# REVEALING THE ROLE OF MED14

# IN

# POL-II TRANSCRIPTION REGULATION

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By

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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# ABSTRACT


REVEALING THE ROLE OF MED14 IN POL-II TRANSCRIPTION REGULATION

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Transcription of protein coding genes by RNA polymerase II (Pol II) is a multi-step process each of which requires a series of factors including coactivators. 30-subunit (the subunits are organized into discrete modules – head, middle, tail, and kinase) -2mDa human Mediator complex is the key coactivator in transcription not only for facilitating the establishment of pre initiation complex (PIC) by acting as a bridge between gene-specific activators and Pol II but also and more importantly for regulating the Pol II activity at all pre-initiation, elongation and re initiation steps. Although the diversity in the function of Mediator lies in its flexible conformation and variable subunit organization (for instance, four subunit CDK8 module can both bind to and disassociate from Mediator), detailed mechanistic studies regarding how Mediator interacts with different activators/repressors to regulate transcription, how Mediator facilitates basal transcription through interactions with Pol II and general transcription factors (GTFs) to establish a proper PIC on promoter DNA and how the regulation of subunit exchanges/rearrangements on the Mediator results in architectural and functional outcome are not well characterized. By using Multibac baculovirus expression system, the previous studies have shown that the reconstitution of a functional 15-subunit human core Mediator complex (composed of head and middle modules together with Med14) is sufficient to support basal transcription as well as selective activator-dependent transcription *in vitro* both with purified factors and with nuclear extracts (in the presence of Med26) as the source of GTFs. This study has uncovered a mechanism by which human core Mediator facilitates transcription by directly interacting with Pol II via its Med14 subunit and recruiting Pol II to target gene promoters.

This is the first time that i unraveled the mechanism of how Pol II binds to Mediator and how it is being recruited to target gene promoters for proper PIC assembly and transcription. This way, i shed light into how protein coding genes are universally regulated via the Mediator complex and in the

future , I will be in a better position to specifically target selected genes by reconstituting the entire Mediator complex and characterizing activator-Mediator-Pol II crosstalk.

*Key Words: Human Mediator Complex, Med14, RNA polymerase II, transcription, GTFs*

# ÖZET

## POL-II TRANSKRİPSİYON REGÜLASYONUNDA MED14 FONKSİYONUNUN BELİRLENMESİ

Yasemin Barış

Moleküler Biyoloji ve Genetik, Yüksek Lisans

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Protein kodlayan genlerin RNA polimeraz-II aracığıyla transkripsiyonu, koaktivatörleri de kapsayan bir çok faktörün katılımını gerektiren, çok aşamalı bir süreçtir.30 alt-ünitelik 2mDa insan Mediator kompleksi, Pol II aktivitesini transkripsiyonun tüm aşamalarında kontrol edebilen ve gene-özgü aktivatörlerle transkripsiyon başlama öncesi kompleks arasında bir köprü görevi gören, en önemli koaktivatördür. Mediator kompleksinin fonksiyonel çeşitliliği her ne kadar değişebilen alt-ünite kompozisyonuna ve esnek yapısına bağlı olsa da, Mediator'ın transkripsiyonda görevli proteinlerle olan etkileşimi, transkripsiyon öncesi kompleksi ilgili DNA promotörlerine nasıl getirdiği, ve aynı zamanda farklı kompozisyonlarda bulunmasının sebebi ve yarattığı sonuçlara ilişkin detaylı mekanistik çalışmalar yapılmamıştır. Yakın tarihte Multibac-ekspresyon sistemini kullanarak yapılan çalışmalar, 15 alt –ünitelik fonksiyonel insan Mediator kompleksini (head ve middle modülleriyle birlikte med14 ve med26 altünitelerini kapsayan) *in vitro* sentezleyebilmiş ve çekirdek olarak adlandırılan bu Mediator kompozisyonun basal transkipsiyonu ve aktivatorlerce sağlanan transkripsiyonu sağlayabildiğini göstermiştir. I*n vitro* transkripsiyon deneyleri, hem saflaştırılarak izole edilen genel transkripsiyon faktörleriyle hem de hali hazırda faktörleri barındıran çekirdek özütleriyle yapılmıştır. (Genel transkripsiyon faktörlerini barındıran özütlerle yapılan deneyde, çekirdek Mediator'ın transkripsiyonu gerçekleştirmesi için med26 alt-ünitesi de bağlanmıştır.)

Bu çalışma Mediator-Pol II interaksiyonun Med14 alt birimiyle sağlandığı ve çekirdek Mediator ‘ın bu interaksiyon sonucu transkripsiyonu gerçekleştirebildiğini ilk kez gösteren özgün bir çalışmadır. Böylelikle ilk kez Pol II ‘nun Mediator kompleksine nasıl bağlandığını ve hedef gen promotorlerine nasıl getirildiğini göstermiş oldum. Mediator kompleksinin protein kodlayan genleri nasıl kontrol ettiğini açıklığa kavuşturarak hali hazırda başladığım tüm Mediator kompleksinin invitro senteziyle birlikte aktivator-Mediator – Pol II interaksiyon mekanizmasını detaylı olarak açıklamayı hedeflemekteyim.

*Anahtar Kelimeler: İnsan Mediator Kompleksi, Med14, RNA Polymeraz II, Transkripsiyon, GTFs.*

***To my grandfather who has always been my source of strength, encouraged me with endless hope and has been my well of wisdom.***

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# Abbreviations:

AcNPV Autographa californica nuclear polyhedrosis virus

APS Ammonium persulphate

BAC Artificial bacterial chromosome

BSA Bovine serum albumin

CBB Coomessie Brilliant Blue

CDK7 Cyclin dependent kinase 7

CDK8 Cyclin dependent kinase 8

CRSP Cofactor Required for Sp1 Activation

CTD Carboxyl terminal domain of RNA polymerase-ll

CX-MS Chemical crosslinking coupled to Mass Spectrometry

DSS Disuccinimidyl suberate

ERα Estrogen Receptor Alpha

FBS Fetal Bovine Serum

GTF General Transcription Factor

kDa kilo Dalton

MED Mediator

NAT Negative Regulator of Activated transcription

NC Negative cofactor

NELF Negative elongation factor

NHR Nuclear Hormone Receptors

PC Positive cofactor

PIC Pre-initiation complex

Pol- II RNA Polymerase II

PBS Phosphate buffered saline

PBST Phosphate buffered saline tween20

SDS-PAGE Sodium Dodecyl sulfate polyacrylamide gel electrophoresis

SMCC Human SRB/MED Cofactor Complex

SRB Suppressor of RNA polymerase B

TAF TBP associated factor

TBP TATA box binding protein

TRAP Thyroid hormone associated protein

# CHAPTER 1

# INTRODUCTION

## 1.1 Eukaryotic Transcription and Identification of Functional Elements in the Transcription Machinery:

Transcription of eukaryotic protein-coding genes by RNA polymerase II (Pol II) is a multi-step process including the events [1] : opening up the chromosome by decondensation of the relevant locus, histone modifications that enable the locus to be recognized by activators /repressors, binding of those activators and coactivators to the enhancers and promoters and finally recruitment of the transcription machinery to the core promoter.

In the native stage of DNA, promoters and related regulatory elements are packaged in nucleosomes, the compact structures wrapped around 146 base pairs(bp) DNA and a histone octamer comprising 2 copies of the core histones, H2A,H2B,H3 and H4.[2-3] Since this condensed shape is not accessible to the proteins that will further recruit the transcription machinery to the site of transcription initiation site, nucleosome itself has a repressive role in transcription.[4] This repressive role could be explained in different mechanisms: First, DNA modifying enzymes, activators and general transcription factors(GTFs) are occluded to bind with the template to enable initiation by Pol II and hence the formation of the preinitiation complex.[4] Second, nucleosome chains could be coiled and folded back upon itself repressing the whole region of transcription. [5] Third, nucleosomes could interact with the proteins in heterochromatin region that will further block the gene expression even in a hereditary manner. [6]

Work on chromatin structure and gene activation mechanisms have revealed the important aspects of proteins named as activators /repressors with the advantage of biochemical studies. Activators couple the transcription to a particular need for any cell type by being specific to each gene or any related gene families.

They are activated in response to a physiological stimulus as in the case of nuclear hormone receptors (NHRs) such as estrogen receptor alpha. [7]

Moreover, they could be maintained in an inactive state in the cytoplasm by being associated to an inhibitory protein, then with a particular external signal, they could be released and could enter the nucleus with its fully functional form. [8] When they enter, they bind to their cognate sequence (enhancers) on DNA and execute the recruitment of the transcription machinery to the promoter. As the explanation implies, activators could accomplish those missions by having a distinct DNA binding and activation domains. [9]

Since the initiation of transcription includes two main steps; opening up chromatin followed by recruitment of the factors by activators and the interaction of Pol II with the GTFs, it was noteworthy to speculate how those activators stimulate initiation of transcription with the assistance of transcription machinery. Scientists initially discovered the factors necessary for transcription of Pol II class genes by first using crude cell extracts and then by using intensive chromatographic techniques to purify all such components from the extracts. [10] General transcription factors (TFIIA,TFIIB,TFIIF,TFIID) have been purified and named based on their chromatographic fractions isolated under particular salt concentrations.[10]. After identification of such components, the mechanism by which the activator could promote the transcription initiation through recruiting TFIID to TATA nucleotide sequence and later how this recruitment would act as a scaffold for the pre initiation complex assembly has been proposed.[11] However, later works showed that isolated yeast and metazoan TATA binding protein (TBP) could supplant for TFIID and enhance basal transcription [12] even if its occupation in the extracts are much lower than TFIID itself. Since the works also proved that TBP alone could not satisfy the needs for activator dependent transcription, scientists clarified this functional distinction by discovering the subunits of TFIID (TBP associated factors-TAFs) that were required to bring activators to the transcription machinery and function as coactivators to regulate activator dependent transcription. [13]

Biochemical studies regarding to the mechanism of transcription led to the identification of other accessory elements that regulate the transcription in both negative and positive manners. In vitro transcription assays performed with purified factors (namely GTFs) necessitated other factors that are crucial to promote both basal and activator driven transcription.

The biochemical fractionation of mammalian cellular extracts as well as reconstitution of transcription *in vitro,* elicited another group of coactivators different from TAFs explained above. [14] First, the crude used in the *in vitro* transcription assays were fractionated and this fraction was named as USA 'upstream stimulatory activity'. [15] The fraction contained both positive and negative activities that were later on termed as PCs and NCs (positive and negative co-factors), respectively. [14] NC2 component of USA comprised of two subunits as Dr1 and Drap1 was identified as a repressor of TATA promoters. [15] PC2 and PC4 were also identified as coactivators the latter is defined as the enhancer of activator dependent transcription by repressing basal transcription. PC2, on the other hand was later shown to belong to the multi-subunit Mediator coactivator complex that will be explained in detail under the title of Mediator Complex later on. [16]

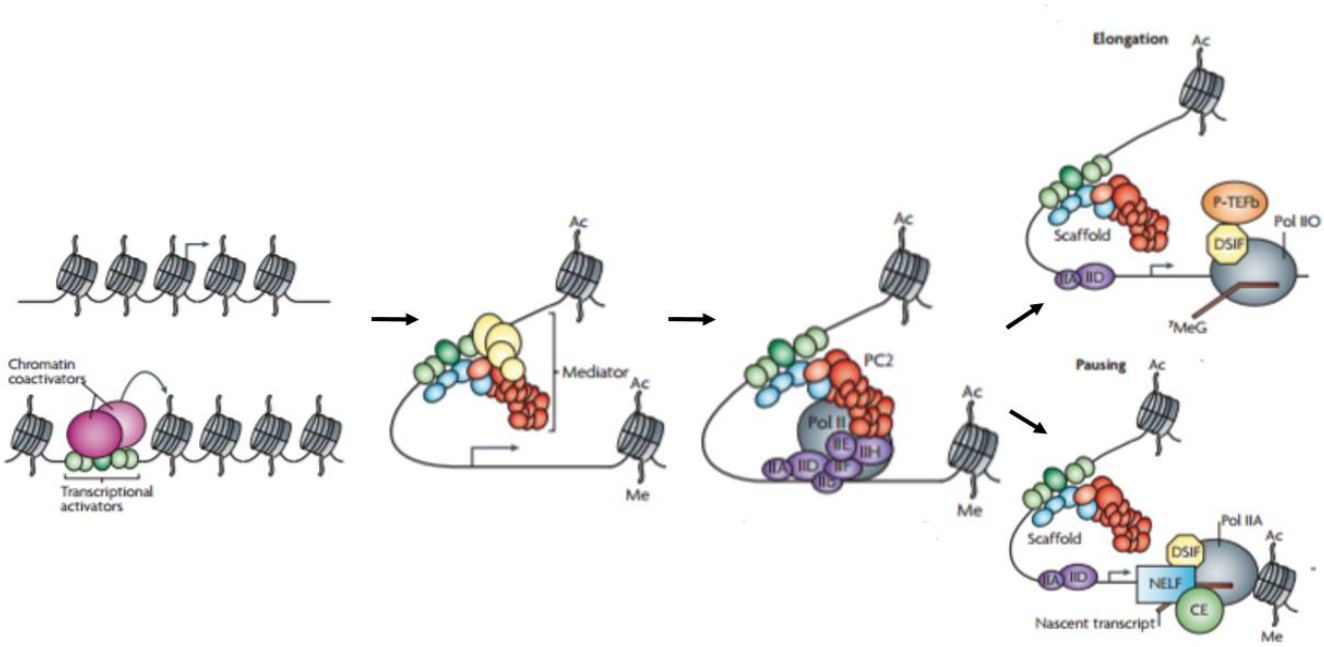



**Figure1.1: The general mechanism of Eukaryotic Transcription [16]**

As depicted in figure 1.1, the chromatin is available for transcriptional activators that recruit a series of chromatin coactivators like histone acetylases that modify the nucleosomes at particular

histone residues and enable the nucleosome to mobilize through ATP-requring reactions to further provide the accessibility to the DNA. When a particular region on the DNA marked by some acetylation(Ac) or methylation(Me) on their histones, it indicates that those regions will be repressed or expressed under specific sets of regulatory proteins. Thereafter, the activators recruit Mediator complex that will subsequently recruit general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH) and Pol II enhancing the formation of PIC. Initiation process will bring a structural change on the Mediator complex, enabling further elongation or pausing of Pol II depending on the needs of the regulatory control and certain physiological conditions. In the elongation process, Pol II is associated with elongation factors including DSIF and P-TEFb and the concominant capping event (7-methyl-guanosine 7MeG) occurs on the newly transcribed (nascent) RNA. Moreover, carboxyl terminal domain (CTD) of RPB1 is phosphorylated at serine2 and serine5 residues, through the actions of TFIIH and PTEF-b. In the case of pausing, Pol II is engaged but not stalled, having the capacity to continue transcription, is approximately +50 nucleotide away from the promoter. As seen, CTD domain is only phosphorylated at serine2 due to blockage of binding of PTEF-b by NELF. [17]

## 1.2 Mediator Complex:

### 1.2.1 Early Studies in the Identification of Mediator Complex as a Coactivator

Mediator Complex was initially studied mostly on yeast and the early studies identified the function of Mediator as an activity in the crude fraction reversing the transcription inhibition in yeast nuclear extracts. [18] When those extracts were included in the transcription reaction performed by using GAL4-VP16 hybrid activator consisting of GAL4 DNA binding domain and the activation domain of VP16 [19], the inhibition by T rich element in the reaction was relieved independent from the actions of other factors importantly TFIID and Pol II were sufficient to induce transcription *in vitro* in the purified system. [18]

It was the first time that Mediator come to the transcription field as a coactivator, yet further studies were needed to identify it as a separate entity.

