An immunoblotting analysis demonstrating the effect of increasing CyclinT1 on CDK9 and FKBP5 interaction *in vitro.* Purified recombinant FKBP5, CDK9 and cyclin T1 proteins were utilized and added as stated

(H) Cartoon diagram depicting the strategy for the *in vitro* dissociation assay.

(I) *In vitro* dissociation assay showing the effect of the addition of FKBP5 in dissociating Cyclint1 from preformed P-TEFb complex. Purified, bead-bound P-TEFb complex obtained through the recombinant expression of FLAG-CDK9 and GST-cyclin T1.

(J) *In vitro* competition assay showing inhibition of P-TEFb complex formation from its constituents in the presence of the FKBP5. Purified recombinant FKBP5, CDK9 and cyclin T1 proteins were utilized and added as stated.

(K) Cartoon diagram depicting the presence of competition between CyclinT1 and FKBP5 for their interaction with CDK9.

4.1.6 FKBP5 regulates expression of diverse sets of genes.

From our initial experiments and observations, we established mechanistic understanding of negative regulation of P-TEFb functions by FKBP5. Next, we were interested to explore the role this regulation on expression of global P-TEFb target genes. In order to study that, we used stable FKBP5 knockdown cell line to perform RNA-seq analysis to identify the set of genes, expressions of which are being affected upon FKBP5 knockdown.

Transcriptomic analysis of FKBP5 knockdown cell line revealed a set of 219 upregulated genes (with $p < 0.5$) and a set of 145 downregulated genes (with $p < 0.5$) when compared with control. A heatmap showing some of these genes presented in Figure 4.21(B).

Interestingly, when we performed gene-ontology analysis (using Database for Annotation, Visualization and Integrated Discovery (DAVID) software) of this RNA-seq data to identify and classify the biological processes affected by these genes, we observed strong association of multiple upregulated genes in several cellular processes including Pol II-mediated transcriptional control, cellular proliferation, cellular differentiation and many other important cellular processes (Figure 4.21(C)). However, the downregulated gene sets were primarily associated with different metabolic processes as observed in our analyses.

These RNA-seq analyses clearly indicate that there is definite role of FKBP5 in regulation of Pol II-mediated transcriptional process for expression of set of those genes which are majorly involved in cellular proliferation and differentiation processes. Thus, our subsequent studies are designed to explore mechanisms of these regulations in details for regulation of target gene expression by FKBP5 through regulation of P-TEFb-mediated phosphorylation activity.

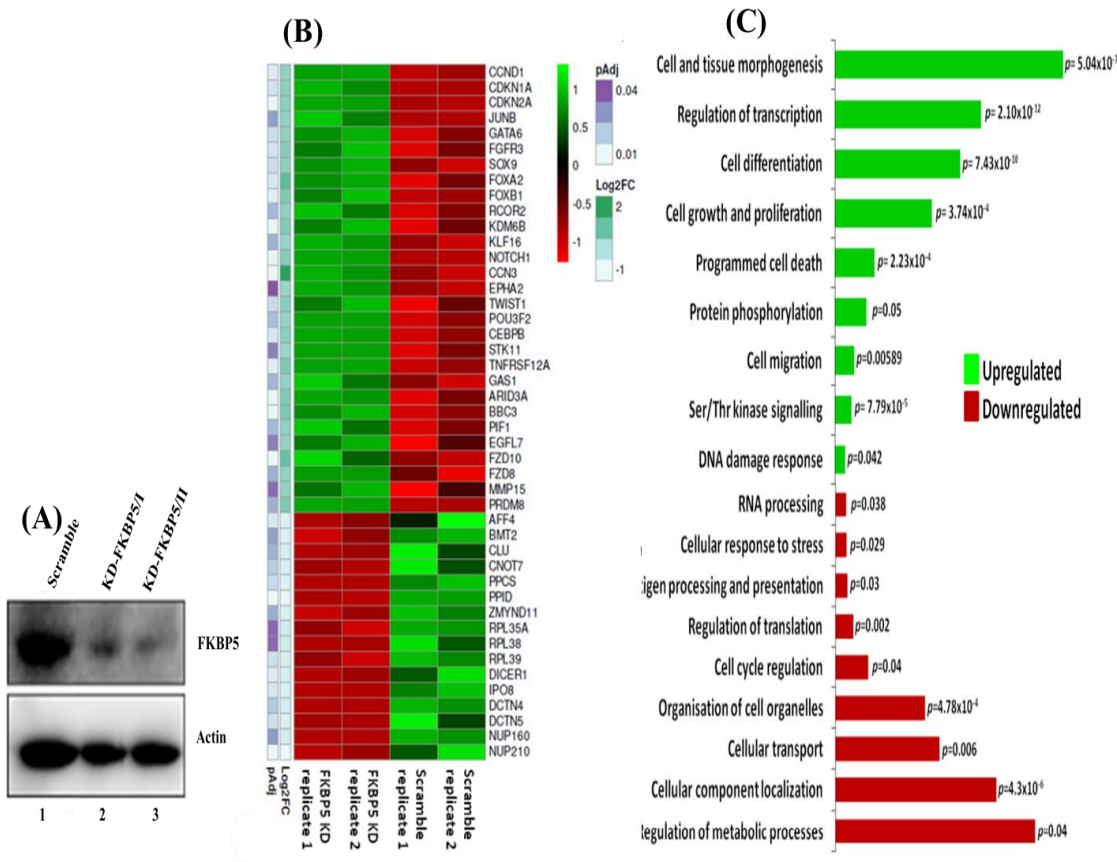


Figure 4.21 FKBP5 knockdown enhances the expression of a large no of proliferation-related genes.

(A) Immunoblot showing sh-RNA mediated FKBP5 knockdown in mammalian 293T cells. These cells were used for RNAseq analysis.

(B) Heatmap demonstrating the effect of FKBP5 knockdown on the expression of selected target genes compared to their expression in control scramble cells. Log2FC denotes fold change on a Log2 scale.

(C) Gene Ontology (GO) analysis predicting the connection of up-regulated and down-regulated genes with diverse biological processes after FKBP5 knockdown.

4.1.6.1 FKBP5 level regulates the expression of P-TEFb target, proliferation-related, genes.

Based on the results obtained from RNA-seq analysis, where we observed that upon FKBP5 knockdown, there was upregulation in a subset of genes which are directly involved in controlling cellular proliferation, cellular differentiation and many other

cellular pathways. We were further interested to decipher the molecular mechanism behind this upregulation of the set of genes (in FKBP5 knockdown condition) which are involved in regulating cellular proliferation. In order to do that, we performed quantitative real time PCR (qRT-PCR) analyses in FKBP5 knockdown condition as well as in condition where FKBP5 was re-expressed in knockdown cells**.**

4.1.6.1.1 Knockdown of FKBP5 in different mammalian cell lines enhances the expression of a set of proliferation related genes.

We performed quantitative real time PCR (qRT-PCR) analyses for multiple proliferation related genes in FKBP5 knockdown condition in HEK293T cells, that confirmed upregulation of multiple genes which are primarily involved in regulation of cellular proliferation. Role of two of these critical genes, *CCND1* and *C-MYC*, which encodes CyclinD1 and c-Myc proteins respectively, have been extensively studied as master regulators of expression of global target genes. Along with these to key regulatory genes, many other key regulatory genes of cellular proliferation including *CDKN1A*, *CDKNIB*, *MDM2*, *E2F2* have been observed to be upregulated upon FKBP5 knockdown. Upregulation of these genes are specific because, at the same time exxpression of several other genes like *RGS2*, *MAP2*, *LDLR* failed to show any significant change in their expression upon FKBP5 knockdown (Figure 4.22A).

Again, to rule out the cell-type specificity of the observed up-regulation of proliferation related genes upon FKBP5 knockdown, we performed quantitative real time PCR (qRT-PCR) analyses for most of the above-mentioned genes in FKBP5 knockdown condition in Prostate Cancer (PC3) cells, which confirmed upregulation of these genes in PC3 cells as well which rules out the cell-type specific effect of FKBP5 knockdown (Figure 4.22B).

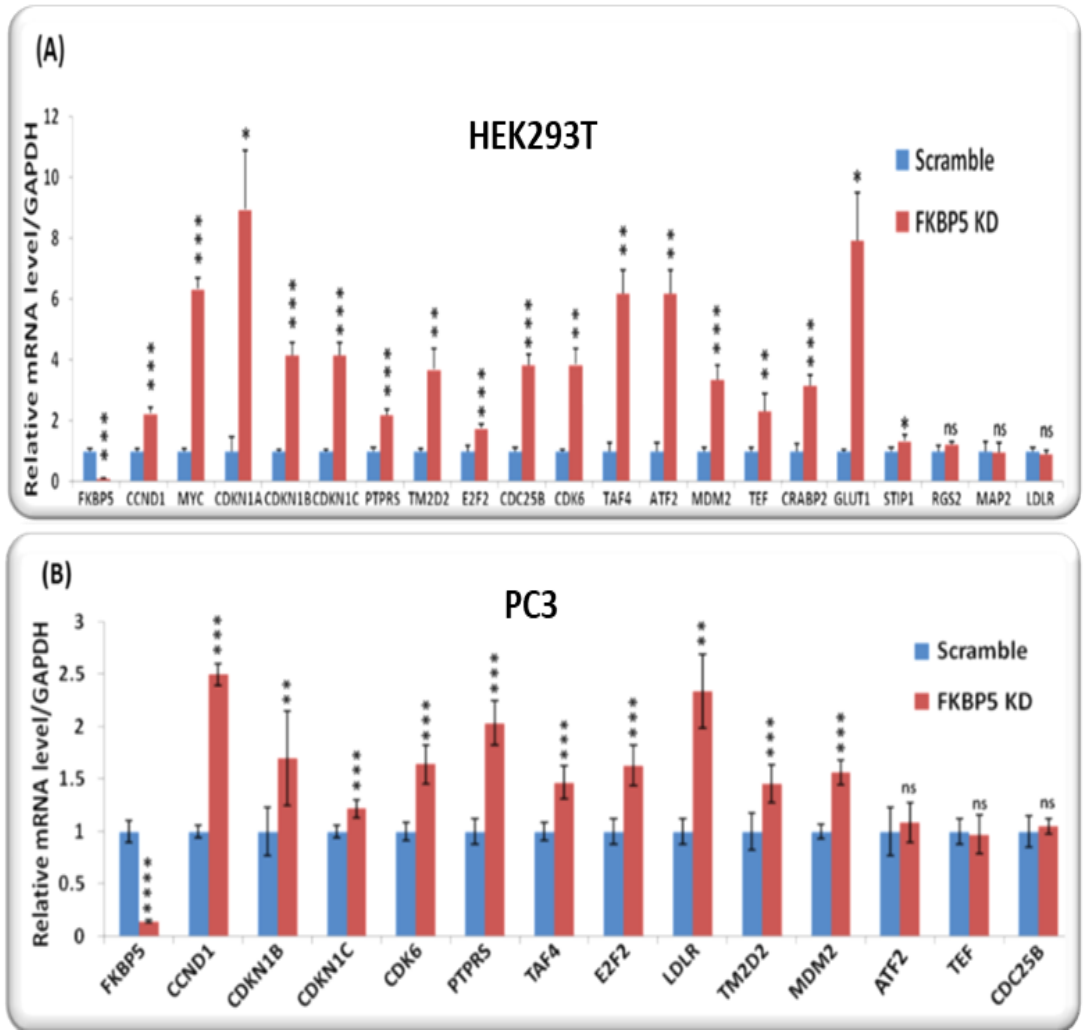


Figure 4.22 FKBP5 knockdown triggers the expression of several p-TEFb target genes in different cell lines.

(A) The qRT-PCR analysis confirmed a significant increase in mRNA expression of target genes involved in cellular proliferation in mammalian 293T cells upon FKBP5 knockdown. Each biological replication has n = 2 biological replicates and 3 PCR replicates.

(B) The qRT-PCR analysis confirmed a significant increase in mRNA expression of target genes involved in cellular proliferation in mammalian PC3 cells upon FKBP5 knockdown. Each biological replication has n = 2 biological replicates and 3 PCR replicates

4.1.6.1.2 Re-expression of FKBP5 reverses the effect of FKBP5 knockdown on expression of target genes.

Subsequently, we wanted to test the specificity of the observed effects on target gene expression in FKBP5 knockdown condition. I order to do that, in FKBP5 knockdown cell-line, we re-expressed WT-FKBP5 in one set, and in other set, we re-expressed the CDK9 interaction-deficient FKBP5 mutant (1-157 amino acids). Western blotting was performed and protein expressions were checked before the RT-PCR analysis (Figure 4.23 A). Interestingly, re-expression of WT- FKBP5 reversed the effect of FKBP5 knockdown as shown for two target genes (*CCND1* and *E2F2*), within 293T cells, whereas, the CDK9 interaction-deficient FKBP5 mutant (1-157 amino acids) failed to do so (Figure 4.23 B). These results clearly show that the overall effect of FKBP5 knockdown on increased target gene expression is specific to the FKBP5 mediated transcriptional control through controlling P-TEFb activity.

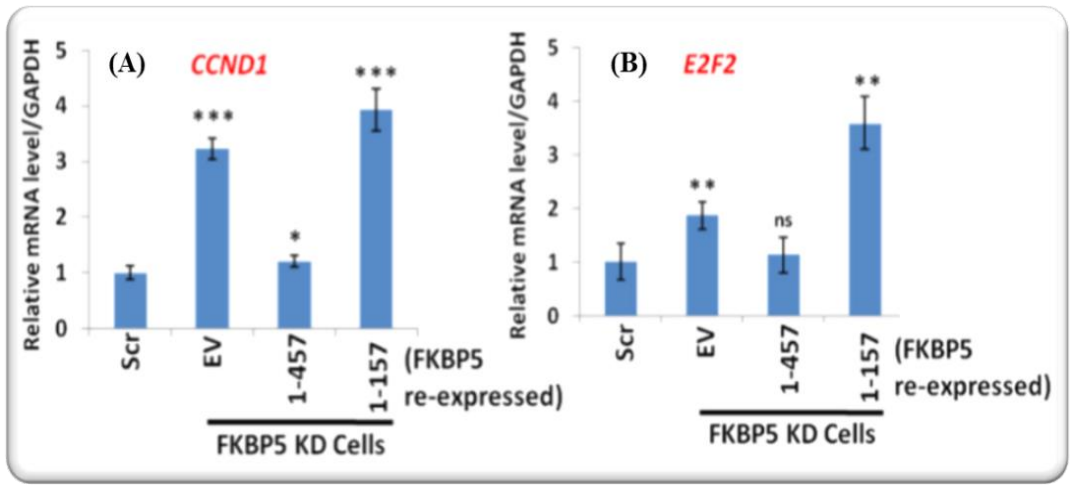


Figure 4.23 Effect of FKBP5 knock-in on target gene expression.

(A) qRT-PCR analysis of the effect of reexpression of full-length and CDK9 interaction-defective FKBP5 proteins on mRNA expression of CCND1 gene in FKBP5 knockdown cells. Each biological replication has two biological replicates and three PCR replicates.

(B) qRT-PCR analysis of the effect of reexpression of full-length and CDK9 interaction-defective FKBP5 proteins on mRNA expression of E2F2 gene in FKBP5 knockdown cells. Each biological replication has two biological replicates and three PCR replicates.

4.1.7 Level of CDK9 within HEK293T controls the expression of a set of proliferation related genes.

To further strengthen and establish hypothesis that overexpression of target gene on FKBP5 knockdown is specific to the FKBP5-mediated transcriptional control through controlling P-TEFb activity, we addressed the set of genes that would show alteration of gene expression upon changes in CDK9 level (i.e., Overall cellular P-TEFb level). qRT-PCR analysis to test the expression level of mentioned target genes.

4.1.7.1 Knockdown of CDK9 in HEK293T suppresses the expression of a set of proliferation related genes.

To further correlate the upregulated target genes (In FKBP5 knockdown condition) with P-TEFb target genes, we used stable CDK9 knock-down cells (protein level was checked through western blotting (Figure 4.24A)). We analysed the expression of target genes through qRT-PCR analysis, which shows a strong correlation (overlap) between the set of genes upregulated upon FKBP5 knockdown and the set of genes being downregulated upon CDK9 knockdown (Figure 4.24B). This observation clearly suggested that the overall negative effect of FKBP5 on expression of P-TEFb-mediated target genes within mammalian cells.

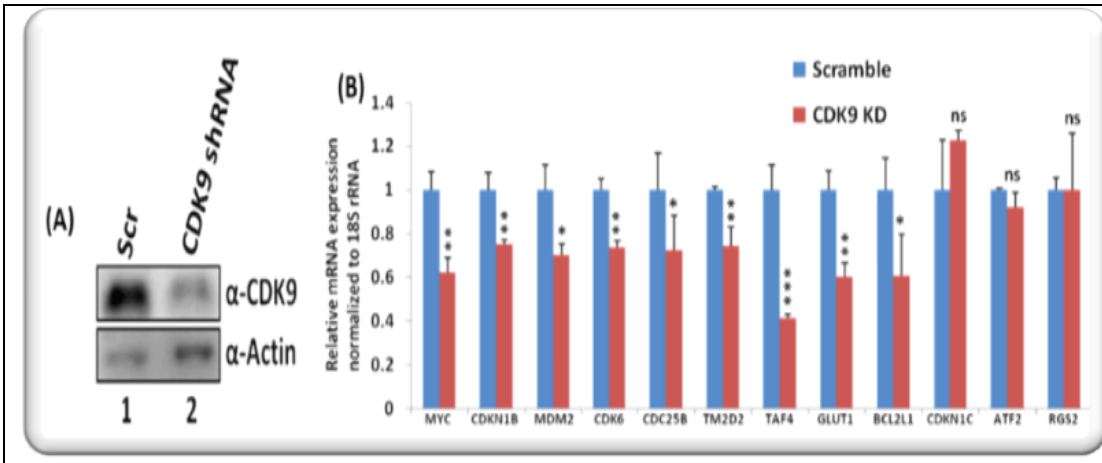


Figure 4.24 Effect of CDK9 knockdown on proliferation related target genes.

(A) Immunoblot analysis showing shRNA-mediated knockdown of CDK9 in mammalian HEK293T.

(B) qRT-PCR analysis of the effect of CDK9 knockdown on the expression of the target genes indicated. In our work, we employed 18Sr RNA expression as a control and for normalization. Each biological replicate has a minimum of two biological replicates and three PCR replicates.

4.1.7.2 Over-expression of CDK9 in HEK293T enhances the expression of a set of proliferation related genes.

Further, we also checked whether similar set of genes would also show increased expression upon over-expression of CDK9. We ectopically over-expressed CDK9 in HEK293T, which increased the phosphorylation of Pol II CTD at both Ser2 and Ser5 positions (Figure 4.25A). Subsequent, RNA analysis through qRT-PCR showed a huge increase in expression of most of the above-mentioned target genes (Figure 4.25B). This data provides additional evidence supporting a negative role of FKBP5 in modulating P-TEFb function inside the cell and ultimately resulting in reduced expression of P-TEFb-dependent target gene.

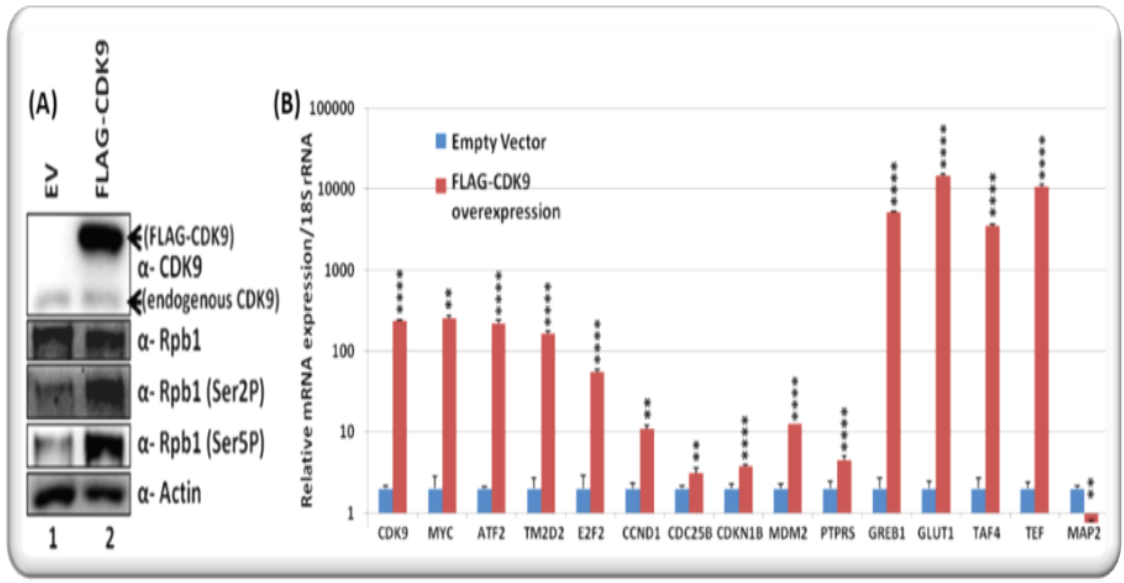


Figure 4.25 Global effect of CDK9 overexpression on target gene expression.

(A) Immunoblot analysis showing effect of overexpression of CDK9 on the phosphorylation of Ser2 and Ser5 residues of polII-CTD.

(B) qRT-PCR analysis of the effect of CDK9 overexpression in mammalian 293T cells on the expression of the indicated target genes. In our work, we employed 18Sr RNA expression as a control and for normalization. Each biological replicate has a minimum of two biological replicates and three PCR replicates.

4.1.8 FKBP5 knockdown enhances recruitment of P-TEFb complex at the target *CCND1* and *C-MYC* genes.

Until now, our results have convincingly demonstrated enough evidences suggesting a negative role of FKBP5 in regulating P-TEFb function within mammalian cells. If we look at the established way of P-TEFb-mediated functions inside the cell, it gets recruited to the promoter proximal region with the help of different recruiting factors, and phosphorylates Ser2, Ser5 of Pol II CTD, NELF complex and DSIF complex which ultimately causes productive transcription of a target gene. Based on our findings, where it has been shown that FKBP5 alters the expression of P-TEFb target genes, we presumed that FKBP5 might be altering P-TEFb recruitment on target genes. Hence, we performed **Ch**romatin

Immuno**p**recipitation (ChIP) assay with FKBP5 knockdown cell line to address the change in P-TEFb recruitment and other related factor's status on the target genes. For this, we chose two most relevant target genes and one nontarget gene as control i.e., *CCND1, C-MYC* as target genes and *RGS2* as nontarget gene. Subsequent, ChIP along with qRT-PCR in FKBP5 knockdown cell line clearly showed significantly enhanced P-TEFb (represented by CDK9) recruitment on the promoter region of target gene when compared with the control scramble knockdown cell line. Whereas, in similar analysis of nontarget (i.e., *RGS2*) gene promoter, we found opposite result i.e., less recruitment of P-TEFb on *RGS2* promoter. Consistent with the established function of P-TEFb, where it is known to phosphorylate Pol II CTD at Ser2 and Ser5 residues, these two modifications of Pol II were significantly high on promoter proximal region when compared to the scramble control. As we know Ser2 and Ser5 phosphorylation triggers the release of promoter proximally paused Pol II, we found a significant decrease in total Pol II level on promoter proximal region when compared to the scramble control. Further normalization of increase in phosphorylated Ser2 and Ser5 form with the total amount of Pol II, also showed significant increase in overall phosphorylation of these two key residues upon enhanced P-TEFb recruitment in the FKBP5 knockdown cells than the control scramble cells (Figure 4.26 A-C).

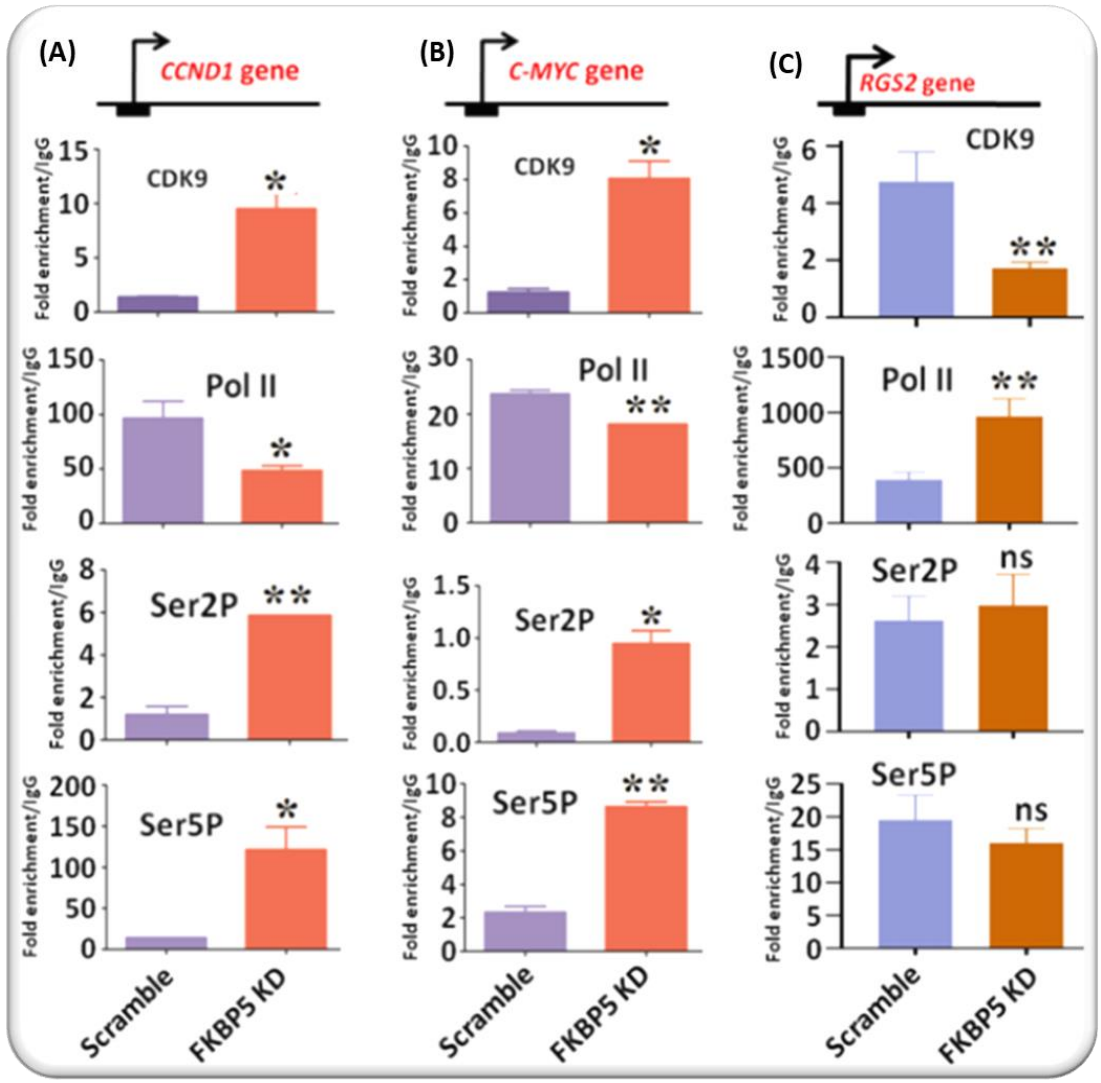


Figure 4.26 ChIP-qPCR revealed increased P-TEFb on TSS of target genes upon FKBP5 knockdown.

(A) Following FKBP5 knockdown, ChIP analysis revealed increased recruitment of the P-TEFb complex (using CDK9 as a candidate), resulting in increased Pol II CTD Ser2 and Ser5 phosphorylation and decreased Pol II on the promoter proximal area of the CCND1 target gene.

(B) Following FKBP5 knockdown, ChIP analysis revealed increased recruitment of the P-TEFb complex (using CDK9 as a candidate), resulting in increased Pol II CTD Ser2 and Ser5 phosphorylation and decreased Pol II on the promoter proximal area of the CMYC target gene.

(C) Following FKBP5 knockdown, ChIP analysis revealed reduced recruitment of the P-TEFb complex (using CDK9 as a candidate), resulting in decreased/unaffected Pol II CTD Ser2 and Ser5 phosphorylation and increased Pol II on the promoter proximal area of the a non-target gene (RGS2).

4.1.8.1 FKBP5 knockdown reduces promoter-proximal pausing of Pol II on target genes.

Again, ChIP analysis to address the status of total Pol II on gene body of both the target genes (i.e., *CCND1*, *C-MYC*) was done, which were found to be significantly increased. This finding was consistent with the earlier ChIP analysis where decreased level of Pol II was found at the TSS region (Figure 4.27A, B). Finally, pausing index (ration of total Pol II present at promoter proximal region/gene body) was calculated which clearly shows decreased pausing of Pol II on promoter proximal region in FKBP5 knockdown condition (Figure 4.27C).

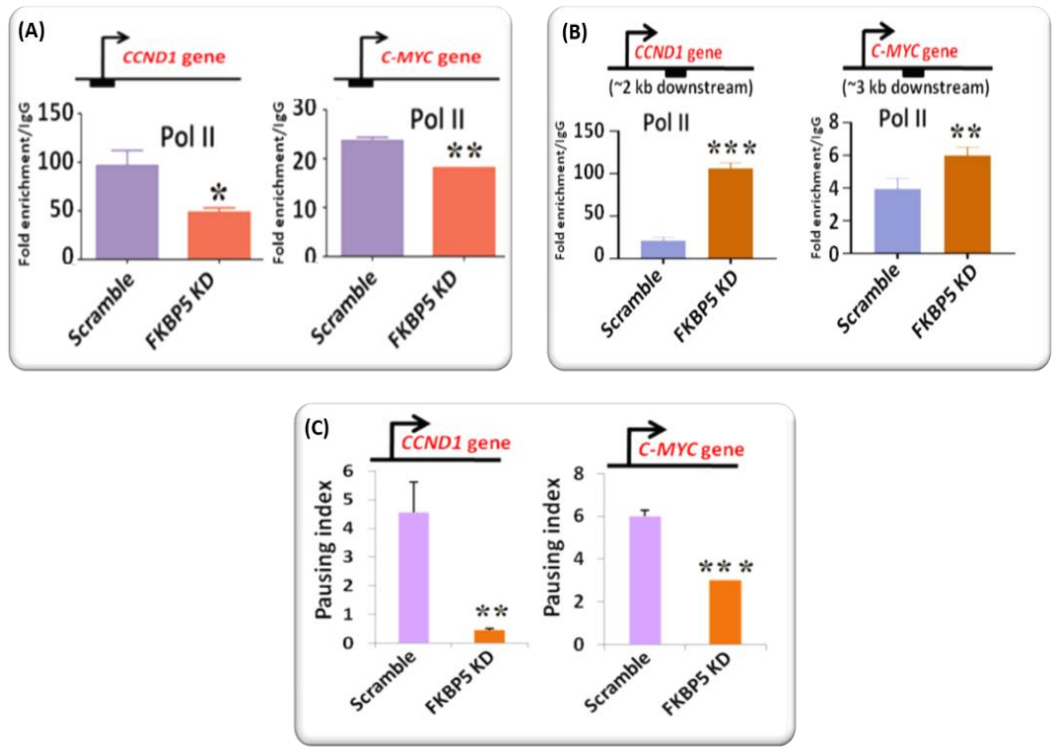


Figure 4.27 Impact of FKBP5 on different phosphorylated forms of polII on target genes.

(A) ChIP-qPCR showing decreased total polII level on TSS of P-TEFb target genes (CCND1 and CMYC genes are taken as candidate).

(B) ChIP-qPCR showing increased total polII level in coding region of P-TEFb target genes (CCND1 and CMYC genes are taken as candidate).

(C) Pausing index analysis showing significant reduction in overall pausing of Pol II at TSS region of both the target genes (CCND1 and CMYC) upon FKBP5 knockdown.

4.1.9 Effect of FKBP5 knockdown on induction of P-TEFb target HIV-LTR-driven luciferase reporter gene inside the cell.

Through different assays, like RNA expression analysis, chromatin immunoprecipitation (ChIP) in FKBP5 knockdown condition, we have shown that FKBP5 knockdown causes more P-TEFb recruitment on target genes resulting in enhanced expression. Further, as HIV1-LTR promoter driven genes are well known to be regulated by P-TEFb, we addressed the induction of HIV1-LTR promoter-driven luciferase gene expression upon FKBP5 knockdown. Here also, in FKBP5 knockdown condition, consistent with our earlier results, we observed a significant increase in overall expression of reporter gene when compared with control scramble knockdown cells (Figure 4.28).

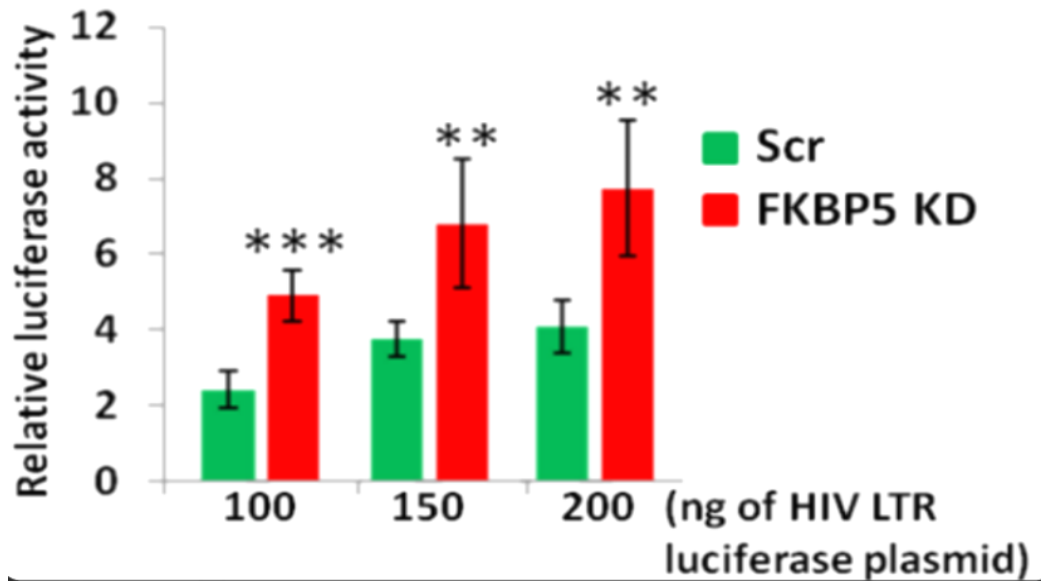


Figure 4.28 Effect of FKBP5 knockdown on P-TEFb target reporter gene expression.

