POLR2A/RPB1 SUBUNIT OF RNA POLYMERASE II INTERACTS WITH NTD-MED14 CONTAINING CORE MEDIATOR COMPLEX TO FACILITATE BASAL AND ACTIVATOR DRIVEN TRANSCRIPTION

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Javaid Jabbar

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June 2020

We certify that we have read this thesis and 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

POLR2A/RPB1 SUBUNIT OF RNA POLYMERASE II INTERACTS WITH NTD-MED14 CONTAINING CORE MEDIATOR COMPLEX TO FACILITATE BASAL AND ACTIVATOR DRIVEN TRANSCRIPTION

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M.Sc in Molecular Biology and Genetics

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June 2020

The Metazoan Mediator is a 2-MDa protein complex that consist of 30 subunits, most of which are evolutionarily conserved from yeast to humans[8]. The maintenance and regulation of the cell is dependent on spatiotemporal control of RNA polymerase II (Pol II) mediated transcription as a result of intrinsic and extrinsic signals. Perturbations caused by the environment and genetics can alter the fate of the cells and can lead to many diseases such as cancer. The role of Mediator is critical in maintaining the cellular environment as it relays signal to RNA polymerase II to regulate homeostasis, cell growth, cell differentiation and development. Thus, it is essential to understand the mechanism by which Mediator regulates the expression of Pol II genes. We have utilized Multibac expression system to synthesize recombinant protein subcomplexes of Mediator and Pol II subunits to elucidate the interaction surface between core Mediator complex and RNA Polymerase II. Our data indicates that POLR2A (RPB1) subunit of Pol II interacts with ~84 kDa N terminal region of Med14 (NTD-Med14) containing core Mediator complex. Furthermore, we also show that other subunits of Pol II including POLR2C (RPB3), POLR2D (RPB4), POLR2E (RPB5), POLR2F (RPB6), POLR2G (RPB7), POLR2H (RPB8), POLR2I (RPB9) POLR2L (RPB10), POLR2J (RPB11) and POLR2K (RPB12) does not interact with core Mediator complex.

The binding assay also demonstrates that the recombinant RPB1 subunit competes with endogenous Pol II for the interaction with core Mediator, forming a stable RPB1-core

Mediator protein complex. The interaction between RPB1 subunit and NTD-Med14 containing core Mediator complex is independent of Med26. We propose a model for Pol II recruitment to the promoter by core Mediator complex which demonstrates that NTD-Med14 of Core Mediator complex interacts with RPB1 subunit of RNA polymerase II and recruits it to the promoter to facilitate basal and activated transcription.

Key words: Mediator complex, Multibac expression system, RNA polymerase II, RPB1, NTD-Med14, transcription.

ÖZET

RNA POLİMERAZ II'YE AİT POLR2A/RPB1 ALT BİRİMİNİN NTD-MED14 İÇEREN KOR MEDİATÖR KOMPLEKSİ İLE ETKİLEŞİME GİREREK BAZAL VE AKTİVATÖR BAĞIMLI TRANSKRİPSİYONU KOLAYLAŞTIRMASI

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Metazoana özgü Meditör, çoğu alt birimi mayadan insana evrimsel süreçte korunmuş olan ve toplamda 30 alt birimden oluşan 2-MDa moleküler ağırlığında bir protein kompleksidir. Hücrenin kendini idame ettirmesi ve düzenlenmesi, içsel ve dışsal sinyaller sonucunda gerçekleşen RNA polimeraz II (Pol II) aracılı transkripsiyonun zamansal ve uzamsal kontrolüne bağlıdır. Çevresel koşulların ve diğer genetik ifadelerin neden olduğu bozulmalar hücrelerin kaderini değiştirebilir ve kanser gibi birçok hastalığa yol açabilir. Mediatör; hücre büyümesini ve farklılaşmasını, homeostazı ve gelişimi düzenlenmek için RNA polimeraz II'ye sinyal gönderiminden sorumlu olması nedeniyle hücresel ortamın korunmasında kritik bir rol oynar. Bu nedenle, Mediatör'ün Pol II genlerinin ekspresyonunu hangi mekanizmalarla düzenlediğinin anlaşılması önemlidir. Kor Mediatör kompleksi ve RNA Polimeraz II arasındaki etkileşim yüzeyinin aydınlatılması için, Multibac ekspresyon sistemini kullanarak Mediatör ve Pol II alt birimlerini içeren rekombinant protein alt komplekslerini sentezledik. Sonuçlarımız, Pol II'ye ait POLR2A (RPB1) alt biriminin 84 kDa büyüklüğündeki Med14'ü içeren Kor Mediatör kompleksi ile etkileşime girdiğini ve bu etkileşimin Med14 alt biriminin N terminal bölgesi (NTD-Med14) aracılığıyla gerçekleştiğini göstermektedir. Ayrıca, Pol II'ye ait POLR2C (RPB3), POLR2D (RPB4), POLR2E (RPB5), POLR2F (RPB6), POLR2G (RPB7), POLR2H (RPB8), POLR2I (RPB9) POLR2L (RPB10), POLR2J (RPB11) ve POLR2K (RPB12) gibi diğer alt birimlerin Kor Mediatör kompleksi ile etkileşmediği gösterilmiştir.

Yaptığımız bağlanma analizi, rekombinant RPB1 alt biriminin, Kor Meditör ile etkileşime girebilmek için endojen Pol II ile rekabet ettiğini ve böylece stabil bir RPB1-Kor Mediatör protein kompleksi oluşturduğunu da göstermiştir. RPB1 alt birimi ile NTD-Med14'ü içeren Kor Mediatör kompleksi arasındaki etkileşim Med26'dan bağımsız olarak gerçekleşmektedir. Bizim modelimiz Pol II'nin Kor Mediatör kompleksi aracılığıyla promotor bölgesine getirildiğini önermektedir. Bu modele göre NTD-Med14 içeren Kor Mediatör kompleksi, RNA polimeraz II'nin RPB1 alt birimi ile etkileşime girmekte ve bazal ve aktivatöre bağımlı transkripsiyonu kolaylaştırmak için promotor bölgesine toplanmasını sağlamaktadır.

Anahtar Kelimeler: Mediatör kompleks, Multibac ekspresyon sistemi, RNA polimeraz II, RPB1, NTD-Med14, transkripsiyon.

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Abbreviations

AcMNPV	*Autographa californica nucleopolyhedrovirus*
BEVS	Baculovirus expression vector system
CDK8	Cyclin dependent kinase 8
cMed	Core Mediator complex
CTD	Carboxyl terminal domain
CycC	Cyclin C
DNA	Deoxyribonucleic acid
DPE	Downstream promoter element
DSSO	Disuccinimidyl sulfoxide
E.coli	*Escherichia coli*
EM	Electron microscopy
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
GTFs	General transcription factors
kDa	Kilo Dalton
MDa	Mega Dalton
mRNA	Messenger RNA
MTE	Motif ten element
NMR	Nuclear magnetic resonance
NPV	Nucleopolyhedrovirus
NTD	N terminal Domain
PIC	Preinitiation complex
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
RNA	Ribonucleic acid
RPB	RNA Polymerase B
rRNA	Ribosomal RNA
S. cerevisiae	S*accharomyces cerevisiae*

S. pombe	S*accharomyces pombe*
Srb	Suppressor of RNA polymerase B
TAFs	Tata binding protein associated factors
TBP	Tata binding protein
TGA	Transposition of the great arteries
tRNA	Transfer RNA
TSS	Transcription start site

CHAPTER 1

Introduction

The Central dogma of molecular biology dictates the flow of genetic information from DNA to RNA through a process known as transcription. The information is translated into proteins that perform distinct functions[1]. The Eukaryotic transcription begins with the formation of the pre-initiation complex (PIC) that consists of RNA Polymerase II, general transcription factors (GTFs) such as Transcription Factor IIA (TFIIA), Transcription Factor IIB (TFIIB), Transcription Factor IID (TFIID), Transcription Factor IIE (TFIIE), Transcription Factor IIF (TFIIF) and Transcription Factor IIH (TFIIH) and the Mediator Complex. Mediator complex acts as a centralised hub, transmitting the activator/repressor signal to the RNA polymerase II that helps to regulate various functions including transcription and long term epigenetic silencing[2].

1.1 The Mediator Complex

In early 1990s, the Mediator Complex was first identified in yeast independently by two groups; Kornberg and Young[3]. The Kornberg and colleagues used crude yeast fractions that stimulated activator dependent transcription i*n vitro* to isolate Mediator complex[4,5]. On the other hand, the Young and colleagues identified first Mediator genes using yeast genetic screens and coined them as suppressor of truncation of RNA polymerase II Carboxyl-terminal domain (CTD). These genes were termed suppressor of RNA polymerase B (Srb) and the four dominant suppressors; Srb2, Srb4, Srb5 and Srb6[4] were found to be a part of a large multi-subunit complex that was tightly bound to RNA Polymerase II (RNA Pol II)[6]. Later, it was shown that the 20-subunit protein complex containing Srb2, Srb4, Srb5 and Srb6 stimulated *in vitro* transcription[7].

The Metazoan Mediator is a 2-MDa protein complex that consist of 30 subunits, most of which are evolutionarily conserved from yeast to humans[8]. Since the metazoan transcription is more complex than yeast, the homology between metazoan and yeast Mediator complex ranges from 50% for the few most conserved subunits such as Med7

and Med31 to a substantially weaker relationship for the other remaining subunits[8,9]. Moreover, the metazoan Mediator complex contains metazoan specific subunits such as Med26 and Med30. For both humans and yeast, the subunits of the Mediator Complex are organised into four distinct modules namely head, middle tail and the kinase module (Figure 1)[2]. The components of the head and middle modules are known to be tightly associated with each other to form a stable core complex and interact with RNA Pol II machinery. On the other hand, the subunits of the tail module are loosely associated with each other and are target for many activators[10,11]. The subunits in the kinase module are mobile and associate reversibly with the Mediator complex enabling repressive properties of the protein complex[11].

Over the past years, many studies have been conducted to determine the structure and architecture of the Mediator Complex. However, the large size, heterogeneity and multiple subunit configuration makes it very challenging to determine its' structure at high resolution. Early electron microscopy (EM) studies laid out a low resolution architecture of Mediator complex as well as the Mediator-Pol II holoenzyme complex. It also identified the modules within the yeast Mediator complex; Head, Middle and Tail[22-24].

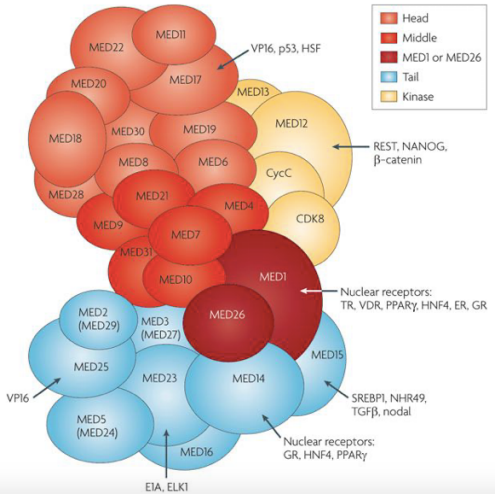

Figure 1: The Mediator complex organised within modules and its interactions with various factors adapted from Malik et al[2].

1.1.1 The Head Module

The head module of S*accharomyces cerevisiae* Mediator consists of seven subunits; Med6, Med8, Med11, Med17, Med18, Med20 and Med22. Together with the middle module, it is known to play an essential role in the assembly of preinitiation complex[12]. In 2006, the seven subunit head module from S*accharomyces cerevisiae* was recombinantly expressed in insect cells[13] that enabled first negative stain analysis by EM[14]. Later after five years, the crystal structure of head module of S*accharomyces cerevisiae* was determined at 4.3 Å resolution[15]. This structure resembled a wrench containing three major domains that were named as neck, fixed jaw and movable jaws[13-15]. The structure of the head module of *S. pombe* at 3.4 Å resolution confirmed the wrench like organisation of the subunits of the head module[16]. The head module is stabilised by the neck domain formed by five subunits; Med6, Med8, Med11, Med17 and Med22[17]. Moreover, these studies suggest that upon recombinant expression of the individual subunits, the proteins tend to be insoluble but constitute a soluble head module when co-expressed together[18-22].

The structure determination of the head module was the first milestone towards the characterisation of the Mediator at high resolution. The year 2014 was a very important year for the Mediator studies as two important studies were published which reported the cryo-electron microscopy (cryo-EM) data on Mediator complex, completely redefining its' modular organisation[23,24]. Previously, the head module was allocated on one side and the middle and tail folded on top of each other to form the opposite side of the Mediator structure[25]. These studies revealed the cryo-EM structure of the Mediator Complex at 20-40 Å resolution showing that the head and middle modules were forming the structure on the Mediator complex that was previously thought to be the middle and the tail module. Moreover, the large opposite domain that was thought to be the head module corresponded to the tail module of the Mediator[23,24]. A low resolution structure of human Mediator complex was also obtained that confirmed the similarity between human and yeast Mediator despite their evolutionary differences. Furthermore, the unassigned subunits of the Mediator complex that were metazoan specific such as

Med27, Med 28, Med29 and Med30 showed multiple contacts with the head module whereas, Med26 associated with the middle module of the Mediator complex[23]. For the very first time, a recombinant head module of the human Mediator complex was recombinantly expressed in the insect cells with an addition of a metazoan specific subunit Med30, making it a 8 subunit head module. Moreover, a functional 15 subunit core-Mediator was reconstituted that consists of Head and middle module being held together by Med14[10].

1.1.2 The Middle Module

The yeast middle module consists of 8 subunits that includes Med1, Med4, Med7, Med9, Med10, Med19, Med21 and Med31. In metazoan, there is an additional subunit Med26 that is a part of the middle module. Until 2017, the structure of the middle module was unknown and the information was limited to two small individual sub-complexes known as Med7N/Med31[26] and Med7C/Med21[19]. In 2010, Cramer and colleagues used the heterologous co-expression strategy to purify a 7-subunit middle module that lacked Med19 similar to the previous 7 subunit endogenous middle module from Δmed19 strain[27]. The protein interaction map formed at that time was very limited and solely based on the previous published data[28-31] and pulldown experiments[27]. Later, cross-linking experiments of six subunit middle module lacking Med1 was co-expressed in bacteria that enabled a three-dimensional model of the middle module of Mediator complex[32]. In 2014, middle module of human Mediator complex was reconstituted using insect cells that consist of five subunits; Med4, Med7, Med10, Med21 and Med31 respectively[10]. These studies did not reveal three dimensional structural data of middle module of the human Mediator complex. The available structural data of middle module of human Mediator is limited to Med26 N terminal domain serving as a an overlapping docking site for ELL/EAF family containing super-elongation complexes and TFIID[33].