The term RNA polymerase holoenzyme was introduced after the discovery of such coactivator function in the yeast crude fraction. [20] Multi-protein holoenzyme consisted of Pol II, GTFs and other regulatory elements that were previously purified as yeast SRB proteins [21], stoichiometrically. In the holoenzyme model, the complex consisting of Pol II, TFIIB (yeast factor e),TFIIH ( yeast factor h) , TFIIF(yeast factor g) and SRB regulatory proteins (4,5,2,6) can be formed before the binding to the DNA, and in the transcription assays, it was capable of initiating transcription with the addition of TBP and factor a (TFIIF).[21] The holoenzyme was very stable and existed as a single complex on the gel filtration without losing its intact form after many purification steps. It also precipitated against antibodies in the holoenzyme confirming that the complex was indeed a –single separate entity. [20,21] The role of SRB regulatory proteins, later on identified as Mediator subunits, was to somewhat stabilize the interaction between GTFs and pol II or more importantly to regulate activators in the initiation of transcription. [20]

20 subunit-yeast mediator complex was first identified in the course of transcription system performed with basal transcription factors and Pol II. [22] It was shown that, mediator complex was not only responsive to Gal4-VP16 activator but also was able to promote basal transcription and phosphorylation of C-terminal repeat domain(CTD) of the largest subunit of pol II suggesting a possible Mediator-pol II interaction. Since Mediator alone did not show any CTD kinase activities and TFIIH was absolutely required for phosphorylation, the possibilities other than a direct interaction between Pol II-Mediator remained to be elucidated such as a latent kinase activity of Mediator waiting for an activation by TFIIH or a flexible subunit(s) of Mediator that could be disassociated from the complex or could function independently. [22] Since Mediator was shown to be an essential component of the transcription machinery, further works were conducted on its structure, the newly discovered subunits' functions *in vivo* and the interactions among the Mediator-activator(s)-GTFs and Pol II.

Functional studies after the identification of 20-subunit yeast Mediator complex was centered on certain deletion of Mediator subunits from the yeast strains. It was shown that deletion of mediator subunits such as SRB4 resulted in the inhibition of transcription from all promoters, suggesting a general requirement of Mediator for transcription. [23] On the other hand, mutations in the yeast SRB2 did not show any global effects on transcription rather showed defects in particular processes such as DNA repair or homologous recombination. [24] The different purification methods , the choice of purification of Mediator alone or with the holoenzyme complex and the yeast strains that were used in the studies created the possibility to obtain different compositions of Mediator.[23] Since the holoenzyme bound Mediator was different from purified Mediator complex and the mutations on certain subunits may not affect viability , the idea of different compositions of Mediator could be found in the cells remained to be elucidated until the characterization of Human Mediator complex and deep structural studies.

The studies regarding to the function of the subunits of yeast Mediator have come with the discovery of new subunits that were necessary for CTD phosphorylation *in vivo.* [25] The Srb10 (human homolog of cdk8) and Srb11 (human homolog of ccnc) has formed a kinase-cyclin pair in Pol II holoenzyme, its absence in the complex was resulted in defective CTD phosphorylation *in vivo.* However, purified holoenzyme lacking the kinase-cyclin pair was still able to initiate transcription with purified factors, the *in vivo* studies suggested that this pair had regulatory roles under physiological conditions that were not needed in the purified system. [25] Even if the exact role of CTD phosphorylation in transcription was not known at that time, it was found that the kinase-cyclin pair of Mediator had regulatory roles that enabled pol II to convert *in vivo* signals to a transcriptional output. [25] Later on, two additional Mediator subunits functioning together with kinase-cyclin pair have identified. [26] Srb8(human med12 homolog) and Srb9 (human med13 homolog) together with the srb10/11 were belong to the family of SSN genes that were related to transcriptional repression rising the possibility that these 4-subunit complex together with the Mediator might have repressive role in transcription. It was argued that Mediator was involved in regulating responses of the transcriptional machinery to repressors such as SSN6/TUP1 as well as to the activators. [26]

Another possibility was that SRB proteins in Mediator had dual roles regulating activation in the context of Mediator and mediating repressive signals by some other mechanisms. [26]

Characterization of human Mediator complex was coincided with the studies regarding to yeast system resulted in a purification of cofactors together with particular activators. Although the nuclear receptors have been identified based on their interactions with the components of basal transcription machinery [27,28] and specific coactivators may involved in regulating those interactions indirectly, [27-29] it remained unknown how and which cofactors have regulated the signals between activators and the transcription apparatus.

Biochemical studies with the human thyroid hormone receptor (hTRa) have shown that, activated hTRa by thyroid hormone (T3) could be pull down with a group of proteins named as thyroid hormone receptor associated proteins(TRAPs). [30] Moreover, activated or liganded TR/TRAP complex could facilitate transcription from a template including T3-response elements(TREs*) in vitro* and purified hTRa from Hela cells grown without T3 could not activate transcription due to the absence of TRAPs in that complex. [30] Therefore, T3 induced TR/TRAP complex has suggested a role for the TRAPs as positive cofactors of transcription.

The isolation of human Srb10/(CDK8) –containing complex named as NAT complex (Negative Regulator of Activated Transcription) due to its repressive function in activated transcription have elucidated the mechanism that CTD phosphorylation of Pol II by NAT was different from TFIIH –associated CTD kinase in which the latter phosphorylates the serine5 residue of CTD domain. [31] Furthermore, CTD domain was not the sole target of NAT complex rising the possibility of an interaction between NAT-Pol II independent from CTD domain. [31]

Other Mediator complexes including SRB and MED proteins- SMCC- (Human SRB/MED Cofactor Complex) have been purified independently in human and mouse cells. [32] The significancy of isolation of such complex was the presence of three subunits in SMCC also found in TRAP complex (TRAP220, TRAP100 and TRAP170). [30]

TRAP220 and TRAP100 were shown to be interactor partners for ligand activated nuclear receptors Tyrosine hormone receptor(TR) and Vitamin D receptor(VDR), [33,34] respectively.

Moreover, SMCC was also shown to interact with p53 through its subunit RB18A equivalent to TRAP220. [35] Overall, those interactions with particular activators indicated that, different activators could be regulated by different subunit compositions of Mediator that in turn is also affected by the signals that the specific activators are induced. [32]

*In vitro* transcription systems with purified factors have created limits in the identification of additional factors required in a more physiological conditions such us the use of nuclear extracts as a source of basal transcription factors. In the previous studies, it was shown that glutamine-rich activation domain of Sp1 activator must interact with Taf II subunit of TFIID in order to accomplish in vitro activation of transcription.[36].Since those works were only conducted with the purified factors ,the question of whether or not other accessory proteins like cofactor(s) could be required to enhance the activation by Sp1,completely.It was later on proven that , a new human cofactor , namely CRSP(Cofactor Required for Sp1 Activation) is essential together with Taf II to activate transcription by Sp1 [37] .The subunit composition of CRSP has revealed that it shared many common subunits with TRAP and SMCC complexes. For instance, CRSP contains yeast Mediator homologues such as CRSP33,CRSP77 and CRSP150. [22] However, some yeast mediator subunits did not show any homologies to CRSP130, CRSP70 and p200 subunits of CRSP complex. Furthermore, CRSP has subunits homologues to NAT (NAT19 with CRSP150) [31] and DRIP/TRAP complexes (p200 subunit of CRSP). [33]

The identification of the NAT, SMCC, TRAP, DRIP and CRSP complexes demonstrated the presence of SRB/MED-like complexes in mammalian cells. Since those purified complexes show substantial homolog subunits, the situation highlighted the conserved nature of pol II transcription apparatus among eukaryotes.

Even the functional studies regarding to the pol II- Mediator interaction and subsequent activation/repression have been explained, the reasons for why different forms of mediator existed in different cells or even in the same cell, does Mediator complex have a de novo assembly after a ligand induced activator function or exist as pre-formed complex and its interaction with the transcription machinery remained to be further identified. Regardless, the action of diverse activators through common Mediator coactivators has facilitated the concept of transcriptional activation at that time. [38]

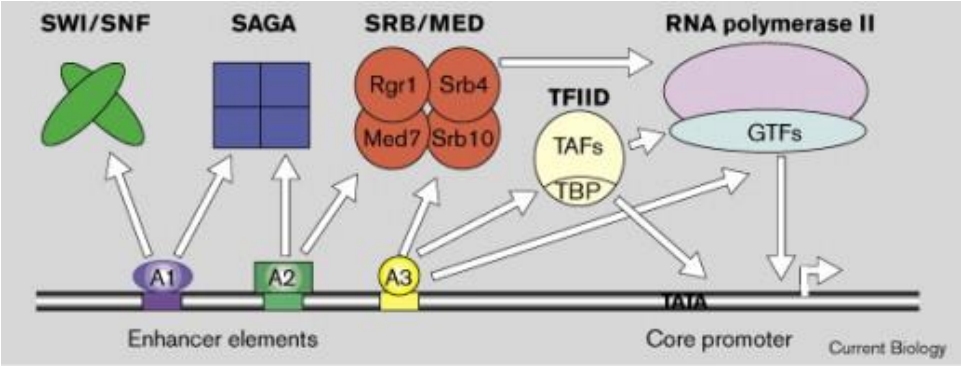



**Figure 1.2.1 Mediator Dependent Transcription Activation [38]**

As shown in figure 1.2.1, activators indicated as A1, A2 and A3 bind to their response elements(enhancer elements) and interact with the core transcriptional machinery consisting of Pol II and GTFs and also with the coactivators.The given coactivators are SWI/SNF , SAGA, SRB/MED (Mediator) and the TAF subunits of TFIID. SRB/MED interacts with CTD domain of Pol II to form a holoenzyme complex in yeast system. Most activators enhances transcriptionby recruiting TBP to the promoter, the process occurs synergestically with the core factors compromising Pol II, TFIIB and SRB/MED.

### 1.2.2 Structural and Functional studies of Human and Yeast Mediator Complex:

With the improvements in Electron Microscopy, X-ray crystallography , identification of protein-protein interactions with Mass-speq coupled with chemical crosslinking have enabled scientists to study large molecular complexes in a more detailed and accurate way. Three dimensional structure of isolated yeast Pol II holoenzyme complex has provided many details about the subunit architecture and the intramolceular interaction of Mediator complex.[39] According to the electron microscopy images, The yeast Mediator consists of three discrete domains named as 'Head','Middle' and 'Tail' and has multiple interactions with Pol II primarily extended from the Head module.[39-40] Later on , a flexible four subunit regulatory 'Kinase' module composed of cdk8/cyclinC plus med12 and med13 was identified as a repressor of ttanscription in yeast.[41]

### 1.2.3 Mediator Architecture and Structure-Function Releationship

The mechanistic studies regarding how Mediator regulates transcription require a deep understanding of its subunit composition, conformational flexibility and intramolecular interactions within the complex. Due to its large size and heterogeneity in the cell, it was challenging to resolve its structure and rearrrangements at high resolution. Yeast Head module was characterized by different groups [42,43,44] Both *S. cerevisiae* and *S. pombe* shared very similar Head module structure, the latter resembled the head of a crocodile with eight separate entities (Shoulder, Spine, Arm, Finger, Joint, Tooth, Nose, Moveable jaw) the four of them as being mobile. [43]

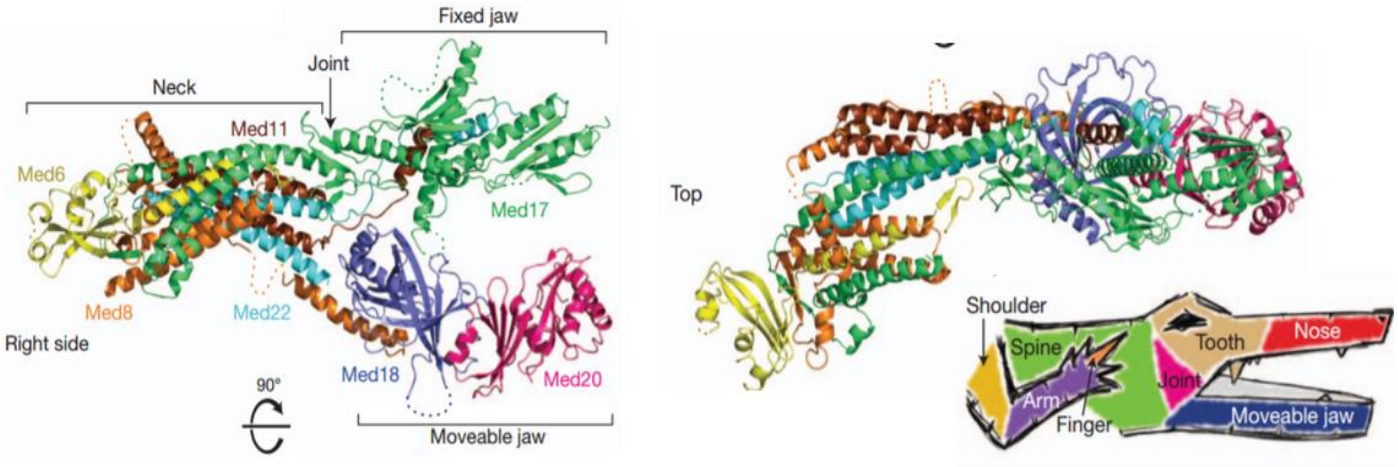



**Figure 1.2.3 Structure of *S.pombe* Mediator Head Module [43]**

The neck submodule consists of Med6, Med8, Med17 Med22 and parts of Med11.The arm binds to the shoulder, which contains Med6 and the fixed and moveable jaw include Med17, Med1, Med22 and also Med8, Med18 and Med20.[45] The shoulder and the arm were the highest conserved regions in the module and deletion of shoulder causes inhibition of transcription globally. Moreover, the jaws and the central joint are responsible for interactions with Pol II and CTD domain and the joint is essential for transcription *in vitro.* [43]

The partial yeast Middle module only lacking Med1 subunit structure was also identified by designing homology models of subunits based on lysine-lysine chemical crosslinking coupled to Mass spectrometry analysis. [46] In the given model, a central tetramer formed by the heterodimers of Med7/Med21 and Med4/Med9 and the highly flexible nature of the middle module made it impossible to study its crystal structure. [46]

Although, the structural studies have underlined the composition of the modules as well as rearrangements and subunit shifts on the complex, no work was conducted on functional assays especially to define a minimal Mediator complex that is capable of initiate transcription both *in vivo* or *in vitro*.

Moreover, there has been a need for understanding a detailed structure-function relation of human Mediator complex since previous studies only characterized Mediator mostly with i*n vitro*

transcription assays using nuclear extracts of HeLa cells stably expressing particular subunits of the complex. In order to gain a deep structure-function relationship, it was inevitable to examine Mediator complex at the subunit level and to make deductions about how each subunit is important in the transcription process. 14 subunit human Mediator complex –named as core Mediator complex – was reconstituted by using MultiBac baculovirus expression system and its architecture was obtained by CX-MS. [47] It was shown that reconstituted Head and Middle alone were not sufficient to initiate transcription *in vitro* both with purified factors and Mediator depleted nuclear extracts although they were stably connected to each other.Med14 was shown to be the critical factor in the transcriptionally responsive form of Mediator by connecting head and middle modules and creating a functional core complex that was able to initiate basal and activator dependent transcription *in vitro* with purified factors.[47] In addition, in vitro transcription assays performed with Mediator depleted nuclear extracts has required med26 in the core complex [47], further explaining an important role of med26 apart from the previous studies that showed the interaction between TFIID and med26 facilitating transcription elongation by creating a docking site for elongation factors.[48] However, it is still unclear how med26 enhance transcription under physiological conditions or the inability of the core complex to initiate transcription is whether or not because of a repressor in the extracts and its overcome by med26. Since med26 containing Mediator complex and Kinase module have shown to be in different population of Mediator [49] and kinase module is also associated with transcriptional repression [50] one possibility is the competition of med26 and kinase module for the same subunit on Mediator complex.

The cryo-EM structure of *S.pombe* Mediator complex was recently shown near-atomic resolution(4.4Å). [51] The structure was also compatible to previous study regarding to Med14 [47] showing that Med14 provides the interactions between modules, and has a distinctive structural motives that enables it to function as a framework in the Mediator complex. [51]

### 1.2.4 Mediator Complex in the regulation of PIC structure and Function

Human and Yeast Mediator has shown to be able to regulate PIC formation by stabilizing it through making physical contacts within the PIC or through functional links that drive other contacts between activators and transcriptional apparatus [52-54] So far, each factor within PIC (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and pol II itself) has been connected to Mediator underlying the importance of Mediator as a fundamental regulator of transcription.