Luciferase reporter assay confirming the inductive effect of FKBP5 knockdown on HIV LTR promoter-driven P-TEFb target gene expression. Data represents n=3 biological replicates and 2 technical replicates of each biological replicate.

4.1.10 Knockdown of FKBP5 enhances cellular proliferation of different human-cell lines.

As we observed through several assays that upon FKBP5 knockdown in HEK293T and in PC3 cells, several genes that control cellular proliferation, show enhanced expression because of more P-TEFb recruitment at promoter proximal regions of these genes. So, finally we wanted to check the change in proliferation rate of the cells upon FKBP5 knockdown. Consistent with the above observations, where proliferation-related genes were upregulated upon FKBP5 knockdown, the rate of proliferation was significantly enhanced upon FKBP5 knockdown when compared with the scramble control in HEK293T (Figure 4.29 A). Further, we also checked the change in proliferation rate of the prostate cancer (PC3) cells upon FKBP5 knockdown. Here also, we observed enhanced cellular proliferation rate upon FKBP5 knockdown when compared with the scramble control (Figure 4.29 B).

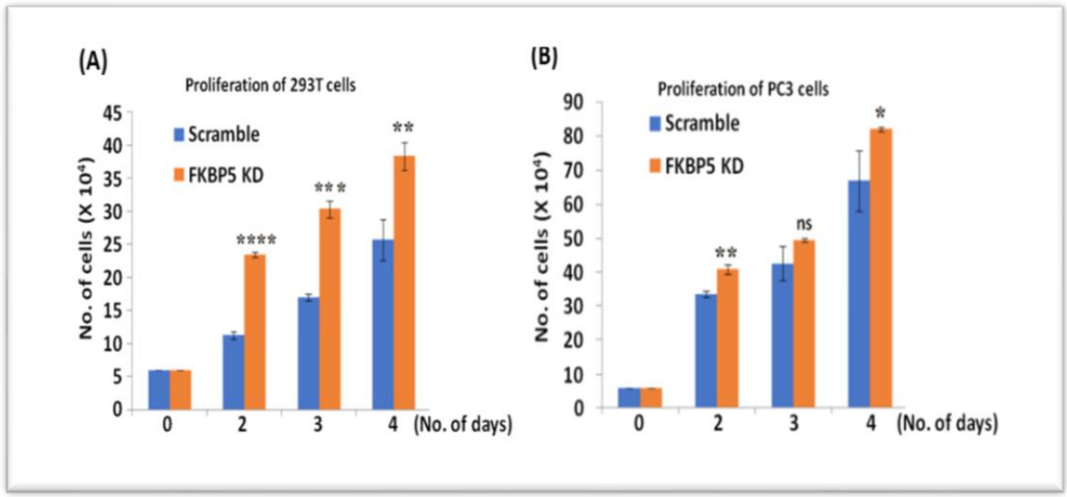


Figure 4.29 Effect of FKBP5 knockdown on proliferation rate.

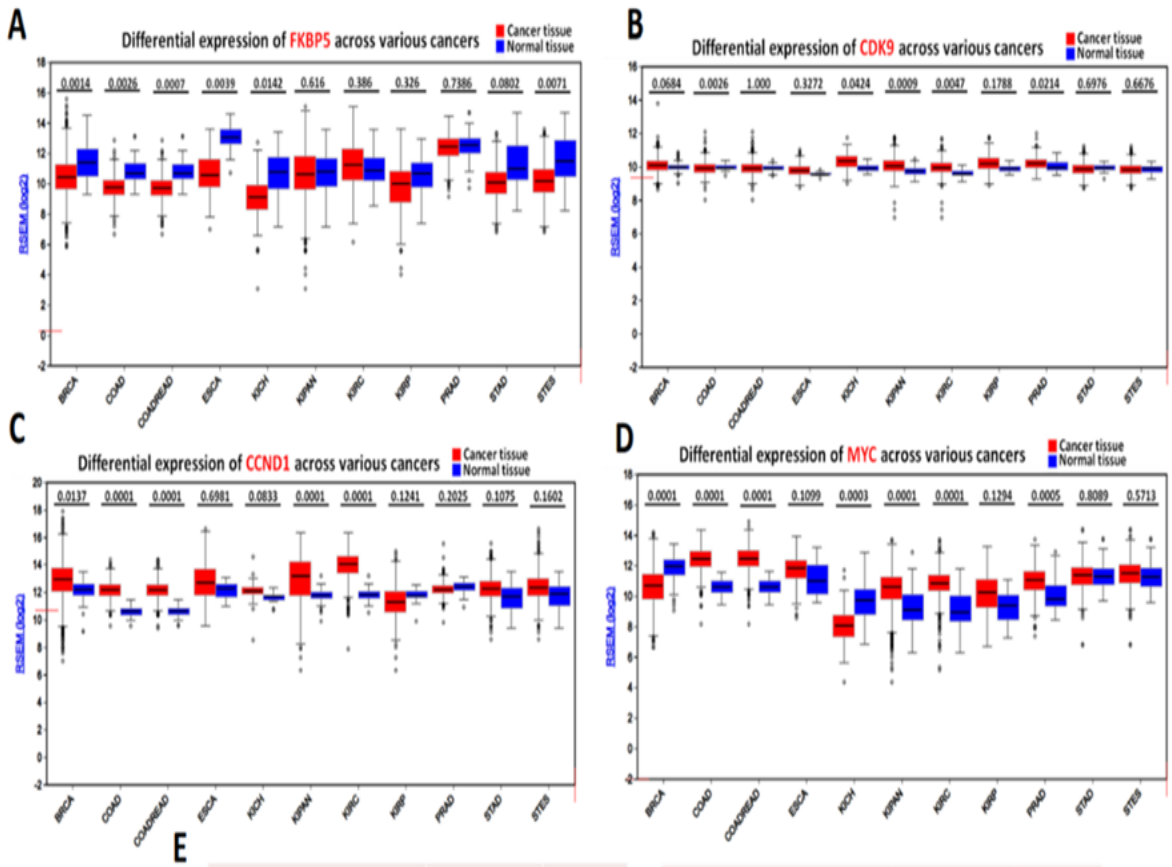
(A, B) Cell proliferation assay showing increased proliferative rate of mammalian 293T (A) and mammalian PC3 (B) cells in FKBP5 knockdown condition. Number of scramble cells was compared with number of FKBP5 knockdown cells at different time points. Data represents n=2 biological replicates and minimum of 3 technical replicates of each biological replicate.

4.1.11 Strong correlation between downregulation of FKBP5 and upregulation of proliferation-related genes in several human cancers.

Few earlier studies have indicated a negative correlation between FKBP5 gene expression and severity of many cancers. Our results as shown in this study also clearly indicate an antiproliferative role of FKBP5 in controlling cell growth of different cell lines. Based on these observations, we assumed that the role of FKBP5 may also be important for cancer pathogenesis and control. To understand role of FKBP5 in cancer progression and related target gene expression, we analyzed expression level of FKBP5 mRNA in patient cohort sample of different cancer cases and its correlation with proliferation related genes using the publicly available TCGA datasets. We observed a significant correlation between downregulation of FKBP5 and upregulation of proliferation-related genes such as *CCND1* and *C-MYC* as well as *CDK9* (Figure 4.30 A-E).

Our present study deciphers a detailed mechanistic understanding of above-mentioned type of cancer progression and the functional role of FKBP5 in this process through enhanced expression of key cancer-causing proto-oncogenes such as *CCND1* and *C-MYC*. Interestingly, we observed the downregulation of FKBP5 and upregulation of related target genes like *CCND1 and C-MYC* in several forms of kidney cancers which further strengthen our observations in HEK293T.

Taken all observations of the present study together, we established a role of FKBP5 as a tumor suppressor in several forms of cancer.



Cancer Types	Abbreviation	Cohort size
Breast invasive carcinoma	BRCA	1098
Colon adenocarcinoma	COAD	460
Colorectal adenocarcinoma	COADREAD	677
Esophageal carcinoma	ESCA	185
Kidney Chromophobe	KICH	113
Pan-kidney cohort (KICH+KIRC+KIRP)	KIPAN	973

Cancer Types	Abbreviation	Cohort size
Kidney renal clear cell carcinoma	KIRC	537
Kidney renal papillary cell carcinoma	KIRP	323
Prostate adenocarcinoma	PRAD	499
Stomach adenocarcinoma	STAD	443
Stomach and Esophageal carcinoma	STES	628

Figure 4.30 RNA expression analyses of FKBP5, CDK9, CCND1, and MYC in multiple types of cancer patient samples.

(A-D) FKBP5, CDK9, CCND1, and MYC mRNA expression in many kinds of cancer patient samples as shown in The Cancer Genome Atlas (TCGA) datasets (data version 2016_01_28) held at the Broad Institute of MIT and Harvard TCGA Government Data Analytics Center (GDAC) (www.gdac.broadinstitute.org). The estimated p values for the examined datasets are given above each box plot.

(E) Table listing the acronyms and patient cohort sizes for various cancer types as found in TCGA databases and examined in our research.

CHAPTER V

Conclusion

CONCLUSION:

Our study deciphers another level of functional regulation of P-TEFb complex for regulation of transcription at the prmoter proximal region through a completely novel way that involves FKBP5. The available literature shows that FKBP5 expression level is one of the major drivers of a normal cell to be cancerous. It has been shown through immunohistochemical analysis that FKBP5 can behave either as an oncogene or as a tumour suppressor, depending on histological conditions (L. Li, Lou, and Wang 2011). However, several questions regarding the mode of FKBP5 action, in different histological conditions, still remained unexplained. Our study gives a possible explanation for the role of FKBP5 as a tumour suppressor.

Here, in this part of the thesis we have presented the whole findings of this study briefly under four subheadings.

- **FKBP5 interacts strongly with CDK9 component of P-TEFb complex.**

  Using mass spectrophotometric analysis and immunoprecipitation assays we have identified FKBP5 as a strong interactor of CDK9. Subsequently, through different *in vitro* and cell-based interaction assays, we clearly showed that FKBP5 interacts very specifically with CDK9 component of P-TEFb complex and not with other similar kinases like CDK7 and CDK8.

- **FKBP5 inhibits the kinase activity of P-TEFb complex *in vitro* and *in vivo*.**

  We performed different *in vitro* and *in vivo* biochemical assays to understand the functional importance of FKBP5 interaction with CDK9. Throughin *in vitro* kinase assay, we showed that FKBP5 has a great potential to inhibit the kinase activity of P-TEFb complex towards Pol II CTD phosphorylation at Ser2 and Ser5 residues. This effect of FKBP5 on P-TEFb is not substrate specific, as this effect was also observed for other substrates of P-TEFb like NELF and DSIF complex in our *in vitro* kinase assays.To test the scenario inside the cell, we made stable FKBP5 knockdown cell lines. A significant global increase in phosphorylation level of P-TEFb substrates, Ser2 and Ser5 residues of heptad repeat "YSPTSPS" of Pol II CTD was observed upon knockdown of FKBP5 which further substantiates our observation. This effect of FKBP5 knockdown was reversed when FKBP5

was re-expressed in knockdown cells, confirming that the observed effect was mediated by FKBP5 only.

- **FKBP5 level affects P-TEFb target gene expression and their physiological effects.**

In order to find out the FKBP5 target genes, we performed RNA-seq analysis using FKBP5 knockdown cell line and observed significant no. of genes being upregulated and downregulated. Gene ontology analysis of upregulated gene fraction found out that a significant number of genes are known to regulate cell proliferation rate and majority of these genes are known as P-TEFb target genes. We selected some of those target genes and performed qRT-PCR to test their expression in FKBP5 knockdown condition. qRT-PCR data also supported the our earlier RNA-seq analysis. When we analysed the data of different cancer cohorts, we found out a significant correlation that in many cancers where the level of FKBP5 was down, the expression of CDK9 and some of the P-TEFb target proliferation-related genes (e.g., *CCND1* and *C-MYC*) was significantly high, which further supports our RNA-seq and qRT-PCR analysis.We preformed cell-proliferation assay in to test the effect of FKBP5 knockdown on overall proliferation of cells. We observed that low level of FKBP5 expression causes enhanced proliferation potential of HEK-293T and PC-3 cell lines.

Our results indicated that these effects on target gene expression and their physiological effects upon FKBP5 knockdown is probably because of the global increase in recruitment of P-TEFb on target genes at cognate promoter proximal regions.

- **FKBP5 negatively regulates P-TEFb recruitment at promoter proximal regions.**

To understand the underlaying mechanism behind the inhibitory functions of FKBP5 on P-TEFb-mediated target gene expression, we performed ChIP qRT-PCR and addressed P-TEFb recruitment at the promoter proximal regions of target genes as well as resulting Pol II CTD Ser2P and Ser5P status upon FKBP5 knockdown. ChIP qRT-PCR data suggested that the reduced expression of FKBP5 inside the cell increases the occupancy of CDK9 on TSS region of target genes and resulting in increased level of Ser2P, and Ser5P phosphorylation of Pol II level along with reduced presence of total Pol II at the promoter

proximal region. Finally, pausing index analysis showed a decreased Pol II pausing upon FKBP5 knockdown resulting in increased expression of target genes.

❖ FKBP5 is one of the major regulators for maintaining level of P-TEFb pool inside the cell.

We deciphered the underlaying mechanism behind the inhibitory action of FKBP5 on P-TEFb kinase activity. In order to do so, we performed colorimetric phosphatase assay, autoradiography-based phosphatase assay, both of which ultimately failed to show any phosphatase activity of FKBP5. Several of our analyses also failed to show any evidence supporting a substrate blocking mechanism of FKBP5 that could explain its inhibitory functions towards P-TEFb kinase activity. For this we did interaction analysis of FKBP5 with some of the P-TEFb substrates like NELF-A, NELF-E and Pol II CTD. We observed that FKBP5 does not interact with the P-TEFb substrates. Available literature shows that CyclinT1 interacts with extreme N-terminus of CDK9 (Anshabo et al. 2021). Consistent with this, we also observed the same result in our domain analysis of CDK9 for interaction with CyclinT1. Further domain analysis of CDK9 for interaction with FKBP5 clearly showed that the same domain of CDK9 is responsible for interaction with FKBP5 and CyclinT1. This observation led us to hypothesize that there could possibly be competition between FKBP5 and CyclinT1 to interact with CDK9 inside the cell and this competition ultimately decides the level of P-TEFb pool within mammalian cells at any given point of time. In order to address the hypothesis of competition model, we performed *in vitro* sequestration assay and we found that FKBP5 has a potential to sequester out CDK9 from P-TEFb complex and thus causes its dissociation. To rule out the artefacts of *in vitro* analyses, we performed cell-based competition assay in HEK-293T cell line. Our analyses showed upon FKBP5 knockdown and enhanced interaction between CDK9 and CyclinT1 was observed. Further, upon over-expression of FKBP5, we observed that equal pull-down of FLAG-CDK9 showed reduced CyclinT1 pulldown with increasing expression of FKBP5 in HEK-293T, whereas in FKBP5 knockdown condition caused increased pulldown of CyclinT1 by endogenous. These observations further substantiate the competition model resulting in FKBP5-mediated inhibition of P-TEFb functions.

Finally, based on our findings, it can be concluded that FKBP5 can affect the global transcriptional output via affecting the level of active P-TEFb pool inside the cell. Present study firmly establishes FKBP5 as a transcriptional repressor that involves negative regulation of P-TEFb functions.

An model depicting functional regulation of transcription by FKBP5 involving maintenance of P-TEFb pool within mammalian cells is presented (Figure 5.1).

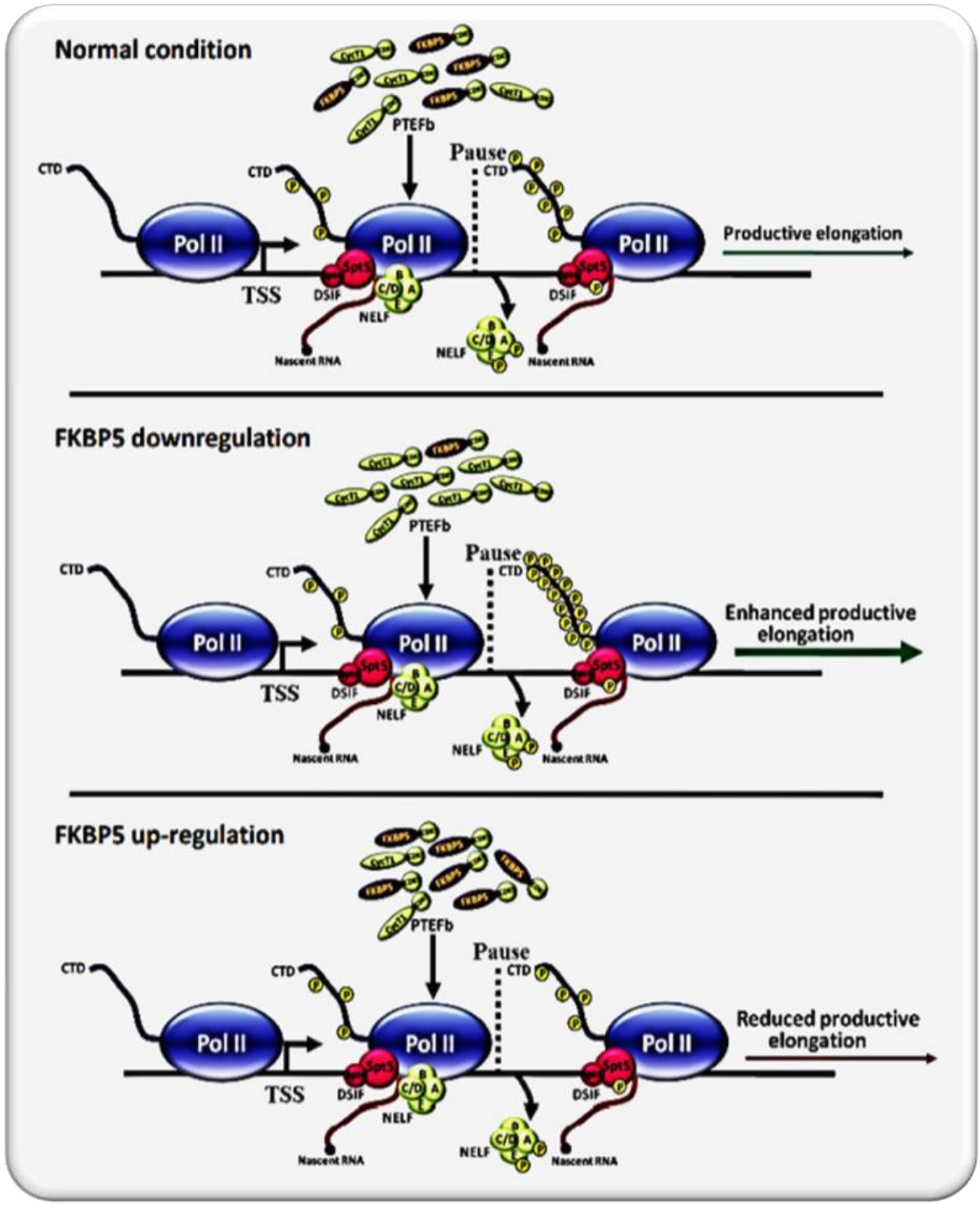


Figure 5.1 Model depicting mechanism of action of FKBP5 mediated P-TEFb regulation.

Here upper panel shows normal cellular level of FKBP5 where balanced FKBP5 as well as P-TEFb level is shown. Middle panel shows FKBP5 downregulated cellular condition resulting in enhanced cellular P-TEFb level causing enhanced transcription of target genes. Lower panel shows normal cellular level of FKBP5 where balanced FKBP5 as well as P-TEFb level is shown. Middle panel shows FKBP5 upregulated cellular condition resulting in lowered cellular P-TEFb level resultind in reduced transcription of target genes.

CHAPTER VI

Discussion

Discussion:

The current work elucidates a unique role of FKBP5 demonstrating that it negatively regulates the P-TEFb function and thus establishing FKBP5 as a negative regulator of transcription. Here we find that FKBP5 and CyclinT1 share the same binding site on CDK9 for their interaction, hence they compete with each other to interact with CDK9. This competition determines the overall P-TEFb pool inside the mammalian cell and, as a result, regulates the expression of P-TEFb-target genes. This regulatory role of FKBP5 answers many unanswered questions about a variety of cellular states many of which are linked to several disorders. Here, we have discussed some of the longstanding questions that can be answered by the findings of this study.

❖ Role of FKBP5 in controlling transcriptional events at the promoter-proximal region and its implications.

Previously, HSP90 chaperone protein was shown to have a function in controlling pausing events at the promoter-proximal region of global target genes (Sawarkar, Sievers, and Paro 2012). In this study, HSP90 has been shown to stabilize NELF complex at the promoter proximal region of the target genes which ultimately helps NELF complex to pause Pol II at the promoter proximal region. But this study lacks in providing a deeper understanding of mechanistic insights. Because, FKBP5 is a cochaperone of the HSP90 chaperone complex and based on the mechanisms discovered in our study, it's very likely that FKBP5 could play additional roles within the HSP90 chaperone complex for achieving overall pausing through negative regulation of functions of the P-TEFb complex which is important for releasing the paused Pol II from the promoter-proximal region. Further our ChIP study of the target *CCND1* and *C-MYC* genes revealed a substantial drop in total chromatin-associated Pol II levels in the promoter-proximal region when FKBP5 was knocked down. To fully understand this process of global pausing of Pol II at promoter proximal region involving HSP90 and FKBP5 inside mammalian cells, further studies would be required.

❖ Involvement of FKBP5 mediated transcriptional repression in controlling cancer progression:

When RNA expression analysis in different cancer cases was done using the data obtained from The Cancer Genome Atlas (TCGA) database, interestingly we observed a significant correlation between the downregulation of FKBP5 and increased expression of many genes including *CCND1*,

C-MYC, CDKN1A etc. that are known as protooncogenes and control cellular proliferation rate. In our present study, we provided the mechanistic understanding of these phenomenon and its implication in cancer pathogenesis. We showed that reduced expression of FKBP5 enhances the active pool of P-TEFb inside the cell which ultimately triggers overexpression of most of these protooncogenes and thus helping in cancer progression. Our overall study establishes FKBP5 as a strong tumor suppressor protein. Our study provides a mechanistic explanation of this tumor suppressive role of FKBP5. Earlier reports have shown strong correlation of low level of FKBP5 breast cancer pathogenesis through regulation of AKT activation(L. Li, Lou, and Wang 2011). As deciphered in our study, the combined role of FKBP5-mediated regulation of AKT and P-TEFb functions may play a significant role in establishment and progression of cancer which is a subject for future research.

- **Involvement of FKBP5-mediated transcriptional repression in type 2 diabetes pathogenesis.**

Earlier it has been reported that FKBP5 is implicated in glucose metabolism and altered level of FKBP5 has been reported in Type 2 diabetes pathogenesis (Sidibeh CO et al., 2018). In our study, we also have seen significantly high *GLUT1* expression upon FKBP5 knockdown. GLUT1 is a well known glucose transporter. GLUT1 overexpression has earlier been reported in Type 2 diabetes pathogenesis (Mogyoró and Ziyadeh2 1999). Recently, it has been shown that AKT signalling pathway is involved in phosphorylation of target AS160 upon insulin stimulation which facilitates the translocation of GLUT4 glucose uptake protein to the cell-membrane and ultimately increases the overall glucose uptake leading to homeostasis (Ni WJ et al., 2022). To our interest, when FKBP5 activities are knocked down or blocked by a pharmacological antagonist, GLUT4 expression rises. Our present study can clearly explain the mechanistic part of above-mentioned effects where GLUT1 and GLUT4 overexpression has been shown to promote type 2 diabetes. Here, in this study we have shown that FKBP5 regulates the overall pool of P-TEFb inside a cell. It is quite possible that overall increased P-TEFb pool upon ablation of FKBP5 stimulates the rate of transcription of *GLUT1* and *GLUT4* genes that ultimately promotes type 2 diabetes.

- **Involvement of FKBP5 mediated transcriptional repression in neuronal pathogenesis.**

FKBP5 has been shown to be highly expressed in the hippocampus and amygdala(Scharf et al. 2011), the two major areas regulating stress-related processes. Other than that, it has also been shown to be implicated in the pathophysiology of numerous neurological diseases, including PTSD, by a number of studies (Talarowska 2020). The majority of these research have suggested that FKBP5 has a role in modulating the glucocorticoid receptor (GR) signalling pathway, in which FKBP5 competes with glucocorticoids for GR binding and subsequent downstream signalling for transcriptional activation of target genes. All these mentioned studies suggest an indirect role of FKBP5 in regulation of transcriptional output. However, no other studies have revealed direct molecular insights into the role of FKBP5 in transcriptional activity. Thus, our research has the potential to uncover crucial mechanistic insights into the involvement of FKBP5 and general neuronal pathophysiology that previous researches have been unable to uncover. Future studies would be focused on answering some of these crucial questions.

- **Involvement of FKBP5 mediated transcriptional repression in adipogenesis**.

There are many studies suggesting a vital role of FKBP5 in adipogenesis. When compared to other tissues, the highest levels of FKBP5 expression have been found in adipocytes, skeletal muscles, and lymphocytes (Pereira et al. 2014). Interestingly, FKBP5 functions have been linked to adipocyte differentiation from white adipose tissue (WAT) to brown adipose tissue (BAT). The expression of the main adipogenesis regulators UCP1 and PRDM16 has been shown to be increased when FKBP5 is knocked down (Stechschulte et al. 2016). However, the underlying mechanisms of this increased expression remained unknown. Our present study where we are showing FKBP5 as a transcriptional repressor that negatively regulates P-TEFb activity inside the cell, can provide a mechanistic explanation of this effect of FKBP5 knock down on increased expression of the main adipogenesis regulators UCP1 and PRDM16. However, in this regard detailed study needs to be done to establish this role of FKBP5 involvement in adipogenesis.

Overall, the mechanistic insights provided through our present study have the potential to enhance our understanding of the underlying mechanisms of pathogenesis of several FKBP5-related

diseases, such as cancer and metabolic disorders, as well as several neuronal disorders that have remained unanswered by several earlier studies.

References:

Akhtar, Md Sohail, Martin Heidemann, Joshua R. Tietjen, David W. Zhang, Rob D. Chapman, Dirk Eick, and Aseem Z. Ansari. 2009. "TFIIH Kinase Places Bivalent Marks on the Carboxy-Terminal Domain of RNA Polymerase II." *Molecular Cell* 34 (3): 387–93. https://doi.org/10.1016/J.MOLCEL.2009.04.016.

Alessi, Dario R., Mirjana Andjelkovic, Barry Caudwell, Peter Cron, Nick Morrice, Philip Cohen, and Brian A. Hemmings. 1996. "Mechanism of Activation of Protein Kinase B by Insulin and IGF-1." *EMBO Journal* 15 (23): 6541–51. https://doi.org/10.1002/j.1460-2075.1996.tb01045.x.

Anshabo, Abel Tesfaye, Robert Milne, Shudong Wang, and Hugo Albrecht. 2021. "CDK9: A Comprehensive Review of Its Biology, and Its Role as a Potential Target for Anti-Cancer Agents." *Frontiers in Oncology* 11 (May): 1573. https://doi.org/10.3389/FONC.2021.678559/BIBTEX.

Asamitsu, Kaori, Koh Fujinaga, and Takashi Okamoto. 2018a. "HIV Tat/P-TEFb Interaction: A Potential Target for Novel Anti-HIV Therapies." *Molecules* 23 (4). https://doi.org/10.3390/MOLECULES23040933.

———. 2018b. "HIV Tat/P-TEFb Interaction: A Potential Target for Novel Anti-HIV Therapies." *Molecules* 23 (4). https://doi.org/10.3390/MOLECULES23040933.

Bacon, Curtis W., and Iván D'Orso. 2018. "CDK9: A Signaling Hub for Transcriptional Control." *Https://Doi.Org/10.1080/21541264.2018.1523668* 10 (2): 57–75. https://doi.org/10.1080/21541264.2018.1523668.

Bai, Lu, Gilles Charvin, Eric D. Siggia, and Frederick R. Cross. 2010. "Nucleosome-Depleted Regions in Cell-Cycle-Regulated Promoters Ensure Reliable Gene Expression in Every Cell Cycle." *Developmental Cell* 18 (4): 544–55. https://doi.org/10.1016/J.DEVCEL.2010.02.007.

Bandukwala, Hozefa S., John Gagnon, Susan Togher, Jason A. Greenbaum, Edward D. Lamperti, Nigel J. Parr, Amy M.H. Molesworth, et al. 2012. "Selective Inhibition of CD4+ T-Cell Cytokine Production and Autoimmunity by BET Protein and c-Myc Inhibitors." *Proceedings of the National Academy of Sciences of the United States of America* 109 (36): 14532–37. https://doi.org/10.1073/PNAS.1212264109.

Baptista, Tiago, Sebastian Grünberg, Nadège Minoungou, Maria J.E. Koster, H. T.Marc Timmers, Steve Hahn, Didier Devys, and László Tora. 2017. "SAGA Is a General Cofactor for RNA Polymerase II Transcription." *Molecular Cell* 68 (1): 130-143.e5. https://doi.org/10.1016/J.MOLCEL.2017.08.016.

Barboric, Matjaz, Robert M. Nissen, Satoshi Kanazawa, Nabila Jabrane-Ferrat, and B. Matija Peterlin. 2001. "NF-KB Binds P-TEFb to Stimulate Transcriptional Elongation by RNA Polymerase II." *Molecular Cell* 8 (2): 327–37. https://doi.org/10.1016/S1097-2765(01)00314-8.

Barent, Richard L, Satish C Nair, Damon C Carr, Ying Ruan, Ronald A Rimerman, Jennifer Fulton, Yan Zhang, and David F Smith. 1998. "Analysis of FKBP5/FKBP52 Chimeras and Mutants for Hsp90 Binding and Association with Progesterone Receptor Complexes." https://academic.oup.com/mend/article/12/3/342/2754337.

Barilla, D., B. A. Lee, and N. J. Proudfoot. 2001. “Cleavage/Polyadenylation Factor IA Associates with the Carboxyl-Terminal Domain of RNA Polymerase II in Saccharomyces Cerevisiae.” *Proceedings of the National Academy of Sciences of the United States of America* 98 (2): 445–50. https://doi.org/10.1073/PNAS.98.2.445.

Bartholomeeusen, Koen, Koh Fujinaga, Yanhui Xiang, and B. Matija Peterlin. 2013. “Histone Deacetylase Inhibitors (HDACis) That Release the Positive Transcription Elongation Factor b (P-TEFb) from Its Inhibitory Complex Also Activate HIV Transcription.” *Journal of Biological Chemistry* 288 (20): 14400–407. https://doi.org/10.1074/JBC.M113.464834.

Bartholomeeusen, Koen, Yanhui Xiang, Koh Fujinaga, and B. Matija Peterlin. 2012. “Bromodomain and Extra-Terminal (BET) Bromodomain Inhibition Activate Transcription via Transient Release of Positive Transcription Elongation Factor b (P-TEFb) from 7SK Small Nuclear Ribonucleoprotein.” *Journal of Biological Chemistry* 287 (43): 36609–16. https://doi.org/10.1074/JBC.M112.410746.

Basu, Subham, Mahesh Barad, Dipika Yadav, Arijit Nandy, Bidisha Mukherjee, Jit Sarkar, Partha Chakrabarti, Satinath Mukhopadhyay, and Debabrata Biswas. 2020. “DBC1, P300, HDAC3, and Siah1 Coordinately Regulate ELL Stability and Function for Expression of Its Target Genes.” *Proceedings of the National Academy of Sciences of the United States of America* 117 (12): 6509–20. https://doi.org/10.1073/PNAS.1912375117.

Bauer, Uta Maria, Sylvain Daujat, Søren J. Nielsen, Karl Nightingale, and Tony Kouzarides. 2002. “Methylation at Arginine 17 of Histone H3 Is Linked to Gene Activation.” *EMBO Reports* 3 (1): 39–44. https://doi.org/10.1093/EMBO-REPORTS/KVF013.

Baughman, Gail, Gregory J Wiederrecht, N Faith Campbell, Mary M Martin, and Suzanne Bourgeois. 1995. “FKBP5, a Novel T-Cell-Specific Immunophilin Capable of Calcineurin Inhibition.” *MOLECULAR AND CELLULAR BIOLOGY*. Vol. 15.

Bieniasz, Paul D., Therese A. Grdina, Hal P. Bogerd, and Bryan R. Cullen. 1998. “Recruitment of a Protein Complex Containing Tat and Cyclin T1 to TAR Governs the Species Specificity of HIV-1 Tat.” *EMBO Journal* 17 (23): 7056–65. https://doi.org/10.1093/EMBOJ/17.23.7056.

Binder, Elisabeth B., Daria Salyakina, Peter Lichtner, Gabriele M. Wochnik, Marcus Ising, Benno Pütz, Sergi Papiol, et al. 2004. “Polymorphisms in FKBP5 Are Associated with Increased Recurrence of Depressive Episodes and Rapid Response to Antidepressant Treatment.” *Nature Genetics* 36 (12): 1319–25. https://doi.org/10.1038/NG1479.

Biswas, Debabrata, Thomas A. Milne, Venkatesha Basrur, Jaehoon Kim, Kojo S.J. Elenitoba-Johnson, C. David Allis, and Robert G. Roeder. 2011. “Function of Leukemogenic Mixed Lineage Leukemia 1 (MLL) Fusion Proteins through Distinct Partner Protein Complexes.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (38): 15751–56. https://doi.org/10.1073/PNAS.1111498108.