1.1.3 The Tail Module

The least conserved subunits of the Mediator complex between yeast and human lie in the tail module. Tail module of *S. cerevisiae* is composed of Med2, Med3, Med5, Med15 and Med16. Among these subunits, Med15 and Med16 are the most conserved subunits. In metazoan module, three more subunits including Med23, Med24 and Med25 exists among which Med24 is known to be a divergent ortholog of Med5 of the yeast Mediator Complex. Moreover, there is evidence which indicates that Med 27 and Med29 are also distant orthologs of Med3 and Med2 of yeast Mediator complex, respectively[9]. Thus, these subunits are referred as Med2/29, Med3/27 and Med5/24 in the literature. More studies are required for their specific assignment alongside with the other metazoan specific subunit Med28 and Med30. Med27, Med28, Med29 and Med30 has previously shown to make numerous contact with head module. Whereas, in *S.pombe*, Med27 shows a tail connection[34]. Med14 plays a key role in the architectural backbone of the Mediator as it makes numerous contact with tail as well as head and middle module[10,23,35].

The heterogeneity in the conformation of the tail module has rendered the structure to be unresolved[36]. One of the study reported a rotation in the tail module of *S. cerevisiae* in the presence of transcription factor Gcn4[23]. It has been hypothesised that these structural transitions take place, making a stable association of Mediator with the transcription machinery[37]. Thus, it can be extrapolated that such transcription factors that are capable of inducing changes in the conformation of Mediator might have a critical role in regulating the transcription process. The first structure of the largest subunit of tail module of Mediator was fully characterised in 2018. The crystal structure at 2.8 Å resolution paved the way for better understanding of Mediator with activators[38]. As reported previously, the truncations in the C terminus of Med14 resulted in loss of interaction between the tail subunits of Mediator[23,35]. The precise organisation of metazoan tail subunits is not yet obtained despite the fact that Med16, Med23 and Med24 has been reported to form a stable sub-complex[39,40]. Many studies have reported the interaction of various activators with the tail module of the Mediator[41]. The nuclear magnetic resonance (NMR) analysis has identified the interactions between the activator binding domain of the tail subunits of Mediator with transactivation domains of several

transcription factors. These include VP16/Med25[42,43], sterol regulatory element binding protein/Med15[44], Gcn4/Med15[45,46], and ATF6α/Med25[47]. A detailed structural architecture of the tail module is required to have a deeper insight into activator mediated transcription of Mediator complex. This would enable the development of specific drugs to interfere the interaction surface of the Mediator-Activators[48] and cure diseases such as cancer.

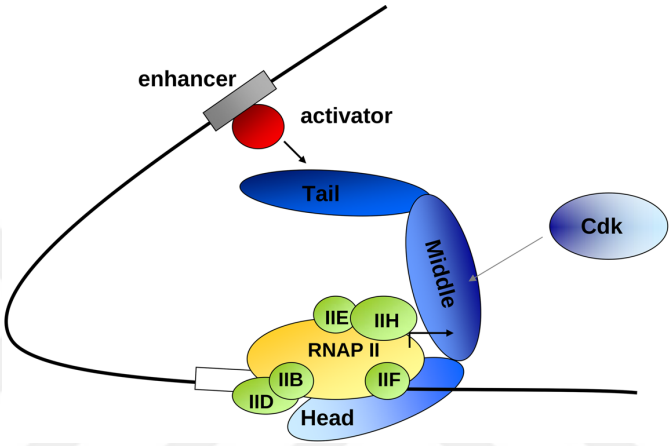

Figure 2: The Mediator acts as a transmission hub, transmitting activator information to the transcription machinery adapted from Toth-Petroczy et al[49].

1.1.4 The Kinase Module

The four subunit kinase module comprising of CycC, CDK8, Med12 and Med13 dissociates reversibly from the head, middle and tail of the Mediator complex[50]. The kinase module is primarily known for its repressive function but some suggest that it can take part in activation of transcription as well[52]. The dissociation of kinase module is essential for the PIC assembly such that Mediator can join the PIC complex[52].The structure of two subunit of kinase module of human Mediator has been determined; CDK8 and CycC, respectively[53]. However, no structural data regarding Med12 and Med13 exists. The basic structure of yeast kinase module was determined at 15 Å using cryo-EM analysis in 2013. It showed that Med12 is situated at the centre forming a central lobe, connecting Med13, CDK8 and CycC on the opposite end[54-55]. Structural data available at the moment suggests that Mediator with kinase module does not interact with the RNA polymerase II[56]. High resolution cryo-EM analysis is required to

determine the inhibitory surface that prevents the Mediator from interacting with RNA Polymerase II.

1.1.5 The Core Mediator

The term Core Mediator has been used in a variety of context in the past. However, it is dedicated to the minimum number of Mediator subunits required that are essential to induce transcription. These subunits belong to head and middle modules with an addition of Med14[10,57]. One of the largest subunit of the Mediator complex, Med14 was characterised to be a part of middle or the tail module[58-60]. Subsequent studies revealed that Med14 had numerous contacts with various subunits of middle module that includes Med1, Med4, Med7, Med9 and Med21 and for the tail module including Med2, Med3, Med15 and Med16. As a result, it was hypothesized that the role of Med14 is very central in connection to different modules of the Mediator. It was confirmed in 2014 when a study demonstrating Cryo-EM analysis of the yeast Mediator at 18 Å resolution revealed the role of Med14 as a scaffold protein[23,24]. It was also found that the critical role of Med14 as a scaffold protein exist in human Mediator as well thus, it is a conserved function[10]. At 3.4 Å resolution, the Cramer laboratory reported the crystal structure of *S.pombe* 15-subunit core Mediator complex (cMed) in 2017. The core structure was divided into 13 submodules; 8 submodules in the head and 5 in the middle module. It was also shown that N terminus of Med17 and C terminus of Med6 interacts with Med14 that brings the head and middle module together[61]. As a result of these studies, our understanding of the Mediator structure has improved. However, The organisation of Med1 within the Mediator could not be confirmed and remains a mystery to be resolved[10,61].

1.2 The Role of Mediator Complex in Human Diseases

The maintenance and regulation of the cell is dependent on spatiotemporal control of RNA polymerase II mediated transcription as a result of intrinsic and extrinsic signals[62]. Perturbations caused by the environment and other genetic comments can alter the fate of the cells and can lead to many diseases such as cancer. The role of Mediator is critical

in maintaining the cellular environment as it relays signal to RNA polymerase II (Figure 2) to regulate homeostasis, cell growth, cell differentiation and development[2,64]. The aberration in Mediator complex subunits has known to cause many diseases such as cancer, neurodevelopmental disorders, and cardiovascular diseases[62]. Charcot-Marie tooth disease also known as hereditary motor and sensory neuropathy is a peripheral neuropathy that is commonly inherited[64]. A missense mutation in MED25 gene (A335V) has been implicated as a likely cause of this disease. As a result, it disrupts the Med25-Mediator interface necessary for transcriptional regulation of genes pertaining to function of peripheral nervous system[62]. Another missense mutation L371P in MED17 gene is associated with infantile cerebral and cerebellar atrophy[65]. This mutation was identified as a result of screening of 79 individuals of Caucasus Jewish origin. However, none of the 113 Arab Muslim or 110 Ashkenazi Jewish carried this mutation, signifying that it is a founder mutation in Caucasus Jewish community[66]. The missense mutations R961W and N1007S in Med12 gene results in syndromal X-linked mental retardation including FG and Lujan syndromes[67,68].

Approximately 1% of the live births exhibit congenital heart disease, making it the most common birth defect in humans. The most common cyanotic heart defect in new-borns is the transposition of the great arteries (TGA) that account for 7% of all the congenital heart diseases[69].With regard to the disease, MED13L, a paralog of MED13 was identified in a patient with TGA. Analysis of 97 additional patients revealed 3 mutations in MED13L that were categorised as missense. These mutations are E251G, R1972H and D2023G that were absent in 400 control samples[70]. DiGeorge syndrome occurs in approximately 1 in every 3000 live births and is categorised as the most recurrent multiple congenital anomaly syndrome[71]. It results in cardiac defects, immune dysfunction, psychoses, schizophrenia, palatal anomalies, characteristic facial dysmorphism, and hypocalcaemia[72]. This disease is caused by the deletion of approximately 3Mb region on chromosome 22 that contains 60 genes including the MED15 gene[73]. Med15 in humans is categorised as transducer of SREBP1α and TGFβ-activated SMAD2/3 that regulates the metabolism of lipid and developmental programs[44,74]. The critical function of Med15 in these signal transduction pathways

indicates that 22q11.2 deletion syndrome/DiGeorge syndrome can be the result of reduced expression of Med15[62].

The Mediator complex plays a wide role in several signalling pathways regulating growth, differentiation and development. Many developments have been made recently that linked its' subunits to variety of cancers. The Med1 subunit of the Mediator was among the first subunits to have a clear link established with the breast cancer. Breast cancer is the most common type of cancer diagnosed in women and is the leading cause of cancer related death worldwide among women[75]. Animal studies in the past have revealed that steroid hormone estrogen (17-β-estradiol; E2) induces and promotes breast cancer therefore, using drugs to oppose these affects or using estrogen ablation therapy can reduce the severity of breast cancer[76,77]. The effects of E2 are mediated through two receptors that are similar in structure; estrogen receptor α (ERα) and β (ERβ) respectively[78]. It is estimated that 70% of the breast cancer patients are scored positive for ERα upon diagnosis[79], hence a valuable target for breast cancer therapy. *In vivo* chromatin association studies and *in vitro* transcription based assays identified the recruitment of Mediator by ERα to promote the formation of pre-initiation complex and transcription of ERα target genes[80-83]. Moreover, mutant mice for defective Med1-ERα-binding resulted in mammary gland development defects and reduced expression of ERα responsive genes[84]. This indicates that Med1 of the Mediator complex is a critical co-activator of ERα. The study by Zhu et al, investigated the role of Med1 in ERα positive breast tumours and found that Med1 mRNA was overexpressed in more than 50% of the breast tumour[85]. Another study by Vijayvargia et al determined that Med1 is overexpressed in approximately 50% prostate cancer, indicating its' role in the progression of prostate cancer[86]. Apart from Med1, Med28 has also been found to be associated with breast cancer. Med28 expression analysis on samples from breast cancer patients based on clinicopathological variables, histopathological subtypes and disease outcome exhibited an increase in protein level in ductal and invasive ductal carcinoma of the breast tissue as compared to non-malignant breast epithelium of ductal and glandular origin. Furthermore, the expression level of Med28 protein also correlated with the disease outcome signifying a higher expression of the protein with elevated risk of death

in early and late stage of breast cancer[87], demonstrating its role as a critical prognostic marker.

Colon cancer is among the leading causes of cancer related death. It is the second most common type of cancer in women and third most common among men[75]. Colon tumours originate in the intestinal crypts, where the progenitor derived epithelial cells start differentiation and ascend towards the intestinal villi[88]. The homeostasis of crypt progenitor phenotype depends on gene expression of Wnt/β-catenin pathway. When the pathway becomes constitutively active, it provides the driving force for the proliferation of intestinal epithelial cells that results in colon cancer[88-91]. The kinase module of the Mediator was thought to be involved in Wnt/β-catenin signalling as the C terminal transactivation domain of β-catenin targets Med12 in Mediator complex to activate transcription[92]. The architecture of the Mediator complex links Cdk8/CyclinC to the Med12 since the depletion of Med12 leads to reduced level of CDK8/CyclinC incorporation to the Mediator complex[93]. The biochemical assays suggests that Med12 activates the Cdk8 kinase[94]. The study by Firestein et al demonstrates the important modulators of β-catenin pathway for colon cancer. Based on the high throughput RNAi, loss of function screens were conducted to determine kinases and phosphatases essential for the transactivation of β-catenin and the proliferation of colon cancer cells. The study found 9 candidate genes that were critical for both functions. However, only Cdk8 was found to be residing in the region of copy number gain in a notable proportion of colon cancer. It was also found that Cdk8 activity was required for colon cancer proliferation, β-catenin mediated gene transcription and cell transformation[95]. As shown earlier, β-catenin does not bind to CylinC or Cdk8 but instead binds directly to Med12[92]. Thus, it is very likely that the transduction of β-catenin signalling is mediated through Med12 to Cdk8. Another study by Kapoor et al revealed Cdk8 role in melanoma progression[96]. Although not very common, melanoma is categorised as the most deadly type of skin cancer with approximately 4% of skin cancer cases and 75% skin cancer related deaths[97]. The identification of macroH2A (mH2A) knockdown cell lines gene expression revealed Cdk8 as a mH2A repressed gene, which indicates that it can be a possible mediator of melanoma malignancy[96]. Further investigations are required to fully

understand the role of individual Mediator subunits in order to develop therapeutic to treat wide range of human diseases.

1.3 RNA Polymerases

The expression of protein coding genes is pivotal for the biological processes carried out within the cell which is regulated predominantly at the level of transcription. Sam Weiss is the first discoverer of the RNA polymerase activity as he showed the incorporation of all four nucleoside triphosphate into a nucleotide in rat liver nuclei in the year 1959[98]. In 1961, Jacob and Monod published the paper on lac operon that explained the landmark of mechanism and regulation of transcription[99]. Although many studies about bacterial RNA polymerase were being conducted in the late 1960, RNA polymerase was purified to homogeneity first time in 1969 by Richard Burgess who later showed that the dissociable sigma subunit was regulating the initiation of transcription[100]. During that time, the understanding of eukaryotic transcription was very limited. The biggest milestone in understanding the eukaryotic mechanism of transcription was reached in 1969 with the discovery of three distinct classes of RNA polymerases by Roeder and colleagues[101].

1.3.1 RNA Polymerase I

The RNA polymerase I (Pol I) synthesises approximately 60% cellular RNA as it transcribes many copies of ribosomal RNA (rRNA) genes. A microscopic structure that depicts a 'Christmas tree' is produced as each gene of rRNA is produced by many Pol I enzymes[102]. RNA polymerase I regulates the levels of ribosomal components and cell growth[103]. Furthermore, deregulation of Pol I results in several diseases that includes cancer[104]. Pol I is a 590 kDa enzyme that consists of 14 subunits[105]. Several attempts had been made to solve the structure of Pol I. In 2013, Christoph et al deciphered the crystal structure of yeast *Saccharomyces cerevisiae* Pol I at 2.8 Å resolution. The purified Pol I was active in both DNA templated RNA extension and cleavage. The structure consist of a Pol I dimer that contains 8681 amino acids, lacking the mobile A49 WH domain

and some other surface loops[105]. The Core of Pol I consist of 10 subunits; A190, A135, AC40, AC19, A12.2, Rpb5, Rpb6, Rpb8, Rpb10, and Rpb12[106]. Furthermore, RNA polymerase I and III contain a TFIIF like sub-complex which stabilises Pol II. In addition to that, Pol I and II also use TFIIB like factors and contain TFIIE related domains. This indicates that the core of transcription limitations of Pol I, II and III is conserved both functionally and structurally[106].