Immobilized template recruitment assays using GAL4-VP16 as an activator and in vitro transcription experiments showed that purified human Mediator is recruited to the immobilized-biotinylated DNA templates by enhancing the recruitment of purified TFIID in an activator dependent manner. [55] Moreover, TFIID was also shown to facilitate mediator recruitment, explaining a synergistic and reciprocal interactions among activator (GAL4-VP16), Mediator and TFIID complex further regulating the stability of high-order PIC formation. [55] In addition to the naked DNA templates used both in the assays explained above, biotinylated chromatin was also used in that assays to investigate the role of Mediator in the coordinated recruitment of chromatin remodelers and TFIID in an activator dependent manner. [56] The cooperative interaction of p300 histone acetyltransferase with activator and Mediator prevents TFIID access to the template blocking nucleation of PIC. It is the checkpoint mechanism ensuring that before proper chromatin opening, TFIID has no access to the DNA and once the chromatin is acetylated, p300 dissociates from chromatin and TFIID binds to Mediator to direct PIC formation. [56]

Along with TFIID, biochemical studies have also showed Mediator dependent recruitment of TFIIB and TFIIE together with pol II to the promoter. [57] In the related study, Mediator and TFIIB were together required for Pol II recruitment to the promoter, suggesting a step wise model of PIC assembly rather than Pol II holoenzyme concept. [57] Overall, the studies were identified Mediator as the key factor in the preinitiation step of transcription.

Studies have linked Mediator to the transcriptional elongation from initiation. Phosphorylation of Pol II CTD domain on serine5 by TFIIH complex has shown to disrupt CTD-Mediator interaction by dissociating Pol II holoenzyme leading to pol II promoter clearance and its enter to elongation step. [58]

Different groups have studied on both human and yeast mediator revealing the subunits of Mediator that mediates TFIIH function and early elongation steps. Yeast med15 tail module subunit was found to be required for recruitment of TFIIE subsequently enhancing TFIIH mediated phosphorylation of CTD.[59] A direct interaction between TFIIH and Med11 of Yeast Mediator was also shown by the experiments both including the use of mutant yeast strains (defective in Med11 as well as in other subunits) and the global gene expression and recruitment profiles of TFIIH in those mutant yeast strains .[60,61]

### 1.3 Baculovirus Expression System for Recombinant Protein Complex Production:

Eukaryotic organisms have many large multi-subunit protein complexes which regulate most if not all specific cellular events. [62] Thus, revealing the structure, function and interaction properties of these complex cellular tools is fundamental for understanding the biological processes. Due to the low amounts of protein complexes in their native environment or in some cases, in low activity in isolation procedure, recombinant protein production have been an emerging area and inevitable for investigating these complexes at the molecular level. [63]

Bacterial protein expression is widely used method to purify recombinant proteins for functional and structural studies due to its cheapness and being an easy host for handling.[64] Scientists have developed several techniques to introduce the gene of interest(s) to bacterial cells by co-expressing multiple vectors in the host or delivering one vector containing multiple genes coding for the complexes .However, those methods have some obstacles such as, in the case of coexpression, expression of one gene could mask the other gene's expression or other gene can be downregulated. More importantly, the complex could not be formed due to additional required post translational modifications lack in prokaryotic system. Furthermore, expression of the multiple genes from one promoter could yield unstable mRNAs that their stability depends on the length of the transcripts produced and the yield &efficiency are further contigent on the order of the genes that the vector contains. Yet, the prokaryotic system does not allow to produce large protein assemblies. [65]

The baculovirus expression system has gained considerable importance in the production of recombinant protein complexes. Their safety use in laboratory (baculoviruses do not replicate in eukaryotic cells except insect cells as being their hosts in the technique) , their high yield production capacity vs low amount host cell requirements (1-100mg protein yield approximately from 1 liter insect cells) and their authentic modification possibilities have paved the way for baculovirus system use in pharmaceuticals, vaccines and in gene therapy vectors [66]

Autographa californica nuclear polyhedrosis virus (AcNPV) was the first expression vector for human beta interferon production by using baculovirus expression system. [67] The logic was to insert the protein coding sequences of interferon to the AcNPV promoter for the gene encoding for polyhedrin by using specially constructed plasmid. The interferon gene was linked to the several regions to polyhedrin transcriptional and translational signals, and subsequently those hybrid genes were transferred to infectious AcNPV plasmids. Hence, expression cassettes consisting of the interferon gene flanked by baculoviral sequence of the polyhedrin were introduced to the transfer vector and incorporated into the circular baculovirus genome via homologous recombination in *Spodoptera frugiperda* insect cells (generally sf9 or sf21 cell lines).The purification yielded active interferon proving that AcNPV was an appropriate eukaryotic expression vector choice for the production of proteins using baculovirus system.[67] However, the experimental procedure introduced in the first baculovirus system practice has resulted only about 0.1 recombination frequency and difficult isolation process of recombinant clones have forced the scientist to find more efficient and less laborious ways to express recombinant proteins.[68]

An important progress in the integration of DNA of interest in to the baculoviral genome was the use of linearized rather than circular baculoviral DNA in the transfection procedure. Companies have offered commercial linearized baculoviruses and transfer plasmids resulted in a better recombination frequency than previous ones.

Later on, the researchers have also overcome the laborious isolation of recombinant clones by engineering an artificial bacterial chromosome (BAC) that was used in the integration of particular DNA pieces via Tn7 transposition *in vivo* and further selection of recombinant BACs having the LacZ gene in bacteria by staightforward blue/white screening. A helper plasmid supplies the Tn7 transposon enzyme for catalyzing the transposition. [69]

While the improvements circumvented many obstacles regarding to enhanced recombination frequency and quick isolation of recombinant clones, scientists were still uncapable of obtaining protein complexes from a single baculovirus that is used in infection. Coinfection of insect cells with baculoviruses having different genes encoding proteins, in other words, with each viruses expressing one subunit of the complex had some drawbacks. It was realized that, coinfection is not always resulted in the complex formation, especially in large scale protein purification, since all the infecting viruses may not be maintained at high titer. [70] Furthermore, it is not completely clear that all the viruses could infect the cells at the same ratio, some of them could mask the expression of others. The worst scenario was that, the high number of viruses could be fatal to the cells, resulted in no protein production at all. [70] Another challenge come from the size(s) of the subunits constituting the protein complex. The companies' offers for plasmids have large in size rendering the cloning of large genes impossible. Therefore, the next step was to eliminate the coinfection procedure, coalescing the genes in one vector, as many as possible and finally to create baculoviruses that express multi-subunit proteins.

In 2004, Multibac baculovirus expression system has offered many superiorities to the previous baculoviruses system. [71] They filled the gap for efficient transfer vectors allowing the assembly of the genes in a flexible and nonsequential way that eases the creation of suitable restriction sites.

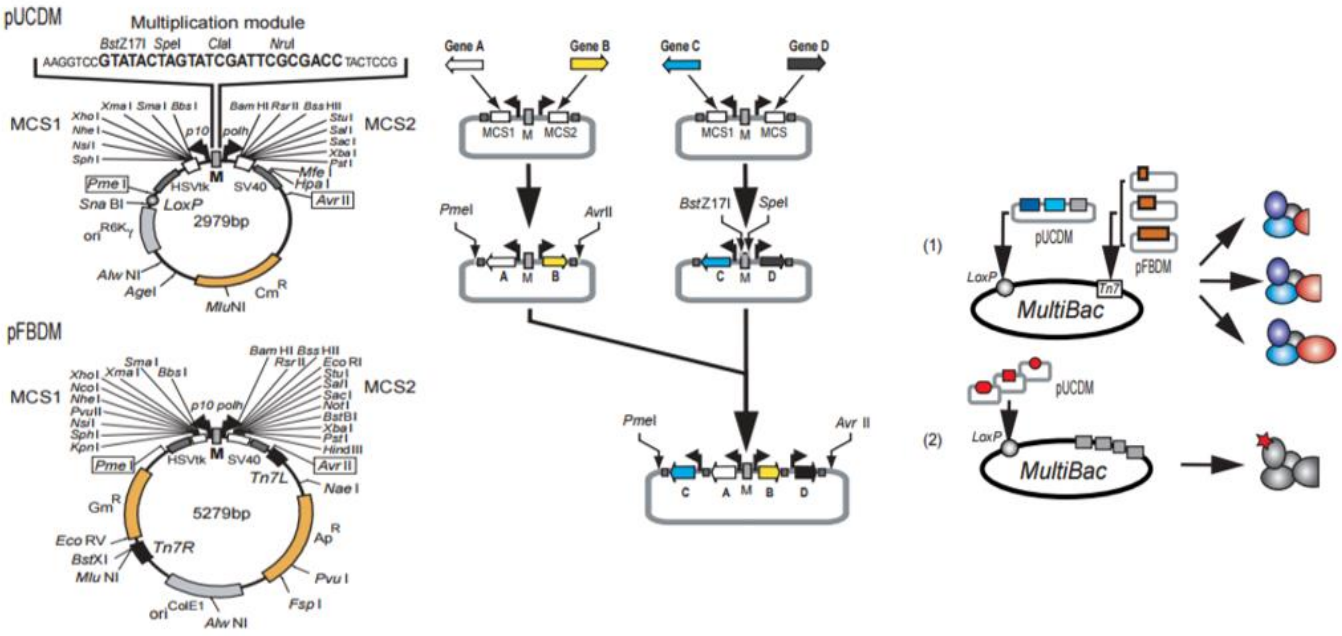



**Figure 1.3 Multibac Expression system strategy showing the cloning approach of the system which enables high yield production of protein complexes. [71]**

In multibac system given in figure above, two transfer vectors namely-pFBDM and pUCDM having a multiplication module (M) between two expression cassettes were designed and those cassettes were planned to driven under the control of p10 and polh viral promoters. PmeI and AvrII are the restriction sites that are used in the integration of expression cassettes in to another cassette containing more genes. In the assembly of the cassettes, PmeI& AvrII are compatible to BstZ17I&SpeI or NruI&SpeI restriction enzymes pairs so that one can assemble as many as possible genes in the transfer vector, theoretically. [71] Apart from this, two transfer vectors were constructed as having different mechanisms for gene integration to the bacmid. The clones are selected with simple blue/white screening according to resistant markers found in two vectors.

The production of multi-subunit protein complexes is inevitable in structural studies such as X-ray crystallography, functional domain-interaction studies and in exploring interaction surface determination. Multibac baculovirus system enables scientists to purify large-scale protein-complexes with optimum efficiency in terms of stoichiometry, functionality and purity.[1] In addition to allowing proper protein folding and optimum gene expression from one baculovirus containing multiple genes, this system also allows the use of viruses in the mammalian cells to examine multi gene delivery. It is plausible to modify those transfer vectors (pFBDM, pUCDM) with mammalian promoters to drive expression in those cells as an alternative way to gene thèrapie studies. [71]

# CHAPTER 2

# MATERIALS:

## 2.1 BUFFERS and SOLUTIONS:

### 2.1.1 Western Blot, Staining and Nuclear Extract buffers:

| | |
|---|---|
| 1X SDS-PAGE Running Buffer | 25 mM Tris, 192 mM glycine, 0.1% SDS |
| 1X TAE | 40 mM Tris (pH 7.6), 20 mM acetic acid, 1 mM EDTA |
| 1X PBST | 8mM $Na_2HPO_4$, 150mM NaCl, 2mM $KH_2PO_4$, 3mM KCl, 0.05% Tween20, pH 7.4 |
| 1X Tris-Glycine Transfer Buffer | 25 mM Tris, 192 mM glycine |
| Western Blot stripping Buffer | 62.5mM Tris-HCl (pH 6.7), 100mM beta-mercaptoethanol, 2% SDS |
| BC1000 (‘‘BC’’ followed by a numerical designation indicates the(variable) KCl concentration of buffers that, otherwise, have the same composition. eg:BC1000=1M KCl containing buffer) | 20mM Tris– HCl (pH 7.9 at 4°C), 20% glycerol, 0.1mM EDTA, 0.5mM PMSF, 0.5 mM DTT, 1M KCl |
| BC0 | 20mM Tris–HCl (pH 7.9 at 4°C), 20% glycerol, 0.1mM EDTA, 0.5mM PMSF, 2 mM DTT |
| Comassie Brilliant Blue (CBB) Staining Solution | 0.1% CBB R-250 (w/v), 50% methanol(v/v), 10% glacial acetic acid(v/v), 40% $H_2O$ |

| | |
|---|---|
| Destaining solution | 50% $H_2O$, 40% methanol, 10% glacial acetic acid |
| Acrylamide/Bisacrylamide solution (30%) | 292g/L acrylamide 7.8g/L bisacrylamide |
| 10% Ammonium Persulfate (APS) | 100g/L APS (w/v) |
| 4X SDS-PAGE sample loading buffer | 240mM Tris-HCl (pH 6.8), 8% SDS(w/v) 40% glycerol(v/v), 0.04% bromophenol blue, 5% beta-mercaptoethanol |
| | |
| | |

### 2.1.2 Buffers for Immobilized Template Recruitment Assay using Streptavidin Dynabeads

| | |
|---|---|
| 2X B&W buffer | 10mMTris-HCl (pH:7.5), 1mM EDTA(pH:0.5), 2M NaCl |
| 10X Assay Mix | 0.2M HEPES-KOH (pH 8.2), 50mM $MgCl_2$ |
| Blocking Buffer | 10X Assay Mix, 5mg/ml BSA, 5mg/ml PVP, 12.5mM DTT, 3% np-40 |
| Wash Buffer | 40mM HEPES, 4mM $MgCl_2$, 4mM DTT, 100mM KCl, 0.1% np-40 |
| | |

### 2.1.3 His-Tag Dynabeads Pulldown Buffers:

| | |
|---|---|
| 2X B&W | 80mM HEPES KOH(pH:8.2), 600mM NaCl, 0.02% Tween-20 |
| His-Elution Buffer | 300mM Imidazole, 40mM HEPES KOH pH:8.2, 10mM NaCl |

## 2.2 MATERIALS

### 2.2.1 Cell Culture Media, Supplements and Culture Equipment:

| | |
|---|---|
| Grace's Insect Media | (TNM-FH) Lonza Biowhittaker Cat. No: 04-649F |
| Gentamicin | |
| Poloxamer | SIGMA 16758 |
| DMEM | (1X) Gibco Life technologies Cat. No: 31885-023 |
| Fetal Bovine Serum (FBS) | Biowest Cat. No: S181T-500 |
| Penicillin/Streptomycin | Gibco Cat. No: 15140-122 |
| 100mm and 145 mm dishes | Corning Coster |
| 6-well and 96 well plates | Corning Coster |
| Plastic Pipettes (5/10/25 ml ) | Corning Coster |
| Spinner Flasks for Insect Cell Culture(10ml, 250 ml , 500ml ) | |

### 2.2.2Antibodies Used in Western Blot and Immunoprecipitation

| | | | |
|---|---|---|---|
| Med4 | Home Made | Med26 | Home Made |
| Med6 | Home Made | Med27 | Santacruz sc-390296 |
| Med7 | Home Made | Med28 | Home Made |
| Med12 | Home Made | Med29 | (B-1) Santa Cruz Biotechnology SC393800 |
| Med13 | Home Made | Med30 | Home Made |
| Med14 | Abcam # ab170605 | Rpb1(8WG16) | Home Made |
| Med15 | Proteintech 115661AP | Rpb5 | Home Made |
| Med16 | Santa Cruz | Rpb6 | Home Made |
| Med17 | Home Made | P53 | Home Made |
| Med22 | Home Made | P62 | Home Made |
| Med23 | Home Made | TAF100 | Home Made |
| Med24 | Home Made | CDK7 | Cell signaling |
| Med25 | (A-7) Santa Cruz Biotechnology SC393759 | Anti-Flag | Sigma F7425 |
| ERα | Cell Signaling mAb #8644 | Cdk8 | Home Made |
| ccnc | Home Made | | |