Boffo, Silvia, Angela Damato, Luigi Alfano, and Antonio Giordano. 2018. “CDK9 Inhibitors in Acute Myeloid Leukemia.” *Journal of Experimental and Clinical Cancer Research* 37 (1). https://doi.org/10.1186/S13046-018-0704-8.

Bonnet, Jacques, Chen Yi Wang, Tiago Baptista, Stéphane D. Vincent, Wei Chun Hsiao, Matthieu Stierle, Cheng Fu Kao, László Tora, and Didier Devys. 2014. "The SAGA Coactivator Complex Acts on the Whole Transcribed Genome and Is Required for RNA Polymerase II Transcription." *Genes & Development* 28 (18): 1999–2012. https://doi.org/10.1101/GAD.250225.114.

Bouwmeester, Tewis, Angela Bauch, Heinz Ruffner, Pierre Olivier Angrand, Giovanna Bergamini, Karen Croughton, Cristina Cruciat, et al. 2004. "A Physical and Functional Map of the Human TNF-Alpha/NF-Kappa B Signal Transduction Pathway." *Nature Cell Biology* 6 (2): 97–105. https://doi.org/10.1038/NCB1086.

Brasier, Allan R. 2008. "Expanding Role of Cyclin Dependent Kinases in Cytokine Inducible Gene Expression." *Cell Cycle* 7 (17): 2661–66. https://doi.org/10.4161/CC.7.17.6594.

Bulger, Michael, and Mark Groudine. 2011. "Functional and Mechanistic Diversity of Distal Transcription Enhancers." *Cell* 144 (3): 327–39. https://doi.org/10.1016/J.CELL.2011.01.024.

Buratowski, Stephen. 1994. "The Basics of Basal Transcription by RNA Polymerase II." *Cell* 77 (1): 1–3. https://doi.org/10.1016/0092-8674(94)90226-7.

Burke, Thomas W., and James T. Kadonaga. 1997. "The Downstream Core Promoter Element, DPE, Is Conserved from Drosophila to Humans and Is Recognized by TAFII60 of Drosophila." *Genes & Development* 11 (22): 3020–31. https://doi.org/10.1101/GAD.11.22.3020.

Cary, Daniele C., Koh Fujinaga, and B. Matija Peterlin. 2016. "Molecular Mechanisms of HIV Latency." *Journal of Clinical Investigation* 126 (2): 448–54. https://doi.org/10.1172/JCI80565.

Chen, Hung Ta, Linda Warfield, and Steven Hahn. 2007. "The Positions of TFIIF and TFIIE in the RNA Polymerase II Transcription Preinitiation Complex." *Nature Structural & Molecular Biology* 14 (8): 696–703. https://doi.org/10.1038/NSMB1272.

Chen, Ruichuan, Zhiyuan Yang, and Qiang Zhou. 2004. "Phosphorylated Positive Transcription Elongation Factor b (P-TEFb) Is Tagged for Inhibition through Association with 7SK SnRNA." *Journal of Biological Chemistry* 279 (6): 4153–60. https://doi.org/10.1074/JBC.M310044200.

Chen, Yexi, Yuki Yamaguchi, Yuta Tsugeno, Junichi Yamamoto, Tomoko Yamada, Mitsuhiro Nakamura, Koji Hisatake, and Hiroshi Handa. 2009. "DSIF, the Paf1 Complex, and Tat-SF1 Have Nonredundant, Cooperative Roles in RNA Polymerase II Elongation." *Genes and Development* 23 (23): 2765–77. https://doi.org/10.1101/GAD.1834709.

Cheng, Yulan, Zhe Jin, Rachana Agarwal, Ke Ma, Jian Yang, Soibrahim Ibrahim, Alexandru v. Olaru, et al. 2012. "LARP7 Is a Potential Tumor Suppressor Gene in Gastric Cancer." *Laboratory Investigation* 92 (7): 1013–19. https://doi.org/10.1038/LABINVEST.2012.59.

Chomonta, Nicolas, Sandrina DaFonsecaa, Claire Vandergeetena, Petronela Ancuta, and Rafick Pierre Sékaly. 2011. "Maintenance of CD4+ T-Cell Memory and HIV Persistence: Keeping Memory, Keeping HIV." *Current Opinion in HIV and AIDS* 6 (1): 30–36. https://doi.org/10.1097/COH.0B013E3283413775.

Citri, Ami, Daniel Harari, Galit Shohat, Parameswaran Ramakrishnan, Judith Gan, Sara Lavi, Miriam Eisenstein, et al. 2006. “Hsp90 Recognizes a Common Surface on Client Kinases.” *The Journal of Biological Chemistry* 281 (20): 14361–69. https://doi.org/10.1074/JBC.M512613200.

Contreras, Xavier, Matjaz Barboric, Tina Lenasi, and B. Matija Peterlin. 2007. “HMBA Releases P-TEFb from HEXIM1 and 7SK SnRNA via PI3K/Akt and Activates HIV Transcription.” *PLoS Pathogens* 3 (10): 1459–69. https://doi.org/10.1371/JOURNAL.PPAT.0030146.

Cramer, Patrick. 2019. “Eukaryotic Transcription Turns 50.” *Cell* 179 (4): 808–12. https://doi.org/10.1016/J.CELL.2019.09.018.

Creyghton, Menno P., Albert W. Cheng, G. Grant Welstead, Tristan Kooistra, Bryce W. Carey, Eveline J. Steine, Jacob Hanna, et al. 2010. “Histone H3K27ac Separates Active from Poised Enhancers and Predicts Developmental State.” *Proceedings of the National Academy of Sciences of the United States of America* 107 (50): 21931–36. https://doi.org/10.1073/pnas.1016071107.

Dahabieh, Matthew S., Emilie Battivelli, and Eric Verdin. 2015. “Understanding HIV Latency: The Road to an HIV Cure.” *Annual Review of Medicine* 66 (January): 407–21. https://doi.org/10.1146/ANNUREV-MED-092112-152941.

Darcis, Gilles, Benoit van Driessche, and Carine van Lint. 2017. “HIV Latency: Should We Shock or Lock?” *Trends in Immunology* 38 (3): 217–28. https://doi.org/10.1016/J.IT.2016.12.003.

Das, Amit K., Patricia T.W. Cohen, and David Barford. 1998. “The Structure of the Tetratricopeptide Repeats of Protein Phosphatase 5: Implications for TPR-Mediated Protein-Protein Interactions.” *The EMBO Journal* 17 (5): 1192–99. https://doi.org/10.1093/EMBOJ/17.5.1192.

Dawson, Mark A., Andrew J. Bannister, Berthold Göttgens, Samuel D. Foster, Till Bartke, Anthony R. Green, and Tony Kouzarides. 2009. “JAK2 Phosphorylates Histone H3Y41 and Excludes HP1α from Chromatin.” *Nature* 461 (7265): 819–22. https://doi.org/10.1038/nature08448.

DeJong, J., and R. G. Roeder. 1993. “A Single CDNA, HTFIIA/Alpha, Encodes Both the P35 and P19 Subunits of Human TFIIA.” *Genes & Development* 7 (11): 2220–34. https://doi.org/10.1101/GAD.7.11.2220.

Delmore, Jake E., Ghayas C. Issa, Madeleine E. Lemieux, Peter B. Rahl, Junwei Shi, Hannah M. Jacobs, Efstathios Kastritis, et al. 2011. “BET Bromodomain Inhibition as a Therapeutic Strategy to Target C-Myc.” *Cell* 146 (6): 904–17. https://doi.org/10.1016/J.CELL.2011.08.017.

Denny, Wesley B, Donna L Valentine, Philip D Reynolds, David F Smith, and Jonathan G Scammell. 2000. “Squirrel Monkey Immunophilin FKBP5 Is a Potent Inhibitor of Glucocorticoid Receptor Binding*.” https://academic.oup.com/endo/article/141/11/4107/2987711.

Devaiah, Ballachanda N., Hanxin Lu, Anne Gegonne, Zeynep Sercan, Hongen Zhang, Robert J. Clifford, Maxwell P. Lee, and Dinah S. Singer. 2010. “Novel Functions for TAF7, a Regulator of TAF1-Independent Transcription.” *Journal of Biological Chemistry* 285 (50): 38772–80. https://doi.org/10.1074/jbc.M110.173864.

Dey, Anwesha, Sheng Hao Chao, and David P. Lane. 2007. “HEXIM1 and the Control of Transcription Elongation: From Cancer and Inflammation to AIDS and Cardiac Hypertrophy.” *Cell Cycle* 6 (15): 1856–63. https://doi.org/10.4161/CC.6.15.4556.

Diribarne, Gaelle, and Olivier Bensaude. 2009. “7SK RNA, a Non-Coding RNA Regulating P-TEFb, a General Transcription Factor.” *RNA Biology* 6 (2): 122–28. https://doi.org/10.4161/RNA.6.2.8115.

Dobin, Alexander, Carrie A. Davis, Felix Schlesinger, Jorg Drenkow, Chris Zaleski, Sonali Jha, Philippe Batut, Mark Chaisson, and Thomas R. Gingeras. 2013. “STAR: Ultrafast Universal RNA-Seq Aligner.” *Bioinformatics* 29 (1): 15–21. https://doi.org/10.1093/bioinformatics/bts635.

Dunyak, Bryan M., and Jason E. Gestwicki. 2016. “Peptidyl-Proline Isomerases (PPIases): Targets for Natural Products and Natural Product-Inspired Compounds.” *Journal of Medicinal Chemistry* 59 (21): 9622–44. https://doi.org/10.1021/acs.jmedchem.6b00411.

Duyne, Gregory D. van, Robert F. Standaert, P. Andrew Karplus, Stuart L. Schreiber, and Jon Clardy. 1991. “Atomic Structure of FKBP-FK506, an Immunophilin-Immunosuppressant Complex.” *Science (New York, N.Y.)* 252 (5007): 839–42. https://doi.org/10.1126/SCIENCE.1709302.

Eberhardy, Scott R., and Peggy J. Farnham. 2002. “Myc Recruits P-TEFb to Mediate the Final Step in the Transcriptional Activation of the Cad Promoter.” *Journal of Biological Chemistry* 277 (42): 40156–62. https://doi.org/10.1074/JBC.M207441200.

Eisele, Evelyn, and Robert F. Siliciano. 2012. “Redefining the Viral Reservoirs That Prevent HIV-1 Eradication.” *Immunity* 37 (3): 377–88. https://doi.org/10.1016/J.IMMUNI.2012.08.010.

Engelman, Jeffrey A., Ji Luo, and Lewis C. Cantley. 2006. “The Evolution of Phosphatidylinositol 3-Kinases as Regulators of Growth and Metabolism.” *Nature Reviews Genetics* 7 (8): 606–19. https://doi.org/10.1038/nrg1879.

Espinoza-Derout, Jorge, Michael Wagner, Louis Salciccioli, Jason M. Lazar, Sikha Bhaduri, Eduardo Mascareno, Brahim Chaqour, and M. A.Q. Siddiqui. 2009. “Positive Transcription Elongation Factor b Activity in Compensatory Myocardial Hypertrophy Is Regulated by Cardiac Lineage Protein-1.” *Circulation Research* 104 (12): 1347–54. https://doi.org/10.1161/CIRCRESAHA.108.191726.

Espinoza-Derout, Jorge, Michael Wagner, Katayoun Shahmiri, Eduardo Mascareno, Brahim Chaqour, and M. A.Q. Siddiqui. 2007. “Pivotal Role of Cardiac Lineage Protein-1 (CLP-1) in Transcriptional Elongation Factor P-TEFb Complex Formation in Cardiac Hypertrophy.” *Cardiovascular Research* 75 (1): 129–38. https://doi.org/10.1016/J.CARDIORES.2007.03.019.

Fairley, Jennifer A., Rachel Evans, Nicola A. Hawkes, and Stefan G. E. Roberts. 2002. “Core Promoter-Dependent TFIIB Conformation and a Role for TFIIB Conformation in Transcription Start Site Selection.” *Molecular and Cellular Biology* 22 (19): 6697–6705. https://doi.org/10.1128/MCB.22.19.6697-6705.2002.

Franco, Lia Carolina, Fátima Morales, Silvia Boffo, and Antonio Giordano. 2018a. “CDK9: A Key Player in Cancer and Other Diseases.” *Journal of Cellular Biochemistry* 119 (2): 1273–84. https://doi.org/10.1002/JCB.26293.

———. 2018b. “CDK9: A Key Player in Cancer and Other Diseases.” *Journal of Cellular Biochemistry* 119 (2): 1273–84. https://doi.org/10.1002/jcb.26293.

Fries, Gabriel R., Nils C. Gassen, and Theo Rein. 2017. “The FKBP5 Glucocorticoid Receptor Co-Chaperone: Regulation, Function, and Implications in Health and Disease.” *International Journal of Molecular Sciences 2017, Vol. 18, Page 2614* 18 (12): 2614. https://doi.org/10.3390/IJMS18122614.

Fruman, David A., Steven J. Burakoff, and Barbara E. Bierer. 1994a. “ Immunophilins in Protein Folding and Immunosuppression 1 .” *The FASEB Journal* 8 (6): 391–400. https://doi.org/10.1096/fasebj.8.6.7513288.

———. 1994b. “ Immunophilins in Protein Folding and Immunosuppression 1 .” *The FASEB Journal* 8 (6): 391–400. https://doi.org/10.1096/fasebj.8.6.7513288.

Fujinaga, Koh, Ran Taube, Jörg Wimmer, Thomas P. Cujec, and B. Matija Peterlin. 1999. “Interactions between Human Cyclin T, Tat, and the Transactivation Response Element (TAR) Are Disrupted by a Cysteine to Tyrosine Substitution Found in Mouse Cyclin T.” *Proceedings of the National Academy of Sciences of the United States of America* 96 (4): 1285–90. https://doi.org/10.1073/PNAS.96.4.1285.

Fujita, Naoyuki, Shin-ichiro Takebayashi, Katsuzumi Okumura, Shinichi Kudo, Tsutomu Chiba, Hideyuki Saya, and Mitsuyoshi Nakao. 1999. “Methylation-Mediated Transcriptional Silencing in Euchromatin by Methyl-CpG Binding Protein MBD1 Isoforms.” *Molecular and Cellular Biology* 19 (9): 6415–26. https://doi.org/10.1128/MCB.19.9.6415.

GALAT, Andrzej. 1993. “Peptidylproline Cis-trans-isomerases: Immunophilins.” *European Journal of Biochemistry* 216 (3): 689–707. https://doi.org/10.1111/j.1432-1033.1993.tb18189.x.

Garber, Mitchell E., Ping Wei, Vineet N. KewalRamani, Timothy P. Mayall, Christine H. Herrmann, Andrew P. Rice, Dan R. Littman, and Katherine A. Jones. 1998. “The Interaction between HIV-1 Tat and Human Cyclin T1 Requires Zinc and a Critical Cysteine Residue That Is Not Conserved in the Murine CycT1 Protein.” *Genes and Development* 12 (22): 3512–27. https://doi.org/10.1101/GAD.12.22.3512.

Gargano, Barbara, Stefano Amente, Barbara Majello, and Luigi Lania. 2007. “P-TEFb Is a Crucial Co-Factor for Myc Transactivation.” *Cell Cycle* 6 (16): 2031–37. https://doi.org/10.4161/CC.6.16.4554.

Garriga, Judit, and Xavier Graña. 2014. “CDK9 Inhibition Strategy Defines Distinct Sets of Target Genes.” *BMC Research Notes* 7 (1). https://doi.org/10.1186/1756-0500-7-301.

Garriga, Judit, Junmin Peng, Matilde Parreño, David H. Price, Earl E. Henderson, and Xavier Graña. 1998. “Upregulation of Cyclin T1/CDK9 Complexes during T Cell Activation.” *Oncogene* 17 (24): 3093–3102. https://doi.org/10.1038/SJ.ONC.1202548.

Garriga, Judit, Hongbo Xie, Zoran Obradovic, and Xavier Graña. 2010. “Selective Control of Gene Expression by CDK9 in Human Cells.” *Journal of Cellular Physiology* 222 (1): 200–208. https://doi.org/10.1002/JCP.21938.

Gassen, Nils C., Gabriel R. Fries, Anthony S. Zannas, Jakob Hartmann, Jurgen Zschocke, Kathrin Hafner, Tania Carrillo-Roa, et al. 2015. “Chaperoning Epigenetics: FKBP5 Decreases the Activity of DNMT1 and Mediates Epigenetic Effects of the Antidepressant Paroxetine.” *Science Signaling* 8 (404). https://doi.org/10.1126/SCISIGNAL.AAC7695/SUPPL_FILE/8_RA119_SM.PDF.

Gaszner, Miklos, and Gary Felsenfeld. 2006. “Insulators: Exploiting Transcriptional and Epigenetic Mechanisms.” *Nature Reviews. Genetics* 7 (9): 703–13. https://doi.org/10.1038/NRG1925.

Ghosh, Koushik, Ming Tang, Nidhi Kumari, Arijit Nandy, Subham Basu, Dheerendra Pratap Mall, Kunal Rai, and Debabrata Biswas. 2018. “Positive Regulation of Transcription by Human ZMYND8 through Its Association with P-TEFb Complex.” *Cell Reports* 24 (8): 2141-2154.e6. https://doi.org/10.1016/j.celrep.2018.07.064.

Giacinti, Cristina, Luigi Bagella, Pier Lorenzo Puri, Antonio Giordano, and Cristiano Simone. 2006. “MyoD Recruits the Cdk9/Cyclin T2 Complex on Myogenic-Genes Regulatory Regions.” *Journal of Cellular Physiology* 206 (3): 807–13. https://doi.org/10.1002/JCP.20523.

Giacinti, Cristina, Antonio Musarò, Giulia de Falco, Isabelle Jourdan, Mario Molinaro, Luigi Bagella, Cristiano Simone, and Antonio Giordano. 2008. “Cdk9-55: A New Player in Muscle Regeneration.” *Journal of Cellular Physiology* 216 (3): 576–82. https://doi.org/10.1002/jcp.21361.

Gilchrist, Daniel A., David C. Fargo, and Karen Adelman. 2009. “Using ChIP-Chip and ChIP-Seq to Study the Regulation of Gene Expression: Genome-Wide Localization Studies Reveal Widespread Regulation of Transcription Elongation.” *Methods* 48 (4): 398–408. https://doi.org/10.1016/J.YMETH.2009.02.024.

Giraud, Sandrine, Adam Hurlstone, Sylvie Avril, and Olivier Coqueret. 2004. “Implication of BRG1 and Cdk9 in the STAT3-Mediated Activation of the P21waf1 Gene.” *Oncogene* 23 (44): 7391–98. https://doi.org/10.1038/SJ.ONC.1207972.

Gnatt, A. L., P. Cramer, J. Fu, D. A. Bushnell, and R. D. Kornberg. 2001. “Structural Basis of Transcription: An RNA Polymerase II Elongation Complex at 3.3 A Resolution.” *Science (New York, N.Y.)* 292 (5523): 1876–82. https://doi.org/10.1126/SCIENCE.1059495.

Goldman, Seth R., Richard H. Ebright, and Bryce E. Nickels. 2009. “Direct Detection of Abortive RNA Transcripts in Vivo.” *Science (New York, N.Y.)* 324 (5929): 927–28. https://doi.org/10.1126/SCIENCE.1169237.

Gomes, Nathan P., Glen Bjerke, Briardo Llorente, Stephanie A. Szostek, Beverly M. Emerson, and Joaquin M. Espinosa. 2006. “Gene-Specific Requirement for P-TEFb Activity and RNA Polymerase II Phosphorylation within the P53 Transcriptional Program.” *Genes and Development* 20 (5): 601–12. https://doi.org/10.1101/GAD.1398206.

Grummt, Ingrid. 1998. “Regulation of Mammalian Ribosomal Gene Transcription by RNA Polymerase I.” *Progress in Nucleic Acid Research and Molecular Biology* 62 (C): 109–54. https://doi.org/10.1016/S0079-6603(08)60506-1.

Gurumurthy, Aishwarya, Yong Shen, Eliot M. Gunn, and Jörg Bungert. 2019. “Phase Separation and Transcription Regulation: Are Super-Enhancers and Locus Control Regions Primary Sites of Transcription Complex Assembly?” *BioEssays : News and Reviews in Molecular, Cellular and Developmental Biology* 41 (1). https://doi.org/10.1002/BIES.201800164.

Hargreaves, Diana C., Tiffany Horng, and Ruslan Medzhitov. 2009. “Control of Inducible Gene Expression by Signal-Dependent Transcriptional Elongation.” *Cell* 138 (1): 129–45. https://doi.org/10.1016/J.CELL.2009.05.047.

Harrison, Richard K., and Ross L. Stein. 1990. “Mechanistic Studies of Peptidyl Prolyl Cis-Trans Isomerase: Evidence for Catalysis by Distortion.” *Biochemistry* 29 (7): 1684–89. https://doi.org/10.1021/bi00459a003.

Hawkes, Nicola A., and Stefan G.E. Roberts. 1999. “The Role of Human TFIIB in Transcription Start Site Selection in Vitro and in Vivo.” *Journal of Biological Chemistry* 274 (20): 14337–43. https://doi.org/10.1074/jbc.274.20.14337.

Hebbes, T. R., A. W. Thorne, and C. Crane-Robinson. 1988. “A Direct Link between Core Histone Acetylation and Transcriptionally Active Chromatin.” *The EMBO Journal* 7 (5): 1395–1402. https://doi.org/10.1002/J.1460-2075.1988.TB02956.X.

Hou, Tieying, Sutapa Ray, and Allan R. Brasier. 2007. “The Functional Role of an Interleukin 6-Inducible CDK9·STAT3 Complex in Human γ-Fibrinogen Gene Expression.” *Journal of Biological Chemistry* 282 (51): 37091–102. https://doi.org/10.1074/JBC.M706458200.

Huang, Da Wei, Brad T. Sherman, and Richard A. Lempicki. 2009. “Systematic and Integrative Analysis of Large Gene Lists Using DAVID Bioinformatics Resources.” *Nature Protocols* 4 (1): 44–57. https://doi.org/10.1038/nprot.2008.211.

Jadlowsky, Julie K., Masanori Nojima, Takashi Okamoto, and Koh Fujinaga. 2008. “Dominant Negative Mutant Cyclin T1 Proteins That Inhibit HIV Transcription by Forming a Kinase Inactive Complex with Tat.” *Journal of General Virology* 89 (11): 2783–87. https://doi.org/10.1099/VIR.0.2008/002857-0.

Jadlowsky, Julie K., Masanori Nojima, Antje Schulte, Matthias Geyer, Takashi Okamoto, and Koh Fujinaga. 2008. “Dominant Negative Mutant Cyclin T1 Proteins Inhibit HIV Transcription by Specifically Degrading Tat.” *Retrovirology* 5 (July). https://doi.org/10.1186/1742-4690-5-63.

Jeang, Kuan Teh. 1998. “Tat, Tat-Associated Kinase, and Transcription.” *Journal of Biomedical Science* 5 (1): 24–27. https://doi.org/10.1007/BF02253352.

Ji, Xiaodan, Huasong Lu, Qiang Zhou, and Kunxin Luo. 2014. “LARP7 Suppresses P-TEFb Activity to Inhibit Breast Cancer Progression and Metastasis.” *ELife* 3: e02907. https://doi.org/10.7554/ELIFE.02907.

Jiang, Huimin, and B. Matija Peterlin. 2008. “Differential Chromatin Looping Regulates CD4 Expression in Immature Thymocytes.” *Molecular and Cellular Biology* 28 (3): 907–12. https://doi.org/10.1128/mcb.00909-07.

Jinwal, Umesh K., John Koren, Sergiy I. Borysov, Andreas B. Schmid, Jose F. Abisambra, Laura J. Blair, Amelia G. Johnson, et al. 2010. “The Hsp90 Cochaperone, FKBP5, Increases Tau Stability and Polymerizes Microtubules.” *Journal of Neuroscience* 30 (2): 591–99. https://doi.org/10.1523/JNEUROSCI.4815-09.2010.

Jirtle, Randy L., and Michael K. Skinner. 2007. “Environmental Epigenomics and Disease Susceptibility.” *Nature Reviews. Genetics* 8 (4): 253–62. https://doi.org/10.1038/NRG2045.

Jones, Katherine A. 1997. “Taking a New TAK on TAT Transactivation.” *Genes and Development* 11 (20): 2593–99. https://doi.org/10.1101/GAD.11.20.2593.

Jonkers, Iris, and John T. Lis. 2015. “Getting up to Speed with Transcription Elongation by RNA Polymerase II.” *Nature Reviews Molecular Cell Biology 2015 16:3* 16 (3): 167–77. https://doi.org/10.1038/nrm3953.

Kanazawa, Satoshi, Takashi Okamoto, and B. Matija Peterlin. 2000. “Tat Competes with CIITA for the Binding to P-TEFb and Blocks the Expression of MHC Class II Genes in HIV Infection.” *Immunity* 12 (1): 61–70. https://doi.org/10.1016/S1074-7613(00)80159-4.

Kanazawa, Satoshi, Laura Soucek, Gerard Evan, Takashi Okamoto, and B. Matija Peterlin. 2003. “C-Myc Recruits P-TEFb for Transcription, Cellular Proliferation and Apoptosis.” *Oncogene* 22 (36): 5707–11. https://doi.org/10.1038/SJ.ONC.1206800.

Kang, Cong Bao, Ye Hong, Sirano Dhe-Paganon, and Ho Sup Yoon. 2008. “FKBP Family Proteins: Immunophilins with Versatile Biological Functions.” *Neurosignals* 16 (4): 318–25. https://doi.org/10.1159/000123041.

Karn, Jonathan. 1999. “Tackling Tat.” *Journal of Molecular Biology* 293 (2): 235–54. https://doi.org/10.1006/JMBI.1999.3060.

———. 2011. “The Molecular Biology of HIV Latency: Breaking and Restoring the Tat-Dependent Transcriptional Circuit.” *Current Opinion in HIV and AIDS* 6 (1): 4–11. https://doi.org/10.1097/COH.0B013E328340FFBB.

Khaperskyy, Denys A., Michelle L. Ammerman, Robert C. Majovski, and Alfred S. Ponticelli. 2008. “Functions of Saccharomyces Cerevisiae TFIIF during Transcription Start Site Utilization.” *Molecular and Cellular Biology* 28 (11): 3757–66. https://doi.org/10.1128/MCB.02272-07.

Kim, Tae Kyung, Richard H. Ebright, and Danny Reinberg. 2000. “Mechanism of ATP-Dependent Promoter Melting by Transcription Factor IIH.” *Science (New York, N.Y.)* 288 (5470): 1418–21. https://doi.org/10.1126/SCIENCE.288.5470.1418.

Kim, TK, M Hemberg, JM Gray, AM Costa, DM Bear - Nature, and undefined 2010. n.d. “Widespread Transcription at Neuronal Activity-Regulated Enhancers.” *Nature.Com*. Accessed February 27, 2022. https://www.nature.com/articles/nature09033.

Kim, Young Kyeung, Uri Mbonye, Joseph Hokello, and Jonathan Karn. 2011. “T-Cell Receptor Signaling Enhances Transcriptional Elongation from Latent HIV Proviruses by Activating P-TEFb through an ERK-Dependent Pathway.” *Journal of Molecular Biology* 410 (5): 896–916. https://doi.org/10.1016/J.JMB.2011.03.054.

Kimata, Jason T., Andrew P. Rice, and Jin Wang. 2016. “Challenges and Strategies for the Eradication of the HIV Reservoir.” *Current Opinion in Immunology* 42 (October): 65–70. https://doi.org/10.1016/J.COI.2016.05.015.

Kloet, Susan L., Jennifer L. Whiting, Phil Gafken, Jeff Ranish, and Edith H. Wang. 2012. “Phosphorylation-Dependent Regulation of Cyclin D1 and Cyclin A Gene Transcription by TFIID Subunits TAF1 and TAF7.” *Molecular and Cellular Biology* 32 (16): 3358–69. https://doi.org/10.1128/MCB.00416-12.

Kohoutek, Jiri. 2009a. “P-TEFb- The Final Frontier.” *Cell Division* 4 (September): 19. https://doi.org/10.1186/1747-1028-4-19.

———. 2009b. “P-TEFb- the Final Frontier.” *Cell Division* 4 (September): 19. https://doi.org/10.1186/1747-1028-4-19.

Kornberg, Roger D. 2007. “The Molecular Basis of Eukaryotic Transcription.” *Proceedings of the National Academy of Sciences of the United States of America* 104 (32): 12955–61. https://doi.org/10.1073/PNAS.0704138104.

Kozany, Christian, Andreas März, Christoph Kress, and Felix Hausch. 2009. “Fluorescent Probes to Characterise FK506-Binding Proteins.” *ChemBioChem* 10 (8): 1402–10. https://doi.org/10.1002/cbic.200800806.

Kuehner, Jason N., Erika L. Pearson, and Claire Moore. 2011. “Unravelling the Means to an End: RNA Polymerase II Transcription Termination.” *Nature Reviews. Molecular Cell Biology* 12 (5): 283–94. https://doi.org/10.1038/NRM3098.

Kuzmina, Alona, Nina Verstraete, Sigal Galker, Maayan Maatook, Olivier Bensaude, and Ran Taube. 2014. “A Single Point Mutation in Cyclin T1 Eliminates Binding to Hexim1, Cdk9 and RNA but Not to AFF4 and Enforces Repression of HIV Transcription.” *Retrovirology* 11 (1). https://doi.org/10.1186/1742-4690-11-51.

Lamb, JR, S Tugendreich, P Hieter - Trends in biochemical sciences, and undefined 1995. n.d. “Tetratrico Peptide Repeat Interactions: To TPR or Not to TPR?” *Elsevier*. Accessed February 27, 2022. https://www.sciencedirect.com/science/article/pii/S0968000400890374.

Langelier, Marie France, Diane Forget, Andrés Rojas, Yanie Porlier, Zachary F. Burton, and Benoit Coulombe. 2001. “Structural and Functional Interactions of Transcription Factor (TF) IIA with TFIIE and TFIIF in Transcription Initiation by RNA Polymerase II.” *Journal of Biological Chemistry* 276 (42): 38652–57. https://doi.org/10.1074/jbc.M106422200.

Lawrence, Michael, Wolfgang Huber, Hervé Pagès, Patrick Aboyoun, Marc Carlson, Robert Gentleman, Martin T. Morgan, and Vincent J. Carey. 2013. “Software for Computing and Annotating Genomic Ranges.” *PLoS Computational Biology* 9 (8). https://doi.org/10.1371/JOURNAL.PCBI.1003118.

Lee, Cheol Koo, Yoichiro Shibata, Bhargavi Rao, Brian D. Strahl, and Jason D. Lieb. 2004. “Evidence for Nucleosome Depletion at Active Regulatory Regions Genome-Wide.” *Nature Genetics* 36 (8): 900–905. https://doi.org/10.1038/NG1400.

Lee, Dong Kun, Hai Ou Duan, and Chawnshang Chang. 2001. “Androgen Receptor Interacts with the Positive Elongation Factor P-TEFb and Enhances the Efficiency of Transcriptional Elongation.” *Journal of Biological Chemistry* 276 (13): 9978–84. https://doi.org/10.1074/JBC.M002285200.

Li, L., Z. Lou, and L. Wang. 2011. “The Role of FKBP5 in Cancer Aetiology and Chemoresistance.” *British Journal of Cancer*. https://doi.org/10.1038/sj.bjc.6606014.

Li, Qintong, Jason P. Price, Sarah A. Byers, Dongmei Cheng, Junmin Peng, and David H. Price. 2005. “Analysis of the Large Inactive P-TEFb Complex Indicates That It Contains One 7SK Molecule, a Dimer of HEXIM1 or HEXIM2, and Two P-TEFb Molecules Containing Cdk9 Phosphorylated at Threonine 186.” *Journal of Biological Chemistry* 280 (31): 28819–26. https://doi.org/10.1074/JBC.M502712200.

Liao, Yang, Gordon K. Smyth, and Wei Shi. 2019. “The R Package Rsubread Is Easier, Faster, Cheaper and Better for Alignment and Quantification of RNA Sequencing Reads.” *Nucleic Acids Research* 47 (8). https://doi.org/10.1093/nar/gkz114.

Lin, Chengqi, Edwin R. Smith, Hidehisa Takahashi, Ka Chun Lai, Skylar Martin-Brown, Laurence Florens, Michael P. Washburn, Joan W. Conaway, Ronald C. Conaway, and Ali Shilatifard. 2010. "AFF4, a Component of the ELL/P-TEFb Elongation Complex and a Shared Subunit of MLL Chimeras, Can Link Transcription Elongation to Leukemia." *Molecular Cell* 37 (3): 429–37. https://doi.org/10.1016/J.MOLCEL.2010.01.026.

Liou, Li-Ying, Richard E. Haaland, Christine H. Herrmann, and Andrew P. Rice. 2006. "Cyclin T1 but Not Cyclin T2a Is Induced by a Post-Transcriptional Mechanism in PAMP-Activated Monocyte-Derived Macrophages." *Journal of Leukocyte Biology* 79 (2): 388–96. https://doi.org/10.1189/JLB.0805429.

Liou, Li-Ying, Christine H. Herrmann, and Andrew P. Rice. 2002. "Transient Induction of Cyclin T1 during Human Macrophage Differentiation Regulates Human Immunodeficiency Virus Type 1 Tat Transactivation Function." *Journal of Virology* 76 (21): 10579–87. https://doi.org/10.1128/JVI.76.21.10579-10587.2002.

Lis, John T. 2019. "A 50 Year History of Technologies That Drove Discovery in Eukaryotic Transcription Regulation." *Nature Structural & Molecular Biology 2019 26:9* 26 (9): 777–82. https://doi.org/10.1038/s41594-019-0288-9.

Liu, Hongbing, and Christine H. Herrmann. 2005. "Differential Localization and Expression of the Cdk9 42k and 55k Isoforms." *Journal of Cellular Physiology* 203 (1): 251–60. https://doi.org/10.1002/JCP.20224.