1.3.2 RNA Polymerase II

The RNA polymerase II (Pol II) is a 12 subunit enzyme that transcribes mRNA from the protein coding genes. It regulates many cellular processes such as cell differentiation, homeostasis, and maintenance of cell identity. These regulations occur throughout the transcription process with most important one being the initiation stage of transcription. It is fundamental to understand the structure of the Pol II initiation complex to be able to understand the mechanism by which Pol II regulates gene transcription[107]. The general transcription factors TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, along with the Pol II and Mediator complex assemble at the Promoter DNA to form pre-initiation complex (PIC)[2].

About two decades ago, the structural analysis of PIC started. The free form structures of Tata binding protein (TBP)[108,109] and DNA bound[110] demonstrated that TBP is a molecule shaped like a saddle that binds to the minor groove of DNA and bends it by 90 degrees. Further investigations revealed that TFIIA and TFIIB transcription factors flank the DNA-TBP complexes[111,112]. The complete structure of 12 subunit TFIIB bound Pol II was elucidated in 2009 which highlighted the location of TFIIB on Pol II wall. Consequently, a model of Pol II-TFIIB-TBP-DNA complex in an open and closed promoter DNA conformation was established[113]. In 2013, TFIIE, TFIIF, and TFIIH were added to Pol II, TFIIB, TBP, DNA and TFIIA containing human PIC and visualised using electron microscope[114]. The structure appeared to be quite similar to the existing yeast core initiation complex. Moreover, it showed the TFIIE location on the clamp domain of Pol II, which was coherent with the homologous yeast factor location[115,116].

The specific binding of the Tata-binding protein to TATA box is the first step in the canonical assembly of PIC. In humans, the consensus sequence of the TATA box is located approximately 30 base pair upstream of the transcription start site and is denoted by TATAWAWR[117]. The consensus sequence of the TATA box is conserved from yeast to humans as the evidence suggests[118]. A variety of promoters lack the TATA boxes and are referred as TATA-less promoters. In yeast, TBP along with other general factors are known to be bound throughout its genome, suggesting that the architecture of the PIC overall is similar for both TATA box containing and TATA-less promoters[119-120]. The mode of binding of TBP in yeast[121], plant[110] and human[122] is highly conserved as exhibited by the structures formed by TBP bound to the TATA box containing DNA. The factor TFIIA stabilises the TBP-DNA complexes but is not essential for basal transcription[123]. It is a Pol II specific transcription factor that enables constitutive and activator driven transcription[124]. The structure of human[125] and yeast[18] TFIIA-TBP-DNA complexes showed a boot shaped TFIIA heterodimer which does not change the structure of TBP-DNA[112]. There are two conserved domains in TFIIA; a 4-helix bundle and 12 stranded β-barrel that enables TFIIA to bind underside of TBP saddle and the upstream region of TATA box, stabilising the TBP-DNA complex[126].

One of the fundamental factor required for the recruitment of Pol II to the promoter is the transcription factor TFIIB[127,128]. It also promotes the binding of TBP to DNA and enables the bending of DNA[54]. The N and C terminal domains of TFIIB are responsible for the recruitment of Pol II and the interaction of TBP with Promoter, respectively[129].TFB is a TFIIB homologue in archaea. TFB and TBP are the only required initiation factors for the transcription system in archaea[130]. The Pol II -TFIIB complex structure elucidated the role of B-ribbon of TFIIB in binding to the docking domain of Pol II in order to recruit it to the promoter[131]. These structure also reveal the region of TFIIB that B-ribbon and B-core domains transversing the cleft of Pol II, forming B-reader and B-linker[113]. If you follow the N-terminus of B-ribbon, the polypeptide chain goes along with the exit channel of RNA on Pol II and continues towards the opposite direction of exiting RNA all the way to the cleft and forms a B-reader and B-linker helix. The B-linker helix enables opening of DNA and maintenance

of the transcription bubble[113]. The B-reader binds to the template DNA strand and position it for synthesis of RNA chain, and it helps with the recognition of the initiator sequence on the DNA[132]. In general, TFIIB initiates the Pol II mediated RNA synthesis and stabilises the initiation complex that contains a five nucleotide RNA strand[131].

TFIIF is another transcription factor that was previously identified in mammalian cells due to its interaction with Pol II[133]. It is a heterodimer consisting of TFIIFα and TFIIFβ also known as RAP74 and RAP30, respectively[134]. In yeast, Tfg1 and Tfg2 are homologous pairs of TFIIFα and TFIIFβ, respectively[135].Furthermore, yeast contains an additional TFIIF element Tfg3 that is not required for transcription[136]. In yeast, about 50% of Pol II is coupled to TFIIF[137]. TFIIF stabilises PIC[138] and prevents the non-specific interaction of Pol II with DNA[139]. Moreover, it also plays a role in the selection of TSS[140], prevents Pol II pausing[94], promotes formation of phosphodiester bond and early synthesis of RNA[141]. TFIIF also stabilises the transcription bubble and plays a very important role in transcription as it can be initiated to an extent in the absence of TFIIE and TFIIH *in vitro*, but it cannot in the absence of TFIIF[142]. TFIIF has several domains and most of their structures have been determined. The N terminal region of both TFIIFα and TFIIFβ dimerises, forming a triple barrel fold and a β-hairpin that is termed as an 'arm'[143]. The winged helix domain is located at the C terminal end of both subunits[144]. The winged helix domain connect to the dimerization module using a charged region on TFIIFα and linker region on TFIIFβ[145,146]. The structural studies elucidated that the dimerization module of TFIIF anchors it to Pol II and binds to the lobe of Pol II on one side of the cleft[116,145,147]. The winged helix domain of Tfg2 is found upstream of DNA[148].This domain is located close to DNA in the initiation complex and is mobile in Pol II- TFIIF complex[145]. Nevertheless, the concurrent data does not support the existing EM model of Pol II-TFIIF complex[149], thus, more studies are required to get a better understanding of Pol II-TFIIF complex.

TFIIE and TFIIH are among other essential factors for PIC formation and are critical for the opening of promoter DNA. The TFIIH factor encompasses DNA dependent ATPase activity that is critical for the initiation of transcription[150-151]. Furthermore, studies

involving transcription assays using linear, supercoiled or mismatched DNA demonstrated that TFIIH is involved in the opening and escape of Promoter DNA[152-153]. The TFIIE factor acts like a bridge between TFIIH and Pol II as it helps in to recruit TFIIH to initiation complex[153,154]. TFIIE is a heterodimer comprising of two subunits TFIIEα and TFIIEβ[155]. These subunits are distant homologs of bacteria initiation factor sigma[156]. The N terminus of TFIIEα contains winged helix domain and a zinc finger domain, which is enough to interact with TFIIEβ to facilitate transcription[157]. The cross-linking experiments pertaining to TFIIEβ suggests that it is located upstream in the proximity of transcription start site[158].TFIIE binds to the clamp domain of Pol II[116], which is similar to archaea homolog TFE[159]. The 10 subunits transcription factor TFIIH comprise of a six subunit core module; ATPase XPD, p62, p52, p34, p8, and p44 which are known as Rad3, Tfb1, Tfb2, Tfb4, Tfb5, and Ssl1 in yeast, respectively. ATPase XPB which is known as Ssl2 in yeast and a kinase module consisting of three subunits CDK7, cyclin H and MAT1 known as Kin28, Ccl1 and Tfb3 in yeast[160,161]. For transcription to occur, a complete 10 subunit TFIIH is required but the DNA repair does not require the kinase module of TFIIH[162]. The mutations in the catalytic ATPase subunits XPB and XPD are associated with diseases such as Cockayne syndrome, xeroderma pigmentosum and trichothiodystrophy[163]. While XPB is required for the opening of promoter during transcription *in vitro* and *in vivo*[164,165], XPD is essential for opening of DNA during DNA repair[166]. There are many subunits of TFIIH for which the structures are available. These include cyclin H, CDK7, a part of MAT1, and the homologs of XPB and XPD in archaea[167-169]. The structural information identified the location of TFIIH within the PIC and it demonstrated the mechanism by which TFIIH opens the DNA. TFIIH rotates the DNA from a fixed protein complex at the TATA box acting like a ‘wrench’, hence it creates sufficient torque to melt the DNA[170].

Lastly, the transcription factor TFIID is multifunctional transcription factor that is conserved and plays a role in the promoter recognition, assembly of PIC and chromatin remodelling[171]. TFIID is approximately 1.2MDa protein complex, consisting of TBP and 13-14 TBP associated factors (TAFs), among which 13 are conserved throughout yeast and humans[172]. Studies using yeast have demonstrated that TFIID contributes

significantly to most of the genes transcribed by Pol II[173]. The primary role of TFIID is the recognition of core promoter sequence. Previously, it was thought that the recruitment of TFIID to Pol II promoters was through TATA binding activity of TBP. However, only 10-20% of human and yeast promoters contain a TATA box[174]. Investigation of core promoter sequence identified many core promoter elements that are being recognised by TAFs[175], including motif ten element (MTE) and downstream promoter element (DPE) which were discovered in *Drosophila melanogaster* and were later attributed as human promoters[176-178]. The TFIID can fine tune its binding to DNA using different composition of elements. The structural studies for holo-TFIID has been very challenging due to large size, low amount of intact complex and heterogeneity in subunit composition. A recent study was published that recombinantly expressed core TFIID complex[179] and enabled the structure to be resolved at 12 Å resolution. The gaps in the structure were filled with the existing data about the TFIID structure[107]. All in all, the transcription of Pol II genes is a complex process that relies on a variety of proteins and protein complexes that work in a cooperative manner to locate the TSS and induce transcription.

1.3.3 RNA Polymerase III

The RNA polymerase III (Pol III) is responsible for transcription of majority of RNAs in the eukaryotic cells that includes transfer RNAs (tRNAs) and many other noncoding RNA that are associated with ribosomes and protein synthesis[180]. RNA polymerase III consists of 10 subunit core that is a common feature of all nuclear RNA polymerases. The transcription of tRNA genes via Pol III requires TFIIIB; a 3 subunit initiation factor that consist of TBP and TFIIB paralog Brf1 and Bdp1, and TFIIIC; a six subunit assembly factor that binds to intragenic A and B block promoter elements. The 17 subunit Pol III is recruited to stably assembled TFIIIB upstream of TSS[181]. Transcription of ribosomal RNA requires a different approach as the assembly of PIC on 5S RNA gene recruits an additional factor TFIIIA that guides the binding of TFIIIC. In metazoans, a third class of Pol III promoters are found that do not rely on intragenic sequence elements thus, the assembly of initiation complex relies on SNAPc instead of

TFIIIA and TFIIIC. Transcription of SNAPc mediated genes uses Brf2 (different paralog of TFIIB) in the initiation factor[182183]. Since the late 1970, many studies had been focused on the ability of yeast to respond to nutrient level by altering ribosome and tRNA synthesis[184,185]. It became more clear upon the identification of Maf1 which is a master regulator of transcription in-response to variable nutrition, cellular stress and change in environment[186,187]. Although Maf1 knockout yeast cells are viable, its' ability to repress Pol III mediated gene transcription was hindered drastically. This process is conserved in higher eukaryotes as MAF1 is essential for Pol III mediated transcription repression in mammalian cells upon serum starvation, treatment with DNA damaging agents and TORC1 inhibitors[188,189]. There is abundant evidence that deregulation of Pol III transcription machinery can causes complex diseases including cancer. Since the tumour cells proliferate faster as compared to normal cells, up-regulation of tRNA levels and its' iso-acceptors is observed in breast cancer and myeloma cell lines when measured using microarray[190,191]. It is important to study nuclear RNA polymerases as they play a vital role in regulating critical processes within the cell and understanding the structure would shed light into the mode and mechanism by which these enzymes carry out their function in a cooperative manner.

1.4 Baculovirus Expression System

The baculoviruses are also known as nucleopolyhedrovirus (NPV) as they form inclusions in the nucleus of the infected cells. These viruses are widely used in pest control and production of recombinant proteins. The baculovirus expression vector system also known as BEVS was developed by two laboratories in the early 1980[192,193]. A mixture of viral DNA were co-transfected along with a donor vector to Sf21 cells to replace the polyhedrin gene from *Autographa californica nucleopolyhedrovirus* (AcMNPV) to generate recombinant viruses which were isolated from the viral plaques[192]. The technology has improved significantly since then as many improvements have been made to obtain high yield of expressed proteins.

The most important breakthrough in the generation of recombinant baculoviruses was made in 1993 upon development of a vector that contained the entire genome of AcMNPV (referred as bacmid) and could be propagated in *Escherichia coli* (E.coli)[194]. This is known as Bac-to-Bac® system which is now commercialised by Invitrogen. In this system, the shuttle vector containing gene of interest is introduced into the genome of bacmid in the host bacteria using the transposition of shuttle vector via Tn7-recombinase. There are many advantages of this system such as selection and isolation of bacmids is easier and high yield of recombinant baculovirus[195]. Moreover, more than one protein could be expressed using this technique. Previously, co-infection of multiple baculoviruses was required to express multiple proteins[196]. However, this method was very inefficient since simultaneous infection of all baculovirus cannot be guaranteed[197]. The problem was overcome by development of new shuttle vectors that can accept more than one gene. These vectors include pFastBacDual (Invitrogen) that are used to express two protein and the Multibac system that can facilitate the expression of multi-subunit protein complexes[198]. Other vectors include *flash*BAC™ system; a combination of bacmid technology and insect homologous recombination for the generation of baculoviruses[199], OmniBac vector that can use both homologous recombination and bacmid technology to generate baculoviruses[200], Bac-2-the-Future that has the essential features of Bac-to-Bac system but it reduces the time and labour to generate viruses[201], and biGBac[202] that utilises Gibson assembly method[202] to assemble many DNA fragments to generate baculoviruses.

The recombinant proteins expression can be attenuated by promoters, enhancers or regulatory cis elements. Most widely used promoters used for production of recombinant proteins using BEVS are p*olh* (polyhedrin) and *p10*. These two promoters are very strong corresponding to high rate of transcription but start expressing proteins at a later stage of infection[203]. More recently, a combination of two promoters is being utilised to increase the yields of protein. One such example would be combination of *polh* with *vp39* or *p*SeL[204,205]. There are many insect cell lines that are used for the expression of recombinant proteins. The most widely used ones are IPLB-SF21-AE (known as Sf21) derived from pupal ovarian tissue of *Spodoptera frugiperda*[206], Sf9 which is a subclone

of Sf21 and BT1-Tn5B1-4 (commonly known as Hi5) derived from adult ovarian tissue of *Trichoplusia ni* [207]. The choice of cell line depends on the type of protein that is going to be expressed. For example, Sf21 and Sf9 are highly vulnerable to viral infection therefore high titre of virus can be obtained. These cells can be grown as monolayers without CO_2 at 27°C or as a suspension and are adaptable to serum free media[208]. The Sf9 cells as compared to Sf21 cells grow faster and in high density, are more tolerant to sheer stress, pH and osmotic changes[209]. The Hi5 cells are usually used for protein production as they are more resistant to nutrient stress and expresses recombinant proteins in high level due to its large size. A limitation of using this cell line is that as compared to Sf9 and Sf21, it produces three times more proteases which results in degradation of the target protein[210]. New improvements in the engineering of BEVS and host cell lines are being made to enhance the quality and quantity of target proteins. Advancements in genome editing techniques such as CRISPR-Cas9 will enable induction of specific mutations, deletions and insertions to improve the expression of recombinant proteins using baculovirus expression system[211].