### 2.2.3 Kits and other Tools :

| | |
|---|---|
| BCA Protein Assay Kit | Pierce BCA protein assay kit ThermoFisher Scientific Cat. No: 23227 |
| Silver Stain Kit | Pierce Silver Stain for Mass Spectrometry ThermoFisher Scientific Cat. No: 24600 |
| Anti-flag M2 Affinity Agarose Beads | Sigma #A4596 |
| Flag Peptide | Sigma #F3290 |
| Dynabeads Histag Isolation Pulldown | Novex Life Technologies Cat. no: 10103D |
| Dynabeads M-280 Streptavidin | Invitrogen ThermoFisher Scientific Cat. No: 11205D |
| Anti-HA M2 affinity Agarose Beads | Thermofisher #88836 |
| HA peptide | Anaspec #AS-63764 |

# CHAPTER 3

# METHODS:

### 3.1 Reconstitution and Purification of Partial Human Mediator complexes using Baculovirus Expression System

Mediator subunit cDNAs were cloned into pFBDM and pUCDM transfer vectors. Different tags (HA, histidine or Flag) were inserted into different subunits of the Mediator in order to enhance downstream purification. The individual cDNAs for subunits of the Mediator head module (MED6, MED8, MED11, MED18, MED19, MED20, MED22, and MED30) were inserted into the pFBDM and pUCDM transfer vectors and individual cDNAs for subunits of the middle module were also inserted into pFBDM and pUCDM transfer vectors (HA-MED7, MED4, MED21, His: MED10, MED31, MED9 and MED26). MED17 and Flag MED14 were integrated into different bacmids separately. Single viruses were generated by integrating the vectors in to bacmids followed by transfection of Sf9 cells with those bacmids. First generation (Po) viruses were amplified in Sf9 cells to second and third generation high titer P1 and P2 respectively. For protein production, Hi5 cells were infected with the particular viruses. (e.g :100 ml Hi5 cells were infected with ($e^6$/ml) 1ml Head P2 +1ml f:Med17,18 to purify Head Module) In order to obtain near - stoichiometric complexes (one to one ratio proteins in the partial complexes) virus titrations were adjusted in each infection. 60-72 hours after infection, depending on the number of infected and dead cells, cells were collected and spin at 1500 rpm for 5 minutes. The pellet was resuspended in BC500 buffer (500mM KC, 20mM Tris– HCl (pH 7.9 at 4°C), 20% glycerol, 0.1mM EDTA, 0.5mM PMSF, 0.5 mM DTT) and homogenized by douncing 3 times using glass douncers. The lysate was centrifuged at 14000 rpm for 15 minutes. After centrifugation, the lysate was diluted to 300mM KCl by drop-wise addition of BC0.

The cell extracts were incubated with either anti-HA or anti-flag M2 agarose beads to pull down the partial complexes. Incubation was done overnight at 4°C by rotating. After overnight incubation, the beads were washed with 1ml BC300 containing 0.1% np-40 5 times. The complexes were eluted using 0.5mg/ml corresponding Flag or HA peptides at 4°C for 45 minutes by rotating. Elutions were repeated 3 times and each elute was analysed by CBB staining and WB.

### 3.2 The purified protein complexes using Baculovirus Expression System

| **Proteins/ Complexes** | **Number of Subunits in the complex** | **Amount of Infected Hi5 cells** | **Amount of infected Viruses** | **Protein yield** |
|---|---|---|---|---|
| **Head (H)Module** | MED6, MED8, MED11, MED18, MED19, MED20, MED22, MED30, f:MED17,18 | 150 ml Hi5 ($e^6$/ml) | 750ul f:MED17,18 P2 + 1.5ml HEAD P2 | 1.2 ug/ul |
| **Middle(M) Module** | HA:MED7, MED4, MED21,His:MED10,MED31, MED9,MED26, f:MED14 | 100 ml Hi5 ($e^6$/ml) | 500ul f:MED14 P2 + 1ml MIDDLE P2 | 0.9ug/ul |
| **H+M Module** | MED6, MED8, MED11, MED18, MED19, MED20, MED22, MED30, HA:MED7, MED4,MED21, His:MED10, MED31, MED9, MED26, f:MED17,18 | 150 ml Hi5 ($e^6$/ml) | 1 ml f:MED17/18 P2, 1.5 ml HEAD P2, 1.5 ml MIDDLE P2 | 1ug/ul |
| **H+M+f:FLMED14 (core complex)** | H+M subunits, f:MED14, MED17,18 | 200 ml Hi5 ($e^6$/ml) | 1.5ml f:MED14 P2, 2.5 ml HEAD P2, 2.5 ml MIDDLE P2, | 1.3ug/ul |

| | | | | |
|---|---|---|---|---|
| | | | 2.5 ml MED17/18 P2 | |
| **H+M+f:C'MED14 (core variant)** | H+M subunits, f:C'MED14, MED17,18 | 200 ml Hi5 ($e^6$/ml) | 1.5ml f:C'MED14 P2, 2.5 ml HEAD P2, 2.5 ml MIDDLE P2, 2.5 ml MED17/18 P2 | 1.2ug/ul |
| **H+M+f:N'med14 (core variant)** | H+M subunits, f:N'MED14, MED17,18 | 200 ml Hi5 ($e^6$/ml) | 1.5ml f:N'MED14 P2, 2.5 ml HEAD P2, 2.5 ml MIDDLE P2, 2.5 ml MED17/18 P2 | 1.3ug/ul |
| **Kinase Module** | HA:CDK8,MED13,MED12, CCNC | 200 ml Hi5 ($e^6$/ml) | 1ml HA:CDK8 P2, 2 ml MED13 P2, 2 ml MED12 P2, 2 ml CCNC P2 | 0.6ug/ul |
| **Tail Module** | MED15,MED16,MED23,MED24, MED25,MED27/29, f:MED14 | 200 ml Hi5 ($e^6$/ml) | 1 ml from each subunits' P2 viruses | 0.6ug/ul |
| **f:p53** | f:p53 | 150 ml Hi5 ($e^6$/ml) | 1.5 ml f:P53 P3 | 1.4ug/ul |
| **f:full length(FL)MED14** | f: FL MED14 | 150 ml Hi5 ($e^6$/ml) | 2 ml f: FL MED14 P2 | 0.7ug/ul |
| **F:N'MED14** | f:N'MED14 | 150 ml Hi5 ($e^6$/ml) | 2 ml f: N'MED14 P2 | 0.7ug/ul |
| **f:C'MED14** | f:C'MED14 | 150 ml Hi5 ($e^6$/ml) | 2 ml f: C' MED14 P2 | 0.6ug/ul |

| | | | | |
|---|---|---|---|---|
| **f:ERα** | f:ERα | 150 ml Hi5 ($e^6$/ml) | 2 ml f: ERα P2 | 1.1ug/ul |

### 3.3 Purification of Endogenous Polymerase II and Human Mediator Complexes from HeLa Cells Stably Expressing f:rpb9 and f:nut2 Respectively

#### 3.3.1 Cell Culturing:

HeLa cells stably expressing one of the subunits of Mediator complex(f:Med10/Nut2) and Pol II (f:rpb9) were kindly gifted from Roeder Lab (Rockefeller University, New York) The cells were grown in DMEM containing 10% FBS and 1% P/S also 300ug/ml geneticin for maintaining the stable cells in 145mm petri dishes in a 37°C incubator with 5 % $CO_2$. Passaging was done by washing the cells with 10ml 1X PBS after aspirating the media then trypsinizing with 2ml trypsin in 37°C incubator for 3 minutes. Cells were grown until they reached the sufficient amount for nuclear extract preparation.

#### 3.3.2 Nuclear Extract Preparation:

Approximately 1.5 liter of HeLa cells stably expressing each f:rpb9 and f:nut2 were collected separately and centrifuged at 1500 rpm for 10 minutes at 4°C. The pellet was washed with ice-cold PBS two times, at each washing, centrifugating for 5 minutes at 1500 rpm at 4°C. The cells were lysed by douncing in the buffer containing 10mM Tris (pH:7.9) , 1.5mM $MgCl_2$ , 10mM KCl, 0.5mM DTT and 0.5 mM PMSF for three times. After that, lysate was centrifuged for 10minutes at 14000 rpm at 4°C and the pellet was saved. Nuclear pellets were resuspended in the buffer containing 20mM Tris (pH:7.9), 1.5 mM $MgCl_2$ , 25% glycerol, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF and 0.3mM NaCl. Preparations were rocked for 30 minutes at 4°C by rotating and centrifuged for 15 minutes at 14000rpm at 4°C. The supernatant was quick frozen and store at -80°C for further purification.

### 3.3.3 Pull Down of Endogenous Pol II and Human Mediator Complex

Approximately 20 ml of each f:rpb9 and f:Nut2 nuclear extract were incubated with 150 ul anti-flag M2 agarose beads for overnight at 4°C by rotating. Before incubation, the beads were washed with 1ml BC300 + 0.1% np-40 for 5 times. After overnight incubation, the extracts were centrifuged at 1500 rpm for 5 minutes. The flow through was kept for Western Blot analysis. The beads were washed with BC300 +0.1% np-40 4 times. The last wash was done with BC200. The complexes were eluted by using 0.5 mg/ml flag peptide in BC200 for 45 minutes at 4°C by rotating. Elutions were repeated three times and each step, 0.5mg/ml flag peptide was used. Three elutions were pooled for each Mediator and Pol II complexes and analyzed by Silver Staining.

## 3.4 Silver Staining:

All steps in silver staining was performed in a clean tray with constant gentle shaking. Staining was done by using Pierce Silver Stain for Mass Spectrometry Kit according to manufacturer's instructions. Before running the gel on SDS-PAGE, all apparatus were cleaned with ultrapure water in order to prevent any dirt during the staining procedure. After running the gel, it was washed with ultrapure water for 5 minutes two times. The gel was fixed with the solution containing 30% ethanol, 10% acetic acid and 60% water for 15 minutes. Fixing step was repeated two times and the gel was washed with 10% ethanol for 5 minutes two times. The gel was washed with ultrapure water for 5 minutes. Sensitizer working solution was prepared by mixing 1part Silver Stain Sensitizer with 500 parts ultrapure water (25ml water+50ul sensitizer). The gel was incubated in sensitizer solution for exactly 1 minute then washed with ultrapure water for 5 minutes two times. Enhancer solution was prepared by mixing 100 parts Silver Stain with 1part silver Stain Enhancer (0.25 ml of enhancer with 25 ml of stain) and incubated with the gel for 5 minutes. After enhancer, the gel was washed with ultrapure water for 20 seconds and then incubated with developer solution containing 0.25 ml of enhancer with 25 ml developer for 2-3 minutes until the bands appeared. When the desired band intensity was reached the developer was stopped by adding 5% acetic acid.

### 3.5 Protein Quantification

Protein quantification was done by using the BCA Assay Kit according to manufacturer's instructions. 0.1 mg/ml BSA was prepared by diluting 2 mg/ml BSA stock. BSA standard solutions were prepared at eight different concentrations ranging from 0 to 0.1 ug/ul. 400 ul standard solutions were prepared as triplicates with a dilution factor of 100. The samples also diluted by 1:100 (4ul protein sample+ 396 ul ultrapure $H_2O$) and prepared as triplicates. 100ul from standards and samples were loaded to 96 well plate as triplicates. Working BCA solution was prepared by mixing reagents A and B in a 50:1 ratio and 100ul of it was mixed with the standards and with the samples. The plate was incubated at 37°C for 1-1.5 hours and absorbances were measured by using the SynergyHT microplate reader (Biotek,VT,USA) at 562nm. Using the absorbance values obtained from BSA standards and samples, a calibration curve was drawn and sample concentrations were quantified by using the standard curve equation.

### 3.6 SDS-PAGE

Before loading on the gel, concentrations of each protein sample was equalized by adding required amount(s) of dd$H_2O$ and 4X sample loading dye. The samples were denatured by boiling for 5 minutes at 95°C . Polyacrylamide stacking and separating gels were prepared by using the ingredients given in the table 3.6

**Table 3.6 Reagents used in SDS-PAGE gel preparation**

| **Reagent (for two gels )** | **7% separating gel** | **10% separating gel** | **12% separating gel** | **4% Stacking Gel** |
|---|---|---|---|---|
| $H_2O$ | 7.65 ml | 5.925 ml | 4.875 ml | 3 ml |
| 1.5M Tris-HCl, pH8.8 | 3.75 ml | 3.75 ml | 3.75 ml | 1.25 ml 0.5 M Tris-HCl pH6.8 |
| 20%(w/v) SDS | 0.075 ml | 0.075 ml | 0.075 ml | 0.025 ml |

| | | | | |
|---|---|---|---|---|
| Acrylamide:bisacrylamide (30% /0.8% w/v) | 3.45 ml | 4.95 ml | 6ml | 0.67 ml |
| 10%(w/v) APS | 125ul | 125ul | 125 ul | 50 ul |
| TEMED | 10ul | 10ul | 10ul | 5 ul |

Separating gel was poured between thick and thin glasses. In order to prevent any bubbles, the top of the gel was covered with isopropanol until the gel was fully polymerized. After polymerization, isopropanol was decanted and stacking gel was poured on top of the resolving gel and 15-well 1mm comb was placed to the stacking gel. Protein samples and appropriate protein marker was loaded to the gel and it was run first at 70V and after the samples entered to the resolving gel, it was run at 110 V for about 90 minutes.

### 3.7 Western Blotting:

After running the samples on SDS-PAGE, the proteins were transferred to PVDF membrane. The cassette was prepared by putting 4 layers of whatman paper on top of the sponge and then the gel and the membrane were placed on the cassette. Before transfer, PVDF membrane was activated by sinking in to 100% methanol for 1 minute. After setting the cassette, the transfer was performed at 330mA for 3 hours at 4°C. The membrane was blocked in 5% milk for 2 hours at room temperature by gently shaking. Primary antibody incubation (antibodies n 1% milk) was done either at 4°C overnight or 1 hour at room temperature by gently shaking. Membrane was washed with 1X PBST for 4 times with 5 minute intervals than secondary antibody (in 1% milk) incubation was done at room temperature for two hours with gentle agitation. The membrane was incubated with 1:1 ratio ECL solution for 2 minutes and development was performed in the dark room. The membrane was exposed to X-ray films with different periods of time ranging from 3 seconds to 30 minutes.

### 3.8 Immunoprecipitation for Pol II - Mediator Interaction by Using Purified Full length and truncated f:Med14 proteins

Purified full length f:Med14, amino terminus f:Med14 and carboxyl terminus f:Med14 were used in IP . Four reactions were designed by using 20ul A/G sepharose beads per reaction. Before starting, the beads were washed with 1ml BC300+0.1% np-40 for 5 times then 20ul beads were incubated with 3ul pol II 8WG16 antibody in 100 ul BC200 +0.025 % np-40.

After 4 hours at 4°C antibody incubation done in a rotator, the beads were washed with BC300+0.1% np-40 for 5 times. 12 ul purified Pol II was added to three reaction and 200ng full length and truncated f:Med14 proteins were added to remaining reaction in 100ul BC150+0.025% np-40 as a control. After 4 hours at 4°C incubation done in a rotator, the beads were washed with BC200+0.1%np-40 for four times at 4°C. The control beads were taken and 20ul 2X SDS sample loading buffer was added and kept at -20°C for WB analysis. 200 ng full length and truncated f:Med14 proteins added to three reactions sepearately in 100ul BC150+0.025 % np-40 and incubated for 4 hours at 4°C by rotating. After incubation, the beads were washed with BC200+0.05% np-40 for 5 times at 4°C and 20ul 2X SDS sample loading buffer was added to each reaction and kept at -20°C for WB analysis.