Liu, Pingyang, Yanhui Xiang, Koh Fujinaga, Koen Bartholomeeusen, Kyle A. Nilson, David H. Price, and B. Matija Peterlin. 2014. "Release of Positive Transcription Elongation Factor b (P-TEFb) from 7SK Small Nuclear Ribonucleoprotein (SnRNP) Activates Hexamethylene Bisacetamide-Inducible Protein (HEXIM1) Transcription." *Journal of Biological Chemistry* 289 (14): 9918–25. https://doi.org/10.1074/JBC.M113.539015.

Liu, Zhe, and Robert Tjian. 2018. "Visualizing Transcription Factor Dynamics in Living Cells." *Journal of Cell Biology* 217 (4): 1181–91. https://doi.org/10.1083/jcb.201710038.

Louder, Robert K., Yuan He, José Ramón López-Blanco, Jie Fang, Pablo Chacón, and Eva Nogales. 2016. "Structure of Promoter-Bound TFIID and Model of Human Pre-Initiation Complex Assembly." *Nature* 531 (7596): 604–9. https://doi.org/10.1038/NATURE17394.

Love, Michael I., Wolfgang Huber, and Simon Anders. 2014. "Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2." *Genome Biology* 15 (12). https://doi.org/10.1186/S13059-014-0550-8.

Lu, Huasong, Zichong Li, Yuhua Xue, Ursula Schulze-Gahmen, Jeffrey R. Johnson, Nevan J. Krogan, Tom Alber, and Qiang Zhou. 2014. "AFF1 Is a Ubiquitous P-TEFb Partner to Enable Tat Extraction of P-TEFb from 7SK SnRNP and Formation of SECs for HIV Transactivation." *Proceedings of the National Academy of Sciences of the United States of America* 111 (1). https://doi.org/10.1073/PNAS.1318503111.

Lu, Huasong, Dan Yu, Anders S. Hansen, Sourav Ganguly, Rongdiao Liu, Alec Heckert, Xavier Darzacq, and Qiang Zhou. 2018. "Phase-Separation Mechanism for C-Terminal Hyperphosphorylation of RNA Polymerase II." *Nature* 558 (7709): 318–23. https://doi.org/10.1038/S41586-018-0174-3.

Luo, Zhuojuan, Chengqi Lin, and Ali Shilatifard. 2012a. “The Super Elongation Complex (SEC) Family in Transcriptional Control.” *Nature Reviews Molecular Cell Biology* 13 (9): 543–47. https://doi.org/10.1038/NRM3417.

———. 2012b. “The Super Elongation Complex (SEC) Family in Transcriptional Control.” *Nature Reviews Molecular Cell Biology* 13 (9): 543–47. https://doi.org/10.1038/NRM3417.

Maile, Tobias, Simona Kwoczynski, Rebeccah J. Katzenberger, David A. Wassarman, and Frank Sauer. 2004. “TAF1 Activates Transcription by Phosphorylation of Serine 33 in Histone H2B.” *Science (New York, N.Y.)* 304 (5673): 1010–14. https://doi.org/10.1126/SCIENCE.1095001.

Manning, Brendan D., and Lewis C. Cantley. 2007. “AKT/PKB Signaling: Navigating Downstream.” *Cell* 129 (7): 1261–74. https://doi.org/10.1016/j.cell.2007.06.009.

Margolis, David M. 2010. “Mechanisms of HIV Latency: An Emerging Picture of Complexity.” *Current HIV/AIDS Reports* 7 (1): 37–43. https://doi.org/10.1007/S11904-009-0033-9.

Marshall, N F, and D H Price. 1992. “Control of Formation of Two Distinct Classes of RNA Polymerase II Elongation Complexes.” *Molecular and Cellular Biology* 12 (5): 2078–90. https://doi.org/10.1128/MCB.12.5.2078.

Marshall, N. F., and D. H. Price. 1995. “Purification of P-TEFb, a Transcription Factor Required for the Transition into Productive Elongation.” *Journal of Biological Chemistry* 270 (21): 12335–38. https://doi.org/10.1074/JBC.270.21.12335.

Marshall, Renee M., and Xavier Grana. 2006. “Mechanisms Controlling CDK9 Activity.” *Frontiers in Bioscience* 11 (SUPPL. 2): 2598–2613. https://doi.org/10.2741/1994.

Marshall, Renée M., Dominic Salerno, Judit Garriga, and Xavier Graña. 2005. “Cyclin T1 Expression Is Regulated by Multiple Signaling Pathways and Mechanisms during Activation of Human Peripheral Blood Lymphocytes.” *The Journal of Immunology* 175 (10): 6402–11. https://doi.org/10.4049/JIMMUNOL.175.10.6402.

Martinez, Ernest, Cheng Ming Chiang, Hui Ge, and Robert G. Roeder. 1994. “TATA-Binding Protein-Associated Factor(s) in TFIID Function through the Initiator to Direct Basal Transcription from a TATA-Less Class II Promoter.” *EMBO Journal* 13 (13): 3115–26. https://doi.org/10.1002/j.1460-2075.1994.tb06610.x.

Mayer, Andreas, Michael Lidschreiber, Matthias Siebert, Kristin Leike, Johannes Söding, and Patrick Cramer. 2010. “Uniform Transitions of the General RNA Polymerase II Transcription Complex.” *Nature Structural and Molecular Biology* 17 (10): 1272–78. https://doi.org/10.1038/NSMB.1903.

Mbonye, Uri, and Jonathan Karn. 2017. “The Molecular Basis for Human Immunodeficiency Virus Latency.” *Annual Review of Virology* 4 (September): 261–85. https://doi.org/10.1146/ANNUREV-VIROLOGY-101416-041646.

McBride, Anne E., and Pamela A. Silver. 2001. “State of the Arg: Protein Methylation at Arginine Comes of Age.” *Cell* 106 (1): 5–8. https://doi.org/10.1016/S0092-8674(01)00423-8.

McCarthy, Davis J., Yunshun Chen, and Gordon K. Smyth. 2012. “Differential Expression Analysis of Multifactor RNA-Seq Experiments with Respect to Biological Variation.” *Nucleic Acids Research* 40 (10): 4288–97. https://doi.org/10.1093/NAR/GKS042.

Melo, Carlos A., Nicolas Léveillé, and Reuven Agami. 2013. “ERNAs Reach the Heart of Transcription.” *Cell Research* 23 (10): 1151–52. https://doi.org/10.1038/cr.2013.97.

Mertz, Jennifer A., Andrew R. Conery, Barbara M. Bryant, Peter Sandy, Srividya Balasubramanian, Deanna A. Mele, Louise Bergeron, and Robert J. Sims. 2011. “Targeting MYC Dependence in Cancer by Inhibiting BET Bromodomains.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (40): 16669–74. https://doi.org/10.1073/PNAS.1108190108.

Metzger, Brian P.H., Fabien Duveau, David C. Yuan, Stephen Tryban, Bing Yang, and Patricia J. Wittkopp. 2016. “Contrasting Frequencies and Effects of Cis- and Trans-Regulatory Mutations Affecting Gene Expression.” *Molecular Biology and Evolution* 33 (5): 1131–46. https://doi.org/10.1093/MOLBEV/MSW011.

Mikhaylichenko, Olga, Vladyslav Bondarenko, Dermot Harnett, Ignacio E. Schor, Matilda Males, Rebecca R. Viales, and Eileen E.M. Furlong. 2018. “The Degree of Enhancer or Promoter Activity Is Reflected by the Levels and Directionality of ERNA Transcription.” *Genes and Development* 32 (1): 42–57. https://doi.org/10.1101/gad.308619.117.

Mitchell, Pamela J., and Robert Tjian. 1989. “Transcriptional Regulation in Mammalian Cells by Sequence-Specific DNA Binding Proteins.” *Science* 245 (4916): 371–78. https://doi.org/10.1126/science.2667136.

Mogyoró, András, and Fuad N Ziyadeh2. 1999. “Nephrology Dialysis Transplantation Hypothesis GLUT1 and TGF-b: The Link between Hyperglycaemia and Diabetic Nephropathy.” *Nephrol Dial Transplant*. Vol. 14.

Moon, Kyoo Jang, Kazuki Mochizuki, Meisheng Zhou, Ho Sang Jeong, John N. Brady, and Keiko Ozato. 2005. “The Bromodomain Protein Brd4 Is a Positive Regulatory Component of P-TEFb and Stimulates RNA Polymerase II-Dependent Transcription.” *Molecular Cell* 19 (4): 523–34. https://doi.org/10.1016/J.MOLCEL.2005.06.027.

Morales, Fatima, and Antonio Giordano. 2016. “Overview of CDK9 as a Target in Cancer Research.” *Cell Cycle*. Taylor and Francis Inc. https://doi.org/10.1080/15384101.2016.1138186.

Murray, Alexandra J., Kyungyoon J. Kwon, Donna L. Farber, and Robert F. Siliciano. 2016. “The Latent Reservoir for HIV-1: How Immunologic Memory and Clonal Expansion Contribute to HIV-1 Persistence.” *The Journal of Immunology* 197 (2): 407–17. https://doi.org/10.4049/JIMMUNOL.1600343.

Natoli, Gioacchino, and Jean Christophe Andrau. 2012. “Noncoding Transcription at Enhancers: General Principles and Functional Models.” *Annual Review of Genetics* 46: 1–19. https://doi.org/10.1146/ANNUREV-GENET-110711-155459.

Navone, Nora M. 2010. “P-TEFb Joins the Family of Cdks in Oncology, Promotes Cell Growth of Cancer Cells.” *Cell Cycle* 9 (15): 2999–3007. https://doi.org/10.4161/CC.9.15.12738.

Nechaev, Sergei, and Karen Adelman. 2011. “Pol II Waiting in the Starting Gates: Regulating the Transition from Transcription Initiation into Productive Elongation.” *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms* 1809 (1): 34–45. https://doi.org/10.1016/J.BBAGRM.2010.11.001.

Nguyen, Van Trung, Tamás Kiss, Annemieke A. Michels, and Olivier Bensaude. 2001. “7SK Small Nuclear RNA Binds to and Inhibits the Activity of CDK9/Cyclin T Complexes.” *Nature* 414 (6861): 322–25. https://doi.org/10.1038/35104581.

Ni, Li, Chun-Song Yang, Daniel Gioeli, Henry Frierson, David O. Toft, and Bryce M. Paschal. 2010. “FKBP5 Promotes Assembly of the Hsp90 Chaperone Complex and Regulates Androgen Receptor Signaling in Prostate Cancer Cells.” *Molecular and Cellular Biology* 30 (5): 1243–53. https://doi.org/10.1128/MCB.01891-08/ASSET/3C7B3FEC-30CF-461F-8888-937BCEDAB429/ASSETS/GRAPHIC/ZMB9991084880009.JPEG.

Ni WJ, Guan XM, Zeng J, Zhou H, Meng XM, Tang LQ. Berberine regulates mesangial cell proliferation and cell cycle to attenuate diabetic nephropathy through the PI3K/Akt/AS160/GLUT1 signalling pathway.” *J Cell Mol Med* 26(4):1144-1155. https://doi.org/10.1111/jcmm.17167

Ni, Zhuoyu, Abbie Saunders, Nicholas J. Fuda, Jie Yao, Jose-Ramon Suarez, Watt W. Webb, and John T. Lis. 2008. “P-TEFb Is Critical for the Maturation of RNA Polymerase II into Productive Elongation In Vivo.” *Molecular and Cellular Biology* 28 (3): 1161–70. https://doi.org/10.1128/MCB.01859-07.

Nojima, Masanori, Yehong Huang, Mudit Tyagi, Hung Ying Kao, and Koh Fujinaga. 2008. “The Positive Transcription Elongation Factor b Is an Essential Cofactor for the Activation of Transcription by Myocyte Enhancer Factor 2.” *Journal of Molecular Biology* 382 (2): 275–87. https://doi.org/10.1016/J.JMB.2008.07.017.

O’Shea-Greenfield, A., and S. T. Smale. 1992a. “Roles of TATA and Initiator Elements in Determining the Start Site Location and Direction of RNA Polymerase II Transcription.” *Journal of Biological Chemistry* 267 (2): 1391–1402. https://doi.org/10.1016/s0021-9258(18)48443-8.

———. 1992b. “Roles of TATA and Initiator Elements in Determining the Start Site Location and Direction of RNA Polymerase II Transcription.” *Journal of Biological Chemistry* 267 (2): 1391–1402. https://doi.org/10.1016/s0021-9258(18)48443-8.

Ott, Melanie, Matthias Geyer, and Qiang Zhou. 2011a. “The Control of HIV Transcription: Keeping RNA Polymerase II on Track.” *Cell Host and Microbe* 10 (5): 426–35. https://doi.org/10.1016/J.CHOM.2011.11.002.

———. 2011b. “The Control of HIV Transcription: Keeping RNA Polymerase II on Track.” *Cell Host and Microbe* 10 (5): 426–35. https://doi.org/10.1016/J.CHOM.2011.11.002.

Oven, Irena, Naděžda Brdičková, Jiri Kohoutek, Tomaž Vaupotič, Mojca Narat, and B. Matija Peterlin. 2007. “AIRE Recruits P-TEFb for Transcriptional Elongation of Target Genes in Medullary Thymic Epithelial Cells.” *Molecular and Cellular Biology* 27 (24): 8815–23. https://doi.org/10.1128/MCB.01085-07.

Padmanabhan, Srivatsan, Arnab Mukhopadhyay, Sri Devi Narasimhan, Gregory Tesz, Michael P. Czech, and Heidi A. Tissenbaum. 2009. “A PP2A Regulatory Subunit Regulates C. Elegans Insulin/IGF-1 Signaling by Modulating AKT-1 Phosphorylation.” *Cell* 136 (5): 939–51. https://doi.org/10.1016/J.CELL.2009.01.025.

Pal, Mahadeb, Alfred S. Ponticelli, and Donal S. Luse. 2005. “The Role of the Transcription Bubble and TFIIB in Promoter Clearance by RNA Polymerase II.” *Molecular Cell* 19 (1): 101–10. https://doi.org/10.1016/J.MOLCEL.2005.05.024.

Paparidis, Nikolas Ferreira dos Santos, Maxwell Castro Durvale, and Fernanda Canduri. 2017. “The Emerging Picture of CDK9/P-TEFb: More than 20 Years of Advances since PITALRE.” *Molecular BioSystems* 13 (2): 246–76. https://doi.org/10.1039/C6MB00387G.

Pei, Huadong, Liang Li, Brooke L. Fridley, Gregory D. Jenkins, Krishna R. Kalari, Wilma Lingle, Gloria Petersen, Zhenkun Lou, and Liewei Wang. 2009. “FKBP5 Affects Cancer Cell Response to Chemotherapy by Negatively Regulating Akt.” *Cancer Cell* 16 (3): 259–66. https://doi.org/10.1016/J.CCR.2009.07.016.

Peng, Junmin, Yuerong Zhu, Jeffrey T. Milton, and David H. Price. 1998. “Identification of Multiple Cyclin Subunits of Human P-TEFb.” *Genes and Development* 12 (5): 755–62. https://doi.org/10.1101/GAD.12.5.755.

Pereira, Maria J., Jenny Palming, Maria K. Svensson, Magnus Rizell, Jan Dalenbäck, Mårten Hammar, Tove Fall, Cherno O. Sidibeh, Per Arne Svensson, and Jan W. Eriksson. 2014. “FKBP5 Expression in Human Adipose Tissue Increases Following Dexamethasone Exposure and Is Associated with Insulin Resistance.” *Metabolism* 63 (9): 1198–1208. https://doi.org/10.1016/J.METABOL.2014.05.015.

Peterlin, B. Matija, and David H. Price. 2006. “Controlling the Elongation Phase of Transcription with P-TEFb.” *Molecular Cell* 23 (3): 297–305. https://doi.org/10.1016/J.MOLCEL.2006.06.014.

Porrua, Odil, and Domenico Libri. 2015. “Transcription Termination and the Control of the Transcriptome: Why, Where and How to Stop.” *Nature Reviews Molecular Cell Biology* 16 (3): 190–202. https://doi.org/10.1038/nrm3943.

Ptashne, Mark, and Alexander Gann. 1997. “Transcriptional Activation by Recruitment.” *Nature* 386 (6625): 569–77. https://doi.org/10.1038/386569a0.

Pugh, B. F., and R. Tjian. 1991. “Transcription from a TATA-Less Promoter Requires a Multisubunit TFIID Complex.” *Genes and Development* 5 (11): 1935–45. https://doi.org/10.1101/gad.5.11.1935.

Purrello, Michele, C. di Pietro, A. Rapisarda, E. Mirabile, S. Motta, G. Sichel, and K. H. Grzeschik. 1995. “Genetic Characterization of General Transcription Factors TFIIF and TFIIB of Homo Sapiens Sapiens.” *Cytogenetic and Genome Research* 69 (1–2): 75–80. https://doi.org/10.1159/000133942.

Rahl, Peter B., Charles Y. Lin, Amy C. Seila, Ryan A. Flynn, Scott McCuine, Christopher B. Burge, Phillip A. Sharp, and Richard A. Young. 2010. “C-Myc Regulates Transcriptional Pause Release.” *Cell* 141 (3): 432–45. https://doi.org/10.1016/J.CELL.2010.03.030.

Rahl, Peter B., and Richard A. Young. 2014a. “MYC and Transcription Elongation.” *Cold Spring Harbor Perspectives in Medicine* 4 (1). https://doi.org/10.1101/CSHPERSPECT.A020990.

———. 2014b. “MYC and Transcription Elongation.” *Cold Spring Harbor Perspectives in Medicine* 4 (1). https://doi.org/10.1101/CSHPERSPECT.A020990.

Ramakrishnan, Rajesh, Eugene C. Dow, and Andrew P. Rice. 2009. " Characterization of Cdk9 T-Loop Phosphorylation in Resting and Activated CD4 + T Lymphocytes ." *Journal of Leukocyte Biology* 86 (6): 1345–50. https://doi.org/10.1189/JLB.0509309.

Ramakrishnan, Rajesh, and Andrew P. Rice. 2012. "Cdk9 T-Loop Phosphorylation Is Regulated by the Calcium Signaling Pathway." *Journal of Cellular Physiology* 227 (2): 609–17. https://doi.org/10.1002/JCP.22760.

Ramanathan, Y., Sanjay M. Rajpara, Syed M. Reza, Emma Lees, Stewart Shuman, Michael B. Mathews, and Tsafi Pe'ery. 2001. "Three RNA Polymerase II Carboxyl-Terminal Domain Kinases Display Distinct Substrate Preferences *." *Journal of Biological Chemistry* 276 (14): 10913–20. https://doi.org/10.1074/JBC.M010975200.

Rao, Suhas S.P., Miriam H. Huntley, Neva C. Durand, Elena K. Stamenova, Ivan D. Bochkov, James T. Robinson, Adrian L. Sanborn, et al. 2014. "A 3D Map of the Human Genome at Kilobase Resolution Reveals Principles of Chromatin Looping." *Cell* 159 (7): 1665–80. https://doi.org/10.1016/J.CELL.2014.11.021.

Rasmussen, Thomas A., and Sharon R. Lewin. 2016. "Shocking HIV out of Hiding: Where Are We with Clinical Trials of Latency Reversing Agents?" *Current Opinion in HIV and AIDS* 11 (4): 394–401. https://doi.org/10.1097/COH.0000000000000279.

Revyakin, Andrey, Chenyu Liu, Richard H. Ebright, and Terence R. Strick. 2006. "Abortive Initiation and Productive Initiation by RNA Polymerase Involve DNA Scrunching." *Science (New York, N.Y.)* 314 (5802): 1139–43. https://doi.org/10.1126/SCIENCE.1131398.

Rice, Andrew, and Christine Herrmann. 2005. "Regulation of TAK / P-TEFb in CD4+ T Lymphocytes and Macrophages." *Current HIV Research* 1 (4): 395–404. https://doi.org/10.2174/1570162033485159.

Rice, Andrew P. 2017. "The HIV-1 Tat Protein: Mechanism of Action and Target for HIV-1 Cure Strategies." *Current Pharmaceutical Design* 23 (28). https://doi.org/10.2174/1381612823666170704130635.

———. 2019. "Roles of CDKs in RNA Polymerase II Transcription of the HIV-1 Genome." *Transcription* 10 (2): 111–17. https://doi.org/10.1080/21541264.2018.1542254.

Richard, Patricia, and James L. Manley. 2009. "Transcription Termination by Nuclear RNA Polymerases." *Genes & Development* 23 (11): 1247–69. https://doi.org/10.1101/GAD.1792809.

Roeder, Robert G. 2019. "50+ Years of Eukaryotic Transcription: An Expanding Universe of Factors and Mechanisms." *Nature Structural & Molecular Biology 2019 26:9* 26 (9): 783–91. https://doi.org/10.1038/s41594-019-0287-x.

Romano, Gaetano. 2013. "Deregulations in the Cyclin-Dependent Kinase-9-Related Pathway in Cancer: Implications for Drug Discovery and Development." *ISRN Oncology* 2013 (June): 1–14. https://doi.org/10.1155/2013/305371.

Russell, Jackie, and Joost C.B.M. Zomerdijk. 2005. "RNA-Polymerase-I-Directed RDNA Transcription, Life and Works." *Trends in Biochemical Sciences* 30 (2): 87–96. https://doi.org/10.1016/j.tibs.2004.12.008.

Saldi, Tassa, Michael A. Cortazar, Ryan M. Sheridan, and David L. Bentley. 2016. “Coupling of RNA Polymerase II Transcription Elongation with Pre-MRNA Splicing.” *Journal of Molecular Biology* 428 (12): 2623–35. https://doi.org/10.1016/J.JMB.2016.04.017.

Sanders, Steven L., Krassimira A. Garbett, and P. Anthony Weil. 2002. “Molecular Characterization of Saccharomyces Cerevisiae TFIID.” *Molecular and Cellular Biology* 22 (16): 6000–6013. https://doi.org/10.1128/MCB.22.16.6000-6013.2002.

Sano, Motoaki, Maha Abdellatif, Hidemasa Oh, Min Xie, Luigi Bagella, Antonio Giordano, Lloyd H. Michael, Francesco J. DeMayo, and Michael D. Schneider. 2002. “Activation and Function of Cyclin T-Cdk9 (Positive Transcription Elongation Factor-b) in Cardiac Muscle-Cell Hypertrophy.” *Nature Medicine* 8 (11): 1310–17. https://doi.org/10.1038/NM778.

Sarbassov, Dos D., David A. Guertin, Siraj M. Ali, and David M. Sabatini. 2005. “Phosphorylation and Regulation of Akt/PKB by the Rictor-MTOR Complex.” *Science* 307 (5712): 1098–1101. https://doi.org/10.1126/science.1106148.

Sawada, Shinichiro, John D. Scarborough, Nigel Killeen, and Dan R. Littman. 1994. “A Lineage-Specific Transcriptional Silencer Regulates CD4 Gene Expression during T Lymphocyte Development.” *Cell* 77 (6): 917–29. https://doi.org/10.1016/0092-8674(94)90140-6.

Sawarkar, Ritwick, Cem Sievers, and Renato Paro. 2012. “Hsp90 Globally Targets Paused RNA Polymerase to Regulate Gene Expression in Response to Environmental Stimuli.” *Cell* 149 (4): 807–18. https://doi.org/10.1016/J.CELL.2012.02.061.

Scharf, Sebastian H., Claudia Liebl, Elisabeth B. Binder, Mathias v. Schmidt, and Marianne B. Müller. 2011. “Expression and Regulation of the Fkbp5 Gene in the Adult Mouse Brain.” *PLoS ONE* 6 (2). https://doi.org/10.1371/journal.pone.0016883.

Scheufler, Clemens, Achim Brinker, Gleb Bourenkov, Stefano Pegoraro, Luis Moroder, Hans Bartunik, F. Ulrich Hartl, and Ismail Moarefi. 2000a. “Structure of TPR Domain-Peptide Complexes: Critical Elements in the Assembly of the Hsp70-Hsp90 Multichaperone Machine.” *Cell* 101 (2): 199–210. https://doi.org/10.1016/S0092-8674(00)80830-2.

———. 2000b. “Structure of TPR Domain-Peptide Complexes: Critical Elements in the Assembly of the Hsp70-Hsp90 Multichaperone Machine.” *Cell* 101 (2): 199–210. https://doi.org/10.1016/S0092-8674(00)80830-2.

Schiene-Fischer, C, C Yu - FEBS letters, and undefined 2001. n.d. “Receptor Accessory Folding Helper Enzymes: The Functional Role of Peptidyl Prolyl Cis/Trans Isomerases.” *Elsevier*. Accessed February 27, 2022. https://www.sciencedirect.com/science/article/pii/S0014579301023262.

Schosser, Alexandra, and Siegfried Kasper. 2009. “The Role of Pharmacogenetics in the Treatment of Depression and Anxiety Disorders.” *International Clinical Psychopharmacology* 24 (6): 277–88. https://doi.org/10.1097/YIC.0B013E3283306A2F.

Shiekhattar, Ramin, Fred Mermelstein, Robert P. Fisher, Ronny Drapkin, Dynlacht Brian, Holly C. Wessling, David O. Morgan, and Danny Reinberg. 1995. “Cdk-Activating Kinase Complex Is a Component of Human Transcription Factor TFIIH.” *Nature* 374 (6519): 283–87. https://doi.org/10.1038/374283A0.

Shore, Sarah M., Sarah A. Byers, Paul Dent, and David H. Price. 2005. "Characterization of Cdk955 and Differential Regulation of Two Cdk9 Isoforms." *Gene* 350 (1): 51–58. https://doi.org/10.1016/J.GENE.2005.01.015.

Shore, Sarah M., Sarah A. Byers, Wendy Maury, and David H. Price. 2003. "Identification of a Novel Isoform of Cdk9." *Gene* 307 (1–2): 175–82. https://doi.org/10.1016/S0378-1119(03)00466-9.

Sidibeh CO, Pereira MJ, Abalo XM, J Boersma G, Skrtic S, Lundkvist P, Katsogiannos P, Hausch F, Castillejo-López C and Eriksson JW. " FKBP5 expression in human adipose tissue: potential role in glucose and lipid metabolism, adipogenesis and type 2 diabetes." Endocrine 62(1):116-128. https://doi.org/10.1007/s12020-018-1674-5

Siliciano, Robert F., and Warner C. Greene. 2011. "HIV Latency." *Cold Spring Harbor Perspectives in Medicine* 1 (1). https://doi.org/10.1101/CSHPERSPECT.A007096.

Simone, Cristiano, Peter Stiegler, Luigi Bagella, Bruna Pucci, Cristiana Bellan, Giulia de Falco, Antonio de Luca, Ginevra Guanti, Pier Lorenzo Puri, and Antonio Giordano. 2002. "Activation of MyoD-Dependent Transcription by Cdk9/Cyclin T2." *Oncogene* 21 (26): 4137–48. https://doi.org/10.1038/SJ.ONC.1205493.

Sinars, Cindy R., Joyce Cheung-Flynn, Ronald A. Rimerman, Jonathan G. Scammell, David F. Smith, and Jon Clardy. 2003. "Structure of the Large FK506-Binding Protein FKBP5, an Hsp90-Binding Protein and a Component of Steroid Receptor Complexes." *Proceedings of the National Academy of Sciences of the United States of America* 100 (3): 868. https://doi.org/10.1073/PNAS.0231020100.

Smith, Edwin, Chengqi Lin, and Ali Shilatifard. 2011. "The Super Elongation Complex (SEC) and MLL in Development and Disease." *Genes and Development* 25 (7): 661–72. https://doi.org/10.1101/GAD.2015411.

Somesh, Baggavalli P., James Reid, Wei Feng Liu, T. Max M. Søgaard, Hediye Erdjument-Bromage, Paul Tempst, and Jesper Q. Svejstrup. 2005. "Multiple Mechanisms Confining RNA Polymerase II Ubiquitylation to Polymerases Undergoing Transcriptional Arrest." *Cell* 121 (6): 913–23. https://doi.org/10.1016/J.CELL.2005.04.010.

Sonawane, Yogesh A., Margaret A. Taylor, John Victor Napoleon, Sandeep Rana, Jacob I. Contreras, and Amarnath Natarajan. 2016. "Cyclin Dependent Kinase 9 Inhibitors for Cancer Therapy." *Journal of Medicinal Chemistry* 59 (19): 8667–84. https://doi.org/10.1021/ACS.JMEDCHEM.6B00150.

Splinter, E, H Heath, J Kooren, … RJ Palstra - Genes &, and undefined 2006. n.d. "CTCF Mediates Long-Range Chromatin Looping and Local Histone Modification in the β-Globin Locus." *Genesdev.Cshlp.Org*. Accessed February 26, 2022. http://genesdev.cshlp.org/content/20/17/2349.short.

Stechschulte, Lance A., Bin Qiu, Manya Warrier, Terry D. Hinds, Man Zhang, Hao Gu, Yuxue Xu, et al. 2016. "FKBP5 Null Mice Are Resistant to Diet-Induced Obesity and the Ppary Agonist Rosiglitazone." *Endocrinology* 157 (10): 3888–3900. https://doi.org/10.1210/en.2015-1996.

Stewart, Grant S., Bin Wang, Colin R. Bigneli, A. Malcolm R. Taylor, and Stephen J. Elledge. 2003. “MDC1 Is a Mediator of the Mammalian DNA Damage Checkpoint.” *Nature* 421 (6926): 961–66. https://doi.org/10.1038/nature01446.

Storer, Cheryl L., Chad A. Dickey, Mario D. Galigniana, Theo Rein, and Marc B. Cox. 2011. “FKBP5 and FKBP52 in Signaling and Disease.” *Trends in Endocrinology and Metabolism* 22 (12): 481–90. https://doi.org/10.1016/j.tem.2011.08.001.

Stucki, Manuel, Julie A. Clapperton, Duaa Mohammad, Michael B. Yaffe, Stephen J. Smerdon, and Stephen P. Jackson. 2005. “MDC1 Directly Binds Phosphorylated Histone H2AX to Regulate Cellular Responses to DNA Double-Strand Breaks.” *Cell* 123 (7): 1213–26. https://doi.org/10.1016/J.CELL.2005.09.038.

Swigut, Tomek, and Joanna Wysocka. 2007. “H3K27 Demethylases, at Long Last.” *Cell* 131 (1): 29–32. https://doi.org/10.1016/J.CELL.2007.09.026.

Taipale, Mikko, George Tucker, Jian Peng, Irina Krykbaeva, Zhen Yuan Lin, Brett Larsen, Hyungwon Choi, Bonnie Berger, Anne Claude Gingras, and Susan Lindquist. 2014. “A Quantitative Chaperone Interaction Network Reveals the Architecture of Cellular Protein Homeostasis Pathways.” *Cell* 158 (2): 434–48. https://doi.org/10.1016/j.cell.2014.05.039.

Takahashi, Hidehisa, Tari J. Parmely, Shigeo Sato, Chieri Tomomori-Sato, Charles A.S. Banks, Stephanie E. Kong, Henrietta Szutorisz, et al. 2011. “Human Mediator Subunit MED26 Functions as a Docking Site for Transcription Elongation Factors.” *Cell* 146 (1): 92–104. https://doi.org/10.1016/J.CELL.2011.06.005.

Talarowska, Monika. 2020. “Epigenetic Mechanisms in the Neurodevelopmental Theory of Depression.” *Depression Research and Treatment* 2020. https://doi.org/10.1155/2020/6357873.

Tan, Justin L., Rachel D. Fogley, Ryan A. Flynn, Julien Ablain, Song Yang, Violaine Saint-André, Zi Peng Fan, et al. 2016. “Stress from Nucleotide Depletion Activates the Transcriptional Regulator HEXIM1 to Suppress Melanoma.” *Molecular Cell* 62 (1): 34–46. https://doi.org/10.1016/J.MOLCEL.2016.03.013.

Tang, Hong, Xiaoqing Sun, Danny Reinberg, and Richard H. Ebright. 1996. “Protein-Protein Interactions in Eukaryotic Transcription Initiation: Structure of the Preinitiation Complex.” *Proceedings of the National Academy of Sciences of the United States of America* 93 (3): 1119–24. https://doi.org/10.1073/PNAS.93.3.1119.

Taube, Ran, Xin Lin, Dan Irwin, Koh Fujinaga, and B. Matija Peterlin. 2002. “Interaction between P-TEFb and the C-Terminal Domain of RNA Polymerase II Activates Transcriptional Elongation from Sites Upstream or Downstream of Target Genes.” *Molecular and Cellular Biology* 22 (1): 321–31. https://doi.org/10.1128/MCB.22.1.321-331.2002.

Taylor, Paul, Jacqueline Dornan, Amerigo Carrello, Rodney F. Minchin, Thomas Ratajczak, and Malcolm D. Walkinshaw. 2001. “Two Structures of Cyclophilin 40: Folding and Fidelity in the TPR Domains.” *Structure (London, England : 1993)* 9 (5): 431–38. https://doi.org/10.1016/S0969-2126(01)00603-7.

Tokusumi, Yumiko, Ying Ma, Xianzhou Song, Raymond H. Jacobson, and Shinako Takada. 2007. “The New Core Promoter Element XCPE1 (X Core Promoter Element 1) Directs Activator-, Mediator-, and TATA-Binding Protein-Dependent but TFIID-Independent RNA Polymerase II Transcription from TATA-Less Promoters.” *Molecular and Cellular Biology* 27 (5): 1844–58. https://doi.org/10.1128/MCB.01363-06.

Verstraete, Nina, Alona Kuzmina, Gaelle Diribarne, Van T. Nguyen, Lydia Kobbi, Monika Ludanyi, Ran Taube, and Olivier Bensaude. 2014. “A Cyclin T1 Point Mutation That Abolishes Positive Transcription Elongation Factor (P-TEFb) Binding to Hexim1 and HIV Tat.” *Retrovirology* 11 (1). https://doi.org/10.1186/1742-4690-11-50.

Wang, Shudong, and Peter M. Fischer. 2008. “Cyclin-Dependent Kinase 9: A Key Transcriptional Regulator and Potential Drug Target in Oncology, Virology and Cardiology.” *Trends in Pharmacological Sciences* 29 (6): 302–13. https://doi.org/10.1016/J.TIPS.2008.03.003.