1.5 The aim of the study

The primary aim of the study was to identify the interaction surface between human Mediator complex and RNA polymerase II in order to elucidate the mechanism of transcription of RNA polymerase II mediated gene transcription. Multibac expression system was utilised to express the proteins and perform biochemical assays to demonstrate the interaction between core Mediator complex and critical subunits of RNA polymerase II responsible for the interaction with the Mediator. Consequently, minimum subunits of Pol II-core Mediator has been identified that might be facilitating transcription hence, highlighting the critical role of Mediator in Pol II mediated gene transcription.

CHAPTER 2

Materials and Reagents

2.1 Materials for Cell culture, buffers, reagents and glassware

Product Name	Manufacturer	Catalogue Number
DMEM, powder, high glucose, pyruvate	Gibco	12800017
DMEM low glucose, without phenol red	Gibco	11880028
HyClone DMEM/High glucose with L-glutamine	GE Life sciences	SH30022.01
Trypsin-EDTA (0.5%), no phenol red	Gibco	15400054
Fetal Bovine Serum (FBS) heat inactivated	Biowest	S181H-500
Grace Insect Medium, supplemented	Gibco	11605086
Gentamicin (50 mg/mL)	Thermofisher Scientific	15750060
Express Five™ SFM	Gibco	10486025
Sf-900 II SFM	Gibco	10902104
Poloxamer	Sigma-Aldrich	16758
Penicillin/Streptomycin	Gibco	15140-122
Cellfectin II Reagent	Invitrogen	10362-100
Insulin solution from bovine pancreas	Sigma	I0516
Nonessential amino acid (NEAA)	Lonza	BE13-114E
Tamoxifen	Sigma-Aldrich	T5648-1G
10X PBS pH 7.4	Self made	
Corning® tissue-culture treated culture dishes D × H 150 mm × 25 mm	Corning™	430599
BioLite 6 Well Multidish	Thermofisher Scientific	NC-130184
cell culture dish 100X20 mm	Greiner	664160
minisart syringe filter	Sartorius	16555
spinner flask	Wheaton	
2M Calcium Chloride	Self made	

Table 1. List of items used for insect and mammalian cell culture.

2.2 Buffers for protein extraction and purification.

BC1000	BCO
40mM Hepes pH:7.6	40mM Hepes pH:7.6
1M KCl	10% Glycerol
10% Glycerol	4mM MgCl2
4mM MgCl2	0.4mM EDTA
0.4mM EDTA pH 8.0	0.5mM PMSF
0.5mM PMSF	0.5mM DTT
0.5mM DTT	

Table 2: Buffers used for protein extraction from insect cells.

Buffer I	Buffer II
40mM Hepes pH:7.6	40mM Hepes pH:7.6
1.5mM $MgCl_2$	1.5mM $MgCl_2$
10mM KCl	0.2mM EDTA pH 8.0
0.5mM PMSF	300mM NaCl
0.5mM DTT	25% Glycerol
	0.5mM PMSF
	0.5mM DTT

Table 3: Buffers used for protein extraction from mammalian cells.

Product Name	Manufacturer	Catalogue Number
Anti-flag M2 Affinity Agarose beads	Sigma-Aldrich	A4596
Flag Peptide	Sigma-Aldrich	F3290
Dynabeads™ His-Tag Isolation and Pulldown	Thermofisher Scientific	10103D
Superose 6 Prep Grade	GE life sciences	17048901

Table 4: Products used for Immuno-precipitation and protein purification.

2.3 Buffers for SDS-PAGE, Western blot analysis and Coomassie blue analysis.

Buffer	Contents
1X SDS-PAGE Running Buffer	25mM Tris, 192mM Glycine, 0.1%SDS
1X Transfer Buffer	25mM Tris, 192mM Glycine, 20% methanol
Acrylamide/Bisacrylamide Solution (30%)	292g/L Acrylamide, 8g/L bisacrylamide
1X PBS-T	137mM NaCl, 8mM Na_2HPO_4, 2mM KH2PO4, 2.7mM KCl,0.05% Tween20
10% Ammonium Persulfate (APS)	100g/L APS
4X SDS-PAGE sample loading buffer	240mM Tris-HCl (pH 6.8), 8% SDS (w/v), 40% glycerol (v/v), 5% beta-mercaptoethanol, 0.04% bromophenol blue
Coomassie Brilliant Blue Solution	40% H_2O, 50% methanol, 10% glacial acetic acid, 0.1% CBB R-250 (w/v)
Destaining Solution	40% methanol, 10% glacial acetic acid, 50% H_2O

Table 5: Buffers used for SDS-PAGE, Western blot analysis and Coomassie staining and their contents.

2.4 Materials used for Immobilize template recruitment assay.

Dynabeads M-280 Streptavidin	Invitrogen Cat no: 11205D
10X Assay Mix	50mM $MgCl_2$, 200mM HEPES-KOH (pH 8.2)
Blocking Buffer	1X Assay Mix, BSA 5mg/ml, 0.03% NP-40, 12.5mM DTT, PVP 5mg/ml
2X B&W before incubation	10mM Tris-HCl (pH:7.5), 2M NaCl, 1mM EDTA
2X B&W after incubation	10mM Tris-HCl (pH:7.5), 2M NaCl, 1mM EDTA, BSA 1mg/ml, 0.006% NP-40
Wash Buffer	40mM Hepes (pH: 7.5) 150 mM KCl, 4mM $MgCl_2$, 4mM DTT, 0.1% NP-40

Table 6: Materials and Buffers used in immobilize template recruitment assay and their contents.

2.5 Antibodies used in Immunoprecipitation and immunoblotting.

Antibody	Manufacturer	Catalogue Number	Dilution
Pol II (N-20)	Santa cruz Biotechnology	sc-899	1:1000
Pol II (A-10)	Santa cruz Biotechnology	sc-17798	1:1000
Pol II (8WG16)	R.G.R Lab at Rockefeller University	Home made	1:1000
Med14 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Med6 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Med7 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Med8 (A-5)	Santa cruz Biotechnology	sc-365960	1:1000
His-Tag Antibody	Cell Signaling Technology	2365s	1:1000
ANTI-FLAG M2	Sigma-Aldrich	F1804	1:1000
ANTI-FLAG M2	Sigma-Aldrich	F7425	1:1000
HA-Tag	R.G.R Lab at Rockefeller University	Home made	1:1000
Med1 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Med12 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Med13 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Med15 rabbit pAb	Proteintech	115661AP	1:1000
Med17 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Med19 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Med23	R.G.R Lab at Rockefeller University	Home made	1:1000
Med24	R.G.R Lab at Rockefeller University	Home made	1:1000
Med25 (A-7)	Santa cruz Biotechnology	sc-393759	1:1000
Med26 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Med27 (B-7)	Santa cruz Biotechnology	sc-390296	1:1000
Med28 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Med29 (B-1)	Santa cruz Biotechnology	sc-393800	1:1000
Med30 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000

Cyclin C	Abcam	ab85927	1:1000
CDK8	R.G.R Lab at Rockefeller University	Home made	1:1000
Rpb5 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Rpb6 rabbit pAb	R.G.R Lab at Rockefeller University	Home made	1:1000
Anti-rabbit IgG, HRP linked antibody	Cell Signaling Technology	7074S	1:1000
Anti-mouse IgG, HRP linked antibody	Cell Signaling Technology	7076S	1:1000
Estrogen Receptor α (D8H8) rabbit mAb	Cell Signaling Technology	8644	1:1000

Table 7: The antibodies used in immunoprecipitation and western blot analysis.

2.6 Kits utilized during the experiments.

Product Name	Manufacturer	Catalogue Number
GeneJET™ Gel Extraction Kit	Thermofisher Scientific	K0691
GeneJET™ Plasmid Miniprep Kit	Thermofisher Scientific	K0503
Clean-Blot™ IP Detection Kit (HRP)	Thermofisher Scientific	21232
Pierce Silver Stain Kit, 1L kit	Thermofisher Scientific	24612
BCA protein assay kit 500ml kit	Thermofisher Scientific	23227

Table 8: Kits and reagents used throughout the experiments.

CHAPTER 3

Methods

3.1 Construction of Plasmids.

3.1.1 Primer Design for Polymerase Chain Reaction.

The majority of the plasmids used in these experiments were already constructed by former students or Dr. Cevher himself. However, some of them had to be constructed to fulfil the aim of the project. The DNA coding sequence of His-Med14 (141-2420) was amplified using forward 5' GC GAA TTC ATG CAT CAC CAT CAC CAT CAC GCA GCC CCA GTG CAG 3' and reverse 5' GC TCT AGA CTA TCT ACC ACC AAC AGG 3' primer containing EcoRI and XbaI restriction sites, respectively. The DNA coding sequence of His-Med14 (141-1280) was amplified using forward 5' GC GAA TTC ATG CAT CAC CAT CAC CAT CAC GCA GCC CCA GTG CAG 3' and reverse 5' GC TCT AGA CTA AGC TGG CAA AGG AGG 3' primer containing EcoRI and XbaI restriction sites, respectively. The DNA coding sequence of His-Med14 (1341-2420) was amplified using forward 5' GC GAA TTC ATG CAT CAC CAT CAC CAT CAC GCA CTG ATT GAC AGT GTC 3' and reverse 5' GC TCT AGA CTA TCT ACC ACC AAC AGG 3' primer containing EcoRI and XbaI restriction sites, respectively. The DNA coding sequence of His-Med14 (2403-3455) was amplified using forward 5' GC GCT AGC ATG CAT CAC CAT CAC CAT CAC GCA GAG CCT GTT GGT GGT 3' and reverse 5' GC GGT ACC CTA TCG TCC ACT TGG TGA 3' primer containing NheI and KpnI restriction sites, respectively. The DNA coding sequence of His-RPB2 was amplified using forward 5' GC CTC GAG ATG CAT CAC CAT CAC CAT CAC GCA TAC GAC GCG GAT GAG 3' and reverse 5' GC GGT ACC CTA CTA AAC ACT CAT CAT TCG 3' containing XhoI and KpnI restriction sites, respectively. There were many more plasmids that were constructed but they are being used for other projects therefore, they are excluded from this project.

3.1.2 cDNA synthesis.

The total mRNA of HEK 293T cells was used to synthesize cDNA according to the manufacturer's instructions for RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Cat no: K1622).

3.1.3 Protocol for Polymerase Chain Reaction (PCR).

The coding DNA for all of the inserts was amplified using Phusion High-Fidelity PCR Master Mix from Thermofisher Scientific (Cat no: F-5315). The reactions were designed as follows.

2x HF Phusion Master Mix	25 µl
DMSO	1 µl
Forward Primer (0.5mM)	2.5µl
Reverse Primer (0.5mM)	2.5 µl
DNA template (plasmid\cDNA)	15 ng\ 5ul
ddH_2O	make total 50 µl

Table 9: Contents of a PCR reaction using 2x HF Phusion Master Mix.

Step	Temperature	Time	Cycle
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	33
Annealing	variable	30 sec	
Extension	72°C	variable	
Final Extension	72°C	7 mins	1

Table 10: Conditions for PCR.

The PCR amplified DNA fragments were ran on agarose gel (percentage depending upon the size of fragments) and after visualization, the fragments were excised using GeneJET™ Gel Extraction Kit.

3.1.4 Digestion of Vector and PCR products.

FastDigest value pack kit (Cat no: K1991) manufactured by Thermofisher Scientific was used in digestion of PCR amplified inserts and pFBDM vector. The Pack contains most of the commonly used enzymes. FastDigest NheI (Cat no: FD0973) from Thermofisher Scientific was also used for the respective cleavage sites. The reactions were set up as shown in table 11. Afterwards, the samples were incubated at 37°C for 30 minutes.

PCR Product	Volume	Vector	Volume
10X Fast Digest Buffer	3 µl	10X Fast Digest Buffer	3 µl
Enzyme I	1.5 µl	Enzyme I	1.5 µl
Enzyme II	1.5 µl	Enzyme II	1.5 µl
Template DNA	600 ng	Template DNA	2 µg
ddH_2O	make total 30 µl	ddH_20	make total 30 µl

Table 11: Schematics for double digestion of PCR product and pFBDM vector.

The double digested vector and insert were purified using GeneJET™ Gel Extraction Kit after running on the agarose gel. The double digested vector was dephosphorylated using Quick CIP (NEB cat no: M0525S) as shown in table 12. The sample was incubated at 37°C for 10 minutes. After dephosphorylation, the sample was purified using GeneJET™ Gel Extraction Kit.

Template DNA	1 pmol
CutSmart Buffer 10X	2 µl
Quick CIP	1 µl
ddH_2O	make total 20 µl

Table 12: Recipe for dephosphorylation using Quick CIP.

3.1.5 Ligation.

Doubled digested inserts and pFBDM vector were ligated at 3:1 using T4 DNA Ligase (NEB cat no: M0202S) at 16ºC overnight.

3.2 Preparation of competent DH5α cells.

This protocol is based on Inoue H. et al method[219]. DH5α cells are streaked onto LB plate without any antibiotics. Next day, about 10 colonies are used to inoculate 200 ml SOB culture. The cells were grown at 18ºC with vigorous shaking in an adequate flask to ensure good aeration. The cells were grown to an OD_{600} of 0.5. It is very critical to catch the culture at correct OD. Once ready, the cells were split into four 50 ml Falcon tubes and placed on ice for about 10 minutes. The cells were spun at 3500 rpm for 15 minutes at 4ºC. The supernatant was discarded and the pellet was resuspended in 64 ml HTB (16 ml for each tube). HTB consists of 10mM HEPES, 15mM $CaCl_2$, 250mM KCl, 55mM $MnCl_2$ and the pH of solution adjust by KOH to 6.7. After adding HTB, the falcons are places on ice for 10 minutes. The cells are spun again for 15 minutes at 3500 rpm. The pellets are resuspended again in 64 ml HTB with 16 ml in each falcon tube followed by 10 minutes incubation on ice. The cells were spun again and supernatant was discarded. To each falcon tube, 4 ml of HTB was added and cells were pooled together. 1.2 ml of DMSO was added dropwise while swirling the cell suspension. Immediately, the cells were aliquoted into to eppendorfs containing 100 µl cells each and flash frozen using liquid nitrogen. The cells were stored at -80ºC until further use.