### 3.9 Immunoprecipitation (IP) for Pol II – Mediator Interaction by using Partial Mediator Complexes.

In order to see the direct interaction between Pol II and Human Mediator in the context of the complexes, the purified partial Mediator complexes were used in IP experiments. 5 reactions were designed by using Head, Head+Middle, Mediator core complex containing amino terminus of f:Med14, Mediator core complex containing carboxyl terminus of f:Med14 and finally Mediator core complex containing full length f:Med14. Since each complex compromising different number of subunits in them, before doing IP, each complex was checked by western blot and the concentration of each complex was adjusted accordingly. For each reaction, 20ul protein A/G sepharose beads were used. Before starting the experiment, the beads were washed with BC300 +0.1% np-40 for 5 times.

The beads were incubated with α-med6 antibody (since Med6 is in all the partial complexes) in 200ul BC200+ 0.025 % np-40 for 4 hours at 4°C by rotating. (3ul α-med6 + 20ul A/G sepharose) After antibody incubation, the beads were washed with BC300+0.1% np-40 for 5 times. For each reaction, the adjusted concentrations of the partial Mediator complexes were added to the beads in 100ul BC150 + 0.025% np-40 then incubated for 4 hours at 4°C by rotating. After incubation, the beads were washed with 200ul BC200 +0.1% np-40 for 4 times. 20ul of Pol II was added to each reaction and the beads in 100ul BC150 +0.025% np-40 were incubated for 4 hours at 4°C by rotating. After incubation, the beads were washed with 200ul BC150 + 0.025 % np-40 for 4 times at 4°C. 20ul 2X SDS sample loading buffer was added to each reaction beads and store at -20°C for WB analysis.

### 3.10 Immobilized Template Recruitment Assay

In order to reveal the importance of Med14 in recruitment of Pol II to the promoter DNA, immobilized template recruitment assay was performed using Mediator variants. GADD template was chosen and PCR amplified using biotinylated primers. After PCR amplification, the template was run on agarose gel at 100V for 20 minutes then extracted from the gel by using GeneJet Gel Extraction Kit (Thermoscientific).

**Table 3.10.1 PCR Program for GADD Template**

| **Reagent** | **Volume added** | **Final concentration** |
|---|---|---|
| 10X taq Buffer(5U/ul) | 5 ul | 1X |
| dNTP mix(10uM) | 1 ul | 0.2 uM |
| Forward Primer(biotinylated)(10uM) | 1.5 ul | 0.3uM |
| Reverse Primer(10uM) | 1.5 ul | 0.3uM |
| GADD template | 1 ul | 10ng/ul |
| $MgCl_2$ (25mM) | 4 ul | 2mM |
| $ddH_2O$ | 36 ul | - |

**PCR cycle**

95°C >> 4 minutes

95°C >> 30 seconds

68°C >> 1 minute

72°C>> 1minute

} X 32 cycles

72°C >> 7 minutes

4°C>>

### 3.10.2 Preparation of Dynabeads M-280 streptavidin(DynaI)

10 ul beads were used per reaction in the assay. Dynabeads were resuspended by gently shaking the vial to obtain a homogeneous suspension. Since 10 reactions were designed in the assay, approximately 100ul beads were transferred to an eppendorf tube. The tube was put on a magnetic rack for 90 seconds. The beads were washed 5 times with 500ul 1X B&W buffer and in each wash, the tube was put on the magnetic rack for 90 seconds. After washing, the beads were resuspended in 150 ul 2X B&W buffer. In order to immobilize the template to the beads, approximately 8ug of template was used for 100ul beads. Total volume was completed to 300ul by adding dd$H_20$. The beads were incubated for 15minutes at room temperature. In each 5 minutes, the tube was mixed by inverting 2 times in order to mix the beads with the template homogeneously. After that, the tube was put on the magnetic rack for 90 seconds and supernatant was kept for checking how much template was bound to the beads by comparing the concentrations of the first template with after incubation one. The beads were washed with 300ul of 1X B&W containing 0.5mg/ml BSA and 0.003% np-40 for two times. The beads were further washed with 300 ul 1X PBS for 2 times and blocked with 100ul blocking buffer for 15minutes at room temperature. After blocking, the beads were washed with 500ul wash buffer for 2 times. The beads containing GADD template were stored at 4°C in 150ul wash buffer.

### 3.10.3 Immobilized Template Recruitment Assay Design

10 reactions were designed including Mediator depleted nuclear extracts (ΔMed) as well as wildtype HeLa nuclear extracts (WT NE). P53 was used as an activator in order to show the activator enhanced recruitment of Pol II to the promoter DNA. Outline of the assay was given in the table 3.10.3 below.

**Table 3.10.3 Outline of Immobilized Template Recruitment Assay**

| | ΔMed | | | | | ΔMed | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | WT NE | + | + | + | + | WT NE | + | + | + | + |
| **P53 (ng)** | - | - | - | - | - | 100 | 100 | 100 | 100 | 100 |
| **NE (ug)** | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 | 180 |
| **BC100 (ul)** | 15 | 27 | 27 | 27 | 27 | 15 | 27 | 27 | 27 | 27 |
| **BC150 (ul)** | 24 | - | - | - | - | 24 | - | - | - | - |
| **2X assay mix (ul)** | 54 | 54 | 54 | 54 | 54 | 54 | 54 | 54 | 54 | 54 |
| **Mediator variants** | - | - | f:N'Med14 core | f:C'Med14 core | f:FLMed14 core | - | - | f:N'Med14 core | f:C'Med14 core | f:FLMed14 core |

( WT: wild type, NE: Nuclear extract)

After template incubation, the beads were washed with 500 ul 1X assay mix (transcription buffer) containing 0.025% np-40 and 0.25mg/ml BSA for four times. 500 ul 2x assay mix containing 100ug/ml E.*coli* genomic DNA was added to the beads and the beads were divided for 10 reactions. For p53 containing reactions, five tubes were incubated at 30°C for 15 minutes. After activator recruitment to the template, indicated amounts of proteins, extracts and buffers were added to each reaction as given in the table 3.10.3. The reactions were incubated at 30°C for 50 minutes and the beads subsequently washed with 50mM NaCl. 15ul 1X SDS sample loading buffer was added to the beads and the samples were further analyzed by WB.

### 3.11 Pull Down of Pol II- Core Mediator Complex by Using His-Tag Dynabeads

Since both Pol II and Human core Mediator complex were purified using anti-flag M2 agarose and eluted with flag peptide, the proteins remained with many flag peptides in them. In order to characterize the interaction between Med14-pol II, chemical crosslinking coupled to MS was required. To prepare samples for this technique, the samples should have been free of any flag peptide otherwise may interfere with the chemical crosslinking. Therefore, Pol II- Mediator mix was eluted using His-tag Dynabeads to get rid of any flag peptides (Mediator contains His-tagged subunits in it. - his:Med10-). Before protein-bead incubation, approximately 4ug of Pol II + Human Mediator core complex in 110 ul 1X B&W buffer were incubated for 2 hours at 4°C by rotating. 5ul of the beads slurry was transferred to a tube and washed with 200ul 1X B&W buffer for 4 times. The proteins were mixed with the beads and incubated for 15 minutes at 4°C by rotating. The proteins were eluted with 50ul His Elution buffer containing imidazole. For elution, imidazole concentration was adjusted as 1200mM. The elute was analyzed by silver staining.

### 3.12 Chemical Crosslinking of Pol II – Core Mediator Complex

Before crosslinking of Pol II – core Mediator, the concentration of DSS (disuccinimidyl suberate) was adjusted to obtain the optimal crosslink efficiency. In order to do so, His-tag dynabeads eluted core Mediator complex was first crosslinked with varying concentrations of DSS. 50 ul of Mediator core was divided in to 4 tubes each having 9ul of core. One tube was designed as control containing no DSS and other tubes were added 5mM DSS, 2.5 mM DSS and 1mM DSS. Crosslink was done at 25°C for 20 minutes by using an orbital shaker (VWR) in a speed of 450 rpm. Crosslinks were quenched by adding 1ul of 50mM Tris-HCl pH:8.0. The samples were run on SDS-PAGE and crosslink efficiency analyzed by silver staining. 5mM DSS concentration gave the best crosslink of core complex so further Pol II- core Mediator crosslink was performed with the same protocol by using 5mM DSS.

# CHAPTER 4

# RESULTS

## 4.1 Purification of Partial Human Mediator Complexes Using Baculovirus Expression System.

It was previously shown that the reconstituted core Mediator complex was responsive to transcription *in vitro* with purified factors. [47] It was revealed that Med14 was indispensable for transcription *in vitro*, the other reconstituted core failed to promote Pol II transcription [47]. In order to further characterize the function of Med14- the most critical subunit of core Mediator complex- by both *in vivo* and *in vitro* studies, first the partial human Mediator complexes were purified in the forms of modules and multi-modules by using Multi Bac system. The subunits constituting the Head and Middle modules were constructed into a transfer vector –pFBDM-. Generated bacmids were transfected to Sf9 insect cells and Po viruses were collected and further amplified in Sf9 cells to generate high-titer expression viruses. Figure 4.1 shows the outline of purification strategy. Hi5 insect cells were infected with Head(H), Middle(M), H+M and H+M +Med14 viruses and the cell lysates subsequently incubated with M2 beads and the complexes eluted with 0.5mg/ml flag peptide. Since each virus titration was adjusted to obtain a near stoichiometric complex, each purification step was repeated several times to get the optimal cell-virus condition and one-to-one subunit ratio partial complexes. Figure 4.2 shows the coomassie staining of purified H, M, H+M and core Mediator, the numbers indicating the names of subunits in the complexes. (from Cevher et al., 2014)

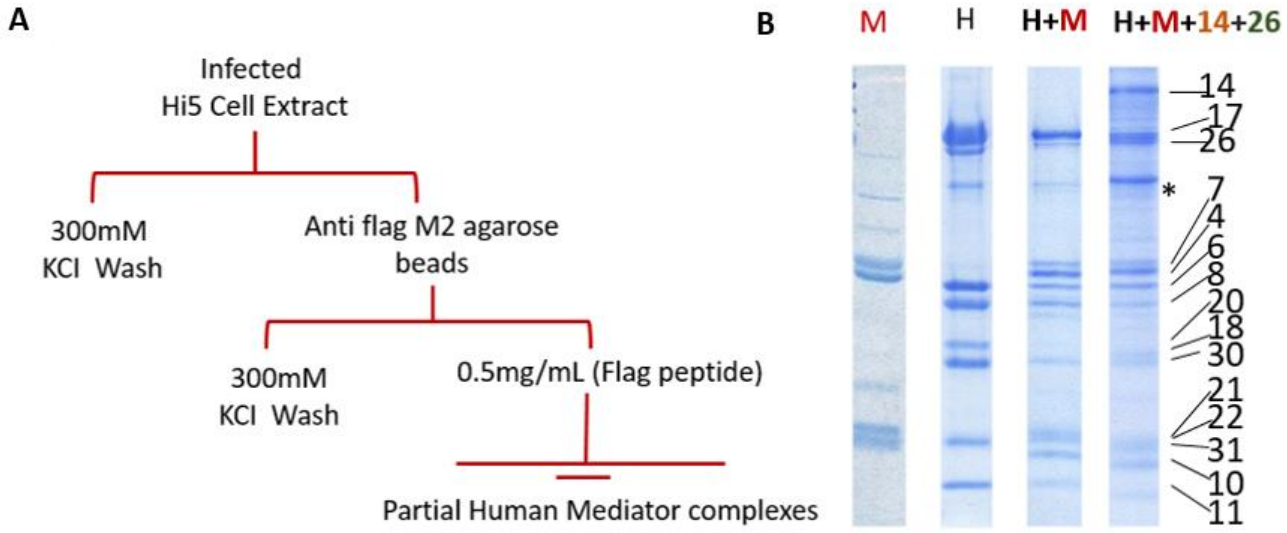
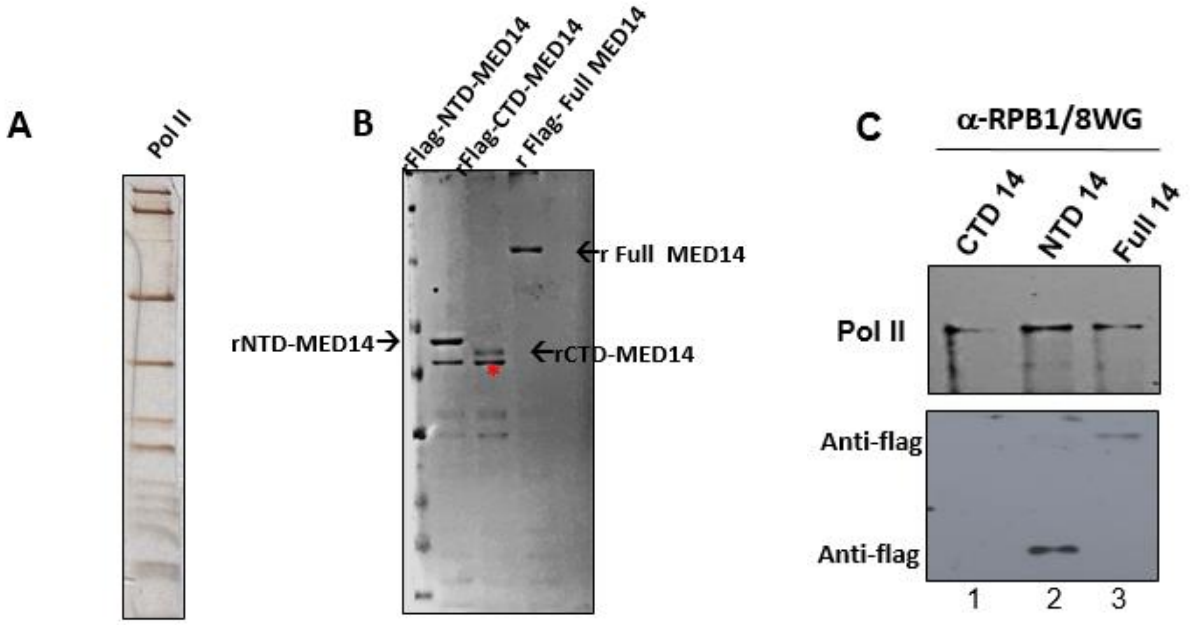



**Figure 4.1 : Reconstituted Head , Middle ,H+M and core Mediator complexes using Multibac system.** ***A)*** *Outline of the purification of Mediator subcomplexes.* ***B****) Head, Middle, H+M and core Mediator viruses infected with Hi5 cells and the cells were collected 3 days after infection. After incubation with M2 beads, the complexes were eluted with 0.5mg/ml flag peptide. Coomassie staining shows the near stoichiometric purified partial complexes. The numbers indicate the subunits in each complex.*

## 4.2 Immunoprecipitation Reveals a Critical Role for Med14 in the Regulation of Pol II activity *in vitro*

Since Mediator is a multi-subunit protein complex, it is not surprising that its surface structure enables many activators and general factors in transcription machinery to bind it and create conformational changes in the complex itself. It was previously shown that yeast Mediator Head module interacts with CTD domain of Pol II to promote transcription.[44] Later on it was shown that Middle module as well as Head module are responsible for the initiation of transcription first by recruiting TBP to the promoter then by interacting with Pol II. [45]

Since both human Head module and Middle module failed to initiate transcription *in vitro* and Med14 was critical in connecting those modules to get a function *in vitro* [47], the function of Med14 in regulating Pol II activity remained to be elucidated. In order to understand the role of Med14, we first tried to recombinantly generate it and use it in the functional assays. Figure 4.2B shows the purification of recombinant full length and truncated forms of Med14 by using MultiBac system. Pol II was purified from HeLa cells stably expressing f:rpb9 (figure4.2A) and immunoprecipitation (IP) was performed using those purified proteins. The IP results shown in figure 4.2C/D have revealed a critical role for Med14 in transcription.We show that Med14 directly interacts with Pol II via its amino terminus. The obtained data is novel and contradictory to the previous yeast Mediator model in which the alterations in the structure of Med14 enhances a large-scale Mediator rearrangement which may be critical for Mediator function. There are no current data showing a direct interaction between Med14-Pol II. As a result, Figure 4.2 shows clearly that Med14 directly interacts with Pol II via its N' terminus and this interaction could pave the way for transcription initiation in functional assays that will be further investigated.