Wei, Ping, Mitchell E. Garber, Shi Min Fang, Wolfgang H. Fischer, and Katherine A. Jones. 1998. “A Novel CDK9-Associated C-Type Cyclin Interacts Directly with HIV-1 Tat and Mediates Its High-Affinity, Loop-Specific Binding to TAR RNA.” *Cell* 92 (4): 451–62. https://doi.org/10.1016/S0092-8674(00)80939-3.

Westover, Kenneth D., David A. Bushnell, and Roger D. Kornberg. 2004. “Structural Basis of Transcription: Nucleotide Selection by Rotation in the RNA Polymerase II Active Center.” *Cell* 119 (4): 481–89. https://doi.org/10.1016/j.cell.2004.10.016.

Whetstine, Johnathan R., Amanda Nottke, Fei Lan, Maite Huarte, Sarit Smolikov, Zhongzhou Chen, Eric Spooner, et al. 2006. “Reversal of Histone Lysine Trimethylation by the JMJD2 Family of Histone Demethylases.” *Cell* 125 (3): 467–81. https://doi.org/10.1016/j.cell.2006.03.028.

Whyte, Warren A., David A. Orlando, Denes Hnisz, Brian J. Abraham, Charles Y. Lin, Michael H. Kagey, Peter B. Rahl, Tong Ihn Lee, and Richard A. Young. 2013. “Master Transcription Factors and Mediator Establish Super-Enhancers at Key Cell Identity Genes.” *Cell* 153 (2): 307–19. https://doi.org/10.1016/J.CELL.2013.03.035.

Wimmer, Jörg, Koh Fujinaga, Ran Taube, Thomas P. Cujec, Yuerong Zhu, Junmin Peng, David H. Price, and B. Matija Peterlin. 1999. “Interactions between Tat and TAR and Human Immunodeficiency Virus Replication Are Facilitated by Human Cyclin T1 but Not Cyclins T2a or T2b.” *Virology* 255 (1): 182–89. https://doi.org/10.1006/VIRO.1998.9589.

Wittmann, Bryan M., Koh Fujinaga, Huayun Deng, Ndiya Ogba, and Monica M. Montano. 2005. “The Breast Cell Growth Inhibitor, Estrogen down Regulated Gene 1, Modulates a Novel Functional Interaction between Estrogen Receptor Alpha and Transcriptional Elongation Factor Cyclin T1.” *Oncogene* 24 (36): 5576–88. https://doi.org/10.1038/SJ.ONC.1208728.

Xing, Sifei, and Robert F. Siliciano. 2013. “Targeting HIV Latency: Pharmacologic Strategies toward Eradication.” *Drug Discovery Today* 18 (11–12): 541–51. https://doi.org/10.1016/J.DRUDIS.2012.12.008.

Yadav, Dipika, Koushik Ghosh, Subham Basu, Robert G. Roeder, and Debabrata Biswas. 2019. “Multivalent Role of Human TFIID in Recruiting Elongation Components at the Promoter-Proximal Region for Transcriptional Control.” *Cell Reports* 26 (5): 1303-1317.e7. https://doi.org/10.1016/j.celrep.2019.01.012.

Yang, Zhiyuan, Jasper H.N. Yik, Ruichuan Chen, Nanhai He, Kyoo Jang Moon, Keiko Ozato, and Qiang Zhou. 2005. “Recruitment of P-TEFb for Stimulation of Transcriptional Elongation by the Bromodomain Protein Brd4.” *Molecular Cell* 19 (4): 535–45. https://doi.org/10.1016/J.MOLCEL.2005.06.029.

Yang, Zhiyuan, Qingwei Zhu, Kunxin Luo, and Qiang Zhou. 2001. “The 7SK Small Nuclear RNA Inhibits the CDK9/Cyclin T1 Kinase to Control Transcription.” *Nature* 414 (6861): 317–22. https://doi.org/10.1038/35104575.

Yates, Andrew D., Premanand Achuthan, Wasiu Akanni, James Allen, Jamie Allen, Jorge Alvarez-Jarreta, M. Ridwan Amode, et al. 2020. “Ensembl 2020.” *Nucleic Acids Research* 48 (D1): D682–88. https://doi.org/10.1093/nar/gkz966.

Yeh, Wen Chen, Barbara E. Bierer, and Steven L. McKnight. 1995. “Rapamycin Inhibits Clonal Expansion and Adipogenic Differentiation of 3T3-L1 Cells.” *Proceedings of the National Academy of Sciences of the United States of America* 92 (24): 11086–90. https://doi.org/10.1073/PNAS.92.24.11086.

Yokomori, Kyoko, Martin P. Zeidler, Jin Long Chen, C. Peter Verrijzer, Marek Mlodzik, and Robert Tjian. 1994. “Drosophila TFIIA Directs Cooperative DNA Binding with TBP and Mediates Transcriptional Activation.” *Genes and Development* 8 (19): 2313–23. https://doi.org/10.1101/gad.8.19.2313.

Yu, Ming, Wenjing Yang, Ting Ni, Zhanyun Tang, Tomoyoshi Nakadai, Jun Zhu, and Robert G. Roeder. 2015. “RNA Polymerase II-Associated Factor 1 Regulates the Release and Phosphorylation of Paused RNA Polymerase II.” *Science* 350 (6266): 1383–86. https://doi.org/10.1126/SCIENCE.AAD2338.

Zhou, Qiang, Tiandao Li, and David H. Price. 2012. “RNA Polymerase II Elongation Control.” *Annual Review of Biochemistry* 81 (July): 119–43. https://doi.org/10.1146/ANNUREV-BIOCHEM-052610-095910.

Zhou, Qiang, and Jasper H. N. Yik. 2006a. “The Yin and Yang of P-TEFb Regulation: Implications for Human Immunodeficiency Virus Gene Expression and Global Control of Cell Growth and Differentiation.” *Microbiology and Molecular Biology Reviews* 70 (3): 646–59. https://doi.org/10.1128/MMBR.00011-06.

———. 2006b. “The Yin and Yang of P-TEFb Regulation: Implications for Human Immunodeficiency Virus Gene Expression and Global Control of Cell Growth and Differentiation.” *Microbiology and Molecular Biology Reviews* 70 (3): 646. https://doi.org/10.1128/MMBR.00011-06.

———. 2006c. “The Yin and Yang of P-TEFb Regulation: Implications for Human Immunodeficiency Virus Gene Expression and Global Control of Cell Growth and Differentiation.” *Microbiology and Molecular Biology Reviews* 70 (3): 646–59. https://doi.org/10.1128/MMBR.00011-06.

Žumer, Kristina, Ana Plemenitaš, Kalle Saksela, and B. Matija Peterlin. 2011. “Patient Mutation in AIRE Disrupts P-TEFb Binding and Target Gene Transcription.” *Nucleic Acids Research* 39 (18): 7908–19. https://doi.org/10.1093/NAR/GKR527.

Click or tap here to enter text.

List of Publications :

1) **Mall, DP.,**Basu, S., Ghosh, K., Kumari, N., Lahiri, A., . . . Biswas, D. (2022). Human FKBP5 Negatively Regulates Transcription through Inhibition of P-TEFb Complex Formation. ***Molecular and Cellular Biology,***42(1):e0034421**.**

2) Ghosh, K., Tang, M., Kumari, N., Nandy, A., Basu, S., **Mall, DP**., . . . Biswas, D. (2018). Positive regulation of transcription by human ZMYND8 through its association with P-TEFb complex. ***Cell reports****, 24*(8), 2141-2154.e2146.

AMERICAN SOCIETY FOR MICROBIOLOGY | Molecular and Cellular Biology®

RESEARCH ARTICLE

Human FKBP5 Negatively Regulates Transcription through Inhibition of P-TEFb Complex Formation

Dheerendra Pratap Mall,[a] Subham Basu,[a] Koushik Ghosh,[a] Nidhi Kumari,[a] Abhishake Lahiri,[b] Sandip Paul,[c] Debabrata Biswas[a]

[a]Laboratory of Transcription Biology, Molecular Genetics Division, CSIR–Indian Institute of Chemical Biology, Kolkata, India
[b]Structural Biology and Bioinformatics Division, CSIR–Indian Institute of Chemical Biology, Kolkata, India
[c]JIS Institute of Advanced Studies and Research, JIS University, Kolkata, India

ABSTRACT Although a large number of recent studies indicate strong association of FKBP5 (aka FKBP51) functions with various stress-related psychiatric disorders, the overall mechanisms are poorly understood. Beyond a few studies indicating its functions in regulating glucocorticoid receptor, and AKT signaling pathways, other functional roles (if any) are unclear. Here, we report an antiproliferative role of human FKBP5 through negative regulation of expression of proliferation-related genes. Mechanistically, we show that, owing to the same region of interaction on cyclin-dependent kinse 9 (CDK9), human FKBP5 directly competes with cyclin T1 for functional positive transcription elongation factor b (P-TEFb) complex formation. *In vitro* biochemical assays, coupled with cell-based assays, showed a strong negative effect of FKBP5 on P-TEFb-mediated phosphorylation of diverse substrates. Consistently, FKBP5 knockdown showed enhanced P-TEFb complex formation that led to increased global RNA polymerase II C-terminal domain (CTD) phosphorylation, expression of proliferation-related genes, and subsequent proliferation. Thus, our results show an important role for FKBP5 in negative regulation of P-TEFb functions within mammalian cells.

KEYWORDS CTD phosphorylation, FKBP5, P-TEFb, RNA polymerase II, transcriptional regulation

The human FKBP5 is a 51-kDa protein of the immunophilin family that binds to immunosuppressants such as rapamycin and FK506 (1, 2). The functions of this protein have largely been described in context of the HSP90 cochaperone complex (3). A recent surge in reports indicates a strong association of FKBP5 functions with several neurological diseases, including posttraumatic stress disorder (PTSD) (4–6). Furthermore, FKBP5 functions have also been correlated with multiple other diseases and processes, including type 2 diabetes, adipogenesis, and fatty acid metabolism, as well as cancers (7). In several cancers, a strong negative correlation has been observed between FKBP5 expression and severity of disease (8–11). However, little is known about the molecular mechanisms of function of this protein and its role in pathogenesis of multiple diseases.

The well-known mechanistic functions of FKBP5 have been described in the context of AKT and glucocorticoid (GC) signaling pathways (3, 7, 9, 10). FKBP5 has been shown to regulate the phosphorylation status of one of the key regulatory residues (Ser473) of AKT1 through regulating the recruitment of PH domain leucine-rich repeat phosphatase (PHLPP) (8). FKBP5 has also been shown to negatively regulate GC signaling by reducing GC binding to cognate receptors and thus reducing nuclear translocation and downstream activation of target genes (12–14). Although a plethora of literature suggests a strong connection of FKBP5 functions with neuronal abnormalities, recent studies have also indicated its role in controlling type 2 diabetes, in part through control of AKT signaling and the downstream effector protein AKT substrate 160 (AS160) (15). Besides, numerous other studies have also shown a negative correlation between

Address correspondence to Debabrata Biswas, dbiswas@iicb.res.in.

The authors declare no conflict of interest.

Received 13 July 2021
Returned for modification 8 August 2021
Accepted 2 November 2021

Accepted manuscript posted online 15 November 2021
Published 20 January 2022

FKBP5 expression and glucose uptake in the plasma membrane (15–17). Reduced levels of FKBP5 have also been strongly correlated with enhanced expression of *UCP-1* and *PRDM16* genes, the key regulators involved in conversion of white adipose tissue (WAT) to brown adipose tissue (BAT) during adipogenesis (18). Several aggressive cancers, including breast and prostate cancers, also show reduced expression of FKBP5 (19). All of these observations strongly point toward a negative role of FKBP5 in regulating the expression of multiple target genes. However, the underlying mechanism(s) of this negative regulation is completely unknown.

Among all the positive regulators of transcription, the human positive transcription elongation factor b (P-TEFb) complex plays an important role in activation of target gene expression (20–22). The human P-TEFb complex is a heterodimer composed of the kinase subunit cyclin-dependent kinse 9 (CDK9) and associated cyclin T1/T2 (23, 24). The majority of the human P-TEFb complex remains in the inactive 7SK small nuclear RNA (snRNA) complex in association with HEXIM1, LARP7, MePCE2, and 7SK snRNA (25, 26). During transcriptional activation, upon exposure to external cues such as stress, the P-TEFb complex dissociates from the inactive 7SK snRNA complex to associate with multiple transcriptional activators, including Brd4, p53, ZMYND8, and components of the super elongation complex (SEC) (22, 27–31). Upon recruitment at the target sites by the associated activator proteins at the promoter-proximal region, the P-TEFb complex has been shown to play a major role in releasing paused RNA polymerase II (Pol II) into the productive elongation step through phosphorylation of target NELF and DSIF complexes, as well as conserved Ser2 and Ser5 residues of the C-terminal domain (CTD) of Pol II (32–35). Therefore, controlling the functions of P-TEFb complex is gradually turning out to be a major paradigm for activation of target gene expression and associated multiple cellular processes.

In an effort to understand the functional regulation of FKBP5 in negative regulation of expression of target genes, we were intrigued by our observation of a strong association of FKBP5 and CDK9 within mammalian cells. Detailed mechanistic analysis showed that human FKBP5 directly competes with cyclin T1, thereby regulating the level of P-TEFb complex formation and thus directly inhibiting P-TEFb complex-dependent phosphorylation of multiple substrates, including Ser2 and Ser5 residues of the Pol II CTD. Inhibition of this phosphorylation activity of the P-TEFb complex directly reduces expression of diverse target genes, including proliferation-related genes such as *CCND1* and *C-MYC*. Consistent with this observation, FKBP5 knockdown cells show enhanced expression of these target genes, which results in increased proliferation. Thus, we describe here a novel transcriptional repression role of human FKBP5 through negative regulation of functions of the P-TEFb complex within mammalian cells.

RESULTS

FKBP5 is a novel interactor of CDK9. The P-TEFb complex has been shown to positively regulate transcription through phosphorylation of NELF and DSIF complexes and the CTD of Pol II (21, 22, 35–37). Multiple other regulators have been described that regulate P-TEFb functions either directly or indirectly. Since P-TEFb plays a major role in activating target gene expression, we initially addressed whether any novel factor, not previously described, would be involved in the functional regulation of P-TEFb within mammalian cells. We purified CDK9-associated protein complex using nuclear extract from a stable cell line expressing FLAG-hemagglutinin (HA)-CDK9 protein (22). Interestingly, and much to our surprise, subsequent mass spectrometric analysis showed the presence of FKBP5, along with that of other known CDK9 interactors (see Fig. S1A in the supplemental material). Subsequent immunoprecipitation and blotting analysis further confirmed CDK9 interaction with FKBP5 along with its known interactors, such as SEC components (Fig. 1A). To address whether a reciprocal interaction would also be observed, we generated another stable cell line expressing FLAG-HA-FKBP5 protein (Fig. S1B). Subsequent purification of FKBP5-associated protein complex showed presence of CDK9 component of the P-TEFb complex only along with its

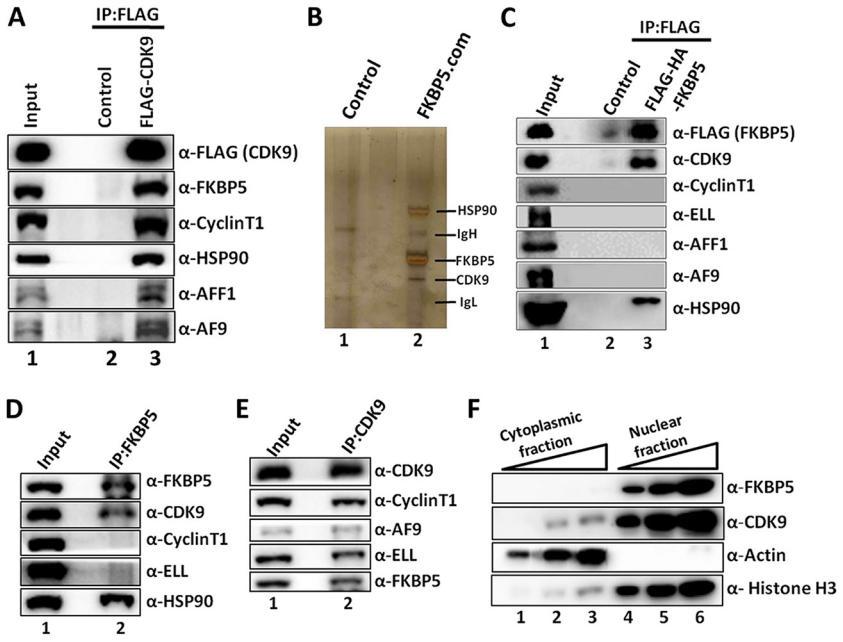


FIG 1 Novel interaction between FKBP5 and CDK9. (A) Immunoprecipitation and subsequent Western blotting showing identification of FKBP5 as a novel CDK9-interacting protein along with other known super elongation complex (SEC) components. (B) Purification of FKBP5-associated protein complex from a stable cell line that ectopically expresses FKBP5 as FLAG-hemagglutinin (HA) tagged. Eluted proteins were run on a 4 to 12% gradient gel and silver stained for visualization. Individual bands were marked based on their size and subsequent confirmation by Western blotting. A similar parallel immunoprecipitation from a control cell line shows the absence of these bands. (C) Immunoprecipitation and subsequent Western blotting showing identification of CDK9 as a novel FKBP5-interacting protein along with its known interactor HSP90. SEC components failed to show any interaction in this immunoprecipitation assay. (D) Immunoprecipitation of endogenous FKBP5 using specific antibody and subsequent Western blotting showing FKBP5 interaction with CDK9 along with HSP90 in endogenous context. (E) Immunoprecipitation of endogenous CDK9 using specific antibody and subsequent Western blotting showing CDK9 interaction with FKBP5 along with SEC components in endogenous context. (F) Western blotting showing copresence of FKBP5 and CDK9 in the nuclear compartment of mammalian 293T cells. As an indicator of this localization, histone H3 protein is used as a nuclear localization marker, whereas the actin protein acts as a cytoplasmic localization marker.

known interactor HSP90 (Fig. 1B). Further immunoprecipitation and blotting analyses confirmed that human FKBP5 associates only with the CDK9 component of the P-TEFb complex and not with other CDK9 interactors, such as SEC components (Fig. 1C).

To rule out artifacts of protein-protein interactions due to overexpression and to address FKBP5 and CDK9 interaction in an endogenous context, we immunoprecipitated endogenous FKBP5 using specific antibody and confirmed its interaction with CDK9 along with its known interactor HSP90 in 293T cells (Fig. 1D). A reverse immunoprecipitation of endogenous CDK9 by using specific antibody also confirmed its interaction with FKBP5, as well as with SEC components, in mammalian cells (Fig. 1E). Therefore, we conclude that FKBP5 and CDK9 interact with each other within mammalian cells and that this interaction is independent of the P-TEFb complex and its associated interactors, such as SEC components. Interestingly, we have observed predominant presence of both FKBP5 and CDK9 within the nuclear compartment of 293T cells (Fig. 1F). This evidence further indicates a functional regulation of CDK9 by FKBP5 through its presence within the nucleus of mammalian cells.

The interaction of FKBP5 with CDK9 is direct and specific. In order to address whether FKBP5 directly interacts with CDK9 or if any other mediator is responsible for this association, we initially checked their interaction through their expression in heterologous Sf9 cells. We coinfected Sf9 cells with baculoviruses expressing FLAG-FKBP5 and cyclin T1 or CDK9 or both. Subsequent immunoprecipitation analysis confirmed that FKBP5 specifically interacts with CDK9 but not with the cyclin T1 subunit of the

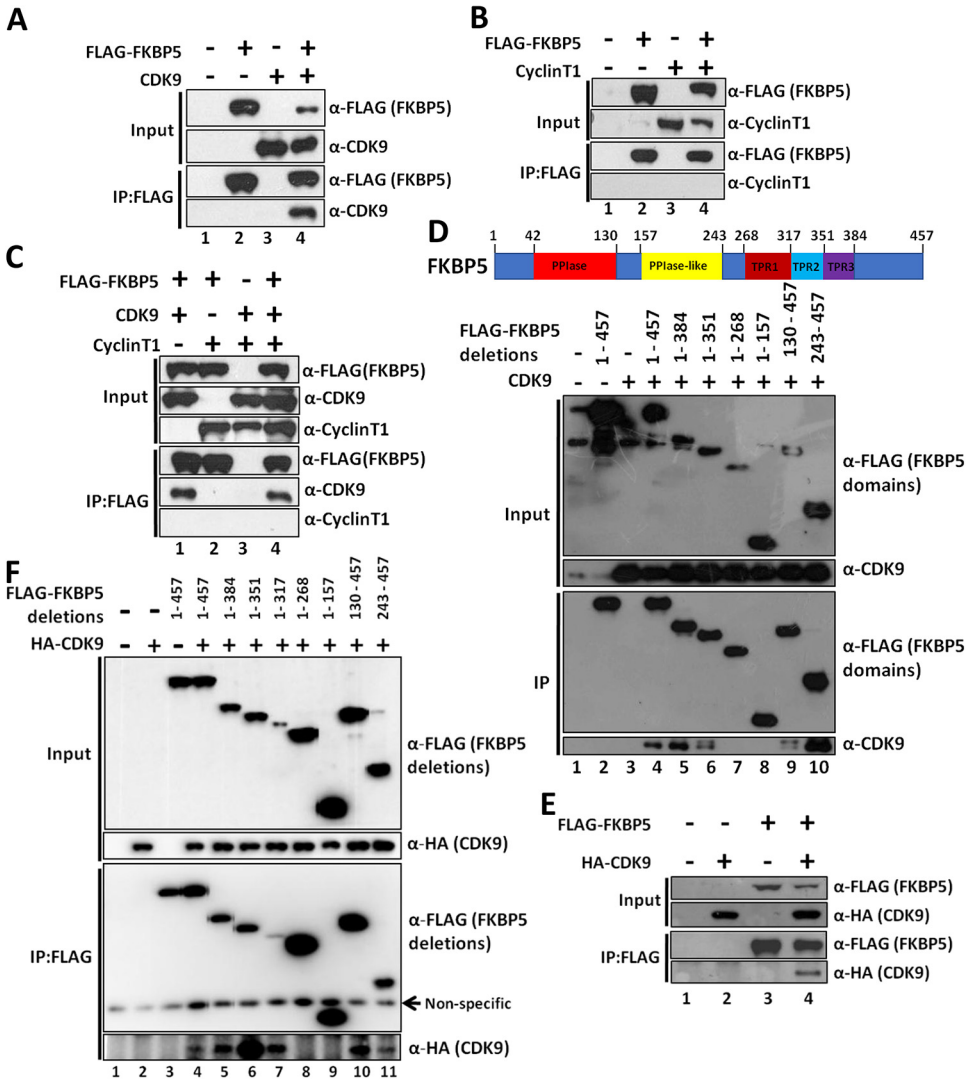


FIG 2 The TPR1 domain of FKBP5 is important for its interaction with CDK9. (A) Immunoprecipitation and subsequent Western blot analysis showing interaction of FKBP5 and CDK9 through their coexpression in heterologous Sf9 cells. (B) Immunoprecipitation and subsequent Western blot analysis showing failure of cyclin T1 to interact with FKBP5 when they are coexpressed in heterologous Sf9 cells. (C) Immunoprecipitation and subsequent Western blot analysis showing specific interaction of FKBP5 with CDK9 through their coexpression in heterologous Sf9 cells. Even though cyclin T1 is expressed, it fails to show any interaction with FKBP5 in this heterologous expression-based interaction assays. (D) Domain analysis of FKBP5 for its interaction with CDK9. (Upper) Cartoon diagram showing the important domains of FKBP5. (Lower) Immunoblots represent FKBP5 domains (as indicated) and their interaction with CDK9 as observed through their expression in heterologous Sf9 cells. (E) Coimmunoprecipitation analysis showing interaction of ectopically expressed FKBP5 and CDK9 within mammalian 293T cells. (F) Domain analysis of FKBP5 for its interaction with CDK9 within mammalian 293T cells. The immunoblots represent FKBP5 domains (as indicated) and their interaction with CDK9 as observed through their expression in mammalian 293T cells and subsequent immunoprecipitation and blotting analyses.

P-TEFb complex (Fig. 2A to C). To further address this interaction *in vitro*, we purified CDK9 and FKBP5 through their expression in mammalian cells and bacterial expression systems, respectively (Fig. S2A and B). Subsequent interaction analysis *in vitro* using these purified proteins clearly showed that human FKBP5 directly interacts with CDK9 without the requirement of any other mediator (Fig. S2C).

To further address whether FKBP5 interaction with CDK9 is specific, we tested its interaction with similar kinases, such as CDK7 and CDK8, through their expression in Sf9 cells. We coinfected Sf9 cells with baculoviruses expressing FLAG-CDK7 and FLAG-CDK8 along with FKBP5. Subsequent immunoprecipitation analysis showed that FKBP5

interacts with neither CDK7 nor CDK8 (Fig. S2D and E). Therefore, based on this evidence, we conclude that human FKBP5 and CDK9 directly interact with each other and that the interaction of FKBP5 with CDK9 is very specific.

The TPR1 domain of FKBP5 plays the most important role for its interaction with CDK9 within mammalian cells. Human FKBP5 is a multidomain protein that includes peptidyl prolyl isomerase 1 (PPIase), peptidyl prolyl isomerase-like (PPIase-like), and tetratricopeptide repeat 1, 2, and 3 (TPR1 to TPR3) domains (Fig. 2D, upper) (38, 39). To identify the domain of FKBP5 that plays an important role in the overall interaction between FKBP5 and CDK9, we coinfected Sf9 cells with baculoviruses expressing different FLAG-FKBP5 fragments along with CDK9 (Fig. 2D). Subsequent immunoprecipitation analyses using FLAG epitope tag showed that while the deletion of TPR3 retained FKBP5 interaction with CDK9 (Fig. 2D, compare lane 4 with lane 6), further deletion of TPR1 and TPR2 abolished this interaction (Fig. 2D, compare lane 6 with lane 7), thus suggesting a role for these domains in the interaction of FKBP5 with CDK9. Consistent with this observation, a C-terminal fragment containing the TPR1 and TPR2 domains (243 to 457, lane 10) fully retained FKBP5 interaction with CDK9. Interestingly, the presence of additional regions within this C-terminal fragment (130 to 242) containing the PPIase-like domain drastically reduced this interaction. This observation thus suggests that while the TPR1 and TPR2 domains are important for FKBP5 interaction with CDK9, presence of the PPIase-like domain inhibits this interaction in this heterologous system.

To further substantiate this interaction within mammalian cells, we initially tested interactions of these two proteins through coimmunoprecipitation analysis. As shown in Fig. 2E, cotransfection and subsequent coimmunoprecipitation analysis clearly showed FKBP5 interaction with CDK9 within mammalian 293T cells (lane 4). Subsequent similar coimmunoprecipitation analysis using different FKBP5 domains (Fig. 2F, same as that of Fig. 2D) further showed that a deletion of the TPR1 domain of FKBP5 completely abolished its interaction with CDK9 within mammalian cells (Fig. 2F, compare lane 7 with lane 8). The deletion of TPR2 and TPR3 had minimal effect on this interaction. Interestingly, unlike our observation within Sf9 cells, a deletion of the PPIase-like domain had minimal effect on FKBP5 interaction with CDK9 within mammalian cells. The overall differences in cell types used and in the experimental setup could explain this discrepancy in our observation. Nevertheless, a deletion of the TPR1 domain completely abolishes FKBP5 interaction with CDK9 and clearly suggests an important role of this domain in overall interaction between these two proteins within mammalian cells.

FKBP5 negatively regulates the phosphorylation activity of P-TEFb *in vitro*. Since FKBP5 shows a strong and direct interaction with CDK9 both *in vitro* and *in vivo* within mammalian cells, we subsequently addressed the role of FKBP5 in regulating functions of the P-TEFb complex. The human P-TEFb complex has been shown to phosphorylate the NELF and DSIF complexes, as well as the conserved serine residues present within the heptad repeat (YSPTSPS) sequences of the CTD of Pol II (21, 32–35). To address the direct role of FKBP5 in regulation of overall P-TEFb complex-mediated phosphorylation, we employed *in vitro* kinase assays. Human DSIF and NELF complexes were purified through their expression in bacterial and baculoviral expression systems (Fig. S3A and B) as described previously (34, 40), whereas the recombinant glutathione *S*-transferase (GST)–CTD protein was purified through its expression in bacterial system (Fig. S3C) (22, 36). Human P-TEFb complex was purified through expression of its subunits in baculovirus-mediated expression in Sf9 cells (Fig. S3D) (22, 36). Our initial analyses showed that the purified P-TEFb complex efficiently phosphorylates its cognate GST-CTD substrate (Fig. S3E), NELF-A and NELF-E subunits (within the NELF complex), and the SPT5 subunit (within the DSIF complex) (Fig. S3F).

Next, we addressed the role of FKBP5 in P-TEFb-mediated phosphorylation of various substrates. Initial analyses showed that addition of purified recombinant FKBP5 to the reaction mixture strongly inhibited P-TEFb-mediated phosphorylation of both the Ser2 and Ser5 residues of Pol II CTD in a dose-dependent manner (Fig. 3A, compare lane 3 versus lanes 4 to 7). It is interesting to note that the dominant function of P-TEFb, Ser2 phosphorylation, is inhibited more strongly than Ser5 phosphorylation in

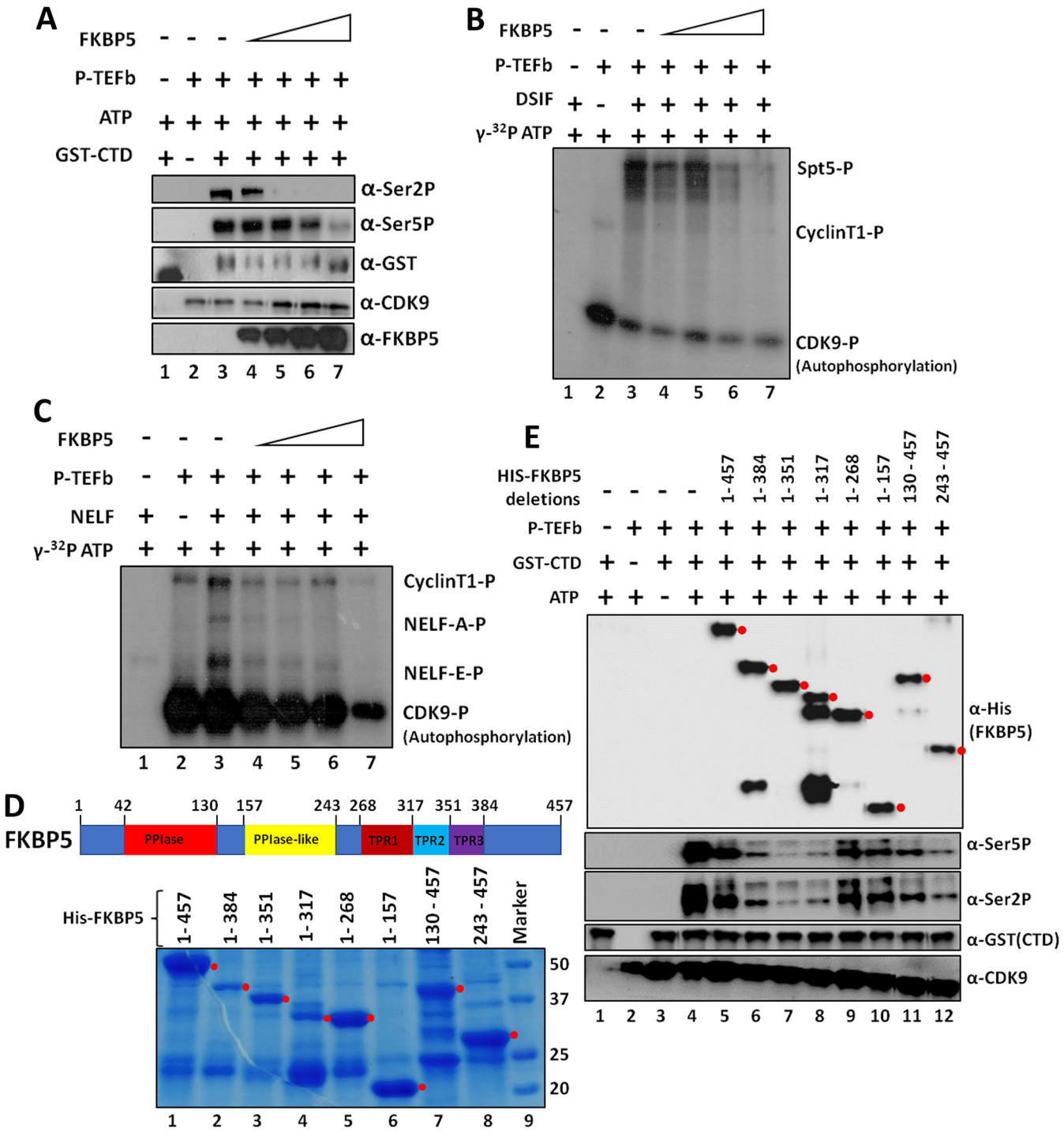


FIG 3 FKBP5 strongly inhibits P-TEFb-mediated phosphorylation of diverse substrates *in vitro*. (A) *In vitro* phosphorylation and subsequent Western blot analysis showing inhibition of P-TEFb-mediated phosphorylation of Ser2 and Ser5 residues of the Pol II C-terminal domain (CTD) by FKBP5. For each reaction mixture, 30 μM glutathione *S*-transferase (GST)–CTD, 15 μM P-TEFb in each lane as appropriate, and 30 μM, 60 μM, 120 μM, and 240 μM (in lanes 4, 5, 6, and 7, respectively) of purified FKBP5 were added. Antibodies specific to phosphorylated Ser2 and Ser5 residues were used in immunoblotting analyses for identifying P-TEFb-mediated phosphorylation of target serine residues present in GST-CTD substrate. (B) *In vitro* phosphorylation and subsequent autoradiography analysis showing inhibition of P-TEFb-mediated phosphorylation of the Spt5 subunit of the DSIF complex by FKBP5 in a dose-dependent manner. γ-^{32}P-ATP was used for labeling the P-TEFb-mediated phosphorylation of Spt5 subunit and subsequently autoradiographed for detecting phosphorylation events. For each reaction mixture, 40 μM purified recombinant DSIF, 15 μM P-TEFb in each lane as appropriate, and 30 μM, 60 μM, 120 μM, and 240 μM (in lanes 4, 5, 6, and 7, respectively) of purified FKBP5 were added. As observed, increasing addition of FKBP5 inhibited P-TEFb-mediated phosphorylation of Spt5 subunit in a dose-dependent manner. Along with Spt5 phosphorylation, we also observed a strong effect of FKBP5 on the autophosphorylation activity of the P-TEFb complex, predominantly on its CDK9 subunit. "P" denotes the phosphorylated form of indicated proteins in our assay. (C) *In vitro* phosphorylation and subsequent autoradiography analysis showing inhibition of P-TEFb-mediated phosphorylation of NELF-A and

(Continued on next page)

our assays (Fig. 3A, compare the immunoblots using antibodies against Ser2P versus against Ser5P). To further address whether this inhibition of FKBP5 toward phosphorylation by the P-TEFb complex is substrate specific, we performed similar analyses using purified NELF and DSIF complexes. As shown in Fig. 3B and C, similar to its effect on P-TEFb-mediated phosphorylation of GST-CTD, purified recombinant FKBP5 efficiently inhibits P-TEFb-mediated phosphorylation of the NELF and DSIF complexes as well. Therefore, based on all these results, we conclude that human FKBP5 negatively regulates P-TEFb-mediated phosphorylation of diverse substrates *in vitro*.