3.3 Preparation of competent DH10b cells.

50 µl of DH10b cells were grown in 5 ml LB brother (overnight). The overnight culture was used to seed 500 ml LB broth at a ratio of 1:100. The culture was incubated for about 2.5 hours at 37ºC until the OD_{600} of the culture reached 0.3. The cell culture was chilled on ice for 10 minutes and spun using 50 ml Falcons at 4000 rpm for 15 minutes at 4ºC. After discarding the supernatant, the cells were resuspended in 10 ml 0.1M $CaCl_2$. The cells were spun at 4000 rpm at 4ºC for 10 minutes and supernatant was discarded. The cells were resuspended again in 4 ml ice cold 0.1M $CaCl_2$. The cells were

left on ice in cold room overnight. The following day, 2 ml of 50% glycerol was added and after resuspension, the cells were aliquoted into eppendorfs each containing 50 µl cell suspension.

3.4 Transformation of competent cells (DH5α and DH10b).

50 µl of cells (DH5α or DH10b) were thawed for each reaction. 50 ng plasmid DNA for DH5α and 100 ng plasmid DNA for DH10b was used for transformation. After adding the plasmid, the cells were incubated on ice for 30 minutes followed by 45 seconds heat shock at 42^{o}C and 1 minute cold shock on ice. 800 µl LB broth was added to cells and left for incubation at 37^{o}C (vigorous shaking for 1 hour for DH5α and no shaking for 8 hours for DH10b). Afterwards, the cells were spun at 2500 rpm for 2-3 minutes and 600 µl of LB was discarded. The cells were resuspended in remaining 200 µl LB broth. 100 µl was spread onto LB-agar plates with appropriate antibiotics (100 µg/ml Ampicillin for DH5α and 10µg/ml tetracycline, 100 µg/ml Ampicillin, 100 µg/ml kanamycin, 0.17mM IPTG and 100 µg/ml X-Gal for DH10b). The plates were incubated at 37^{o}C for 16 hours for DH5α and 36 hours for DH10b. All the plasmids transformed to DH5α were checked for the insert with PCR prior to their transformation to DH10b.

3.5 Recombinant bacmid Isolation from transformed DH10b cells.

DH10b cells already contain a shuttle vector (Bacmid). After transformation with donor plasmid, transposition took place and recombinant bacmid was generated. The colonies that appeared white were the ones that had recombinant plasmids. These colonies were selected and grown overnight in 5 ml LB containing 100 µg/ml ampicillin overnight at 37^{o}C at constant shaking. The next day, 3 ml of bacterial culture was spun at 14000 rpm for 1 minute. The supernatant was discarded and the pellet was resuspended in 0.3ml solution I containing 15mM Tris-HCl of pH 8.0, 10mM EDTA and 100 µg/ml RNaseA. 0.3 ml solution II was added that consist of 0.2N NaOH and 1% SDS and the suspension was mixed by inverting the tube 6-8 times. The translucent suspension was incubated at room temperature for 5 minutes followed by addition of 0.3 ml of 3M potassium acetate of pH 5.5 in a drop wise manner. A thick white precipitate was formed when the

suspension was mixed by inverting 6-8 times. The sample was incubated on ice for 10 minutes followed by centrifugation at 14000 rpm for 15 minutes. The supernatant was carefully transferred to tubes containing 0.8 ml isopropanol avoiding any white precipitate. The sample was inverted several times to ensure a homogenous mix and incubated on ice for 10 minutes. The sample was centrifuged at 14000 rpm for 10 minutes and then the pellet was washed twice with 0.5ml of 70% ethanol. After washing, all the ethanol was removed and sample was left to air dry to prevent residual ethanol contamination. The DNA pellet was dissolved in 30 µl ultra-pure water. The same day, these recombinant bacmids were transfected to Sf9 cells.

3.6 Transfection of Sf9 cells with recombinant bacmids.

$1.1x10^6$ Sf9 cells were seeded to each well in a 6 well plate and allowed to attach to the surface for about 2 hours. 6 µg of recombinant bacmid was added to an Eppendorf containing 200 µl serum free grace insect cell media. 5 µl Cellfectin II reagent was added to the tubes containing recombinant plasmid. The transfection mix was mixed by tapping as pipetting will shear the bacmid DNA. The tubes were covered with aluminum foil and incubated in dark for about 15-20 minutes. The media was aspirated from the Sf9 cells and transfection mix was added on top of the cells in a dropwise manner to ensure it spreads homogenously. 1 ml serum free grace insect cell media was added and the cells were incubated in dark at 27ºC. After 5 hours, the serum free media was replaced with grace media containing 10% FBS, 1% poloxamer and 50 µg/ml gentamicin. After 5 days, the virus was collected (supernatant) and the pellet was used for SDS-PAGE followed by western blot analysis to check the expression of the protein. The viruses obtained after transfection are denoted as P0. These viruses were amplified to P1 and P2 by infecting 100 µl P0 to 50 million Sf9 cells and incubated for 5 days and so on.

3.7 Purification of recombinant proteins using anti-flag M2 agarose beads.

Prior to purification, the Hi5 cells were infected with respective viruses and incubated for about 3 days in a spinner flask. For Head+middle+14+17, 500 µl P3 (head, middle and 17 each) virus were used and 100 µl flag-14 virus was used to infect 100 million Hi5 cells. After 3 days, the cells were collected and the Pellet was lysed in 5 ml BC300 (made using appropriate dilution of BC0 and BC1000) buffer using a douncer homogenizer. The pellet was lysed by douncing it 10 times and incubating on ice for 10 minutes. The process was repeated 3 times followed by centrifugation at 13000 rpm for 15 minutes. The supernatant was pooled together in a 15 ml falcon tube. Meanwhile, 100µl anti-flag M2 agarose beads were washed 5 times with BC500(50:50 ratio of BC0 to BC1000) containing 0.1% NP-40. After washing, the beads were incubated with protein extract overnight at 4^{o}C on a rotator. Next day, the incubated protein extract was centrifuged for 1 minute at 1500 rpm. The supernatant was discarded and beads were washed once with 5 ml BC500 with 0.1% NP-40 followed by 5 times washing with BC300 with 0.1% NP-40 and a final wash with BC100. After discarding the supernatant, 100 µl BC100 with 0.02% NP-40 was added that contained 50 µg/ml flag peptide. The sample was incubated on a rotator for about 45 minutes at 4^{o}C. The sample was centrifuged at 3000 rpm for 1 minutes and the supernatant was collected. 10 µl from the collected fraction was analyzed using SDS-PAGE followed by Coomassie blue staining and western blot analysis if required.

3.8 Immunoprecipitation (IP) using Med30 antibody.

Sepharose protein A beads (10 µl per reaction) were washed 5 times with 1 ml BC500 buffer with 0.1% NP-40 at room temperature. After discarding the wash buffer, 100 µl BC200 solution with 0.02% NP-40 was added followed by 2 µl per reaction Med30 antibody. The beads were incubated on a rotator for 3 hours at 4^{o}C. The beads were centrifuged at 3000 rpm at room temperature for about 1 minute. After discarding the supernatant, beads were washed 4 times with 200 µl BC300 with 0.1% NP-40. The beads were transferred equally to eppendorfs (10 µl per reaction) and after discarding the wash buffer, 100 µl BC200 buffer with 0.02% NP-40 was added followed by addition of equal amounts of respective purified recombinant proteins (core Mediator or head

module). The amount of recombinant protein to be added was an approximation based on Coomassie staining of the purified protein complexes. The proteins were incubated with protein A beads coupled with Med30 antibody for 3 hours in on a rotator at 4°C. After centrifugation, supernatant was discarded and the beads were washed 4 times with 200 µl BC200 with 0.1% NP-40. 100 µl BC200 was added followed by adequate amount of RPB1 proteins (15 µl for flag-RPB1 from HEK 293T cells and 50 µl from His-RPB1 extract). The reactions were incubated at 4°C for 3 hours on a rotator. The beads were washed 4 times with 200 µl BC150 buffer with 0.1% NP-40 and ensured that all the supernatant was removed. 20 µl 2X loading dye was added and the samples were kept at -20°C for analysis using western blot.

3.9 Immunoprecipitation (IP) using anti-flag M2 agarose beads.

Anti-flag M2 agarose beads (10 µl per reaction) were washed 5 times with 1 ml BC500 buffer with 0.1% NP-40 at room temperature. The beads were split into different reaction tubes ensuring same volume (10 µl). The wash buffer was discarded and 100 µl BC200 buffer with 0.02% NP-40 was added. Equal amounts of respective purified recombinant proteins (core Mediator or head module) were added to respective reaction tubes and incubated on a rotator for 3 hours at 4°C. After centrifugation, supernatant was discarded and the beads were washed 4 times with 200 µl BC200 with 0.1% NP-40. Required amount of nuclear extract of recombinant Pol II subunits were added (refer to lab notebook for details) and ensured that each tube has equal volume and 150 mM salt concentration. The samples were incubated on a rotator for 3 hours at 4°C. After centrifugation, supernatant was discarded and the beads were washed 4 times with 200 µl BC200 with 0.1% NP-40. After removing all the supernatant, 20 µl 2X loading dye was added and the samples were kept at -20°C for analysis using western blot.

3.10 SDS-PAGE analysis, western blot analysis, Coomassie staining and silver stain analysis.

The samples were ran on appropriate percent hand casted Tris-Glycine gels. The gels were ran at 120V until the dye ran out. For Coomassie staining, the gel was washed in water for 5 minutes at constant shaking followed by 15-30 minutes incubation with Coomassie stain until the bands started to appear. For silver staining, the gel was washed with ultra-pure water twice for 5 minutes each followed by fixation (2 times for 15 minutes each in 10%Acetic acid, 30% Ethanol solution). The gel was washed twice in 10% ethanol for 5 minutes each followed by 2 times washing with ultra-pure water for 5 minutes each. The gel was sensitized using 20 µl sensitizer solution in 20 ml water (Thermofisher Scientific cat no: 246129) for exactly 1 minute. The gel was rinsed twice with ultra-pure water for 1 minute each followed by incubation with silver stain solution supplemented with enhancer solution (Thermofisher Scientific cat no: 246129) for 30 minutes. The gel was rinsed twice with ultra-pure water for 20 seconds each and developed with developing solution supplemented with enhancer solution (Thermofisher Scientific cat no: 246129). Once the bands appeared, the reaction was stopped using 5% acetic acid. For western blotting, the proteins from the SDS-PAGE were transferred to PVDF membrane using 330mA current for about 2 hours 15 minutes. After transfer, the membrane was blocked using 5% PBS-milk or blocking buffer from Clean blot IP detection Kit (for IP reactions). The membranes were cut and placed in primary antibody solution for the respective proteins at 4^{o}C for overnight incubation. Next day, the membranes were washed 3 times with PBST followed by secondary antibody incubation for 90 minutes in 1% PBS-milk or blocking buffer Clean blot IP detection Kit (for IP reactions). The membranes were washed 3 times with PBST and developed using X-ray film or the Amersham Imager 600 using Pierce ECL plus or ECL western blotting substrate.

3.11 Cell culture.

Sf9 and Hi5 cells were maintained and grown using grace insect cell media with 10% FBS, 50 µg/ml gentamicin and 1% Poloxamer in a spinner flask. Occasionally, Hi5 cells were grown using Express five SFM media supplemented with 18mM L-glutamine, 1% Poloxamer and 50 µg/ml gentamicin. For transfection and recombinant protein

production using Hi5 cells, grace insect cell media with 10% FBS, 50 µg/ml gentamicin and 1% Poloxamer was used. HEK 293T cells were grown in DMEM supplemented with 1% Pen/Strep and 10% FBS. Hela S3 (B9b) cells were grown using DMEM supplemented with 1% Pen/Strep, 10% FBS and 0.5mg/ml Geneticin (G418). The initial stock was grown on cell culture plates but for large scale production, the cells were transferred to petri dishes to provide suspension culture conditions. Consequently, 300 plates were grown at a given time where 250 would be collected and the pellet was frozen for Pol II extraction and the remaining 50 were reused to seed 300 plates. MCF7 wild type and tamoxifen resistant strain were a generous gift from Sahin laboratory (Bilkent University, Turkey). These cells were grown in low glucose phenol red free DMEM with 10% charcoal stripped FBS, 1X non-essential amino acids, 1% Pen/Strep and 10 µg/ml insulin. MCF7 cells were treated with 5µM tamoxifen for 18 hours and processed for nuclear protein extraction to be used for further experiments.

3.12 Nuclear extract preparation from HEK 293T, MCF7 and B9b cells.

The media from the plates was aspirated and the cells were washed and collected using ice cold 1X PBS (pH 7.4). The cell pellet was pooled together in one 15 ml falcon tube. Appropriate amount of Buffer I was added to resuspend the cell pellet which was lysed using a douncer homogenizer for 3 times, each time 10 dounces followed by 10 minutes incubation on ice. The lysate was centrifuged at 13000 rpm for 15 minutes and the supernatant was collected and labelled as cytoplasmic fraction (collected for MCF7 wild type and tamoxifen resistant cells). The pellet was resuspended in adequate amount of Buffer II and incubated on a rotator at 4^{o}C for 45 minutes. The lysate was centrifuged at 13000 rpm for 15 minutes and the supernatant was collected and labeled as nuclear extract. This step with Buffer II was repeated once again to collect a second nuclear fraction.

3.13 UV treatment of HEK 293T cells.

HEK 293T cells were grown to 50% confluency prior to UV treatment. Once the cells were ready, the media was aspirated and cells were washed with 1X PBS (pH 7.4). After aspirating the PBS, cells were treated with 40μJ UV using Stratagene UV crosslinker. The plate was rotated at an angle of 15 degree and was treated again with 40μJ UV. After treatment, DMEM supplemented with 1% Pen/Strep and 10% FBS was added and cells were placed back in the incubator for appropriate amount of time. This process was repeated for adequate time point of treatment. Once the treatment finished, the cells were processed for nuclear extract preparation using method described in 3.12.