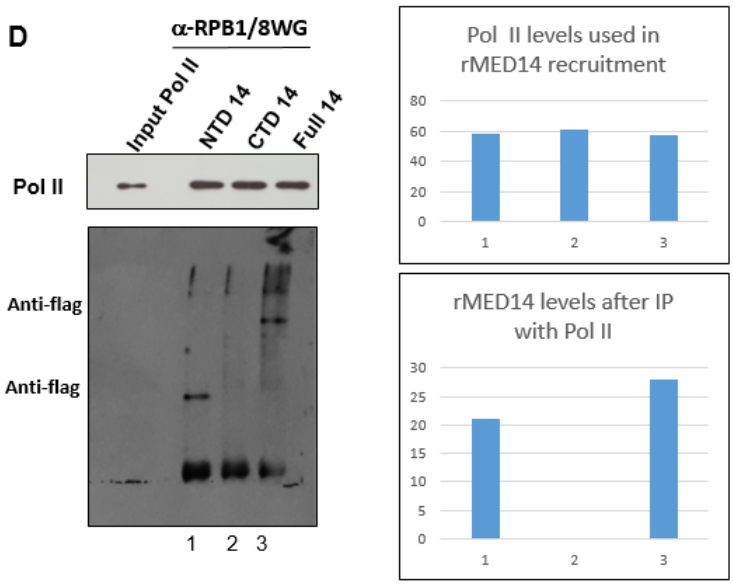
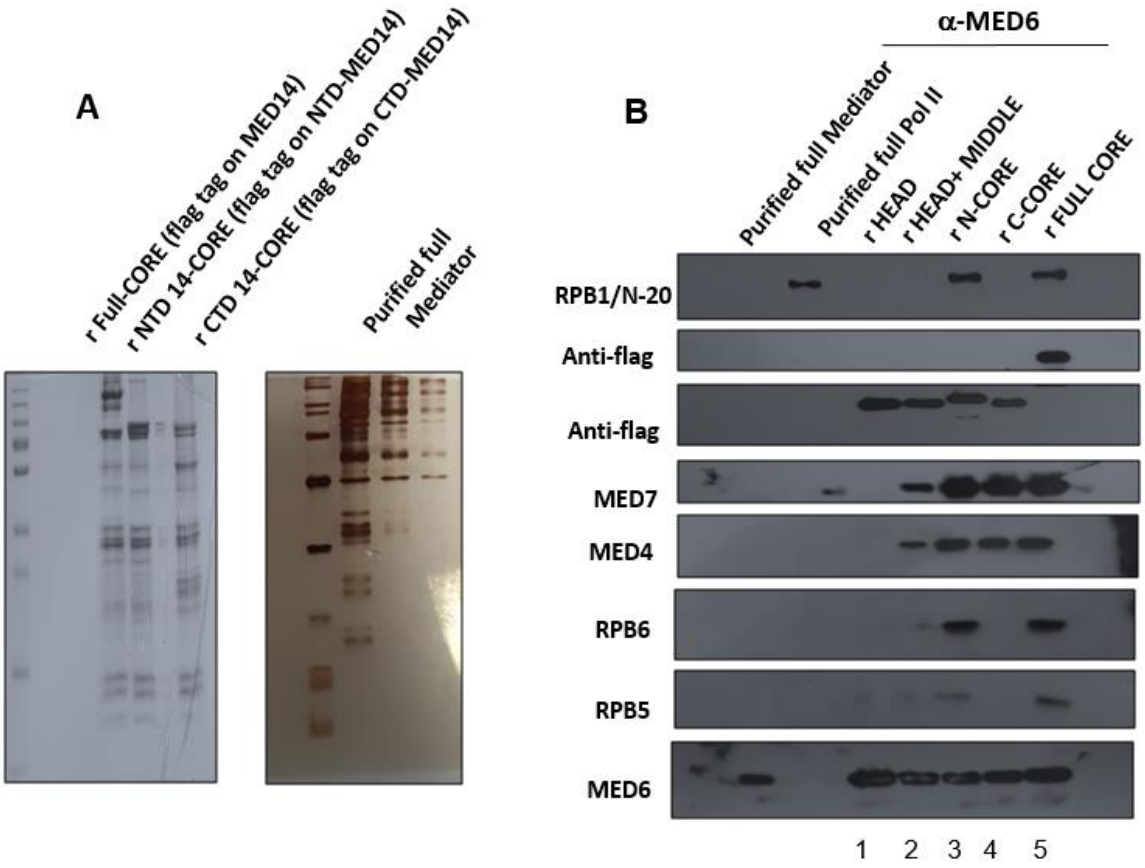



**Figure 4.2 Med14 Directly Interacts with Pol II via its Amino terminus. A-B)** *Silver Staining of purified endogenous Pol II from HeLa cells stably expressing f:rpb9 and coomassie staining of the purification of full length and truncated Med14 variants using Multibac system.* ***C-D)*** *IP experiments revealing a direct interaction between Pol II –N'Med14 and Pol II-full length Med14. The f:Med14 levels normalized to IgG. Med14 recruitment was checked with anti-flag antibody.*

### 4.3 Med14 directly interacts with Pol II via its N' terminus in the context of Mediator

Chemical crosslink coupled to Mass-spectrometry(CX/MS) revealed an enormous intersubunit links between Med14 and Head & Middle modules. It was shown that both the amino and carboxyl termini of Med14 linked to Head & Middle modules, Med14 can co-purify either with Head or middle modules. [47] Since those findings impose Med14 as being an architectural backbone of Mediator, it is critical to show that Med14 also interacts with Pol II in the context of Mediator. In order to do so, the next step was to purify core complexes by using truncated forms of Med14.

Because the truncated forms of Med14 can fold properly (Figure 4.2B) and both N' & C' termini link to Head and Middle modules [47], it was highly probable that Core Mediator can be purified by using those Med14 variants. Figure 4.3A (left) shows the purification of core variants by using full length, N' and C' termini expressed f: Med14 viruses by Multibac system. These complexes were further used in IP to show the interaction between Pol II – Med14 in Mediator context. Furthermore, endogenous Mediator was purified from HeLa cells stably expressing f: nut2 in order to validate the Mediator subunits characterized in IP. As seen from figure 4.3B, neither Head nor Middle modules interacted with Pol II. Most importantly, IP experiment confirmed that N'Med14 and full length Med14 purified core Mediator interacted with pol II whereas C' Med14 core not. Therefore, it was concluded that N'med14 directly interacts with Pol II both with its purified form and in the context of Mediator.


A
r Full-CORE (flag tag on MED14)
r NTD 14-CORE (flag tag on NTD-MED14)
r CTD 14-CORE (flag tag on CTD-MED14)
Purified full Mediator
B
α-MED6
Purified full Mediator
Purified full Pol II
r HEAD
r HEAD+ MIDDLE
r N-CORE
r C-CORE
r FULL CORE
RPB1/N-20
Anti-flag
Anti-flag
MED7
MED4
RPB6
RPB5
MED6
1 2 3 4 5

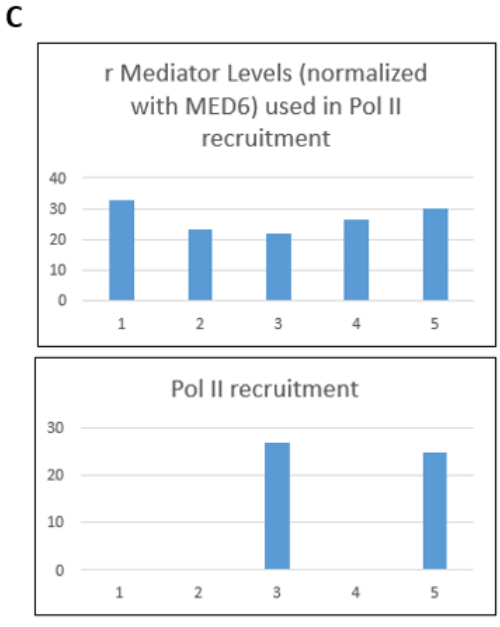

C
r Mediator Levels (normalized with MED6) used in Pol II recruitment
40
30
20
10
0
1 2 3 4 5
Pol II recruitment
30
20
10
0
1 2 3 4 5

**Figure 4.3 Immunoprecipitation validates the direct interaction between Pol II- N'Med14 in the context of Mediator. A*)*** *Coomassie stain of the purified of core variants using truncated forms of Med14 expressing viruses by Multibac system (left). Silver stain of the purified Pol II from f:rpb9 overexpressing HeLa cells.(right)* ***B)*** *Western blot analysis of Mediator-Pol II interaction assay . Med6 antibody was used in IP. Pulldown of Pol II with full length 14 core and N' 14 core were validated by rpb1/n-20, rpb6 and rpb5 antibodies.* ***C)*** *Prior to IP, Mediator subcomplexes levels were adjusted by WB and also normalized with Med6. (upper panel) Pol II recruitment was enriched in full length and N' 14-core as shown in lower panel.*

## 4.4 Pol II interaction with core Mediator is independent from Med26

Since the core Mediator (H+M+14) failed to response to transcription when using Mediator depleted nuclear extracts as a source of GTFs, Med26 was included in these experiments. Previously, Med26 was shown to be required for transcription using nuclear extracts.it was previously hypothesized that med26 may interact with Pol II to facilitate transcription in a more physiological condition.[47]

In order to confirm the interaction of core Mediator with Pol II is not dependent on Med26, IP was performed by using purified f:Med26 and core Mediator including Med26 (as a part of Middle module). As shown in Figure 4.4, Pol II was only pulled down with core Mediator but not with f: Med26 itself. As a result, we can clearly say that Med26 is important for transcription but does not interact with Pol II. The function of Med26 in transcription could be further explained by the experiments that will be done with transcriptional repressors that are more relevant to the elongation complex that was previously shown to be related with Med26. [48]

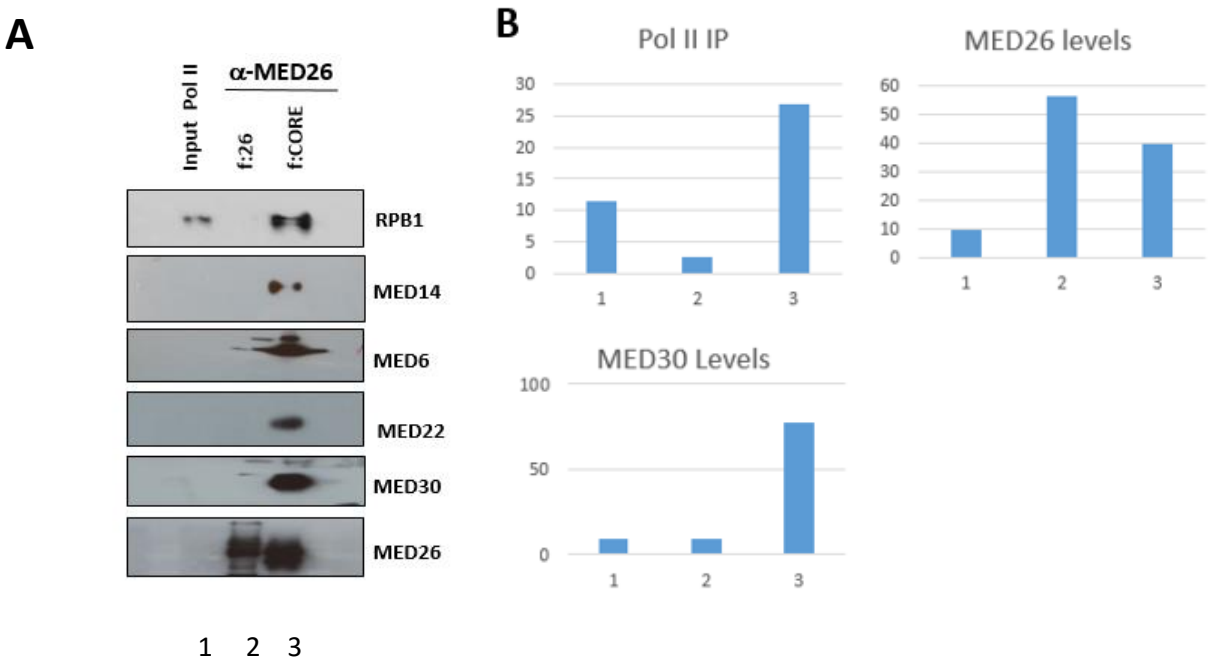



**Figure 4.4 Pol II does not directly interact with Med26** ***A)*** *Western Blot analysis shows the interaction of Pol II with the core Mediator. IP was performed using α-med26 antibody.* ***B)*** *Pol II levels indicate the interaction pattern with Med26 vs core Mediator and Med30 levels were also indicated in purified f:med26 and core Mediator.*

## 4.5 N' Med14 Dependent Pol II recruitment to promoter DNA Revealed by Immobilized Template Recruitment Assay

So far we showed the interaction between Pol II and Mediator using the purified system. However, those studies do not shed light in to how Pol II is being recruited to promoter DNA via the Mediator in the presence of all other factors. In order to show the Pol II recruitment together with the GTFs to the promoter is dependent on Med14, immobilized template recruitment assay was performed by using a p53 responsive GADD template. In order to understand the significance of core Mediator with Med14 in nuclear extracts deficient from the Mediator, we first immunodepleted the endogenous Mediator complex using CNBr-activated sepharose coupled to Med30 antibody. Later, we added recombinantly generated variants of the Mediator and examined the Pol II

recruitment to the promoter. As shown in figure 4.5B, Pol II was recruited to the promoter of the DNA template only in the presence of full length and N' Med14 core complexes. Although there is some non-specific binding observed in the presence of Head, other subcomplexes did not respond to Pol II (e.g: H+M). The recruited Pol II  levels are also showen as bars.

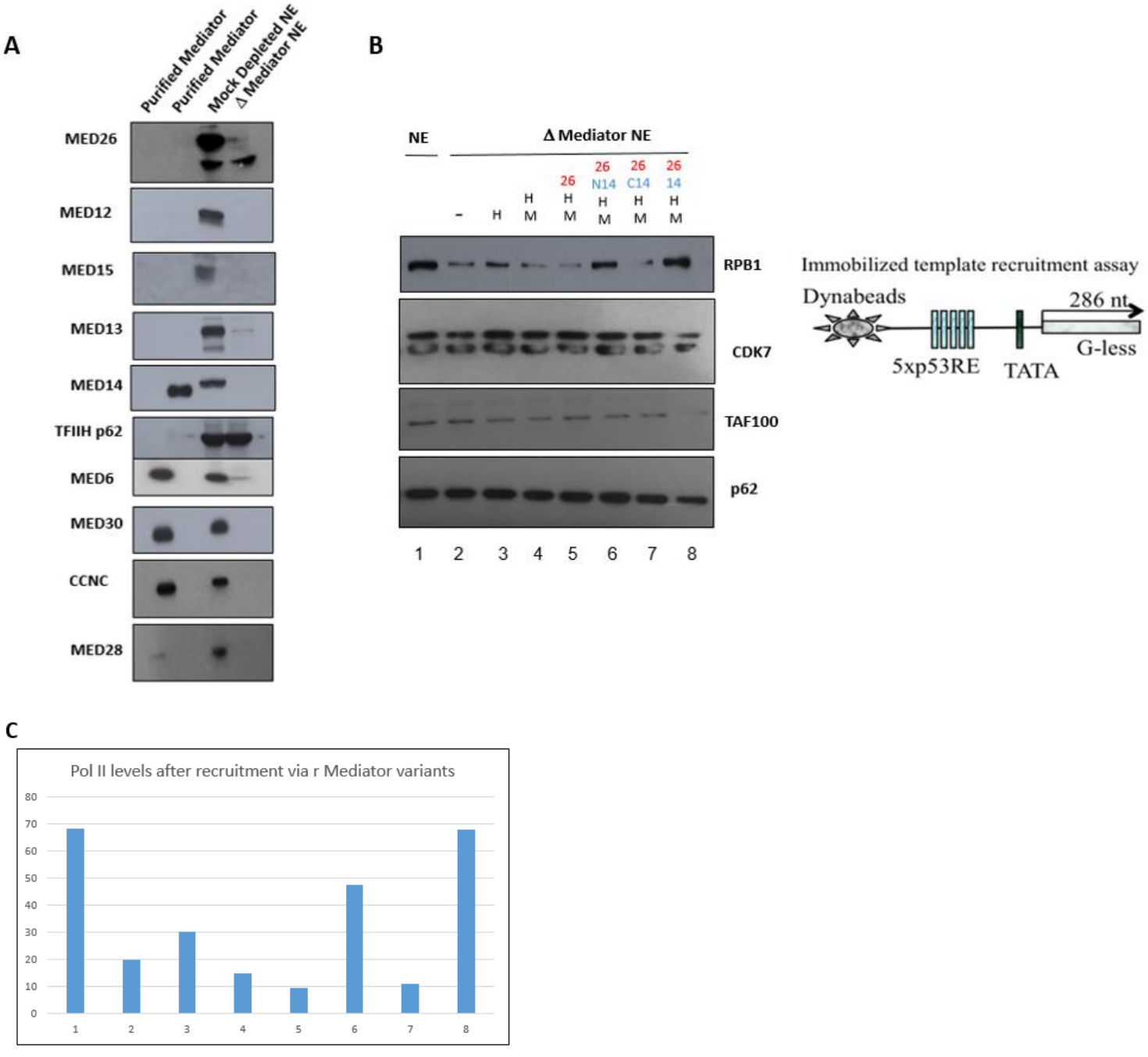
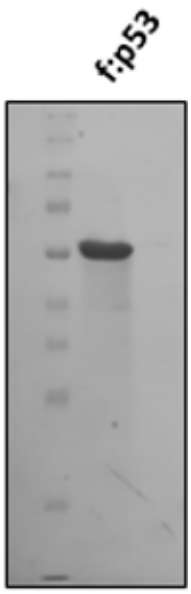