To identify the specific domain within FKBP5 required for its overall inhibitory role in P-TEFb-mediated phosphorylation, we purified several FKBP5 fragments through its expression in bacterial expression system (Fig. 3D). Subsequent kinase assays showed that, consistent with our earlier observation, full-length FKBP5 efficiently inhibits P-TEFb-mediated phosphorylation of GST-CTD substrate in this assay as well (Fig. 3E, compare lane 4 versus lane 5). Interestingly, a deletion of the C-terminal 385 to 457 amino acid residues further enhanced FKBP5-mediated inhibition of phosphorylation by the P-TEFb complex, suggesting a role of this domain in antagonizing the overall inhibitory effect. Furthermore, and consistent with a role of the TPR1 domain in FKBP5-CDK9 interaction (Fig. 2F), a deletion of the TPR1 domain that prevents its interaction with CDK9 significantly reduced the inhibitory effect of FKBP5 on P-TEFb-mediated CTD phosphorylation compared to deletions of other domains that show maximal inhibition (Fig. 3E, compare lanes 6 to 8 versus lane 9). Consistently, a C-terminal fragment containing the TPR1 domain showed efficient inhibition of phosphorylation compared to the fragments without this domain (Fig. 3E, compare lanes 9 and 10 versus lanes 11 and 12). Overall, based on all of these data, we conclude that human FKBP5 efficiently inhibits P-TEFb-mediated phosphorylation of cognate GST-CTD substrate *in vitro*. Furthermore, while the C-terminal domain of FKBP5 (385 to 457 amino acids) negatively regulates this inhibitory function, its TPR1 domain-mediated interaction with CDK9 is important, at least in part, for efficient inhibition of P-TEFb complex-mediated phosphorylation of GST-CTD substrate *in vitro*.

FKBP5 regulates global Pol II CTD phosphorylation within mammalian cells in an HSP90-independent manner. Since our *in vitro* analyses showed a negative role of FKBP5 in P-TEFb-mediated phosphorylation of GST-CTD substrate (Fig. 3), we wanted to address whether similar effects would also be observed within mammalian cells. To address this, we generated short hairpin RNA (shRNA)-mediated stable FKBP5 knockdown 293T cells (Fig. 4A, left, immunoblots; right, mRNA analysis). Subsequent immunoblotting analyses using whole-cell extract clearly showed that upon FKBP5 knockdown, significant increases in global Pol II CTD phosphorylation at both Ser2 and Ser5 residues were observed compared to that in control scramble knockdown cells (Fig. 4B; see Fig. S4A for quantitation). Interestingly, in our analyses, we consistently observed a modest increase in CDK9 level upon FKBP5 knockdown within 293T cells. This could as well be a result of FKBP5 being a member of the HSP90 chaperone complex, which uses CDK9 as a client protein. Furthermore, global levels of Pol II were also increased due to increases in Ser2 and Ser5 phosphorylation that were previously shown to protect it from ubiquitin proteasome-mediated degradation by cognate E3 ligase Def1 (41). Nevertheless, normalization of Pol II levels with the levels of phosphorylated Ser2 and Ser5 form of Pol II further confirmed an overall modest but

FIG 3 Legend (Continued)

NELF-E subunits of the NELF complex by FKBP5. γ^{32}P-ATP was used for labeling the P-TEFb-mediated phosphorylation of both these subunits and subsequently autoradiographed for detecting phosphorylation events. For each reaction mixture, 30 μM purified recombinant NELF, 15 μM P-TEFb in each lane as appropriate, and 30 μM, 60 μM, 120 μM, and 240 μM (in lanes 4, 5, 6, and 7, respectively) of purified FKBP5 were added. As observed, and in this case as well, increasing addition of FKBP5 inhibited P-TEFb-mediated phosphorylation of NELF-A and NELF-E subunits in a dose-dependent manner. "P" denotes the phosphorylated form of indicated proteins in our assay. (D) Purification of recombinant FKBP5 full-length protein and its mutant derivatives through their expression in a bacterial expression system. Protein bands marked with red-filled dots represent the purified protein bands. Others represent either degradation or nonspecific proteins. (E) *In vitro* phosphorylation and subsequent Western blot analysis showing the effect of different domains of FKBP5 on inhibition of P-TEFb-mediated phosphorylation of Ser2 and Ser5 residues of Pol II CTD. For each reaction mixture, 30 μM GST-CTD, 15 μM P-TEFb in each lane as appropriate, and 250 ng of purified FKBP5 (full-length and deletion fragments) were added.

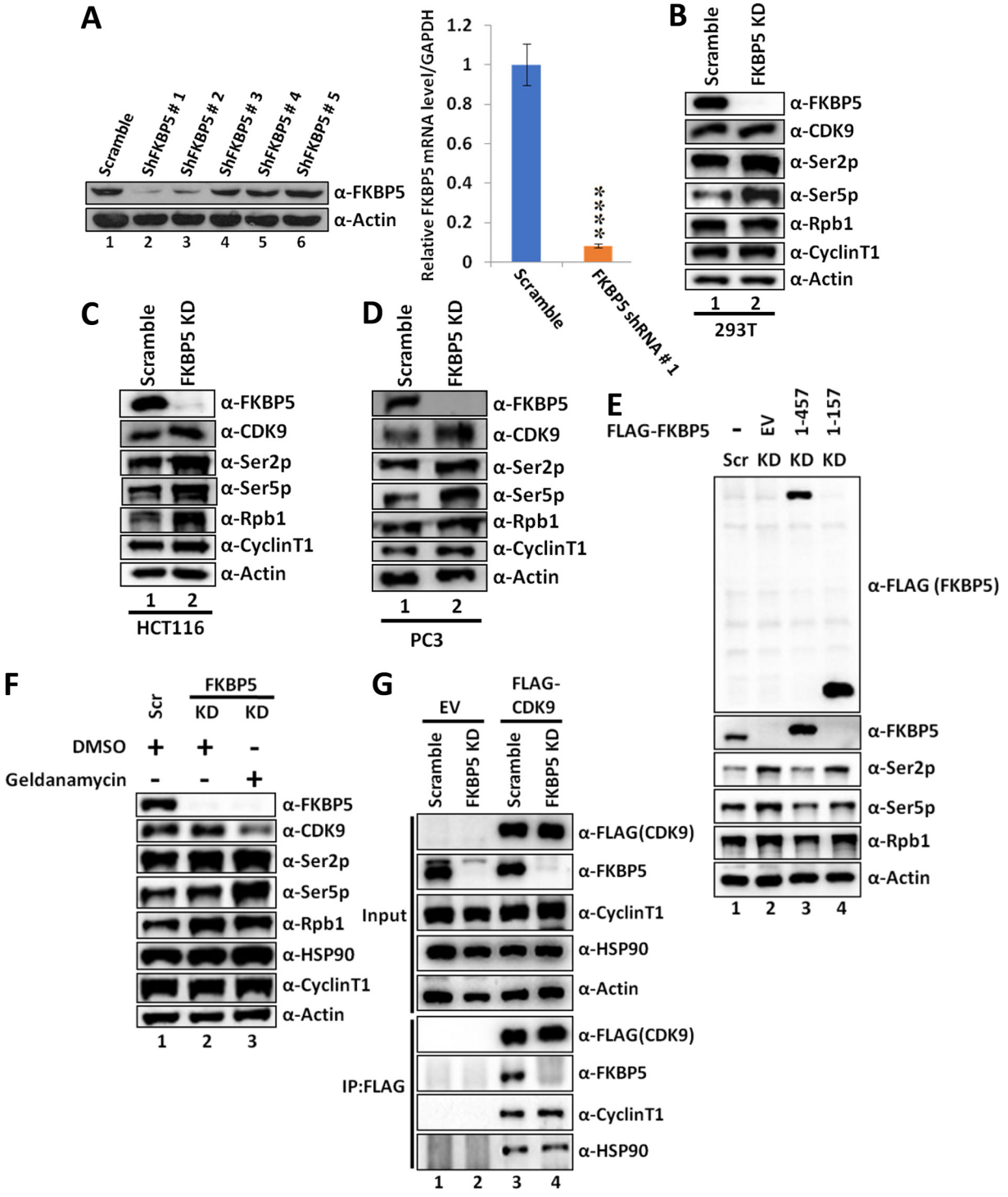


FIG 4 Global increase in Pol II CTD Ser2 and Ser5 phosphorylation upon FKBP5 knockdown in various cell lines. (A) Immunoblot analysis showing short hairpin RNA (shRNA)-mediated stable knockdown of FKBP5 within 293T cells by various target shRNAs (left) and quantitative real-time PCR (qRT-PCR) analysis showing FKBP5 mRNA levels in knockdown cells (right). (B) Immunoblot analysis showing the effect of stable FKBP5 knockdown on global levels of various factors (as indicated) within mammalian 293T cells. (C) Immunoblot analysis showing the effect of stable FKBP5 knockdown on the global levels of various factors (as indicated) within mammalian HCT116 colon carcinoma cells. (D) Immunoblot analysis showing the effect of stable FKBP5 knockdown on global levels of various factors (as indicated) within mammalian PC3 prostate cancer cells. (E) Immunoblot analysis showing the effect of reexpression of full-length and CDK9 interaction-defective FKBP5 proteins in FKBP5 knockdown 293T cells on the global levels of various factors, as indicated. (F) Immunoblot analysis showing the effect of geldanamycin (HSP90 inhibitor) treatment (5 μM final concentration) in FKBP5 knockdown cells on an overall increase in the global level of Pol II CTD phosphorylation in 293T cells. The level of CDK9 was used as a control for geldanamycin treatment. (G) Immunoblot analysis showing the effect of FKBP5 knockdown on interaction between ectopically expressed FLAG-CDK9 and endogenous HSP90 compared to that in control scramble cells.

reproducible increase in global levels of Pol II Ser2 and Ser5 phosphorylation upon FKBP5 knockdown within 293T cells (Fig. S4B). Similar results were also observed in both colon carcinoma (HCT116) (Fig. 4C; see Fig. S4C and D for quantitation) and prostate cancer (PC3) cell lines (Fig. 4D; see Fig. S4E and F for quantitation), thus ruling out an effect of FKBP5 on increasing global Pol II CTD Ser2 and Ser5 phosphorylation in a cell type-specific manner.

To further address whether the effect of FKBP5 knockdown on global increases in Pol II Ser2 and Ser5 phosphorylation is specific to FKBP5 only and not an indirect effect, we reexpressed full-length FKBP5 in the knockdown cells. As shown in Fig. 4E, reexpression of full-length FKBP5 reversed the effect of FKBP5 knockdown on those phosphorylation level (Fig. 4E, compare lane 2 versus lane 3). Further, and more importantly, an FKBP5 fragment (1 to 157) that failed to interact with CDK9 (Fig. 2D and F) and show an effect on reducing overall FKBP5-mediated inhibition of CTD phosphorylation *in vitro* (Fig. 3E) failed to reverse the effect of FKBP5 knockdown on global increases in Ser2 and Ser5 phosphorylation (Fig. 4E, compare lane 3 versus lane 4). Overall, these results further substantiate a negative role of FKBP5 in P-TEFb-mediated phosphorylation both *in vitro* and *in vivo* within mammalian cells. Furthermore, these results also signify a role of FKBP5-CDK9 interaction in the overall inhibitory effect of FKBP5 on P-TEFb-mediated phosphorylation of Pol II CTD.

The functions of FKBP5 have been described in the context of a cochaperone of HSP90 (3). Furthermore, CDK9 has also been described as an HSP90 client protein (42). Therefore, it is possible that an FKBP5 knockdown affects the functions of HSP90 toward its client protein such that enhanced folding of the CDK9 protein, leading to enhanced P-TEFb complex formation, may result in increased Pol II Ser2 and Ser5 phosphorylation, as observed in multiple cell lines. To rule out this possibility and show that an overall effect of FKBP5 knockdown is independent of HSP90 functions, we treated the cells with geldanamycin, a known HSP90 inhibitor. As shown in Fig. 4F, although treatment of cells with geldanamycin results in a reduced level of CDK9 (compare lane 2 versus lane 3), a known effect reported previously (43), it failed to show any effect on global increases in Pol II Ser2 and Ser5 phosphorylation levels (compare lane 2 versus lane 3). Therefore, the observed effect of FKBP5 knockdown on global increases in Pol II Ser2 and Ser5 phosphorylation is independent of the functions of HSP90. Furthermore, in support of this HSP90-independent effect of FKBP5, we also failed to observe any increase in CDK9 interaction with HSP90 upon FKBP5 knockdown compared to that in control scramble cells (Fig. 4G, compare lane 3 versus lane 4).

FKBP5 and cyclin T1 interact with CDK9 through the same domain. Our earlier results have shown that the inhibitory effect of FKBP5 on P-TEFb-mediated phosphorylation activity depends at least in part on its interaction with CDK9 both *in vitro* and *in vivo* within mammalian cells. Towards a deeper mechanistic understanding of this regulation, we initially addressed the specific region within CDK9 that would interact with FKBP5. For providing direct evidence, we initially addressed the CDK9 domain that would interact with FKBP5 through their expression in heterologous Sf9 cells. We generated several plasmids expressing CDK9 fragments deleted at both N and C termini and expressed them through baculovirus-mediated infection of Sf9 cells (see Fig. 5A and B for fragments used). As shown in Fig. 5B, coinfection of these fragments along with full-length FKBP5 showed that while the full-length CDK9 strongly interacts, a deletion of 31 amino acids from the N-terminal end almost abolished CDK9 interaction with FKBP5 (compare lane 5 versus lane 6). Since the N-terminal half of CDK9 was reported previously to interact with cyclin T1 (44), we also addressed whether this amino acid region (1 to 31) of CDK9 would also be required for its interaction with cyclin T1. As shown in Fig. 5C, immunoprecipitation analyses using extract of Sf9 cells expressing the indicated CDK9 fragments and cyclin T1 clearly showed dependence on the same region of CDK9 for its interaction with cyclin T1 within this heterologous system (compare lane 5 versus lane 6). Furthermore, we also addressed whether the same domain of CDK9 would be required for its interaction with both partners within

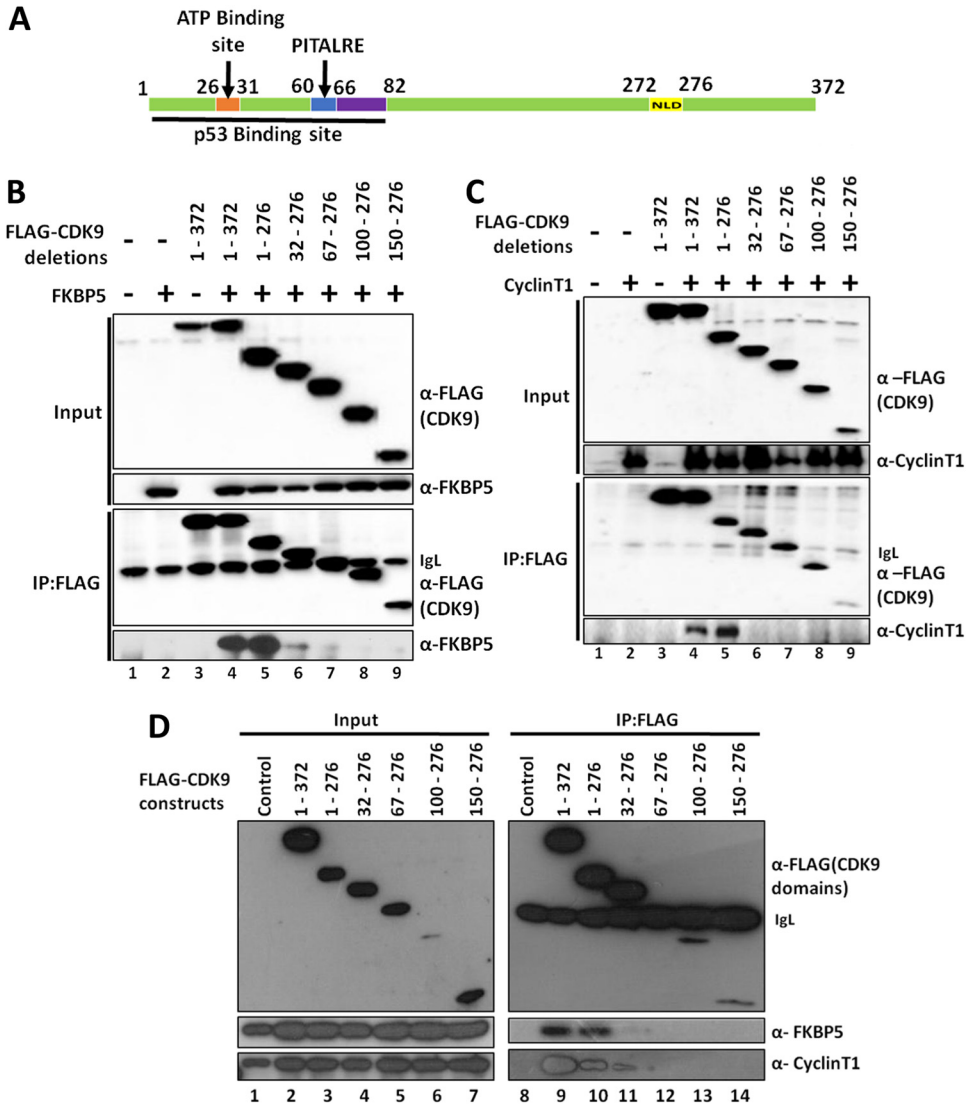


FIG 5 FKBP5 and cyclin T1 interact in the same N-terminal region of CDK9. (A) Cartoon diagram representing various functional domains within CDK9 that are important for its functions. NLD, nuclear localization domain. Amino acids 26 to 31, ATP-binding site; amino acids 60 to 66, PITALRE; amino acids 1 to 82, p53-binding site. (B) Domain analysis of CDK9 (using various domains as indicated) for its interaction with FKBP5 through their baculovirus-mediated expression in Sf9 cells. Sf9 cells were coinfected with the baculoviruses as indicated, and cell lysates were subjected to immunoprecipitation and subsequent Western blotting for identifying their interaction within this heterologous system. (C) Domain analysis of CDK9 (using various domains as indicated) for its interaction with cyclin T1 through their baculovirus-mediated expression in Sf9 cells. Sf9 cells were coinfected with the baculoviruses as indicated, and cell lysates were subjected to immunoprecipitation and subsequent Western blotting for identifying their interaction within this heterologous system. (D) Domain analysis of CDK9 (using various domains as indicated) for its interaction with endogenous FKBP5 and cyclin T1 within mammalian 293T cells. 293T cells were transfected with various CDK9 plasmids as indicated, and cell lysates were subjected to immunoprecipitation and subsequent Western blotting using factor-specific antibodies for identifying their interaction within 293T cells.

mammalian cells. We transfected 293T cells expressing the indicated CDK9 fragments and tested their interaction with endogenous cyclin T1 and FKBP5. As shown in Fig. 5D and consistent with our observations in heterologous Sf9 cells, we also observed a strong dependence on the N-terminal 31 amino acids for CDK9 interaction with both cyclin T1 and FKBP5 (compare lane 10 versus lane 11). Thus, based on all of this evidence, we conclude that the N-terminal 31 amino acids of CDK9 are required for its interactions with both cyclin T1 and FKBP5. These results also further indicate a possibility of existence of competition between FKBP5 and cyclin T1 for their binding at the

N terminus of CDK9, thus providing a mechanistic explanation of the negative role of FKBP5 on P-TEFb-mediated phosphorylation both *in vitro* and *in vivo* within mammalian cells.

FKBP5 and cyclin T1 compete with each other for their binding to CDK9. Since our earlier analyses indicated the presence of competition between FKBP5 and cyclin T1 for their binding to CDK9 owing to their use of the same binding sites (Fig. 5), we subsequently addressed this competitive mode of binding between these factors in details through several experiments. In the initial analysis, we cotransfected 293T cells with plasmids expressing CDK9 and increasing concentrations of FKBP5 to test whether its increased expression and subsequent binding would reduce concomitant CDK9 binding to cyclin T1 within mammalian cells. As shown in Fig. 6A, ectopically expressed CDK9 efficiently bound endogenous cyclin T1 (lane 3). Interestingly, and consistent with our hypothesis of a competition model, increased expression, and thus increased binding, of FKBP5 concomitantly reduced CDK9 interaction with endogenous cyclin T1 (Fig. 6A, compare lane 3 versus lanes 4 and 5). Furthermore, this reduction in interaction requires FKBP5 binding to CDK9, since an FKBP5 fragment (1 to 157 amino acids) that failed to show an interaction with CDK9 (Fig. 2D and F), also failed to show an effect on CDK9 binding with endogenous cyclin T1 (Fig. 6B, compare cyclin T1 binding between lanes 3 and4 versus 5 and 6). In a reverse experiment, increased expression of ectopic cyclin T1 and its increased binding to CDK9 resulted in concomitant reduced binding of FKBP5 as well (Fig. 6C, compare lane 3 versus lanes 4 and 5) within mammalian cells.

For providing direct evidence of this competitive binding, we performed *in vitro* interaction analyses with purified FKBP5, CDK9, and cyclin T1 proteins (Fig. S2A and B and Fig. 6D). The cyclin T1 protein was purified through its expression in a bacterial system. Our initial analysis with purified CDK9 and cyclin T1 clearly showed their direct interaction *in vitro* (Fig. 6E, lane 4). Interestingly, addition of purified FKBP5 to this interaction assay clearly showed reduced cyclin T1 binding to CDK9 upon concomitant increased binding of FKBP5 (Fig. 6F, compare cyclin T1 binding to CDK9 in lane 2 versus lane 4 and 5). In a reciprocal experiment, increased addition of cyclin T1 and its increased binding also reduced concomitant FKBP5 binding to CDK9 (Fig. 6G, compare FKBP5 binding to CDK9 in lane 2 versus lanes 4 to 5).

To provide further evidence of this model of competitive binding in an endogenous context, we used FKBP5 knockdown cells (Fig. 4A). Immunoprecipitation of endogenous CDK9 clearly showed enhanced CDK9 binding with endogenous cyclin T1 upon FKBP5 knockdown compared to that upon control scramble knockdown (Fig. 6H, compare lane 2 versus lane 3). For providing further mechanistic evidence of these functional regulations, we used purified preformed P-TEFb complex (FLAG-CDK9 plus GST-cyclin T1) in our *in vitro* dissociation assay (see Materials and Methods). As shown in Fig. 6I, addition of purified recombinant FKBP5 efficiently dissociated cyclin T1 from the preformed P-TEFb complex such that its overall amount in the supernatant increased with increasing concentration of added FKBP5 (compare lane 5 with lanes 6 to 8). Consistent with this, cyclin T1 bound to FLAG-CDK9 also concomitantly decreased with increasing addition of FKBP5 (Fig. 6I, lane 1 versus lanes 2 to 4). Furthermore, FKBP5 could also efficiently dissociate cyclin T1 from immunoprecipitated P-TEFb complex from mammalian cells upon its binding with CDK9 (Fig. S5A). These data provide evidence of FKBP5 directly modulating cyclin T1 association with CDK9 that can have implications in overall P-TEFb-mediated functional regulations both *in vitro* (Fig. 3) and *in vivo* within mammalian cells (Fig. 4). Based on all of this evidence, we conclude that human FKBP5 and cyclin T1 compete with each other for their binding to CDK9 (Fig. S5B).

FKBP5 regulates expression of diverse sets of genes. Based on our mechanistic understanding of the role of FKBP5 in negative regulation of P-TEFb functions, we were further interested in understanding the implication of this regulation on expression of global target genes. We performed transcriptome sequencing (RNA-Seq) analysis for identifying genes that would be affected upon FKBP5 knockdown (GEO accession no. GSE169461). For the purpose of control scramble cells, we used data sets that were reported in our earlier study (37) (SRA accession no. PRJNA512165), since all of these experiments were performed at the same time. Transcriptome analysis revealed a rather small set of genes (a

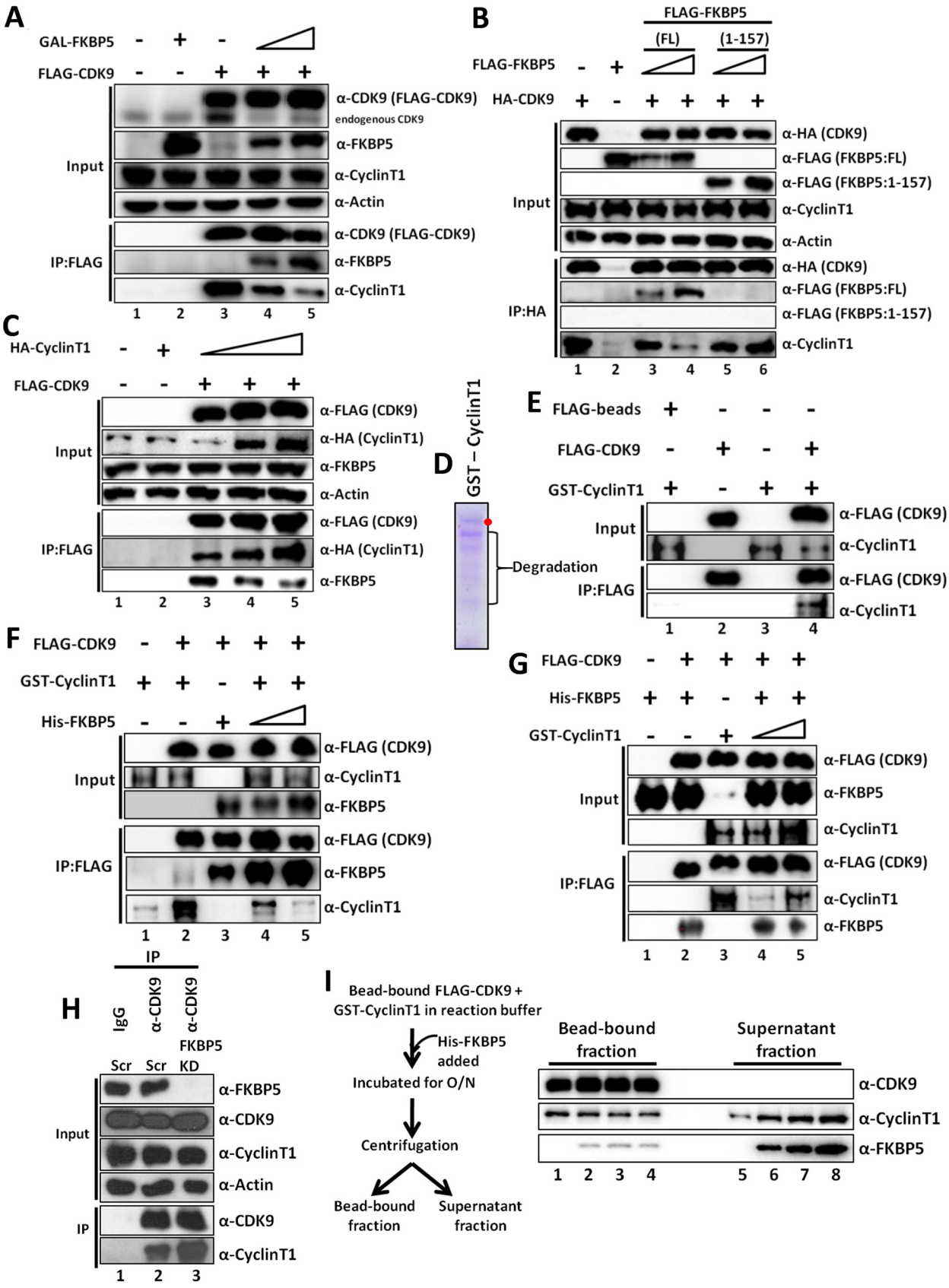


FIG 6 FKBP5 and cyclin T1 compete with each other for their binding to CDK9 both *in vitro* and *in vivo* within mammalian cells. (A) Immunoblotting analysis showing the effect of increasing expression of FKBP5 on the association of ectopically expressed CDK9 and endogenous cyclin T1. 293T cells

(Continued on next page)

total of 219) showing significant ($P < 0.05$) upregulation of expression ($\log_2$ fold change [$\log_2$FC] > 1.5) and a similar number of genes (a total of 145) being downregulated upon FKBP5 knockdown. A heatmap representing expression of some of these genes is shown in Fig. 7A. Interestingly, subsequent gene ontology analyses showed a strong association of some of these upregulated genes with multiple cellular processes, such as Pol II-mediated transcriptional regulation, as well as in cellular differentiation and proliferation-related events (Fig. 7B). In contrast, the downregulated genes showed a strong association with regulation of multiple metabolic processes. The overall effect of FKBP5 knockdown on global target gene expression further indicates a role for FKBP5 in regulation of Pol II-mediated transcriptional processes for expression of genes that are important for regulation of cellular proliferation and differentiation.

Knockdown of FKBP5 enhances expression of proliferation-related genes. Based on our RNA-Seq analysis and an indication of involvement of a subset of these upregulated genes in controlling cellular proliferation, we were interested in identifying molecular mechanism of the upregulation of expression upon FKBP5 knockdown. Subsequent quantitative real-time PCR (qRT-PCR) analyses confirmed significant upregulation of genes that are widely involved in regulation of cellular proliferation upon FKBP5 knockdown (Fig. 7C). Expression of two of these critical genes, *CCND1* and *C-MYC*, encoding cyclin D1 and c-Myc proteins, respectively, have been widely studied for their role as master regulators of expression of global target genes. Along with these two critical regulators, we also observed upregulation of other key regulators of cellular proliferation, including *CDKN1A*, *CDKN1B*, and *MDM2*, among others. This upregulation is specific, since in the same assay, we failed to observe any effect of FKBP5 knockdown on expression of nontarget genes such as *RGS2*, *MAP2*, and *LDLR*. Similar effects are also observed upon FKBP5 knockdown on target gene expression in a prostate cancer cell line (PC3) (Fig. S6A), which thus rules out an effect of FKBP5 knockdown on increasing expression of proliferation-related genes in a cell type-specific manner. The overall effect of FKBP5 knockdown in increasing the expression of target genes is specific, since reexpression of full-length FKBP5 reversed the effect, as shown for two target genes (*CCND1* and *E2F2*), within 293T cells, whereas the CDK9 interaction-deficient FKBP5 mutant (1 to 157 amino acids) failed to do so (Fig. 7D). To further corroborate the overall effect, we tested the expression level of these target genes in CDK9 knockdown cells (Fig. S6B). As shown in Fig. S6C, the majority of these target genes are also downregulated upon CDK9 knockdown, indeed suggesting the overall effect of FKBP5 knockdown on target gene expression through modulation of P-TEFb

FIG 6 Legend (Continued)
were cotransfected with the indicated plasmids, and cell lysates were subjected to immunoprecipitation using epitope tag-specific agarose beads and to subsequent Western blotting using factor-specific antibodies for identifying their interactions. (B) Immunoblotting analysis showing effect of expression of full-length (FL) and CDK9 interaction-defective (1 to 157 amino acids) FKBP5 proteins in 293T cells on association of ectopically expressed CDK9 with endogenous cyclin T1. 293T cells were cotransfected with the indicated plasmids, and cell lysates were subjected to immunoprecipitation using epitope tag-specific agarose beads and to subsequent Western blotting using factor-specific antibodies for identifying their interactions. (C) Immunoblotting analysis showing effect of increasing expression of cyclin T1 on association of ectopically expressed CDK9 with endogenous FKBP5. 293T cells were cotransfected with the indicated plasmids, and cell lysates were subjected to immunoprecipitation using epitope tag-specific agarose beads and to subsequent Western blotting using factor-specific antibodies for identifying their interaction. (D) SDS-PAGE Coomassie staining showing purification of GST-cyclin T1 used in panels E to G. A filled red dot indicates the target protein band, whereas others represent either degradation or nonspecific proteins. (E) Immunoblotting analysis showing direct interaction of CDK9 with cyclin T1. Purified recombinant CDK9 and cyclin T1 proteins were used and added as indicated in the *in vitro* interaction assays. (F) Immunoblotting analysis showing effect of increasing addition of FKBP5 on association of CDK9 with cyclin T1 *in vitro*. Purified recombinant CDK9, cyclin T1, and FKBP5 proteins were used and added as indicated in the *in vitro* interaction assays for identifying the effect of increasing addition of FKBP5 on CDK9 association with cyclin T1 *in vitro*. For each reaction mixture, 60 μM purified FLAG-CDK9, 50 μM GST-cyclin T1 in each lane as appropriate, and 100 μM, 100 μM, and 200 μM (in lanes 3, 4, and 5, respectively) purified FKBP5 were added. (G) Immunoblotting analysis showing the effect of increasing addition of cyclin T1 on association of CDK9 with FKBP5 *in vitro*. Purified recombinant CDK9, cyclin T1, and FKBP5 proteins were used and added as indicated in the *in vitro* interaction assays for identifying the effect of increasing addition of cyclin T1 on CDK9 association with FKBP5 *in vitro*. For each reaction mixture, 60 μM purified FLAG-CDK9, 50 μM His-FKBP5 in each lane as appropriate, and 50 μM, 50 μM, and 100 μM (in lanes 3, 4, and 5, respectively) purified GST-cyclin T1 were added. (H) Immunoblotting analysis showing effect of FKBP5 knockdown on association of endogenous CDK9 with cyclin T1. Endogenous CDK9 was immunoprecipitated using specific antibody, and its association with endogenous cyclin T1 was assessed by Western blot analysis. (I) (Left) Experimental strategy that was employed for our assay as shown in the right subpanel. (Right) *In vitro* dissociation assay demonstrating the effect of addition of purified recombinant FKBP5 in dissociating cyclin T1 from preformed P-TEFb complex obtained through recombinant expression of FLAG-CDK9 and GST-cyclin T1. It can be observed that purified FKBP5 efficiently dissociates cyclin T1 from CDK9, and thus the level of cyclin T1 is increased in the supernatant and is decreased with bead-bound CDK9.