3.14 Immobilized template recruitment assay.

The template fragments were PCR amplified via the 5' biotinylated primers. For the control reaction, pTata-luci vector was used to amplify a fragment consisting of 250 base pairs whereas 2XERE template was amplified using pERE vector gifted by Prof. Mesut Muyan (Middle East Technical University, Turkey) that generated approximately 400 base pair fragment containing ERE sequence. For each condition (control or 2XERE), 10 PCR reactions were set up to obtain sufficient DNA template for the assay. The DNA fragments were purified using GeneJet gel extraction kit. 10 µl per reaction Dynabeads M-280 Streptavidin were obtained after homogenizing the suspension slurry. These beads were divided into two portion; one for control template and other for 2XERE. The reaction was proceeded with two tubes containing 50 µl beads each. These tubes were placed on magnetic rack placed on ice and the supernatant was removed after 2 minutes incubation. The beads were washed 4 times with 250 µl 1X B&W before incubation, each time inverting the tube 10 times. The tubes were placed on magnetic rack and after 2 minutes the supernatant was removed. To immobilize the template, 2500 ng 2XERE template and 2000 ng control template was added to their respective tubes. 75 µl of 2X B&W before incubation was added to each tube followed by addition of water to make the volume of both tubes the same. The tubes were incubated at room temperature, giving it a tap every 5 minutes to ensure beads do not settle down at the bottom. The beads were placed on magnetic rack and the supernatant was removed. The beads were washed twice with 150 µl 1X B&W after incubation followed by a wash

with 150 µl 1X PBS. After removing the PBS, the beads were blocked using blocking buffer for 15 minutes at room temperature, giving it a tap every 5 minutes to ensure beads to do not settle down at the bottom. The beads were washed twice with 200 µl wash buffer and transferred to Eppendorf tubes in equal volume. We had 4 reaction each for 2X ERE and control template. To each tube, 150 µg protein, 1X Assay mix, 10 µg E.coli genomic DNA and required amount of ddH_2O was added to make the final volume 105 µl. The tubes were incubated at 30°C in a water bath for 50 minutes while giving it a tap every 5 minutes to ensure the beads do not settle at the bottom. The beads were placed on magnetic rack and the supernatant was removed followed by 4 times washing with 200 µl wash buffer. The beads were spun at 3000 rpm for 1 minute to ensure all the wash buffer is removed. 15 µl 1X SDS loading dye was added and the samples were boiled for 5 minutes at 95°C. The samples were analyzed using western blot.

3.15 Competition assay using anti-flag M2 agarose beads.

Anti-flag M2 agarose beads (10 µl per reaction) were washed 5 times with 1 ml BC500 buffer with 0.1% NP-40 at room temperature. The beads were split into different reaction tubes ensuring same volume (10 µl). The wash buffer was discarded and 100 µl BC200 buffer with 0.02% NP-40 was added. Equal amounts of purified recombinant proteins (core Mediator for Pol II competition and head+middle+17+14 for kinase subunit competition) was added to each reaction tube and incubated on a rotator for 3 hours at 4°C. After centrifugation, supernatant was discarded and the beads were washed 4 times with 200 µl BC200 with 0.1% NP-40. For Pol II competition, two reactions were set up; one containing Pol II and other containing Pol II + His-RPB1 (refer to lab notebook for amount of protein added). For Kinase module subunits competition with His-RPB1, 5 reactions were set up that consisted of His-RPB1, His-RPB1+Med12, His-RPB1+Med13, His-RPB1+ Cyclin C and His-RPB1+HA:CDK8 (refer to lab notebook for amount of proteins added). The samples were incubated on a rotator for 3 hours at 4°C. After centrifugation, supernatant was discarded and the beads were washed 4 times with 200 µl BC200 with 0.1% NP-40. After removing all the supernatant, 20 µl 2X

loading dye was added and the samples were kept at -20ºC for analysis using western blot.

3.16 Crosslinking using disuccinimidyl sulfoxide (DSSO).

The DSSO cross linker was diluted from 50 µM stock to 10 µM and 1 µM. 1 µl DSSO crosslinker from 10 µM and 1 µM dilutions was added to 9 µl purified His-RPB1+head+middle+14+17 protein subcomplex with or without imidazole. The control reaction had 1 ul ddH_2O. After addition of crosslinker, the tubes were incubated at constant agitation of approximately 400 rpm at room temperature for 20 minutes. The reaction was stopped by addition of 1 µl 50mM Tris followed by 10 minutes incubation at room temperature. Required amount of 4X loading dye was added and the samples were analyzed using SDS-PAGE followed by silver staining. For mass spectrometry, the sample was concentrated using Dynabeads for His tag isolation and dialyzed using Slide-A-Lyzer Dialysis Cassette 20K MWCO 0.5ml (Thermofisher Scientific cat no: 66005) according to the manufacturers instructions. The sample will be crosslinked and analyzed using mass spectrometry to elucidate the structure of RPB1+head+middle+14+17 protein complex.

3.17 Gel filtration for protein purification.

19 ml Superose 6 Prep grade beads (GE life sciences cat no: 17048901) were packed to an empty C column and washed with adequate amount of distilled water to remove residual ethanol. The column was equilibrated with 10 column volumes of BC100 buffer solution. 500 µl His-RPB1 whole cell extract was loaded to the column and purified using ÄKTA pure protein purification system. 500 µl protein fractions were collected and analyzed using western blot.

CHAPTER 4

RESULTS

4.1 Purification of baculovirus expressed reconstituted human Mediator sub-complexes.

The Mediator Complex plays a crucial role in the assembly of PIC as it relays regulatory information to the RNA polymerase II to modulate the gene expression[2]. Thus, it is very important to know which region of the Mediator Complex is mediating the interaction with Pol II. Previous study by Cevher et al highlighted the critical role of Med14 in the core Mediator complex with respect to Mediator interaction with Pol II[10]. The study also signified the role of Med14 in basal and activator driven transcription[10]. Hence, it is very critical to understand the mechanism by which Mediator plays a critical role as a co-activator for RNA polymerase II directed gene transcription. The large size of natural Mediator complex, its heterogeneity and difficulty in purification has limited the number of studies with respect to structure-function relationship. In order to circumvent this problem, Mediator sub-complexes were reconstituted and expressed using baculovirus expression system as mentioned in Cevher et al[10]. The core Mediator complex consists of head module, middle module, Med14 and Med26 as shown in figure 3A. Another preparation of Mediator sub complex was prepared that lacked Med26 and comprised of head and middle module along with Med14 (figure 3B). Figure 3C shows the purified head module pulled via Med14. These preparations were used for the binding assays between purified RNA polymerase II obtained from stable flag-RPB9 expressing Hela cells (figure 3D) or recombinantly expressed Pol II subunits using Hi5 cells. Moreover, we were curious to find whether NTD Med14 can interact with Pol II thus, we purified flag-NTD Med14 via transient expression in HEK 293T cells (figure 3E) and performed immunoprecipitation assay. Since the interaction between Mediator subunits is not one to one, we see sub-stoichiometry between the subunits (figure 3A,B and C).

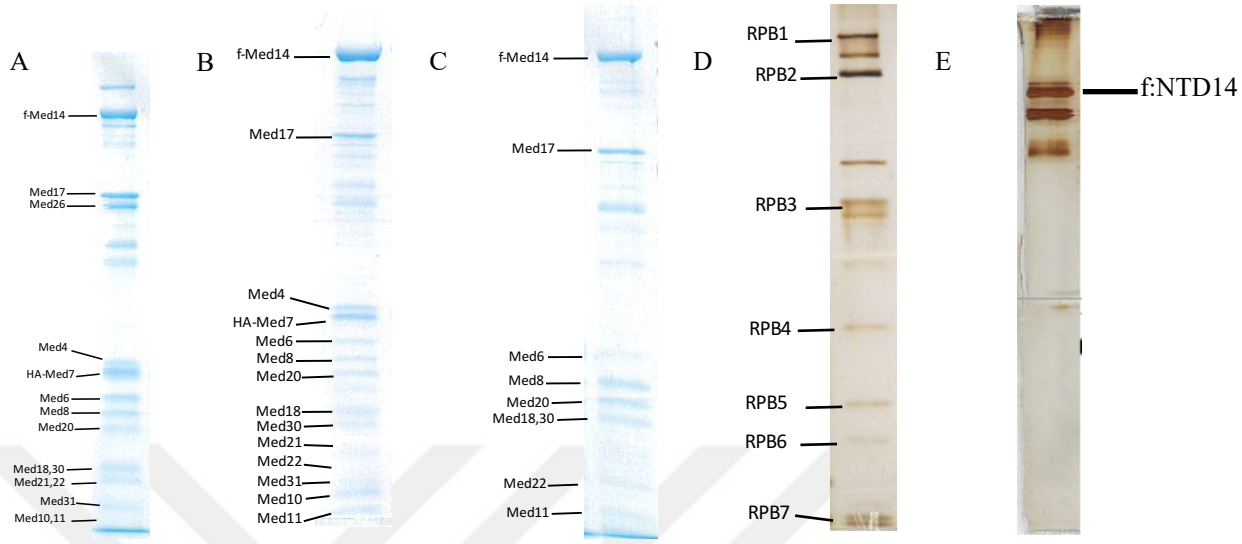

Figure 3: Reconstituted human Mediator sub-complexes and purified human RNA polymerase II. A) SDS-PAGE analysis followed by Coomassie staining for reconstituted core Mediator Complex (H+M+14+26) using baculovirus. **B)** SDS-PAGE analysis followed by Coomassie staining of reconstituted H+M+14 using baculovirus. **C)** SDS-PAGE analysis followed by Coomassie staining of reconstituted head module expressed using baculovirus. **D)** SDS-PAGE followed by silver staining for purified RNA Polymerase II (Pol II) used in the binding assays. **E)** SDS-PAGE followed by silver staining for purified flag-Med14 NTD using transient expression in HEK 293T cells. H stands for head module, M stands for middle module and number signifies individual subunits of human Mediator complex.

4.2 The interaction surface between RNA polymerase II and Mediator complex lies between RPB1 subunit of Pol II and NTD Med14 of core-Mediator complex.

The former members of Cevher lab already demonstrated the critical role of NTD Med14 containing core Mediator complex in direct binding to Pol II. It was also shown that NTD Med14 containing core Mediator complex can recruit Pol II to TATA promoter and induce basal and activator (P53) mediated transcription. However, concurrent literature is inconclusive about the region of Pol II that interacts with the Mediator complex. Thus, we were interested in elucidating the interacting region

between these two protein complexes. Consequently, we decided to test the interaction between 12 subunit of Pol II with core Mediator complex one by one.

As RPB1 is largest of Pol II subunits, we began to test whether it is sufficient to interact with core Mediator complex. The FLAG-Pol-II WT which was a generous gift from Benjamin Blencowe (Addgene cat no: 35175) was transiently expressed in HEK 293T cells using $CaCl_2$ transfection method[212]. Upon anti-flag pulldown of flag-RPB1, it was observed that other subunits of Pol II came alongside RPB1 (figure 4A). The binding capacity of flag-RPB1 pulled down Pol II was tested via IP reactions using anti-Med30 antibody against the head subunit of core Mediator complex. The IP showed that it interacts with core Mediator complex but not with head module of Mediator complex (figure 4B) as shown by Cevher et al[10]. The purpose of the experiment was to demonstrate minimum subunits required by Pol II to interact with core Mediator complex. However, transient expression of FLAG-Pol-II WT[212] brought other subunits alongside (figure 4C). In order to circumvent the problem, Multibac expression system was utilised to highlight the interaction surface of core Mediator and Pol II.

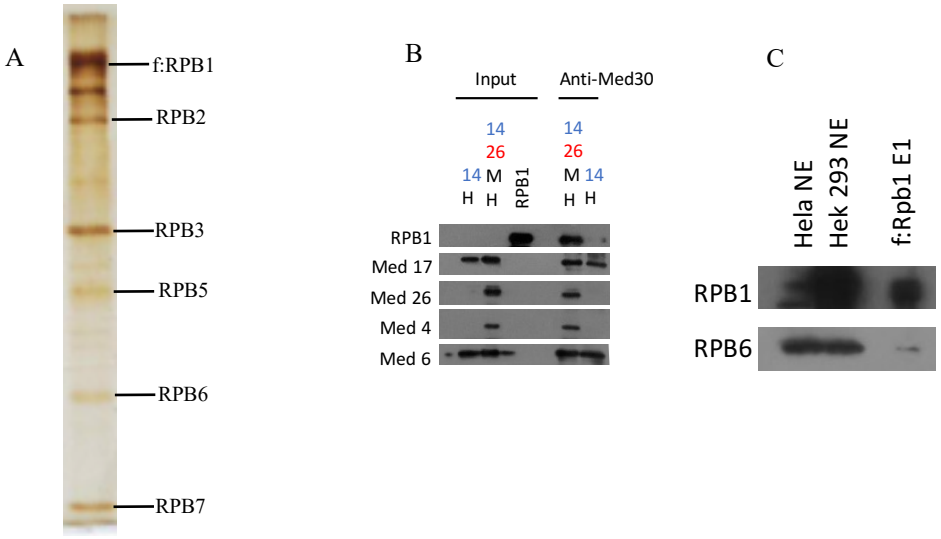

Figure 4: Purification of RPB1 subunit of human RNA polymerase II (Pol II) transiently expressed in HEK 293T cells and its immunoprecipitation (IP) with Mediator sub-complex. A) SDS-PAGE analysis followed by silver stain for flag-RPB1 purified using anti-flag M2 agarose beads. **B)** Western blot of IP between Pol II pulled down via flag-RPB1 and head module and core Mediator complex using anti-

Med30 antibody. **C)** Western blot analysis of Hela nuclear extract, flag-RPB1 expressing HEK 293T nuclear extract and purified flag-RPB1 using anti-flag M2 agarose beads. NE corresponds to nuclear extract. E1 corresponds to first elution using 0.5mg/ml flag peptide.