**Figure 4.5: Immobilized Template Recruitment Assay shows the requirement of N' Med14 in recruitment of Pol II to the promoter DNA by Mediator complex.** ***A)*** *Immuno-depletion of Mediator complex from HeLa nuclear extracts (Δmed) was analyzed by WB with indicated antibodies.* ***B)*** *Immobilized template recruitment assay was performed using ΔMed nuclear extracts and by adding indicated Mediator subcomplexes. WB shows different GTFs and Pol II recruitment to the promoter in the presence/absence of Mediator variants. While Head, Head+Middle, Head+Middle+26 and C'Med14 core failed to recruit Pol II (lanes 2-5 and 7), in the presence of N' core and full length Med14 core, pol II was enriched in promoter. The design of GADD template with 5Xp53 binding site is also depicted in the right panel.* ***C)****The graph for Pol II levels in the promoter was enriched in lanes 6 &8 with N' core and full length Med14 core, respectively.*

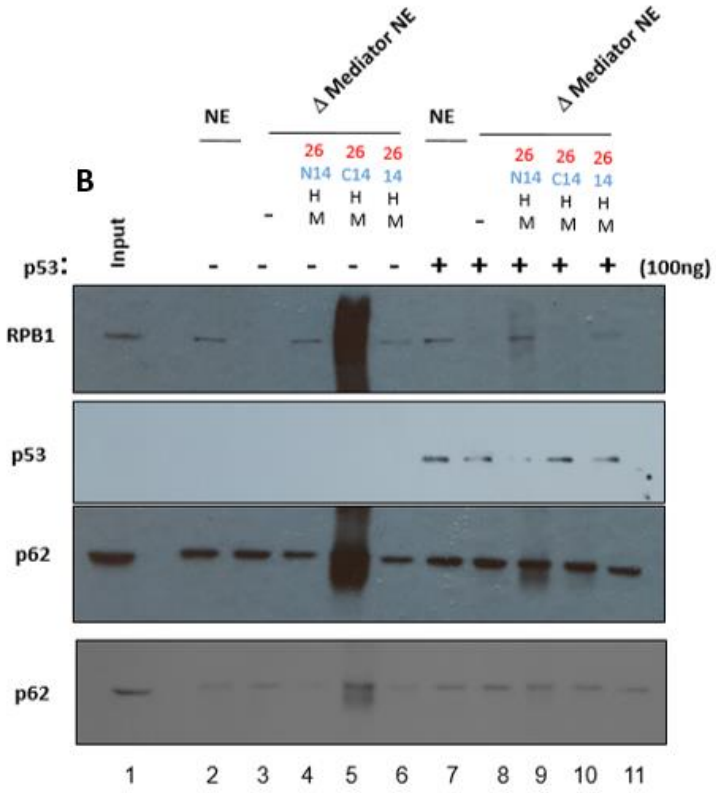
### 4.6 Activator Facilitated Recruitment of Pol II to the promoter by N'Med14 core Complex:

Immobilized template recruitment assay was performed using p53 as an activator. Since the template used in the previous assay has p53 responsive element, the recruitment of Pol II can be augmented by including p53 in the assay. Figure 4.6A shows the purification of f:p53 from Hi5 cells using Multibac system. Figure 4.6B shows p53 enhanced recruitment of pol II and p62 via FL med14 core and N' med14 core complexes. Although the WB analysis is not clearly indicating the activator enhanced recruitment, the bar graph in Figure 4.6C clearly shows the increase in the given proteins. The importance of N'med14 was again confirmed by the recruitment assay performed with an activator.

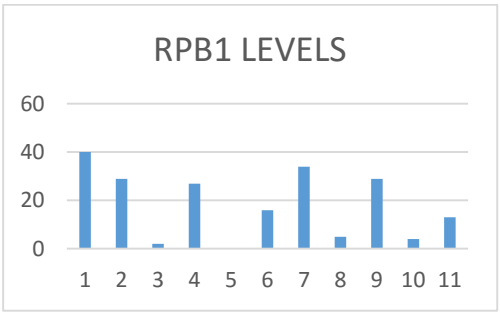
A

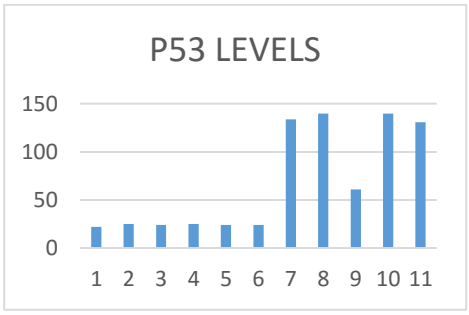

f:p53

B


NE
Δ Mediator NE
NE
Δ Mediator NE
26 N14 H M
26 C14 H M
26 14 H M
Input
p53:
(100ng)
RPB1
p53
p62
p62
1 2 3 4 5 6 7 8 9 10 11

C


RPB1 LEVELS
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11


P53 LEVELS
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11

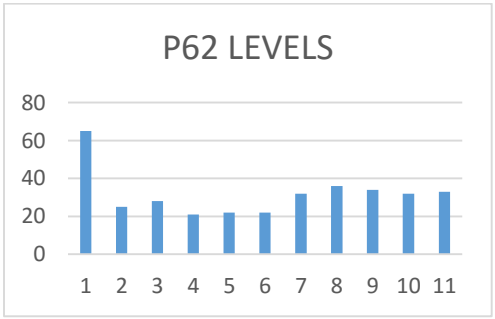

P62 LEVELS
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11

**Figure 4.6: p53 enhanced recruitment of Pol II to the promoter dependent on N'Med14 :**

***A)****Comassie stain shows the purified p53 from f:p53 virus infected Hi5 cell extracts.****B)*** *WB analysis of immobilized template recruitment assay performed with and without p53 as an activator and ΔMed nuclear extracts were used. Recruitments of Pol II, p62 and cdk7 to the promoter were checked with each supplemented Mediator sub complexes. Only full length and N' Med14 core were able to recruit pol II both in the presence or absence of p53, (lanes 4&6 and 9&11)* ***C)*** *Bars showing Rpb1 , p62 and p53 levels.*

## 4.7 Chemical Crosslinking of Pol II- Core Mediator Complex.

In order to identify interaction partner(s) and surface between N'Med14 and Pol II, the samples were prepared to be analysed for CX/MS by our collaborator. Since flag peptide was used in the purification of both Pol II and core Mediator complex, it was important to get rid of the excess flag peptides in those complexes to make chemical crosslink efficient and to get a meaningful result from MS. In order to get rid of the excess peptides in the samples, the mixed Pol II and core Mediator was eluted using His-tag dynabeads (via His-Med10). Figure 4.7A shows the purification of core mediator by using His-tag dynabeads.We need to optimize the imidazole concentrations since the first purification trial of core-Pol II failed to elute whole complex most likely due to the insufficient concentration of Imidazole and/or the excess of the beads used in the purification. After imizadole concentration was adjusted and the amount of the beads were changed, core Mediator was succesfully eluted as shown in figure 4.7B. Another obstacle came from DSS concentration, which also required different optimizations concentrations as well as the adjusments in the incubation and temperature for crosslinking. Figure 4.7C shows the crosslinking of the core with varying concentrations of DSS. Because 0.2M and 0.5 M concentrations of DSS remained with non –crosslinked Middle module subunits in the complex, 1 M DSS was chosen as an optimal DSS concentration for crosslinking of Pol II-Core.

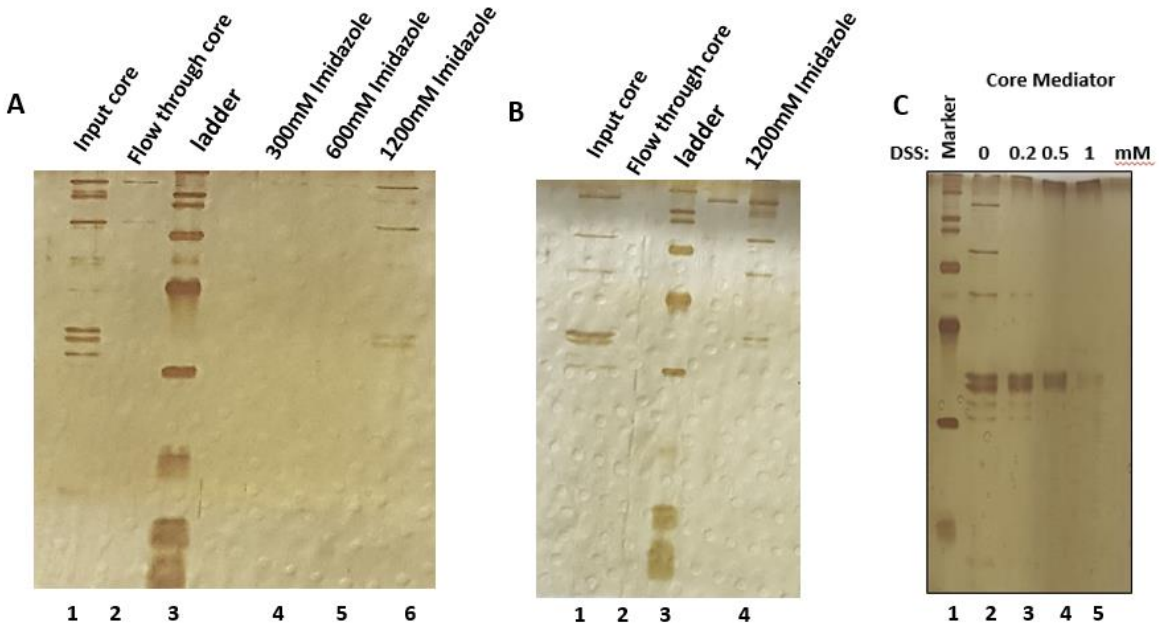



**Figure 4.7: The optimal DSS condition was adjusted for chemical crosslinking of Pol II-core for MS analysis.** ***A-B)*** *Purification of core Mediator by using His-tag dynabeads through His-Med10). 1200 mM imidazole (lane 6 in A) was chosen for the optimal concentration for further purification.* ***C)*** *Crosslinking of core by different concentrations of DSS at 25°C for 20mins. 1mM DSS gave the best crosslinking in the complex (lane 5) and this molarity was used in the subsequent crosslinking experiments.*

# CHAPTER 5

# DISCUSSION

Human Mediator is a multi- subunit complex comprised of 30 discrete proteins, having a fundamental role in the regulation of gene expression by acting as a scaffold between transcription factors and Pol II transcription machinery. Studies to uncover the Mediator function and its mechanism in transcription have been impeded due to the lack of detailed structural analysis regarding to its subunit organization and conformational rearrangements. It is crucial to dissect the subunit interactions as well as inter-modular contacts within Mediator complex in order to elucidate the detailed mechanism of its regulation of activators and PIC in gene expression. Because of its size and heterogeneity, the Mediator itself creates a challenge for high resolution structural studies.

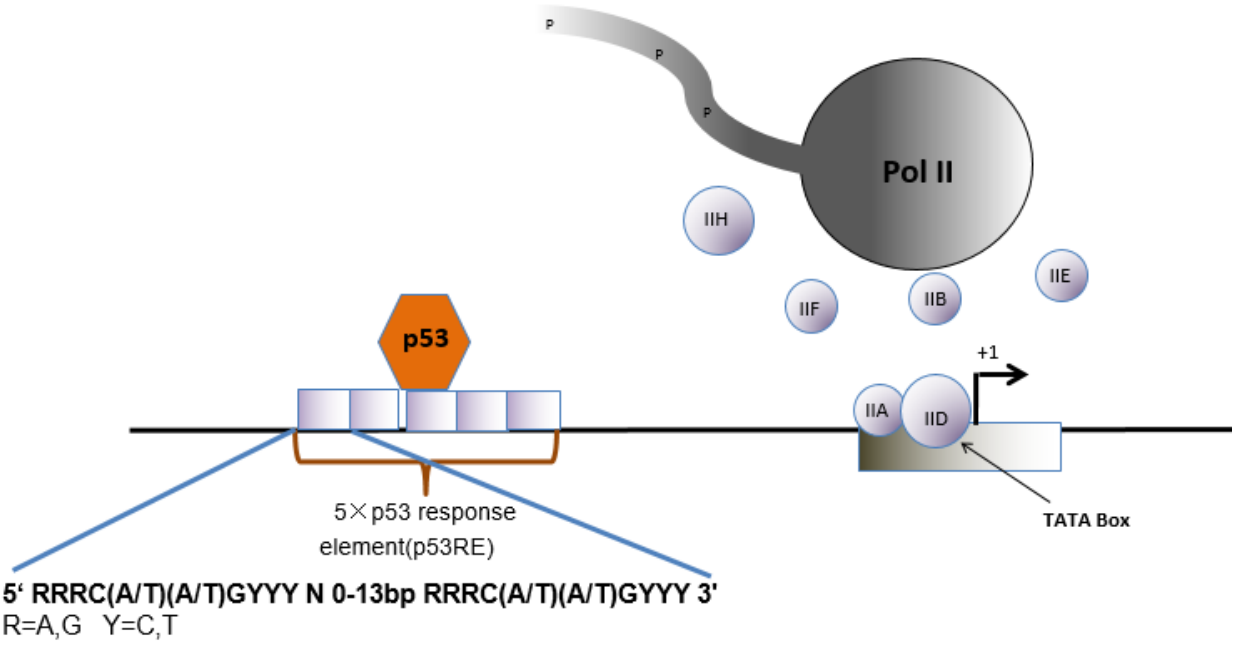
To date, several laboratories have worked on the structure of both yeast and human Mediator complexes [ 42,43,45,46,51,64,65]. Nevertheless, those studies lacked in dept information on the functionality of the Mediator complex. Absence of the functional biochemical studies was stem from the different purification methods that each laboratory used. Overexpression of particular subunits of Mediator in HeLa cells and subsequent EM analysis resulted in numerous interaction surfaces between Mediator subunits [65], yet the study still lacked deep understanding of this interaction network outcome in terms of function. Moreover, because the Mediator is highly heterogeneous in the cells with respect to its subunits, it is difficult to be 100% sure about the stability of its subunits as well as interactions with other factors. That is why, a meaningful dissection of functional mechanism is critical to understand Mediator. Despite the extensive work on the reconstitution based approach for Mediator characterization in yeast system, we still lack of understanding the nature and behavior of human Mediator complex and its function in transcription.