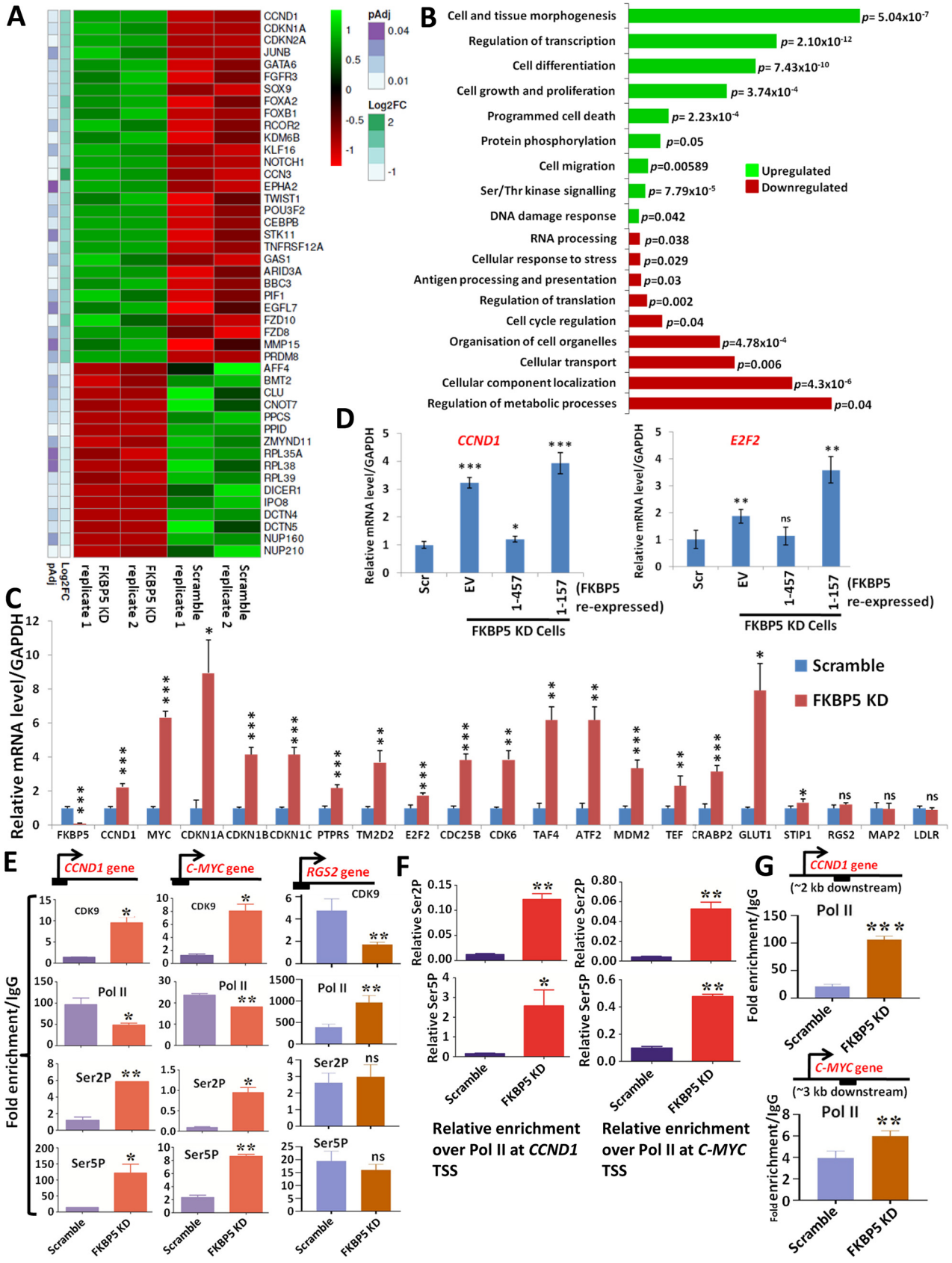


FIG 7 FKBP5 knockdown enhances expression of proliferation-related genes. (A) Heatmap showing the effect of FKBP5 knockdown on expression of selected target genes compared to that in control scramble cells. Log_2FC represents fold change on a log_2 scale. (B) Gene ontology (GO)

(Continued on next page)

activity. Furthermore, overexpression of CDK9 that increased Pol II CTD phosphorylation (Fig. S6D) also caused significant increases in expression of all these target genes (Fig. S6E) and provided additional evidence of functional effect of FKBP5 in modulating P-TEFb-dependent target gene expression within mammalian cells. Also, consistent with a role of P-TEFb in stimulating transcriptional activation of HIV long terminal repeat (LTR) promoter-driven genes, we also observed an increase in the overall expression of HIV LTR promoter-driven luciferase reporter gene upon FKBP5 knockdown compared to that in control scramble cells (Fig. S6F).

FKBP5 knockdown enhances recruitment of P-TEFb complex at the target *CCND1* and *C-MYC* genes. Once we established *CCND1* and *C-MYC* genes as key targets whose expressions are regulated by FKBP5, we were interested in further mechanistic understanding of this regulation. Both of these genes have been shown to be key targets for P-TEFb complex-containing SEC for transcriptional activation (37, 45). Since FKBP5 negatively regulates P-TEFb level and its functions within the cellular system, we wondered whether enhanced recruitment of the P-TEFb complex upon FKBP5 knockdown would correlate with enhanced expression of these genes. As shown in Fig. 7E, our chromatin immunoprecipitation (ChIP) analysis showed a significant increase in CDK9 (representing P-TEFb complex) recruitment at the promoter-proximal region of these target genes. An increase in P-TEFb complex recruitment also results in increased presence of Pol II CTD Ser2 and Ser5 phosphorylation (Fig. 7E), whereas similar analyses with the control *RGS2* gene failed to show any effect. Consistent with a role for P-TEFb-mediated phosphorylation in releasing paused Pol II from the promoter-proximal region, we also observed a significant decrease in the overall level of total Pol II at the promoter-proximal region of these genes. Enhanced P-TEFb recruitment in the FKBP5 knockdown cells compared to that in the control scramble cells (Fig. 7F). Consistent with decreases in Pol II levels at the transcriptional start site (TSS) region, we also observed an increased presence of Pol II in the coding region of both of these genes (Fig. 7G). Pausing index analysis clearly showed significant reduction in overall pausing of Pol II at both of these target genes upon FKBP5 knockdown (Fig. S6G). Therefore, based on all of this evidence, we conclude that human FKBP5 negatively regulates the overall level of the P-TEFb complex within mammalian cells for regulating expression of key proliferation-related target genes such as *CCND1* and *C-MYC*. These results also point out to an antiproliferative role of FKBP5 in regulating cell growth.

Knockdown of FKBP5 enhances cellular proliferation. Since our study has shown a negative role of FKBP5 in P-TEFb-mediated expression of proliferation-related genes, we anticipated that reduced expression of FKBP5 might enhance overall cellular proliferation. Indeed, consistent with this hypothesis, knockdown of FKBP5 significantly enhanced proliferation of 293T cells compared to control scramble knockdown (Fig. S6H). Consistent with a similar effect of FKBP5 knockdown on expression of proliferation-related genes in the PC3 prostate cancer cell line (Fig. S6A), we also observed enhanced cellular proliferation of the PC3 cell line upon FKBP5 knockdown (Fig. S6I), thus ruling out an overall effect of FKBP5 in controlling proliferation in a cell-type-specific manner.

Strong association of downregulation of expression of FKBP5 and upregulation of expression of proliferation-related genes in several human cancers. Since our results clearly indicated an antiproliferative role of FKBP5 in controlling cell growth, we

FIG 7 Legend (Continued)
analysis predicting association of upregulated and downregulated genes upon FKBP5 knockdown with various biological processes. (C) qRT-PCR analysis confirming upregulation of mRNA expression of target genes that are involved in cellular proliferation in 293T cells upon FKBP5 knockdown. Data represent $n = 2$ biological replicates and 3 PCR replicates of each biological replicate. (D) qRT-PCR analysis showing effect of reexpression of full-length and CDK9 interaction-defective FKBP5 proteins in FKBP5 knockdown cells on mRNA expression of indicated target genes. Data represent $n = 2$ biological replicates and 3 PCR replicates of each biological replicate. (E) Chromatin immunoprecipitation (ChIP) analysis showing enhanced recruitment of the P-TEFb complex (using CDK9 as the candidate) upon FKBP5 knockdown resulting in enhanced level of Pol II CTD Ser2 and Ser5 phosphorylation and reduced levels of Pol II in the promoter-proximal region of *CCND1* and *C-MYC* target genes. Data represent $n = 2$ biological replicates and 3 PCR replicates of each biological replicate. (F) Quantitation of relative enrichment of Pol II CTD Ser2 and Ser5 phosphorylation normalized with the amount of total Pol II being present on the promoter-proximal regions of target *CCND1* and *C-MYC* genes. Data represent $n = 2$ biological replicates and 3 PCR replicates of each biological replicate. (G) ChIP analysis showing enhanced presence of Pol II at the indicated coding regions of target *CCND1* and *C-MYC* genes. Data represent $n = 2$ biological replicates and a minimum of 3 PCR replicates of each biological replicate. In all of our assays, statistical analyses were performed using a one-tailed Student's *t* test for obtaining statistical significance of our data. *, $P \leq 0.05$; **, $P \leq 0.01$; ***; $P \leq 0.001$; ns, not significant.

assumed that similar functional significance would also be important for cancer pathogenesis. It is of note that a few earlier studies have indicated a negative correlation between FKBP5 expression and severity of cancers (8, 19). To address FKBP5 functions and target gene expression in cancer pathogenesis, we analyzed mRNA expression of FKBP5 and its correlation with expression of proliferation-related genes in patient cohort samples of multiple cancer types available in The Cancer Genome Atlas (TCGA) data sets. We were intrigued by our observation of significant correlation between downregulation of FKBP5 expression and the increased expression of proliferation-related genes such as *CCND1*, and *C-MYC* (Fig. S7A to E). Thus, based on mechanistic understanding of the functional role of FKBP5 as deciphered by our study, downregulation of FKBP5 expression could play an essential role in overall cancer pathogenesis through enhanced expression of key cancer-causing protooncogenes such as *CCND1* and *C-MYC*. Furthermore, it is interesting to note that several forms of kidney cancer show strong correlations between downregulation of FKBP5 expressions and upregulation of *CCND1* and *C-MYC* genes, which further substantiates our observation in 293T cells. Our observation that FKBP5 downregulation enhances cell proliferation through enhancement of expression of proliferation-related genes further supports the overall role of FKBP5 as a tumor suppressor in these types of cancers.

DISCUSSION

One of the key findings of our study is to decipher a novel function of human FKBP5 in regulation of Pol II-mediated transcription through regulation of P-TEFb levels within mammalian cells. Owing to having the same binding sites on CDK9, FKBP5 competes with cyclin T1 for their interaction and thus regulates the level of P-TEFb complex formation through regulation of cyclin T1 association with CDK9 for transcriptional activation. Consistent with this model, overexpression of FKBP5 reduces CDK9 association with cyclin T1 and transcriptional activation, whereas knockdown of FKBP5 expression increases CDK9-cyclin T1 association and causes transcriptional activation. A working model, as deciphered by our studies, is presented in Fig. S8 in the supplemental material.

Potential implication of role of FKBP5 in regulating pausing events at the promoter-proximal region. An earlier study reported a role for the HSP90 chaperone protein in regulating pausing events at the promoter-proximal region of global target genes (46). This study indicated a role for HSP90 interaction with the NELF complex in overall regulation of pausing without providing deeper mechanistic insights. Since FKBP5 plays a key role as a cochaperone of the HSP90 chaperone complex, it is highly likely that, through the mechanisms as deciphered in our study, FKBP5 could also play additional roles within the HSP90 chaperone complex for attaining overall pausing event through negative regulation of functions of the P-TEFb complex which is important for releasing the paused Pol II from the promoter-proximal region. Consistent with this hypothesis, our ChIP analysis of target *CCND1* and *C-MYC* genes showed a significant decrease in overall Pol II levels upon knockdown of FKBP5 at the promoter-proximal region (Fig. 7E). Further studies are needed for detailed understanding of this mechanism of global pausing event involving FKBP5 within mammalian cells.

FKBP5-mediated transcriptional downregulation and cancer pathogenesis. It is quite interesting to note that in our study, significant number of cancers show a strong correlation between downregulation of FKBP5 and increased expression of key proliferation-related genes (see Fig. S4 in the supplemental material). Mechanistic understanding obtained from our study provides a paradigm for this overall observation, in which enhanced activation of P-TEFb functions upon reduced FKBP5 expression causes upregulation of expression of protooncogenes such as *C-MYC*, *CCND1*, etc. Overactivation of AKT functions is also implicated in breast cancer pathogenesis (8). A strong correlation between downregulation of FKBP5 expression and enhanced expression key proliferation-related genes in multiple cancers further provides a functional role of FKBP5 as a tumor suppressor in multiple types of cancers. The combined role of FKBP5-mediated AKT and P-TEFb functions in overall cancer pathogenesis is a subject for future studies to come.

Transcriptional downregulation by FKBP5 and type 2 diabetes pathogenesis. The human FKBP5 has also been implicated in glucose metabolism and associated type 2 diabetes pathogenesis. FKBP5 knockout mice show improved glucose tolerance associated with leaner body weight when fed with normal chow diet (47, 48). Interestingly, a recent study has shown a role for the AKT signaling pathway, upon insulin stimulation, in phosphorylation of target AS160, which helps in translocation of the GLUT4 glucose uptake protein to the plasma membrane and thus facilitates overall glucose uptake and homeostasis (15). Interestingly, the same study also observed an increase in GLUT4 expression upon ablation of FKBP5 functions by its knockdown or pharmacological antagonist. The results, as deciphered in our study, provide a new mechanistic insight into this overall regulation. It is quite possible that upon FKBP5 knockdown, the overall increase in P-TEFb functions results in increased transcription of GLUT4, leading to its increased expression. Whether pharmacological inhibition would also result in a similar increase in P-TEFb functions remains to be studied. Nevertheless, the important mechanistic insight on functional regulation of FKBP5 would provide a basis for pathogenesis of multiple diseases, including that of type 2 diabetes.

Transcriptional downregulation by FKBP5 and adipogenesis. Few studies have implicated a role for FKBP5 in regulation of adipogenesis. The maximum amount of FKBP5 expression has been reported in adipocytes, as well as in skeletal muscles and lymphocytes, compared to that in other tissues (49). Therefore, not surprisingly, FKBP5 functions have been correlated with adipocyte differentiation from white adipose tissue (WAT) to brown adipose tissue (BAT) (18). FKBP5 knockdown has been shown to increase in expression of the key regulators of adipogenesis UCP1 and PRDM16 (18). However, the underlying mechanisms of this increase are completely unknown. The overall role of FKBP5 in transcriptional regulation through negative regulation of functions of the P-TEFb complex certainly provides a mechanistic explanation of this overall regulation. Functions of the P-TEFb complex have been shown to be involved in regulation of expression of multiple key genes during WAT to BAT differentiation. In this context, whether FKBP5 downregulation and its implication in an increase in P-TEFb-mediated transcriptional upregulation of key *UCP1* and *PRDM16* genes, as well as others, plays an important role in overall adipogenesis would be a subject for detailed studies.

Functions of FKBP5 and neuronal pathogenesis. Consistent with its high expression in the hippocampus and amygdala (50), the two key regions regulating stress-related functions, FKBP5 has been implicated in controlling the pathogenesis of several neuronal disorders, including PTSD, by a significant number of studies (4–6). The majority of these studies have implicated a role for FKBP5 in regulating the glucocorticoid receptor (GR) signaling pathway, in which FKBP5 competes with glucocorticoids for their binding to the GR and subsequent downstream signal transduction for transcriptional activation of target genes. However, no other studies have provided direct mechanistic insights into the functional role of FKBP5 in transcription. Thus, our study has the potential to decipher important mechanistic insights into the role of FKBP5 and overall neuronal pathogenesis that studies have failed to explore until now. Further future studies would be directed toward addressing some of these key questions.

Overall, the mechanistic insights obtained from our study have the potential to increase understanding of underlying mechanisms of pathogenesis of several FKBP5-associated diseases, including cancer and metabolic disorders, as well as several neuronal disorders that studies until now have not been able to address.

MATERIALS AND METHODS

Cell culture. For this study, mammalian cells were cultured in Dulbecco's modified Eagle medium (DMEM) with high glucose (Gibco, USA), supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 U/mL penicillin-streptomycin (Gibco). Mammalian cells were maintained in a 5% CO_2 incubator at 37°C while maintaining proper humid conditions. All insect cell (Sf9) culturing in this study was done in Grace's insect medium (HiMedia, India) supplemented with 10% FBS (Gibco) and 7 μg/mL gentamycin. Sf9 cells were maintained in a bio-oxygen demand (BOD) incubator at 26°C.

Transfection of mammalian and Sf9 cells. Transfection of mammalian cells were done using FuGene transfection reagent and following the manufacturer's protocol. Unless otherwise mentioned, cells were

harvested 48 h after transfection for subsequent assays. To generate baculoviruses for expression of recombinant proteins, Sf9 cells were transfected using Cellfectin II reagent (Invitrogen) per the manufacturer's protocol. Virus particles (supernatant) were collected 72 h posttransfection.

Plasmids, primers, and antibodies. All the plasmids used for this study, primers used for RNA analyses, ChIP analyses, construction of shRNAs, and antibodies used for various experiments are mentioned in Tables 1 to 5.

Construction of plasmids used in this study. We obtained pCMVSPORT6 vector, with the FKBP5 gene cloned into it, from Open Biosystems. The construct was sequenced in its entirety before being used for downstream cloning experiments. Full-length as well as derivatives of different FKBP5 constructs were cloned in multiple expression vectors, including pcDNA5/FRT/TO, pFASTBac, pET-28a(+), and pGEX. Similarly, CDK9, cyclin T1, CDK7, CDK8, Pol II CTD, and other required factors and their fragments were PCR amplified and cloned in different vectors for their expression with epitope tags and in mammalian, Sf9, and bacterial cells. The details of cloning information for any given construct are available through the corresponding author of this study.

Immunoprecipitation analysis. To test the interaction between FKBP5 and CDK9 in an endogenous context, nuclear extracts were prepared from a confluent 10-cm dish of 293T cells. Subsequently, this extract was precleared using protein A-agarose beads for 2 h at 4°C. The precleared extract was subsequently used for overnight immunoprecipitation with 2 μg of indicated antibody. Antibody-bound proteins were pulled down using protein A-agarose beads by incubating for 4 h. After rigorous washing, bound proteins were eluted by boiling in 1× SDS loading dye for 10 min at 95°C. The sample was analyzed by Western blotting using the indicated antibodies.

For all other exogenously expressed epitope-tagged mammalian target proteins, cells were harvested after 48 h of transfection and lysed for 2 h at 4°C by rotating at 16 rpm in BC300 buffer (20 mM Tris-Cl [pH 8.0], 300 mM KCl, 2 mM EDTA, and 20% glycerol) containing 0.1% NP-40 and protease inhibitor cocktail. From the whole-cell extract, target proteins were pulled down using tag-specific-antibody-coated agarose beads. Subsequently, after overnight incubation, the beads were washed three times (10 min each at 4°C) using washing buffer containing BC300 plus 0.1% NP-40 to remove nonspecific interactors. Subsequently, bead-bound proteins were eluted in 1× SDS loading dye by incubating at 95°C for 10 min. The eluted proteins thus obtained were subsequently used for Western blotting.

Generation of stable cells. In this study, we used two different stable cell lines expressing FLAG-HA-tagged FKBP5 and CDK9 proteins. Generation of a CDK9 stable cell line was already been reported in our earlier study (22). To generate a FLAG-HA-FKBP5-expressing stable line, we used the Flp-In method (Invitrogen) following the manufacturer's protocol. Flp-In 293T cells were transfected with the appropriate pCND5-FRT-TO vector containing the FKBP5 open reading frame (ORF). The transfected cells were subjected to hygromycin (200 μg/mL) selection for several weeks to obtain the positive colonies. Individual colonies were picked up, expended, and further screened for the expression of the FLAG-HA-FKBP5 protein by Western blotting using FLAG tag-specific antibody.

Generation of stable knockdown cells. Stable knockdown of FKBP5 in mammalian cell lines was obtained through lentivirus-mediated stable integration of target shRNA sequences against FKBP5 and CDK9. To produce specific lentiviruses, target sequences were first cloned in lentiviral pLKO.1 puro vector. Subsequently we cotransfected the shRNA construct along with psPAX2 (lentivirus packaging plasmid) and pMD2.G (lentivirus envelope plasmid) in 293T cells. Lentivirus particles were produced and collected after 72 to 96 h of transfection. Collected lentivirus particles were used with Polybrene (8 μg/mL) for transduction. Subsequently, transduced cells were selected using puromycin (3 μg/mL), and knockdown efficiency was measured at both RNA and protein levels.

Baculovirus expression-based reconstitution and purification. To reconstitute and purify proteins or protein complex from insect cells, we used a baculoviral expression system. For the purification of expressed proteins/complexes, baculovirus particles were used directly to the 90% confluent Sf9 cells either alone or in combination, per experimental requirement. Cells were harvested after 48 h of infection. Cell lysate was prepared in BC300 buffer (20 mM Tris-Cl, 200 mM KCl, 2 mM EDTA, and 20% glycerol), containing 0.1% Nonidet P-40, 0.7 μL/mL β-mercaptoethanol, and 1× protease inhibitor cocktail for 2 h at 4°C. To get rid of cell debris, cell lysate was centrifuged at 12,000 rpm for 20 min at 4°C. Subsequently, immunoprecipitation was performed using epitope-tag-specific agarose beads to pull down protein-protein complex. After overnight binding, beads were washed three times (10 min each at 4°C) with washing buffer containing BC300 plus 0.1% NP-40. This bead-bound protein-protein complex was either used directly or eluted by competitive elution using epitope-tag-specific peptide.

Baculovirus expression-based interaction analysis. For baculovirus expression-based interaction analysis, Sf9 cells were coinfected with the combination of viruses as indicated. Cells were harvested with phosphate-buffered saline (PBS) after 48 h of infection. Subsequently, cell lysate was prepared using BC300 buffer containing 0.1% Nonidet P-40, 0.7 μL/mL β-mercaptoethanol, and 1× protease inhibitor cocktail for 2 h at 4°C. The prepared lysate was subjected to immunoprecipitation overnight at 4°C using antibody-coated agarose beads. Protein-bound beads were washed three times (10 min each at 4°C) with washing buffer containing BC300 plus 0.1% NP-40. The bound proteins were eluted by incubating at 95°C for 10 min in 1× SDS loading dye. To identify the interactors, Western blot analyses were performed with specific antibodies.

***In vitro* protein-protein interaction assay.** For direct *in vitro* protein-protein interaction assay, we used purified recombinant target factors, as mentioned, either bound to agarose beads or added exogenously. Purified proteins were incubated in a binding buffer containing 20 mM Tris (pH 8.0), 20% glycerol, 2 mM EDTA, 150 mM KCl, 0.1% Nonidet P-40, and 20 to 80 ng/μL bovine serum albumin (BSA) and incubated for overnight at 4°C. Subsequently, protein-bound beads were washed three times (10 min each at 4°C) with

TABLE 1 List of plasmids used in this study

Plasmid name	Description
M219	HA-tagged NELF-A gene cloned into FLAG-pFASTBAC vector
M220	Myc-tagged NELF-B gene cloned into FLAG-pFASTBAC vector
M221	VSVG-tagged NELF-C gene cloned into FLAG-pFASTBAC vector
M222	FLAG-tagged NELF-E gene cloned into FLAG-pFASTBAC vector
M250	CDK9 cloned into FLAG-HA pCDNA5-FRT-TO vector
M384	FKBP5 gene cloned in pCMVSPORT6 vector
M250	CDK9 cloned into FLAG-HA pCDNA5-FRT-TO vector
M452	FKBP5 cloned into FLAG-HA pCDNA5-FRT-TO vector
M493	FKBP5 cloned into 6-His pET-11d vector
M494	FKBP5 cloned into pFASTBAC vector plasmid containing FLAG tag
M495	FKBP5 cloned into pFASTBAC vector plasmid containing Non tag
M502	RNA Pol II CTD cloned into pGEX vector
M503	FKBP5 cloned into FLAG-tagged pcDNA5-FRT-TO vector
M504	FKBP5 cloned into HA-tagged pcDNA5-FRT-TO vector
M619	CDK9 cloned into FLAG-tagged pcDNA5-FRT-TO vector
M620	CDK9 cloned into HA-tagged pcDNA5-FRT-TO vector
M661	FKBP5 (1–384) fragment cloned into FLAG pCDNA5-FRT-TO vector
M662	FKBP5 (1–351) fragment cloned into FLAG pCDNA5-FRT-TO vector
M663	FKBP5 (1–317) fragment cloned into FLAG pCDNA5-FRT-TO vector
M664	FKBP5 (1–268) fragment cloned into FLAG pCDNA5-FRT-TO vector
M665	FKBP5 (1–157) fragment cloned into FLAG pCDNA5-FRT-TO vector
M666	FKBP5 (130–452) fragment cloned into FLAG pCDNA5-FRT-TO vector
M667	FKBP5 (243–452) fragment cloned into FLAG pCDNA5-FRT-TO vector
M411	CDK9 cloned into FLAG-pFASTBAC vector
M251	CDK9 cloned into Non tag pFASTBAC vector
M296	psPAX2 lentivirus packaging plasmid from Addgene
M297	pMD2.G lentivirus envelope plasmid from Addgene
M298	Lentiviral pLKO.1 vector containing scramble sequence
M562	Cyclin T1 cloned into pGEX vector
M669	FKBP5 gene cloned into Gal-DBD pM vector
M781	FKBP5 (1–384) fragment cloned into FLAG-pFASTBAC vector
M782	FKBP5 (1–351) fragment cloned into FLAG-pFASTBAC vector
M783	FKBP5 (1–268) fragment cloned into FLAG-pFASTBAC vector
M784	FKBP5 (1–157) fragment cloned into FLAG-pFASTBAC vector
M785	FKBP5 (130–452) fragment cloned into FLAG-pFASTBAC vector
M786	FKBP5 (243–452) fragment cloned into FLAG-pFASTBAC vector
M808	Human CDK7 gene cloned into FLAG-pFASTBAC vector
M809	Human CDK8 gene cloned into FLAG-pFASTBAC vector
M916	CDK9 (1–276) fragment cloned into FLAG-pFASTBAC vector
M917	CDK9 (32–276) fragment cloned into FLAG-pFASTBAC vector
M918	CDK9 (67–276) fragment cloned into FLAG-pFASTBAC vector
M919	CDK9 (100–276) fragment cloned into FLAG-pFASTBAC vector
M920	CDK9 (150–276) fragment cloned into FLAG-pFASTBAC vector
M928	FKBP5 (1–384) fragment cloned into 6-His pET-11d vector
M929	FKBP5 (1–351) fragment cloned into 6-His pET-11d vector
M930	FKBP5 (1–317) fragment cloned into 6-His pET-11d vector
M931	FKBP5 (1–268) fragment cloned into 6-His pET-11d vector
M932	FKBP5 (1–157) fragment cloned into 6-His pET-11d vector
M933	FKBP5 (130–452) fragment cloned into 6-His pET-11d vector
M934	FKBP5 (243–452) fragment cloned into 6-His pET-11d vector
M935	CDK9 (1–276) fragment cloned into FLAG-tagged pcDNA5-FRT-TO vector
M936	CDK9 (32–276) fragment cloned into FLAG-tagged pcDNA5-FRT-TO vector
M937	CDK9 (67–276) fragment cloned into FLAG-tagged pcDNA5-FRT-TO vector
M938	CDK9 (100–276) fragment cloned into FLAG-tagged pcDNA5-FRT-TO vector
M939	CDK9 (150–276) fragment cloned into FLAG-tagged pcDNA5-FRT-TO vector
M341	pRL-TK-internal *Renilla* luciferase control plasmid
M1008	pLTR-Luc (with HIV LTR promoter) in pGL3 basic vector
S110	shRNA construct cloned into pLKO.1 for human FKBP5 no. 1
S111	shRNA construct cloned into pLKO.1 for human FKBP5 no. 2
S142	shRNA construct cloned into pLKO.1 for human CDK9 no. 1

TABLE 2 List of primers used for RNA analysis

Gene name	5′ forward primer	3′ reverse primer
FKBP5	TCCTTGCTGCCTTTCTG	CTTTCTCAAAGTCACCCTTG
CDK9	GCATCATGGCAGAGATGTG	GTTGTCCACGTTTGGCC
CCND1	CAAACACGCGCAGACCTTC	GATCACTCTGGAGAGGAAGCG
C-MYC	GCTTGTACCTGCAGGATC	GACTCCGTCGAGGAGAG
CDKN1A	GGACAGCAGAGGAAGACCATG	CTGTCATGCTGGTCTGCC
CDKN1B	CGACGATTCTTCTACTCAA	TTACGTTTGACGTCTTCTG
CDKN1C	GCTGCACTCGGGGATTTC	GGACATCGCCCGACGACT
CDC25B	GGCACATCAAGACTGCGG	GGTAGTCGTTGACAGCACG
ATF2	GTACCAGGCCCATTTCCTCTTC	GAACGAGTGGGACTGCAGCTG
E2F2	GGAGCCGGACAGTCCTTC	GCTGTCAGTAGCCTCCAAG
MAP2	AATAGACCTAAGCCATGTGACATCC	AGAACCAACTTTAGCTTGGGCC
TAF4	GACGACAGATATGAGCAGG	GTTGCTGCATCTCCTTTG
PTPRS	AACACAGAAGTGCCCGCAC	GTGACGTGTGGGCCTTGGAG
CDK6	GGAGTGTTGGCTGCATATTTG	CGATATCTGTTACAAACTTC
TM2D2	GGACACTACTTCATAACCAC	AATAAGGTCAACAAACCACC
MDM2	TTGGATCAGGATTCAGTTTC	GAGAGTTCTTGTCCTTCTTC
TEF	GCTCTTCCACAGCATCC	GCACACTGGAGAGCAC
LDLR	CTGGAAATTGCGCTGGAC	GTCTTGGCACTGGAACTCGT
GAPDH	CATCACCATCTTCCAGGAG	GTTCACACCCATGACGAAC
18S rRNA	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
RGS2	AAGATTGGAAGACCCGTTTGAG	GCAAGACCATATTTGCTGGCT
STIP1	GAGAAAATCCTGAAGGAGCAAG	ATGCTTCATGGCCTGGGGATA
GLUT1	GAAGTGTCACCCACAGCCCTTC	GGGCCACAGGTCCTTGTTGC
CRABP2	CCCTACACCAACAAAGAGG	CCCTCAAGTCCCCTTTAG

binding buffer. The bound proteins were eluted by incubating at 95°C for 10 min in 1× SDS loading dye. To identify the interactors, Western blot analyses were performed with specific antibodies as indicated.

***In vitro* kinase assay.** *In vitro* kinase assays were performed to test the effect of FKBP5 on P-TEFb-mediated phosphorylation of its substrates Pol II CTD, DSIF complex, and NELF complex. Phosphorylation reactions were carried out in 1× kinase assay buffer (50 mM Tris-Cl [pH 8.0], 2 mM $MgCl_2$, and 500 μM ATP) at 30°C for 2 h. After completion of phosphorylation reactions, protein phosphorylation was assessed by Western blot analysis using specific antibodies (for GST-CTD phosphorylation) or by autoradiography (for NELF and DSIF complexes). For the purpose of phosphorylation assay of DSIF and NELF complexes, along with the 500 μM ATP, 0.2 μM γ^{32}P-ATP was used additionally.

***In vitro* competitive binding assay.** For the *in vitro* competitive binding assay, we used purified bead-immobilized FLAG-CDK9 along with exogenously added GST-cyclin T1 and His-FKBP5. We used a constant amount (0.5 μg) of bait protein (immobilized FLAG-CDK9) along with a constant amount of one of the proteins (GST-cyclin T1/His-FKBP5), with the other competitor (His-FKBP5/GST-cyclin T1) being added in a gradient fashion. The binding reaction was carried out overnight at 4°C in a binding buffer containing 20 mM Tris (pH 8.0), 20% glycerol, 2 mM EDTA, 100 mM KCl, 0.1% Nonidet P-40, and 80 ng/μL BSA. After overnight incubation, the beads were washed in binding buffer rigorously. Bead-bound proteins were eluted by incubating at 95°C for 10 min in 1 × SDS loading dye. To identify the interactors, Western blots were performed with specific antibodies, as mentioned.