The binding assays showed that recombinant His-RPB1interacts with the core Mediator complex and it does not interact with head module containing Med14 (figure 5B,C and D). While anti-flag pull down of head module did not bring His-RPB1 (figure 5B3, C3 and D), core Mediator interacted with His-RPB1 (top band) which can be seen clearly by comparing figure 5B and C1 with figure 5B and C2, where number 1 corresponds to core Mediator + His-RPB1 and 2 refers to core Mediator. IP experiments using anti-Med30 antibody showed similar result as shown by western blot analysis in figure 6A. The core Mediator interacted with His-RPB1 while head module did not thus, it will serve as a negative control throughout other experiments (figure 6A). The experiment was repeated using anti-flag beads and moderately purified His-RPB1 using Ni-NTA column. It was surprising to see that head+middle+Med14 itself is sufficient to interact

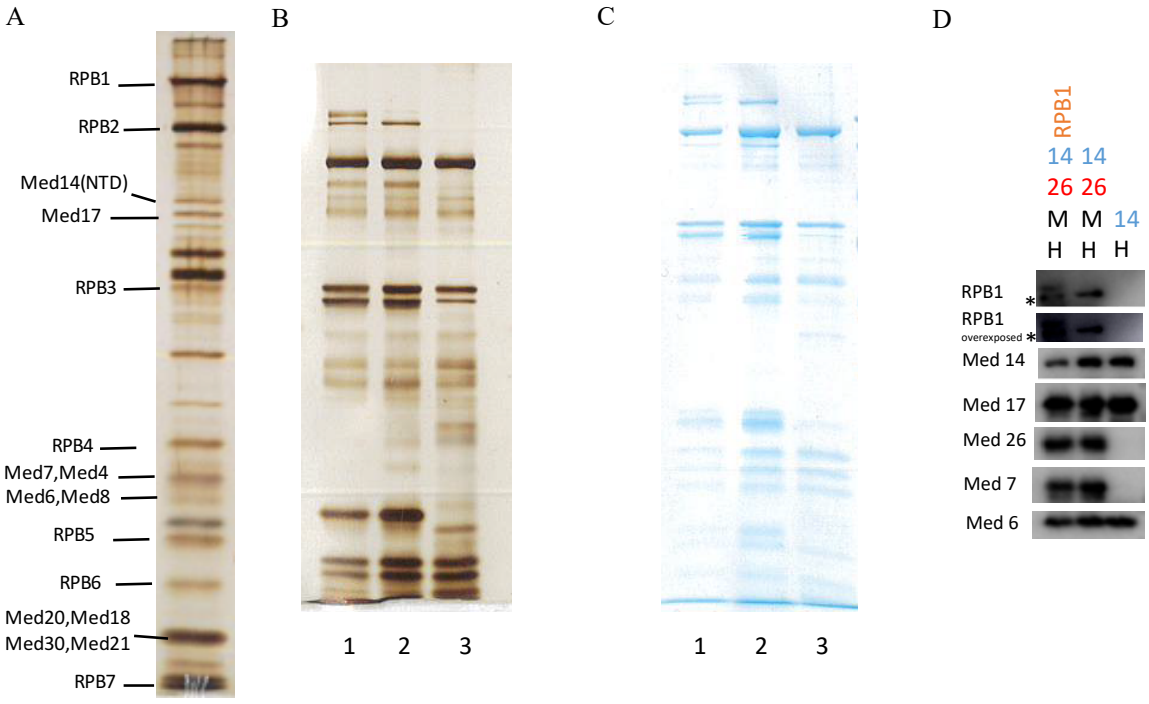

Figure 5: Immuno-precipitation of reconstituted Mediator sub-complexes with Pol II and His-RPB1. A) SDS-PAGE analysis followed by silver staining for purified Pol

II pull down of H+M+14NTD. **B)** SDS-PAGE analysis followed by silver staining for anti-flag Immuno-precipitates of Mediator sub-complexes with His-RPB1 subunit of Pol II. **C)** SDS-PAGE analysis followed by Coomassie staining for anti-flag Immuno-precipitates of Mediator sub-complexes with His-RPB1 subunit of Pol II. **D)** Western blot analysis of anti-flag Immuno-precipitates of Mediator sub-complexes with His-RPB1 subunit of Pol II. The number 1 refers to anti-flag pull down of core Mediator+His-RPB1, 2 refers to core Mediator and 3 refers for Head+Med14.

with His-RPB1 and 200 mM imidazole did not affect the binding affinity of His- RPB1 to core Mediator complex (figure 6B). Other purification methods such as size exclusion chromatography using superose 6 beads was utilised to obtain monomeric His-RPB1 (figure 7A). The fractions were tested for His-RPB1 using western blot analysis and it showed that fractions 8-14 contained His-RPB1. As discussed earlier, NTD Med14 containing core Mediator complex interacted with Pol II and can be seen in figure 5A. The binding assay also demonstrated that His-RPB1interacts with NTD Med14 containing core Mediator complex and not the CTD Med14 containing core Mediator complex (figure 6C and D). The experiment also demonstrated that Med14 by itself, Med14+middle, Med14+middle+Med17 does not interact with His-RPB1.

The study by Cevher et al found that Med26 was required for Pol II interaction with Mediator complex to overcome the suppressive effects of proteins in the nuclear extracts[10]. However, His-RPB1 expression using baculovirus expression system overcomes that problem and enables interaction in an extract based system independent of Med26 requirement. It gives birth to two ideas; the negative regulator of Pol II-Mediator interaction could be binding to other subunits of Pol II complex or the over expression of His-RPB1 can overcome the suppression by itself. The competition between endogenous Pol II and recombinant His-RPB1 for binding to core Mediator revealed that His-RPB1 has a higher binding capacity compared to Pol II as seen by enrichment of RPB1 and depletion of RPB6 (figure 6E). The competition assay between His-RPB1 and subunits of kinase module for its binding capacity to head+middle+14

revealed that Med12 does not interact with core Mediator complex but decreases the binding of His-RPB1 to head+middle+14 sub-complex. While Cdk8 and cyclinC had no effect on the binding capacity, Med13 did not express at all. Since Med14 itself does not interact with His-RPB1, this observation alongside of binding data for other Mediator sub-complexes signifies the critical role of Med14 in restructuring and altering the conformation of Mediator complex in such a way that NTD Med14 can interact with RPB1 subunit of Pol II, recruit it to the promoter and facilitate transcription.

4.3 RPB1 is the only subunit that interacts with core-Mediator complex.

The IP reactions showed that RPB1 subunit of Pol II interacts with core Mediator and NTD Med14 containing core Mediator complex (figure 5B, C and D, figure 6). Studies in yeast[213] report the interaction of Mediator complex with other subunits of Pol II as well therefore, binding assays for interaction between core Mediator and Pol II subunits were performed. The IP reactions performed using anti-flag antibody indicates that RPB3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 subunits of Pol II does not interact with core Mediator as well as the head module of Mediator complex (figure 8). The cross-linking coupled with mass spectrometry for yeast Mediator-PIC complex identified point of contacts between RPB1 and Med9, RPB3 with Med2, Med9 and Med17, RPB4 with Med14, Med17 and Med22, RPB5 with Med15 and Med16, RPB8 with Med4, RPB10 with Med9, RPB11 with Med14 and RPB12 with Med14 and Med17[213]. Other subunits of Pol II did not interact with any of the Mediator complex subunits but what is striking is that there was no contact between RPB1 and Med14[213]. Except Med2, Med9, Med15 and Med16, all other subunits that interacted with Pol II in yeast Mediator PIC crosslink[213] are a part of core Mediator complex and did not interact with any of the subunits mentioned above (figure 8). The points of contact mentioned in the Robinson et al[213] could be the result of full Mediator assembling during PIC. Nevertheless, there is a possibility of false contacts due to close proximity of individual subunits.

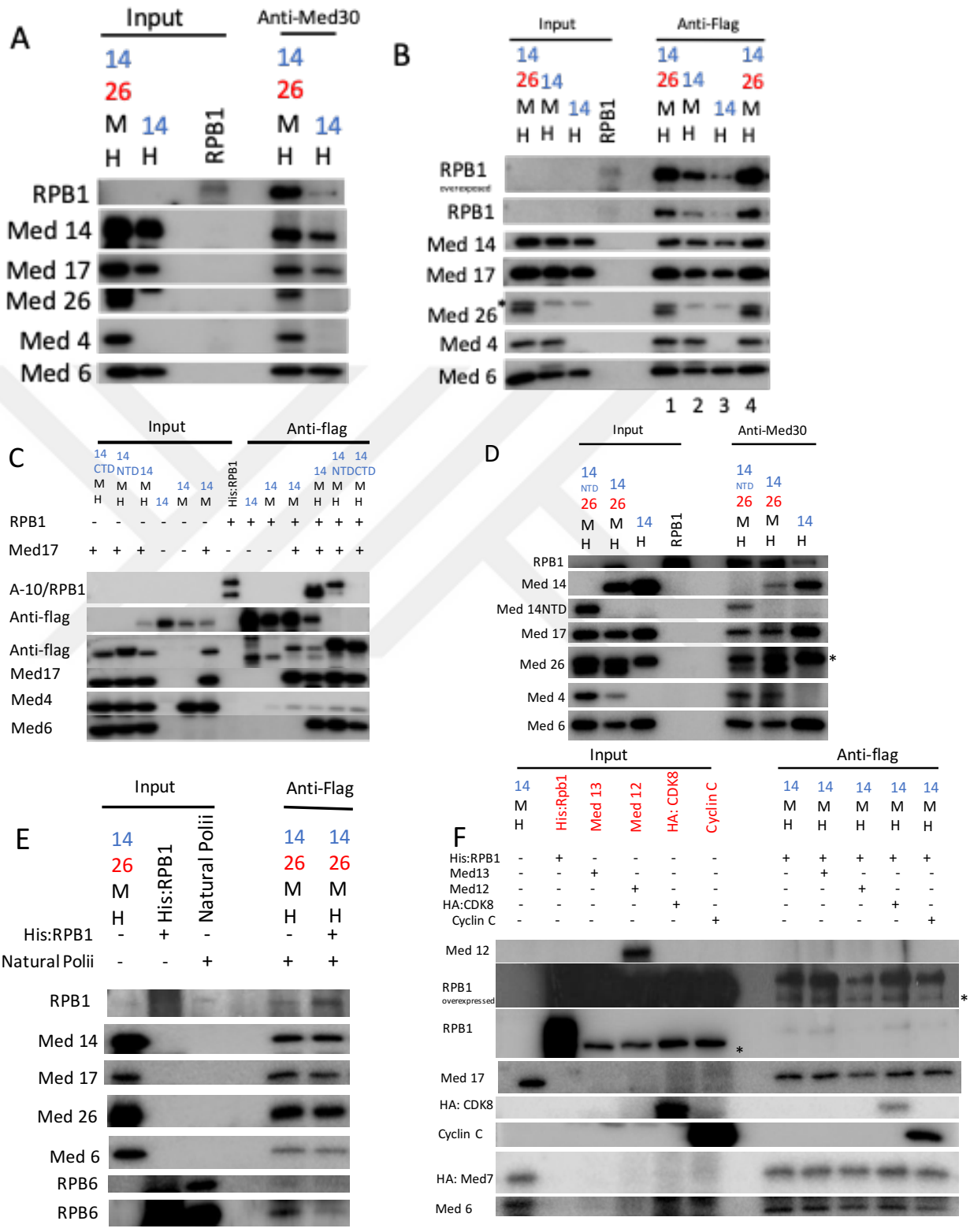

Figure 6: Immunoprecipitation (IP) of His-RPB1 subunit of human RNA polymerase II (Pol II) and natural Pol II with different modules of Mediator

complex. A) Western blot analysis of IP between recombinant His-RPB1 subunit of Pol II with core Mediator complex and head module of Mediator complex using anti-Med30. **B)** Western blot analysis of IP performed between His-RPB1 with head module, head and middle module and core Mediator Complex in the presence (lane number 4) and absence of 200mM imidazole (lane 1,2 and 3). **C)** Western blot analysis of IP between His-RPB1 subunit of Pol II with Med 14, head module, head and middle module and core Mediator complex without Med26. **D)** Western blot analysis of IP conducted between head module, core and NTD-Med14 containing core Mediator complex with His-RPB1. **E)** Western blot analysis of competition assay performed between recombinant His-RPB1 and natural Pol II for its' binding to core Mediator complex. **F)** Western blot analysis of competition assay performed between kinase module subunits of Mediator complex and recombinant His-RPB1 for its' binding to core Mediator complex lacking Med26. * indicates non-specific bands.

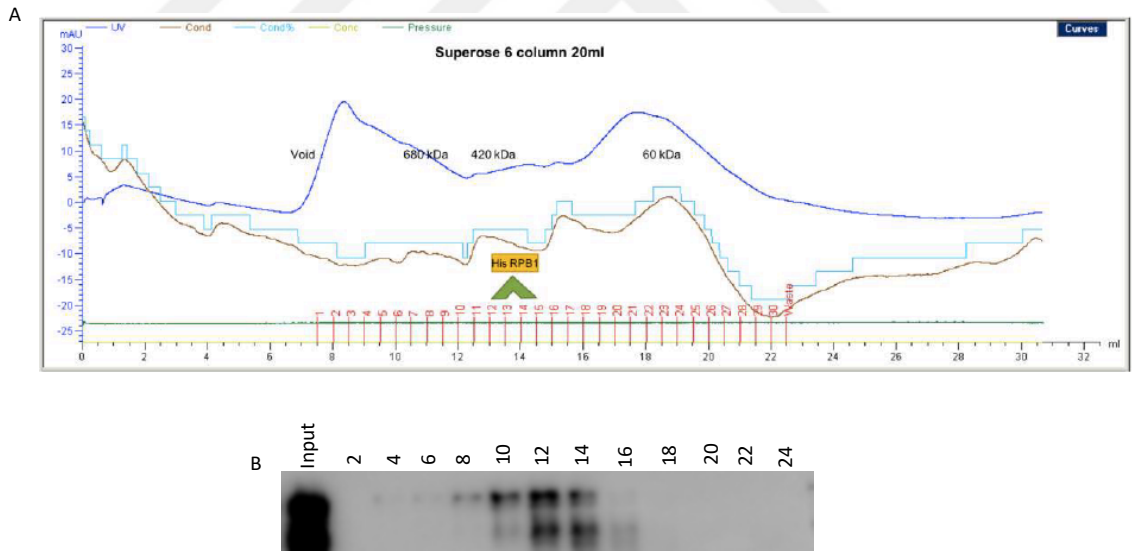

Figure 7: Purification of baculovirus expressed recombinant His-RPB1 subunit of Pol II. A) Size exclusion chromatography of recombinant His-RPB1 subunit of Pol II via 20 ml Superose 6 column using ÄKTA system. The numbers corresponds to the 500 ul fractions collected at a given time point. **B)** Western blot analysis of Superose 6 purified His-RPB1. The numbers reflect the eluate collected during the size exclusion chromatography.

4.4 Characterisation of human Mediator subunits in HEK 293T cells upon UV damage.

The role of Mediator complex with respect to transcription is widely known. For the continuity of Pol II Mediated gene expression, transcription is often coupled with DNA repair[214]. Proteins such as TFIIH have been implicated with their dual role; one being an essential general transcription factor for Pol II transcription and other in nucleotide excision repair[215]. There are two types of nucleotide excision repair; global genome repair that removes any form of lesions in the genome overall and transcription coupled repair which removes the lesions while active transcription of Pol II genes is being carried out[216]. Few years ago, a study identified involvement of Mediator in DNA repair by promoting the recruitment of Rad2/XPG to the actively Pol II transcribed genes. It showed that Med17 interacted with Rad 2 and Med17 yeast mutants sensitive to UV showed reduced Rad2 occupancy to Pol II transcribed genes[214]. Another study found Med23 deficiency prevents UV-induced damage and facilitates DNA repair as Med23 regulated the expression of MITF by modulating its' enhancer function[217]. HEK 293T cells treatment with UV reveals that Med23 expression changes significantly after 12 hours of treatment (figure 9), supporting the argument that Med23 deficiency promotes DNA repair. Furthermore, Med26 level also increases at 12hours as compared to 0 hours (figure 9) which might indicate that it facilitates DNA repair. As Med26 is a metazoan specific subunit[10], its role in DNA repair using yeast has not been studied. Despite the inverse correlation of Med26 expression with Med23, further studies need to be carried out to make a conclusive argument about role of Med26 and Med23 of Mediator complex in DNA repair.