Recently, 15-subunit human Mediator core complex was reconstituted by Dr. Cevher [47] using Multibac expression system. The importance of this study was not only to reconstitutute the largest complex so far, but was also to unravel the mechanism in which the minimal Mediator complex is required to promote transcription.

Here, i have uncovered the role of Med14 in the binding and recruitment of Pol II to the promoter. Our laboratory uses the state of the art Baculovirus expression system which gave me a great opportunity to purify many Mediator complex variants as well as activators in large quantities in order to characterize the interaction between Pol II and Mediator. I purified full length and truncated forms of Med14 and also Mediator sub complexes (Head, Middle, H+M) , p53 and Pol II to further use them in my IP , immobilized template recruitment and in vitro transcription assays.(figure 4.1B, Figure 4.2A-B,data coming soon ) I was also able to purify the core complex with my partial N' terminus and C' terminus Med14s, which were all functional in my immobilized assays in terms of Pol II recruitment to the promoter. (Figure 4.3A) Since the mechanisms shown so far relied mostly on CTD domain of Pol II binding to Head or kinase Module, lacked a direct interaction between interaction between pol II and Med14. Here, I showed that Med14 directly interacts with pol II via its amino terminus (Figure 4.2C-D) and this interaction drives the recruitment of Pol II to the promoter to form a proper proper PIC. (figure 4.5B) All the Mediator sub complexes (Head, Head+Middle and the core variant purified with C' med14) failed to interact with Pol II in my hands (Figure 4.3B), despite the previous finding in the yeast showing that Pol II interacts with Mediator Head module. I have verified this interaction in the context of Mediator by doing IP with core variants and pol II. (Figure 4.3B) Furthermore, my PI previously showed that Med26 was essential for the core Mediator to enhance transcription in vitro performed with nuclear extracts as the source of GTFs [47]. Therefore, the hypothesis at that time was a possible interaction between Med26 and Pol II to overcome the obstacle created by nuclear extracts to further facilitate transcription. However, my IP experiments done with purified Med26 and core Mediator (including med26) showed that Pol II interacts with the core but not with Med26 and this interaction is solely mediated by N'Med14 . (Figure 4.4) Since it was also possible that Med26 interacts with Pol II in the context of Mediator core, my IP experiments done with core variants (N', C' and FL f: Med14 cores all including Med26) did not support this possibility and I only got Pol II interaction with N' and full length Med14 core.

As a result, I was able to show for the first time the direct interaction between N' Med14 and Pol II which results in the recruitment of Pol II to the promoter. Here, we solved the main question asked by leaders in the transcription field. This findings will pave the way for my future studies dissecting the detailed mechanisms of Mediator in the regulation of transcription machinery which i have already started to work on structural studies regarding to Pol II- Mediator interaction. The figure 5 summarizes the proposed mechanism that I am currently working on.

**A**


P
P
P
Pol II
IIH
IIE
IIF
IIB
p53
+1
IIA
IID
5×p53 response
element(p53RE)
TATA Box
5‘ RRRC(A/T)(A/T)GYYY N 0-13bp RRRC(A/T)(A/T)GYYY 3'
R=A,G Y=C,T

**B**

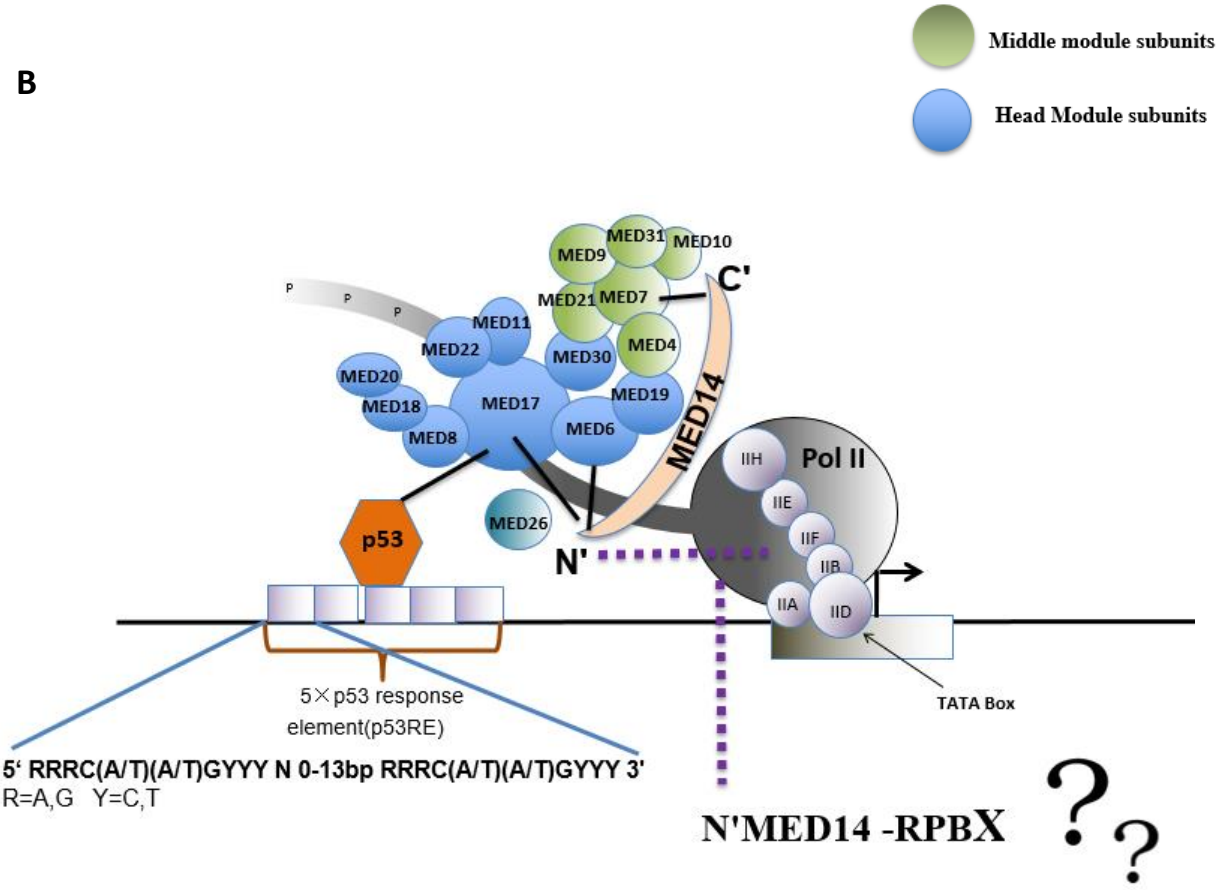

Middle module subunits
Head Module subunits
MED31
MED10
MED9
C'
MED21
MED7
MED11
MED22
MED30
MED4
MED20
MED18
MED17
MED19
MED8
MED6
MED14
IIH
Pol II
IIE
IIF
IIB
IIA
IID
MED26
p53
N'
TATA Box
5×p53 response
element(p53RE)
5‘ RRRC(A/T)(A/T)GYYY N 0-13bp RRRC(A/T)(A/T)GYYY 3'
R=A,G Y=C,T
N'MED14 -RPBX
??

**Figure 5: The proposed mechanism of the regulation of transcription by the Mediator Complex A)** *In the absence of the Mediator, binding of p53 to its cognate element cannot initiate the formation of proper PIC itself. Some of the GTFs and Pol II are thought to be away from the promoter to initiate transcription.* **B)** *Upon binding of Mediator (via Med17) to p53, the Mediator interacts synergistically with IID which in turn nucleates PIC formation. As depicted by crescent, Med14 brings whole core together by interacting Head and Middle modules via its N' and C' termini, respectively. The study here shows that Pol II interacts with N' Med14 to be recruited to the promoter. The proposed mechanism here will further uncover which Pol II subunit is responsible for that interaction as well as how exactly the Mediator recruits the PIC to the promoter by interacting which GTF(s) and activators?*

# CHAPTER 6

# CONCLUSIONS AND FUTURE PERSPECTIVES

Over the last few years, the information obtained from structural studies have uncovered the modular architecture of yeast Mediator and the role of core Mediator on Pol II. Med14 was shown to be part of the architectural backbone of Mediator by linking Head and Middle modules and also essential for facilitating transcription *in vitro* in purified system. Here, I have uncovered the reason of Med14 requirement in transcription by showing a direct interaction between Pol II and N'Med14 as opposed to the current knowledge of Pol II recruitment via Head module. It was surprising to find this interaction since Med14 is a conserved protein among eukaryotes and the previous structural studies regarding to yeast Mediator have insisted on the rearrangements rather than interaction of Med14 in Pol II holoenzyme formation. Even though the secondary structures of *S.pombe* and Human Mediator complex were shown to be conserved with a little difference in their C' terminus, its relation to Pol II is somewhat distinct based on my result and the previous one. This distinction could be due to the lack of showing any functions of Med14 in regulating Pol II activity via biochemical assays since the structural studies have never been confirmed with functional assays. Therefore, while I was doing in vitro transcription with the core variants, I've purified sufficient amount of Pol II and core Mediator and crosslinked them with DSS for CX/MS analysis that will be done by our collaborator. The interaction between the subunit(s) of Pol II and Med14 will be revealed with CX/MS which will enable me to deeply study this surface interaction by using gene editing methods as well as X-ray crystallography. Since the reconstitution of Pol II has not been done yet, it will be a great opportunity for me to get crystal structures of both reconstituted Pol II (reconstitution has been currently started in my laboratory) and Med14.

Since Mediator complex has linked to many activators including nuclear hormone receptors and my laboratory focuses on prevention of ERα mediated breast cancer progression by focusing on blocking ERα-Mediator interaction, my secondary aim is to reconstitute ERα responsive form of Mediator.

The power of Multi bac system enabled me to purify ERα together with Mediator kinase module, each protein was cloned in to a single transfer vector and transfected to Sf9 cells, viruses were

amplified and infected into Hi5 cells and finally resulting protein complex is purified as I have done for Mediator sub complexes previously. Figure 6.1B shows the purification of ERα together with whole kinase module. It was noteworthy to get such a strong pulldown of kinase module with ERα since I did not see any interaction between ERα and Mediator when I did IP with my reconstituted Mediator variants. I only got the interaction with the whole Mediator complex purified from HeLa cells stably expressing f: nut2. Moreover, it was also surprising that core Mediator (including Med26+Med1) did not show any interaction with ERα contrary to previously shown Med1-ERα interaction. (Figure 6.1A) Therefore the probable interaction between ERα and Mediator should be either with kinase (it has a strong potential as I showed and the interaction studies will be done at the subunit level and *in vivo* studies using MCF7 as a model cell line will be conducted through the knockdown(s) of particular kinase subunits) or tail module. In the long run, the aim is to reconstitute both the kinase and the tail modules and to get a function with ERα and to further block the interaction of ERα- Mediator complex in order to prevent ERα mediated tumor progression. Figure 6.2 summarizes my proposed mechanism for ERα-Mediator interaction.

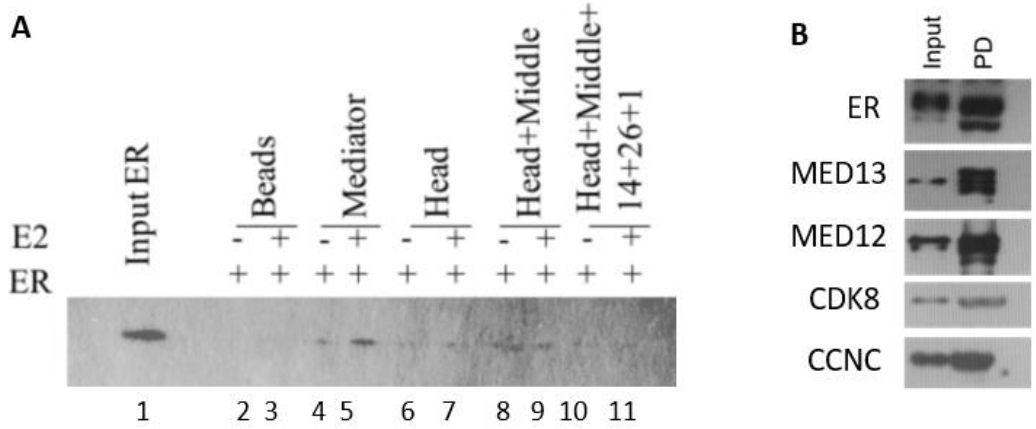



**Figure 6.1: Reconstituted Mediator complex and ERα interaction studies *A)*** *WB analysis showing the ERα- Mediator interaction assay. Upon estrogen treatment (100nm), only the Mediator complex purified from HeLa cells was able to interact with ERα(lane5). No interaction was observed with any reconstituted Mediator sub complexes. (lanes 6-11)* ***B)*** *WB analysis of pulldown of ERα with kinase module. Hi5 cell were infected with kinase module subunits (Med12, Med13, cdk8 and ccnc) and f:ERα viruses and purified with anti-flag M2 agarose. ERα and ccnc enriched in pulldown(PD) compared to other subunits.*

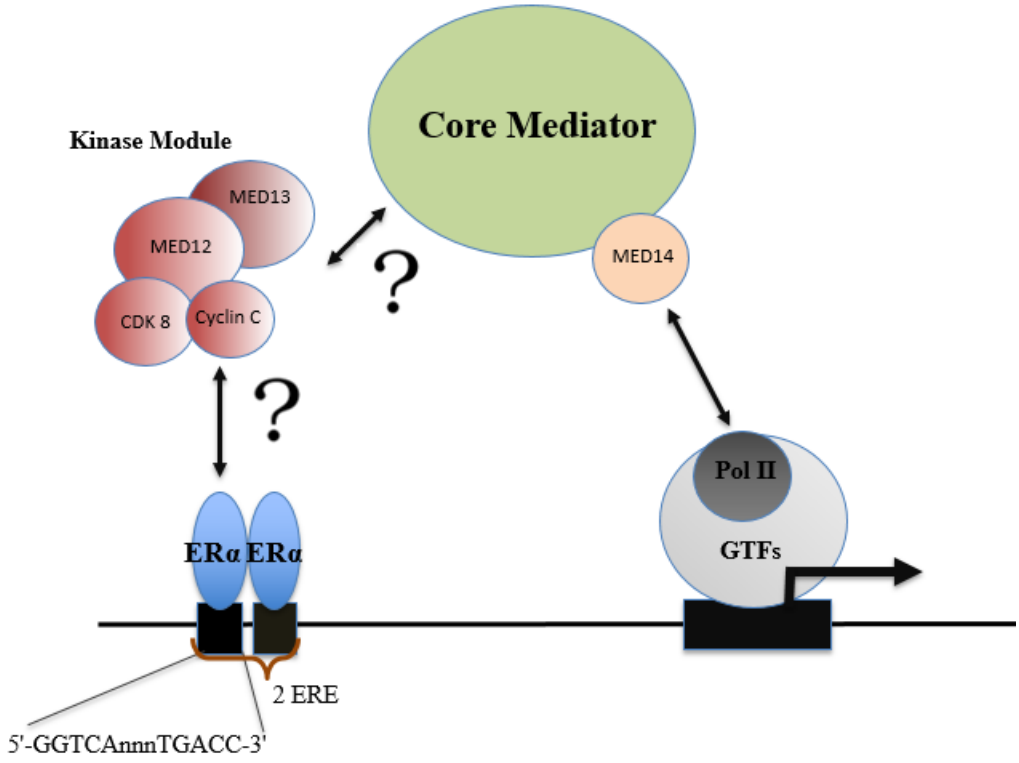



**Figure 6.2: Proposed mechanism for ERα- Mediator interaction.** *Upon binding of ERα to its cognate element, it activates its target genes first by interacting with Mediator complex (with kinase module based on data in Figure 6.1). In turn, Mediator recruits transcription machinery to the target gene promoters and initiate transcription by interacting with Pol II via Med14. The proposed mechanism here identifies a novel interaction between Mediator and ERα unlikely to previously shown models. The question marks depict the future works that will identify the direct interaction between ERα and subunit(s) of Mediator complex and also how kinase module binds to core complex to drive transcription.*

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