***In vitro* dissociation assay.** For the *in vitro* dissociation assay, we used purified bead-immobilized preformed P-TEFb complex composed of FLAG-CDK9 plus GST-cyclin T1. Both of these proteins were individually purified and assembled into the P-TEFb complex by adding them together in assembly buffer (20 mM Tris [pH 8.0], 20% glycerol, 2 mM EDTA, 300 mM KCl, and 0.1% Nonidet P-40) and incubating overnight at 4°C. Bead-bound FLAG-CDK9 was incubated with an excess amount of purified GST-cyclin T1 for fully saturating FLAG-CDK9 binding with GST-cyclin T1. The excess unbound GST-cyclin T1 was removed by extensive washing. This immobilized P-TEFb complex was then used in the reaction setup, along with increasing concentrations of purified FKBP5 protein, as mentioned, in reaction buffer (20 mM Tris [pH 8.0], 20% glycerol, 2 mM EDTA, 100 mM KCl, 0.1% Nonidet P-40, and 80 ng/μL BSA) and then incubated overnight at 4°C. The reaction mixtures were subjected to brief centrifugation, and the supernatant fraction was collected. The bead-bound

TABLE 3 List of primers used in ChIP analyses

Gene name	5′ forward primer	3′ reverse primer
CCND1 (TSS)	CGGGCTTTGATCTTTGCTTA	CTGCTGCTCGCTGCTACT
C-MYC (TSS)	TCCTCTCTCGCTAATCTCCGC	GGGTCCTCAGCCGTCCAGAC
RGS2 (TSS)	CGAGGCCTCATAAATGCTG	CTCTTGTCCATGGGTCTG
CCND1 (~2 kb downstream)	CTGTTTTGATCTGGGATTGCGTGT	CACCTCCGGCTCAGAGCACTG
C-MYC (~3 kb downstream)	GACTCTGGTAAGCGAAGC	TCCAGATCTGCTATCTCTCC

TABLE 4 Oligonucleotide sequences for generation of shRNA constructs

	Sequence	
Gene	**Upper**	**Lower**
FKBP5 sh#1	CCGGACCTAATGCTGAGCTTATATACTCGAGTATATAAGCT CAGCATTAGGTTTTTTG	AATTCAAAAAACCTAATGCTGAGCTTATATACTCGAGTATAT AAGCTCAGCATTAGGT
FKBP5 sh#2	CCGGCGAAGGAGCAACAGTAGAAATCTCGAGATTTCT ACTGTTGCTCCTTCGTTTTTG	AATTCAAAAACGAAGGAGCAACAGTAGAAATCTCGAGATTT CTACTGTTGCTCCTTCG
CDK9 sh#1	CCGGCCGCTGCAAGGGTAGTATATACTCGAGTATATAC TACCCTTGCAGCGGTTTTTG	AATTCAAAAACCGCTGCAAGGGTAGTATATACTCGAGTATA TACTACCCTTGCAGCGG

proteins were washed twice with reaction buffer before eluting in 1× SDS loading dye by incubating them at 95°C for 10 min and were treated as bead-bound fraction. Both the supernatant and bead-bound fractions were loaded in parallel for comparing the amount of target proteins present in both fractions by Western blot analyses with specific antibodies, as mentioned. For immunoprecipitated P-TEFb complex, similar experimental approach was used with bead-bound P-TEFb complex immunoprecipitated from whole-cell extract from mammalian cells along with purified FKBP5 protein.

Dual luciferase assay. To perform this assay, 50,000 cells of both scramble and stable FKBP5-KD cell lines were seeded into a 24-well dish. The next day, we transfected HIV1-LTR-luciferase (firefly) construct in three different concentrations along with equal amount of *Renilla* luciferase construct (as an internal control for transfection) in both of the cell lines. After 40 h of transfection, cells were harvested and proceeded for luciferase assay using the dual-luciferase assay kit from Promega and following the manufacturer's protocol. The firefly luciferase data obtained were normalized with the internal control, *Renilla* luciferase.

qRT-PCR analysis for RNA expression. To perform qRT-PCR analysis for mRNA expression of target genes, total mRNA extraction was done using TRIzol reagent (Invitrogen, Inc.) following the manufacturer's protocol. Subsequently, 1 μg of extracted mRNA was used to synthesize cDNA using the Verso cDNA synthesis kit (Thermo Scientific) by following the manufacturer's protocol. Synthesized cDNA was diluted 50× before being used for qRT-PCR analysis. qRT-PCR analysis was performed using iTaq Universal SYBR green supermix (Bio-Rad) and target gene-specific primers. qRT-PCR data were analyzed using CFX96 real-time PCR system software using GAPDH as an internal control for normalization.

Chromatin immunoprecipitation analysis. ChIP analysis was performed essentially following a previously described protocol (37). Briefly, cells were cross-linked with 1% formaldehyde (Sigma) for 10 min at room temperature, followed by incubation with 125 mM glycine for 5 min to stop the cross-linking. The cross-linked cells were washed three times with ice-cold PBS. Nuclear extract was prepared using these cells for downstream immunoprecipitation analysis by resuspending the cells in ChIP lysis buffer (0.5% NP-40, 1% Triton X-100, 300 mM NaCl, 20 mM Tris [pH 7.5], 2 mM EDTA, and protease inhibitor cocktail) and incubating on ice for 30 min. After syringe passage 8 times through a 23-gauge needle, the lysate was spun down at 5,000 rpm for 10 min at 4°C. The obtained nuclear pellet was resuspended with

TABLE 5 List of antibodies used in this study

Antibody	Source(s)
FKBP5	Bethyl Laboratories, Cell Signaling Technology
CDK9	Santa Cruz Biotechnology
ELL	Bethyl Laboratories, Cell Signaling Technology
CCNT1	Santa Cruz Biotechnology
AFF1	Abcam
NELF-A	Bethyl Laboratories
NELF-E	Bethyl Laboratories
AF9	Bethyl Laboratories
β-Actin	Santa Cruz Biotechnology
Phospho-Rpb1 CTD (Ser2)	Cell Signaling Technology
Phospho-Rpb1 CTD (Ser5)	Cell Signaling Technology
Rpb1	Cell Signaling Technology
FLAG epitope	Sigma
HA epitope	Santa Cruz Biotechnology
His epitope	Santa Cruz Biotechnology
GST epitope	Santa Cruz Biotechnology
Normal rabbit IgG	Cell Signaling Technology
Normal mouse IgG	Cell Signaling Technology
HRP-conjugated secondary antibody (rabbit)	Bio-Rad
HRP-conjugated secondary antibody (mouse)	Cell Signaling Technology
HSP90β	Bio-Rad
Histone H3	Cell Signaling Technology

ChIP sonication buffer (1% SDS, 50 mM Tris [pH 8.0], 10 mM EDTA, and 1× protease inhibitor cocktail). The resuspended nuclear pellet was subsequently sonicated at high efficiency using a Bioruptor UCD-200 instrument for 20 min. The sonicated samples were spun down at 15,000 rpm for 20 min. The obtained supernatant was precleared using 20 μL protein A-agarose beads in 65 μg of sonicated DNA sample for 30 min at 4°C. The precleared lysate was diluted 10 times using ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.1 mM EDTA, 20 mM Tris-Cl [pH 8.0], and 167 mM NaCl). Immunoprecipitation was carried out overnight at 4°C with 2 μg of target antibody for each immunoprecipitation sample, while IgG was used as a negative control. In a parallel reaction, protein G magnetic beads were blocked using 50 μg of salmon sperm DNA for overnight. The next day, 25 μL of preblocked protein G magnetic beads were further added with the immunoprecipitated sample and incubated for 1 h at 4°C. After a short spin at 3,000 rpm, magnetic beads bound to antibody-protein-DNA complex were successively washed twice with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl [pH 8.0], 150 mM NaCl, and protease inhibitor cocktail), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl [pH 8.0], 500 mM NaCl, and protease inhibitor cocktail), and lithium chloride buffer (0.5 M LiCl, 1% NP-40, 1% deoxycholate, 20 mM Tris-Cl [pH 8.0], and 1 mM EDTA). The immunoprecipitated protein-DNA complexes were eluted by incubating for 30 min with elution buffer (1% SDS and 0.1 M $NaHCO_3$) at room temperature. The eluted DNA-protein complex was incubated with 190 mM NaCl at 65°C for de-cross-linking. The remaining proteins were digested by using proteinase K for 45 min at 45°C. The eluted DNA was further purified using the Qiagen PCR purification kit following the manufacturer's protocol. The purified eluted DNA was subsequently used in qRT-PCR analysis using primers specific to the target gene locus.

Cell proliferation assay. To perform the cell proliferation assay, 6×10^4 FKBP5 knockdown and control cells were seeded in a 6-well plate. Cell numbers were counted using a hemocytometer on the 3rd, 4th, and 5th days after seeding of cells. Scramble knockdown cells were used as control.

RNA-Seq analysis. For the purpose of analysis of differential expression of genes between control (scramble) and FKBP5 knockdown cells, raw data were generated following the same protocol mentioned in our earlier study (37). The data obtained in the previously reported control sample (37) were used for reanalyzing the effect of FKBP5 knockdown, reported here, since all these experiments were performed at the same time. For differential gene expression analysis, the raw FASTQ sequences of all 4 samples (as obtained) were mapped to the *Homo sapiens* GRCh38 genome using STAR (v2.27.2b) to create BAM files (51, 52). The BAM files were then processed using the Rsamtools, Rsubread, and GenomicAlignments R packages, and abundance tables were created from the alignments (53, 54). The abundance tables were further normalized and differentially abundant genes were identified using the DESeq2 R package (55). Genes with a $\log_2$ fold change ($\log_2$FC) of 1.5-fold or more, having a P value of <0.05 and an adjusted P value of <0.05 were considered significantly differentiated gene expression. The heatmap of the selected genes was generated using counts-per-million normalization from raw counts by implementing the edgeR package, and subsequent visualization was done with "pheatmap" R function (56).

Gene ontology analysis. Gene ontology (GO) analysis for the identification and classification of biological processes was performed as described previously (37). Functional classification of differentially regulated genes identified from the RNA-Seq data was done utilizing the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (57). GO terms with P values of >0.05 as determined by the EASE score, a modified Fisher's exact P value test integrated into the DAVID workflow, were considered significant.

RNA expression analysis using TCGA data sets. Gene expression data were obtained from The Cancer Genome Atlas (TCGA) database (data version 2016_01_28) stored as part of the Broad Institute of MIT and Harvard TCGA Government Data Analytics Center (GDAC) (www.gdac.broadinstitute.org). mRNA levels of indicated genes in 11 cancers were obtained from a TCGA database of 38 different cancer cohorts containing more than 14,000 sequencing samples. For easy data retrieval and analysis, the Firehose analysis pipeline developed by the Broad Institute itself was utilized through its Java-dependent web application FireBrowse (http://firebrowse.org/) (58). Cohort-specific mRNA abundance was represented as $\log_2$ RNA-Seq by expectation maximization (RSEM) for greater accuracy in quantification (59). Statistical analysis was performed using a one-tailed Student's t test, defining a P value of <0.05 as significant.

Data availability. The full set of mass spectrometry data for identifying FLAG-HA-CDK9-associated proteins purified from mammalian 293T cells can be obtained from Mendeley Data at https://data.mendeley.com/datasets/sxndp9wpbx/1. Original raw images of Western blots used for making the figures shown in this study can also be accessed from Mendeley Data repository at https://data.mendeley.com/datasets/3c9vdxrf3n/1.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

AACKNOWLEDGMENTS

This work was supported by Department of Science and Technology (DST) extramural fund EMR/2016/001593, awarded to D.B. D.P.M. is a recipient of a Department of Biotechnology Senior Research Fellowship (SRF). S.B. and N.K. are recipients of University Grants Commission (UGC) SRFs. K.G. and A.L. are recipients of Council of Scientific and Industrial Research (CSIR) SRFs. S.P. was supported by a Ramanujan Fellowship from DST.

We thank Debashis Mitra, NCCS, Pune, for assistance with the HIV LTR-luciferase. We also deeply acknowledge assistance from other members of the Biswas lab for their critical comments on the manuscript.

D.P.M. performed the majority of the experiments in consultation with D.B. S.B. performed RNA expression analysis in normal and tumor samples available in TCGA data sets, as well GO analysis of upregulated and downregulated genes in FKBP5 knockdown cells. K.G. performed ChIP experiment for factor binding on target genes. N.K. performed a few important experiments. A.L. and S.P. performed data analysis of raw data as obtained for RNA-Seq analysis. D.P.M. and D.B. wrote the manuscript.

We declare no conflicts of interest.

REFERENCES

1. Sinars CR, Cheung-Flynn J, Rimerman RA, Scammell JG, Smith DF, Clardy J. 2003. Structure of the large FK506-binding protein FKBP51, an Hsp90-binding protein and a component of steroid receptor complexes. Proc Natl Acad Sci U S A 100:868–873. https://doi.org/10.1073/pnas.0231020100.
2. Gaali S, Kirschner A, Cuboni S, Hartmann J, Kozany C, Balsevich G, Namendorf C, Fernandez-Vizarra P, Sippel C, Zannas AS, Draenert R, Binder EB, Almeida OF, Ruhter G, Uhr M, Schmidt MV, Touma C, Bracher A, Hausch F. 2015. Selective inhibitors of the FK506-binding protein 51 by induced fit. Nat Chem Biol 11:33–37. https://doi.org/10.1038/nchembio.1699.
3. Fries GR, Gassen NC, Rein T. 2017. The FKBP51 glucocorticoid receptor co-chaperone: regulation, function, and implications in health and disease. Int J Mol Sci 18:2614. https://doi.org/10.3390/ijms18122614.
4. Duman RS, Girgenti MJ. 2019. Molecular and cellular studies of PTSD: postmortem transcriptome analysis and novel therapeutic targets. J Neurosci Res 97:292–299. https://doi.org/10.1002/jnr.24306.
5. Jaworska-Andryszewska P, Rybakowski JK. 2019. Childhood trauma in mood disorders: neurobiological mechanisms and implications for treatment. Pharmacol Rep 71:112–120. https://doi.org/10.1016/j.pharep.2018.10.004.
6. Talarowska M. 2020. Epigenetic mechanisms in the neurodevelopmental theory of depression. Depress Res Treat 2020:6357873. https://doi.org/10.1155/2020/6357873.
7. Hausl AS, Balsevich G, Gassen NC, Schmidt MV. 2019. Focus on FKBP51: a molecular link between stress and metabolic disorders. Mol Metab 29:170–181. https://doi.org/10.1016/j.molmet.2019.09.003.
8. Pei H, Li L, Fridley BL, Jenkins GD, Kalari KR, Lingle W, Petersen G, Lou Z, Wang L. 2009. FKBP51 affects cancer cell response to chemotherapy by negatively regulating Akt. Cancer Cell 16:259–266. https://doi.org/10.1016/j.ccr.2009.07.016.
9. Yu J, Qin B, Wu F, Qin S, Nowsheen S, Shan S, Zayas J, Pei H, Lou Z, Wang L. 2017. Regulation of serine-threonine kinase Akt activation by NAD^+-dependent deacetylase SIRT7. Cell Rep 18:1229–1240. https://doi.org/10.1016/j.celrep.2017.01.009.
10. Daneri-Becerra C, Zgajnar NR, Lotufo CM, Ramos Hryb AB, Piwien-Pilipuk G, Galigniana MD. 2019. Regulation of FKBP51 and FKBP52 functions by post-translational modifications. Biochem Soc Trans 47:1815–1831. https://doi.org/10.1042/BST20190334.
11. Zgajnar NR, De Leo SA, Lotufo CM, Erlejman AG, Piwien-Pilipuk G, Galigniana MD. 2019. Biological actions of the Hsp90-binding immunophilins FKBP51 and FKBP52. Biomolecules 9:52. https://doi.org/10.3390/biom9020052.
12. Denny WB, Valentine DL, Reynolds PD, Smith DF, Scammell JG. 2000. Squirrel monkey immunophilin FKBP51 is a potent inhibitor of glucocorticoid receptor binding. Endocrinology 141:4107–4113. https://doi.org/10.1210/endo.141.11.7785.
13. Scammell JG, Denny WB, Valentine DL, Smith DF. 2001. Overexpression of the FK506-binding immunophilin FKBP51 is the common cause of glucocorticoid resistance in three New World primates. Gen Comp Endocrinol 124:152–165. https://doi.org/10.1006/gcen.2001.7696.
14. Wochnik GM, Ruegg J, Abel GA, Schmidt U, Holsboer F, Rein T. 2005. FK506-binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. J Biol Chem 280:4609–4616. https://doi.org/10.1074/jbc.M407498200.
15. Balsevich G, Hausl AS, Meyer CW, Karamihalev S, Feng X, Pohlmann ML, Dournes C, Uribe-Marino A, Santarelli S, Labermaier C, Hafner K, Mao T, Breitsamer M, Theodoropoulou M, Namendorf C, Uhr M, Paez-Pereda M, Winter G, Hausch F, Chen A, Tschop MH, Rein T, Gassen NC, Schmidt MV. 2017. Stress-responsive FKBP51 regulates AKT2-AS160 signaling and metabolic function. Nat Commun 8:1725. https://doi.org/10.1038/s41467-017-01783-y.
16. DeFronzo RA, Tripathy D. 2009. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care 32:S157–S163. https://doi.org/10.2337/dc09-S302.
17. Leto D, Saltiel AR. 2012. Regulation of glucose transport by insulin: traffic control of GLUT4. Nat Rev Mol Cell Biol 13:383–396. https://doi.org/10.1038/nrm3351.
18. Stechschulte LA, Qiu B, Warrier M, Hinds TD, Jr, Zhang M, Gu H, Xu Y, Khuder SS, Russo L, Najjar SM, Lecka-Czernik B, Yong W, Sanchez ER. 2016. FKBP51 null mice are resistant to diet-induced obesity and the PPARγ agonist rosiglitazone. Endocrinology 157:3888–3900. https://doi.org/10.1210/en.2015-1996.
19. Periyasamy S, Warrier M, Tillekeratne MP, Shou W, Sanchez ER. 2007. The immunophilin ligands cyclosporin A and FK506 suppress prostate cancer cell growth by androgen receptor-dependent and -independent mechanisms. Endocrinology 148:4716–4726. https://doi.org/10.1210/en.2007-0145.
20. Bres V, Yoh SM, Jones KA. 2008. The multi-tasking P-TEFb complex. Curr Opin Cell Biol 20:334–340. https://doi.org/10.1016/j.ceb.2008.04.008.
21. Basu S, Nandy A, Biswas D. 2020. Keeping RNA polymerase II on the run: functions of MLL fusion partners in transcriptional regulation. Biochim Biophys Acta Gene Regul Mech 1863:194563. https://doi.org/10.1016/j.bbagrm.2020.194563.
22. Ghosh K, Tang M, Kumari N, Nandy A, Basu S, Mall DP, Rai K, Biswas D. 2018. Positive regulation of transcription by human ZMYND8 through its association with P-TEFb complex. Cell Rep 24:2141–2154.e6. https://doi.org/10.1016/j.celrep.2018.07.064.
23. Peng J, Marshall NF, Price DH. 1998. Identification of a cyclin subunit required for the function of *Drosophila* P-TEFb. J Biol Chem 273:13855–13860. https://doi.org/10.1074/jbc.273.22.13855.
24. Price DH. 2000. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. Mol Cell Biol 20:2629–2634. https://doi.org/10.1128/MCB.20.8.2629-2634.2000.
25. Yang Z, Zhu Q, Luo K, Zhou Q. 2001. The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. Nature 414:317–322. https://doi.org/10.1038/35104575.
26. Yik JH, Chen R, Nishimura R, Jennings JL, Link AJ, Zhou Q. 2003. Inhibition of P-TEFb (CDK9/cyclin T) kinase and RNA polymerase II transcription by the coordinated actions of HEXIM1 and 7SK snRNA. Mol Cell 12:971–982. https://doi.org/10.1016/S1097-2765(03)00388-5.
27. Sessler RJ, Noy N. 2005. A ligand-activated nuclear localization signal in cellular retinoic acid binding protein-II. Mol Cell 18:343–353. https://doi.org/10.1016/j.molcel.2005.03.026.
28. Yang Z, Yik JH, Chen R, He N, Jang MK, Ozato K, Zhou Q. 2005. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. Mol Cell 19:535–545. https://doi.org/10.1016/j.molcel.2005.06.029.
29. Lu X, Zhu X, Li Y, Liu M, Yu B, Wang Y, Rao M, Yang H, Zhou K, Wang Y, Chen Y, Chen M, Zhuang S, Chen LF, Liu R, Chen R. 2016. Multiple P-TEFbs cooperatively regulate the release of promoter-proximally paused RNA polymerase II. Nucleic Acids Res 44:6853–6867. https://doi.org/10.1093/nar/gkw571.
30. He N, Liu M, Hsu J, Xue Y, Chou S, Burlingame A, Krogan NJ, Alber T, Zhou Q. 2010. HIV-1 Tat and host AFF4 recruit two transcription elongation factors into a bifunctional complex for coordinated activation of HIV-1 transcription. Mol Cell 38:428–438. https://doi.org/10.1016/j.molcel.2010.04.013.

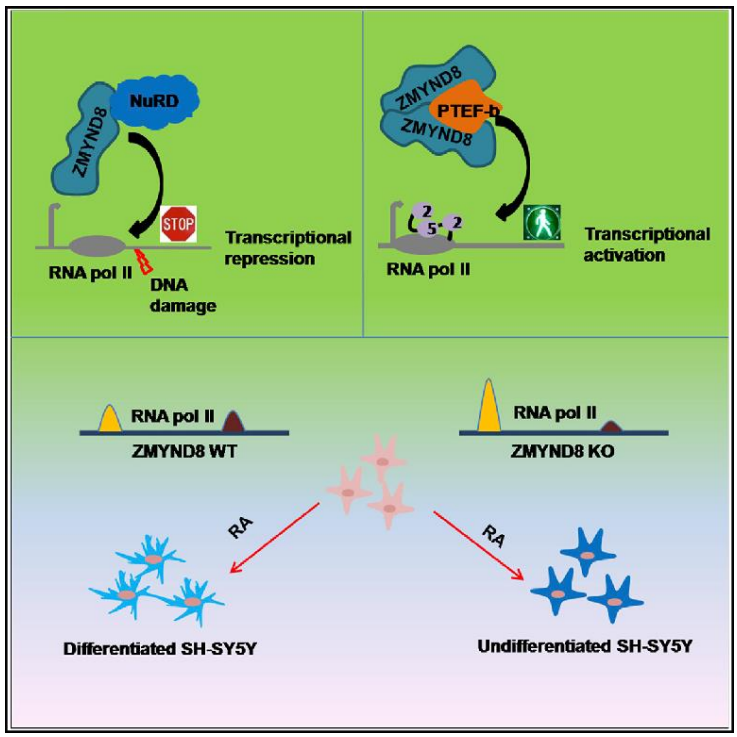
31. Biswas D, Milne TA, Basrur V, Kim J, Elenitoba-Johnson KS, Allis CD, Roeder RG. 2011. Function of leukemogenic mixed lineage leukemia 1 (MLL) fusion proteins through distinct partner protein complexes. Proc Natl Acad Sci U S A 108:15751–15756. https://doi.org/10.1073/pnas.1111498108.
32. Chiba K, Yamamoto J, Yamaguchi Y, Handa H. 2010. Promoter-proximal pausing and its release: molecular mechanisms and physiological functions. Exp Cell Res 316:2723–2730. https://doi.org/10.1016/j.yexcr.2010.05.036.
33. Wu CH, Yamaguchi Y, Benjamin LR, Horvat-Gordon M, Washinsky J, Enerly E, Larsson J, Lambertsson A, Handa H, Gilmour D. 2003. NELF and DSIF cause promoter proximal pausing on the *hsp70* promoter in *Drosophila*. Genes Dev 17:1402–1414. https://doi.org/10.1101/gad.1091403.
34. Yamaguchi Y, Takagi T, Wada T, Yano K, Furuya A, Sugimoto S, Hasegawa J, Handa H. 1999. NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation. Cell 97:41–51. https://doi.org/10.1016/s0092-8674(00)80713-8.
35. Zaborowska J, Isa NF, Murphy S. 2016. P-TEFb goes viral. Bioessays 38 Suppl 1:S75–85. https://doi.org/10.1002/bies.201670912.
36. Kumari N, Hassan MA, Lu X, Roeder RG, Biswas D. 2019. AFF1 acetylation by p300 temporally inhibits transcription during genotoxic stress response. Proc Natl Acad Sci U S A 116:22140–22151. https://doi.org/10.1073/pnas.1907097116.
37. Yadav D, Ghosh K, Basu S, Roeder RG, Biswas D. 2019. Multivalent role of human TFIID in recruiting elongation components at the promoter-proximal region for transcriptional control. Cell Rep 26:1303–1317.e7. https://doi.org/10.1016/j.celrep.2019.01.012.
38. Riggs DL, Cox MB, Tardif HL, Hessling M, Buchner J, Smith DF. 2007. Noncatalytic role of the FKBP52 peptidyl-prolyl isomerase domain in the regulation of steroid hormone signaling. Mol Cell Biol 27:8658–8669. https://doi.org/10.1128/MCB.00985-07.
39. Schulke JP, Wochnik GM, Lang-Rollin I, Gassen NC, Knapp RT, Berning B, Yassouridis A, Rein T. 2010. Differential impact of tetratricopeptide repeat proteins on the steroid hormone receptors. PLoS One 5:e11717. https://doi.org/10.1371/journal.pone.0011717.
40. Narita T, Yamaguchi Y, Yano K, Sugimoto S, Chanarat S, Wada T, Kim DK, Hasegawa J, Omori M, Inukai N, Endoh M, Yamada T, Handa H. 2003. Human transcription elongation factor NELF: identification of novel subunits and reconstitution of the functionally active complex. Mol Cell Biol 23:1863–1873. https://doi.org/10.1128/MCB.23.6.1863-1873.2003.
41. Somesh BP, Reid J, Liu WF, Sogaard TM, Erdjument-Bromage H, Tempst P, Svejstrup JQ. 2005. Multiple mechanisms confining RNA polymerase II ubiquitylation to polymerases undergoing transcriptional arrest. Cell 121:913–923. https://doi.org/10.1016/j.cell.2005.04.010.
42. Bacon CW, D'Orso I. 2019. CDK9: a signaling hub for transcriptional control. Transcription 10:57–75. https://doi.org/10.1080/21541264.2018.1523668.
43. Citri A, Harari D, Shohat G, Ramakrishnan P, Gan J, Lavi S, Eisenstein M, Kimchi A, Wallach D, Pietrokovski S, Yarden Y. 2006. Hsp90 recognizes a common surface on client kinases. J Biol Chem 281:14361–14369. https://doi.org/10.1074/jbc.M512613200.
44. Baumli S, Lolli G, Lowe ED, Troiani S, Rusconi L, Bullock AN, Debreczeni JE, Knapp S, Johnson LN. 2008. The structure of P-TEFb (CDK9/cyclin T1), its complex with flavopiridol and regulation by phosphorylation. EMBO JEmbo J 27:1907–1918. https://doi.org/10.1038/emboj.2008.121.
45. Basu S, Barad M, Yadav D, Nandy A, Mukherjee B, Sarkar J, Chakrabarti P, Mukhopadhyay S, Biswas D. 2020. DBC1, p300, HDAC3, and Siah1 coordinately regulate ELL stability and function for expression of its target genes. Proc Natl Acad Sci U S A 117:6509–6520. https://doi.org/10.1073/pnas.1912375117.
46. Sawarkar R, Sievers C, Paro R. 2012. Hsp90 globally targets paused RNA polymerase to regulate gene expression in response to environmental stimuli. Cell 149:807–818. https://doi.org/10.1016/j.cell.2012.02.061.
47. Hartmann J, Wagner KV, Liebl C, Scharf SH, Wang XD, Wolf M, Hausch F, Rein T, Schmidt U, Touma C, Cheung-Flynn J, Cox MB, Smith DF, Holsboer F, Muller MB, Schmidt MV. 2012. The involvement of FK506-binding protein 51 (FKBP5) in the behavioral and neuroendocrine effects of chronic social defeat stress. Neuropharmacology 62:332–339. https://doi.org/10.1016/j.neuropharm.2011.07.041.
48. Sanchez ER. 2012. Chaperoning steroidal physiology: lessons from mouse genetic models of Hsp90 and its cochaperones. Biochim Biophys Acta 1823:722–729. https://doi.org/10.1016/j.bbamcr.2011.11.006.
49. Pereira MJ, Palming J, Svensson MK, Rizell M, Dalenback J, Hammar M, Fall T, Sidibeh CO, Svensson PA, Eriksson JW. 2014. FKBP5 expression in human adipose tissue increases following dexamethasone exposure and is associated with insulin resistance. Metabolism 63:1198–1208. https://doi.org/10.1016/j.metabol.2014.05.015.
50. Scharf SH, Liebl C, Binder EB, Schmidt MV, Muller MB. 2011. Expression and regulation of the Fkbp5 gene in the adult mouse brain. PLoS One 6:e16883. https://doi.org/10.1371/journal.pone.0016883.
51. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29:15–21. https://doi.org/10.1093/bioinformatics/bts635.
52. Yates AD, Achuthan P, Akanni W, Allen J, Allen J, Alvarez-Jarreta J, Amode MR, Armean IM, Azov AG, Bennett R, Bhai J, Billis K, Boddu S, Marugan JC, Cummins C, Davidson C, Dodiya K, Fatima R, Gall A, Giron CG, Gil L, Grego T, Haggerty L, Haskell E, Hourlier T, Izuogu OG, Janacek SH, Juettemann T, Kay M, Lavidas I, Le T, Lemos D, Martinez JG, Maurel T, McDowall M, McMahon A, Mohanan S, Moore B, Nuhn M, Oheh DN, Parker A, Parton A, Patricio M, Sakthivel MP, Abdul Salam AI, Schmitt BM, Schuilenburg H, Sheppard D, Sycheva M, Szuba M, Taylor K, et al. 2020. Ensembl 2020. Nucleic Acids Res 48:D682–D688. https://doi.org/10.1093/nar/gkz966.
53. Lawrence M, Huber W, Pages H, Aboyoun P, Carlson M, Gentleman R, Morgan MT, Carey VJ. 2013. Software for computing and annotating genomic ranges. PLoS Comput Biol 9:e1003118. https://doi.org/10.1371/journal.pcbi.1003118.
54. Liao Y, Smyth GK, Shi W. 2019. The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. Nucleic Acids Res 47:e47. https://doi.org/10.1093/nar/gkz114.
55. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. https://doi.org/10.1186/s13059-014-0550-8.
56. McCarthy DJ, Chen Y, Smyth GK. 2012. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. Nucleic Acids Res 40:4288–4297. https://doi.org/10.1093/nar/gks042.
57. Huang da W, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57. https://doi.org/10.1038/nprot.2008.211.
58. Deng M, Bragelmann J, Kryukov I, Saraiva-Agostinho N, Perner S. 2017. FirebrowseR: an R client to the Broad Institute's Firehose Pipeline. Database 2017:baw160. https://doi.org/10.1093/database/baw160.
59. Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12:323. https://doi.org/10.1186/1471-2105-12-323.

Article

Cell Reports

Positive Regulation of Transcription by Human ZMYND8 through Its Association with P-TEFb Complex

Graphical Abstract



Highlights

- ZMYND8-mediated P-TEFb complex recruitment activates transcription
- Differentiation of SH-SH5Y cells requires transcriptional activation by ZMYND8
- ZMYND8 exists as monomer as well as dimer
- Preferential association of CHD4 with monomer and P-TEFb with dimer form of ZMYND8

Authors

Koushik Ghosh, Ming Tang, Nidhi Kumari, ..., Dheerendra Pratap Mall, Kunal Rai, Debabrata Biswas

Correspondence

dbiswas@iicb.res.in

In Brief

Ghosh et al. report that coiled-coil domain-dependent dimerization of ZMYND8 positively regulates transcription through P-TEFb complex recruitment, whereas monomeric ZMYND8 associates with NuRD complex for transcriptional repression. ZMYND8-mediated P-TEFb complex recruitment is required for target gene activation during retinoic acid-induced differentiation of SH-SY5Y cells.

Ghosh et al., 2018, Cell Reports *24*, 2141–2154
August 21, 2018
https://doi.org/10.1016/j.celrep.2018.07.064

CellPress

List of conferences attended :

1) *India International Science Festival "Young Scientists' Conference", 2019 - **Poster Presentation***
2) *India/EMBO Symposium "Human microbiome: Resistance and disease", 2019 - **Poster presentation***

Ministry of Science and Technology
Ministry of Earth Sciences
Ministry of Health and Family Welfare
GOVERNMENT OF INDIA

SCIENCE EMPOWERING NATION · RESEARCH INNOVATION

INDIA INTERNATIONAL SCIENCE FESTIVAL 2019

vibha विभा भारती

VIGYAN PRASAR

CERTIFICATE OF PARTICIPATION

THIS IS TO CERTIFY THAT

PROF./DR./MRS./MR./MS./ ***Dheerendra Pratap Mall***

HAS ACTIVELY PARTICIPATED IN THE **YOUNG SCIENTISTS' CONFERENCE**
AS A PART OF "INDIA INTERNATIONAL SCIENCE FESTIVAL – 2019" HELD AT
BISWA BANGLA CONVENTION CENTRE, KOLKATA DURING NOVEMBER 5-8, 2019.
SHE/HE HAS ALSO PRESENTED A PAPER ON THE THEME

Frontier areas of Research

TITLED

The Role of FKBP5 in P-TEFb Mediated Transcriptional Regulation in Eukaryotes.

PROF. ASHUTOSH SHARMA
SECRETARY, DST

DR. VIJAY BHATKAR
PRESIDENT, VIJNANA BHARATI

EMBO Courses & Workshops

CERTIFICATE OF PARTICIPATION

This is to confirm that

DHEERENDRA PRATAP

attended the EMBO event

Human microbiome: Resistance and disease

from 09 – 12 November 2019 in Kalyani, India

Gerlind Wallon, PhD
EMBO Deputy Director

Date and location: 17 November 2019, Heidelberg

European Molecular Biology Organization

Meyerhofstr. 1
69117 Heidelberg
Germany

phone +49 6221 8891 0
fax +49 6221 8891 200

embo@embo.org
www.embo.org

EMBO excellence in life sciences