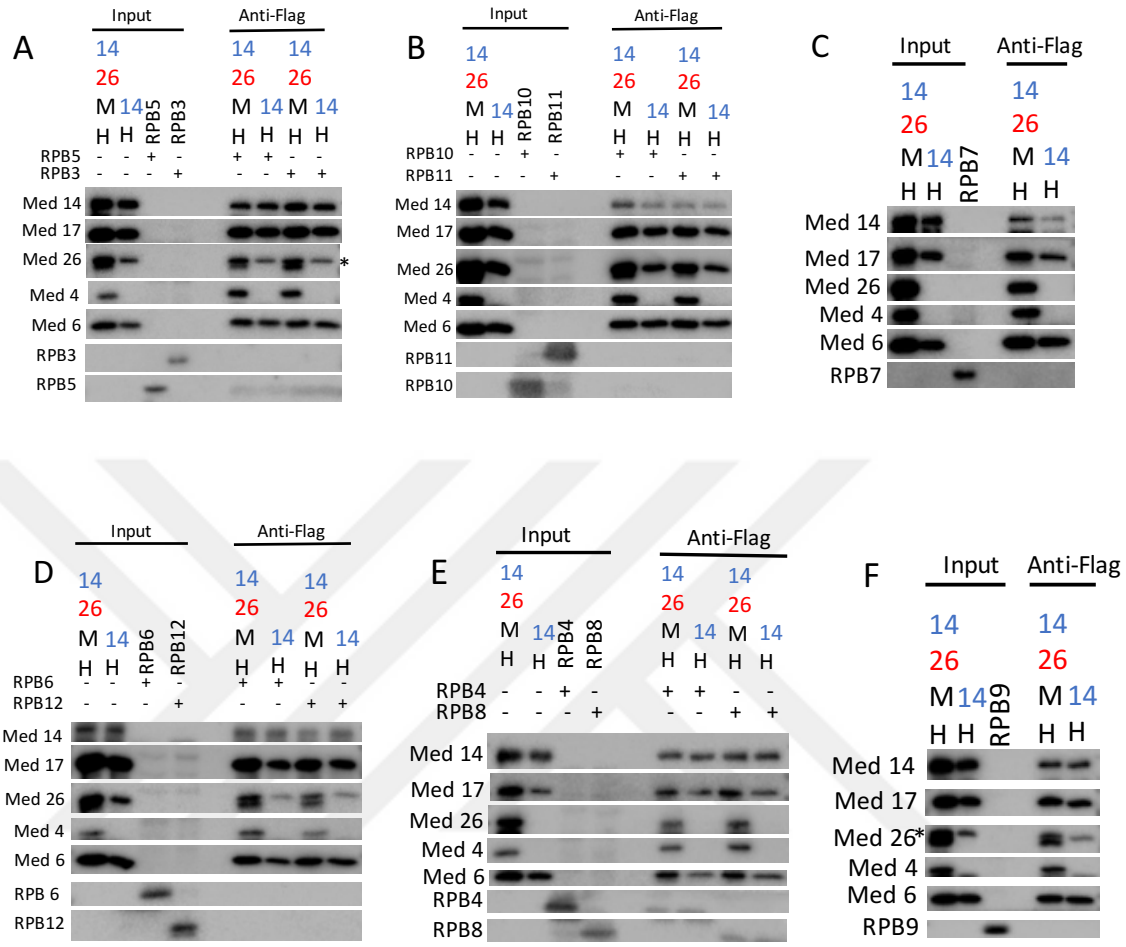

Figure 8: Immunoprecipitation (IP) of human RNA polymerase II subunits with purified core Mediator complex and Head module of Mediator complex. A) Western blot analysis of IP between His-RPB3 and His-RPB5 subunit of human Pol II with core Mediator and head module of Mediator complex. **B)** Western blot analysis of IP between HA-RPB10 and HA-RPB11 with core Mediator and head module of Mediator complex. **C)** Western blot analysis of IP performed between His-RPB7 with head module and core Mediator complex. **D)** Western blot analysis of IP conducted between His-RPB6 and His-RPB12 with head module and core Mediator complex. **E)** Western blot analysis of IP between HA-RPB4 and HA-RPB8 with core Mediator complex and head module of Mediator complex. **F)** Western blot analysis of IP performed between His-RPB9 with head module and core Mediator complex. * indicates non-specific bands.

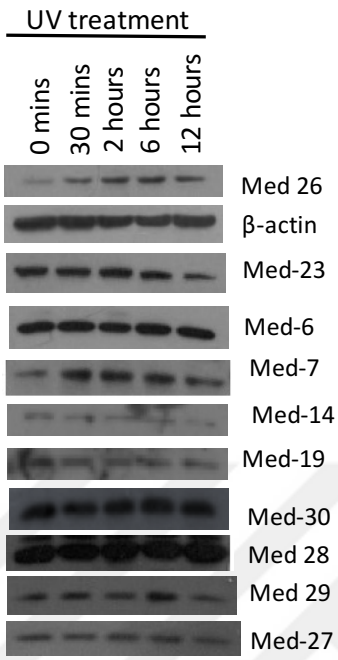

Figure 9: Screening of Mediator complex subunits in HEK 293T cells upon UV treatment. Western Blot Analysis of Mediator complex subunits upon 40uJ UV treatment for up to 12 hours.

4.5 Characterisation of human Mediator subunits in MCF-7 wild type and Tamoxifen resistant cells and their recruitment to the ERE-promoter.

For the past 30 years, tamoxifen had been used to treat breast cancer at various stages of disease. It is a selective estrogen receptor modulator (SERM) that competes with estrogen for binding to estrogen receptor (ER) and acts as an agonist to prevent transcriptional activity downstream of ER[218]. Screening of Mediator complex subunits was carried out on MCF-7 breast cancer cell line to identify if the resistance to SERM such as tamoxifen can be explained using aberrant expression of Mediator subunits. Upon tamoxifen treatment, ERα level for wild type and tamoxifen resistant MCF-7 cells increases which is an expected outcome but many of Mediator subunits' expression level did not change including Cdk8, Med28, Med6, Med27, Med23, Med24, Med29 and CyclinC (figure 10A). Med1, Med12 and Med13 expression level increases when the cells are treated with tamoxifen for both wild type and tamoxifen resistant cells

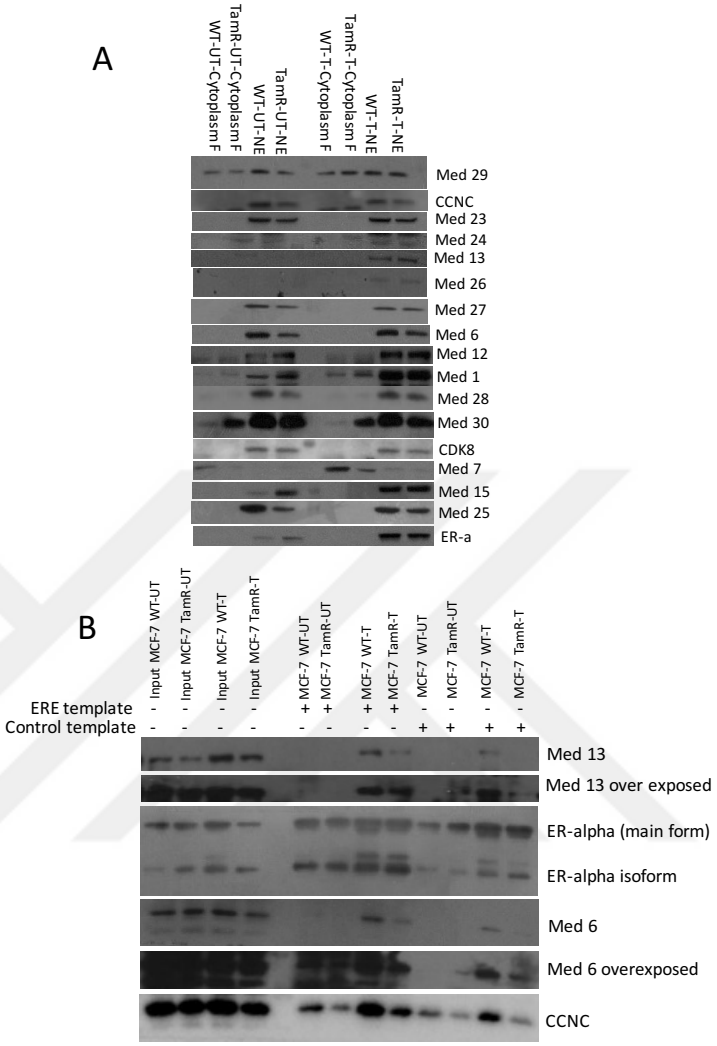

Figure 10: Characterisation and Immobilised template recruitment of Mediator complex subunits in wild-type and tamoxifen resistant MCF7 cells. A) Western blot analysis of Mediator complex subunits in wild-type and tamoxifen resistant MCF7 cells. 40 µg of protein was loaded for each sample. **B**) Western blot analysis of Immobilise template recruitment assay to determine the occupancy of Mediator complex subunits on estrogen response element (ERE). WT denotes wild type, Cytoplasmic F indicates cytoplasmic fraction, UT denotes untreated, T denotes treated with tamoxifen, TamR corresponds to tamoxifen resistant and NE represents Nuclear extract.

(figure 10A). Although Med30 expression does not change when treated with tamoxifen, its' expression profile is very distinct from other subunits as it is highly expressed in the

cytoplasm of tamoxifen resistant cells. Med15 also showed an increase in expression in tamoxifen resistant untreated cells (figure 10A). To understand the mechanism by which Mediator interacts with ER and transmits the regulatory signals to Pol II, Immobilised template recruitment assay was performed. The assay showed that upon tamoxifen treatment, Med13, Med6 and Cyclin C recruitment to the ERE increases (figure 10B). Although these proteins are recruited to the non-ERE template as well, the recruitment is two times more in ERE template which is proportional with the recruitment of ERα as well. Med13, Cyclin C and Med6 is recruited more in MCF-7 wild type cells as compared to tamoxifen resistant cells (figure 10B).

CHAPTER 5

Discussion

This study is continuation of Dr Cevher and his published work in 2014[10]. As mentioned earlier, core Mediator is sufficient to interact with RNA polymerase II and recruit it to the promoter to facilitate basal and activator mediated transcription[10]. Previous work of former graduate students highlighted that N terminus of Med14 containing core Mediator is responsible for the interaction with Pol II. This study sheds light into the individual subunit of Pol II that interacts with NTD Med14 containing core Mediator complex.

To determine the individual subunits of Pol II that interacts with core Mediator complex, Multibac expression system had to be utilised as flag pull down of FLAG-RPB1 from HEK 293T cells or flag-RPB9 expressing HeLa cells brought other subunits of Pol II as well (figure 3D and 4A). The 15 subunit core Mediator that contains Med26 interacts with endogenous RPB1 subunit of insect cells therefore a band above Med14 can be seen (figure 3A). However, this band is not seen when a 14 subunit head+middle+Med14+Med17 is purified using anti-flag agarose beads (figure 3B) signifying an undiscovered mechanism by which Med26 regulates the interaction of Mediator complex with Pol II. As described in Cevher et al[10], Med26 is required for core Mediator interaction with Pol II in an extract based system and is not needed when purified Pol II is used. As nuclear extract contains thousands of other proteins, there must be some suppressor of Pol II-Mediator interaction which can be overcome in the presence of Med26.

The recombinant His-RPB1 has a larger molecular weight as compared to endogenous one thus, it is seen above the endogenous RPB1 (figure 5B1 and C1). While other subunits of Pol II did not interact with core Mediator complex (figure 8), His-RPB1 interacted and competed with endogenous Pol II for the interaction with core Mediator. RPB2 subunit has not been tested for its binding with core Mediator complex hence, it needs to be verified if it has a role in the binding of Pol II with core Mediator complex.

The main aim of the experiment was fulfilled as the interaction surface between NTD14 containing core Mediator complex and Pol II was identified. Whether RPB1-NTD 14 core Mediator complex is functionally active or not remains to be elucidated. Purified RPB1 is required to perform such assays. As there are many proteins that exhibit endogenous His-tag, Ni-NTA purification does not yield a pure His-RPB1 protein (data not shown). While *in vitro* transcription using purified system as explained in Cevher et al[10] can be used to determine if RPB1-NTD Med14 core Mediator complex can yield an RNA transcript, nuclear extract system cannot be used as endogenous Pol II will interfere with reaction. We propose a model of transcription (figure 11) which demonstrates that NTD Med14 core Mediator complex interacts with RPB1 subunit of Pol II and recruits it to promoter to induce transcription (data not shown). Although recruitment and active transcription yet remains to be elucidated, based on previous findings, it can be extrapolated to fit to the model.

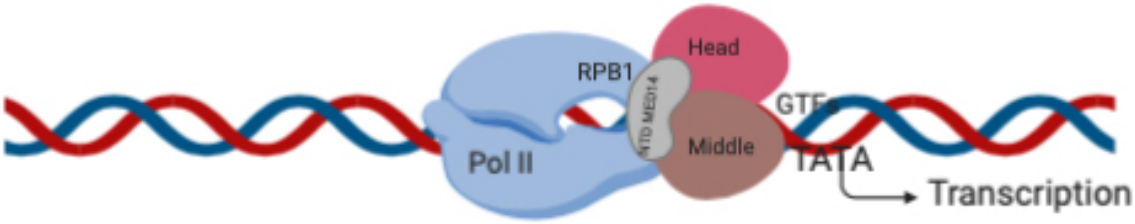

Figure 11: A proposed model for Pol II recruitment to promoter by core-Mediator complex. A model showing RPB1 subunit of Pol II interacts with NTD-Med14 of core Mediator complex, gets recruited to the promoter to facilitate transcription.

Med14 is a critical subunit for the interaction with Pol II. In an effort to understand if it interacts with Pol II, recombinant Med14 was expressed using baculovirus and tested for interaction with Pol II. However, the protein aggregated and did not interact with Pol II. In order to eliminate the possibility of misfolding, flag-Med14NTD was transiently transfected to HEK 293T cells and checked whether it interacts with His-RPB1. The results indicated that it did not interact therefore pointing out the fact that Med14 plays a

role in restructuring of the core Mediator which enables the interaction with Pol II. None of the core-Mediator subunits interact with Pol II since head module, middle module, head+middle, middle+17 were tested for the interaction with His-RPB1. Only head+middle+17+14 (full and NTD) interacts with His-RPB1 which supports the argument of role of Med14 in restructuring of Mediator complex. The mechanism by which this happens remains to be elucidated but with advancements in cryo-EM, it will soon be possible.

The Mediator complex also plays a vital role in other cellular processes such as transcription coupled DNA repair. Our data indicates that Med23 expression decreases upon 12 hours of UV treatment which is coherent with previous studies signifying that Med23 expression is inversely correlated with DNA repair[217]. Med17 has already been shown to play a critical role in DNA repair as it interacts with Rad2 and facilitates repair mechanism[214]. This needs to be investigated further as an insight into Mediator complex with respect to DNA repair would enable better understanding of the mechanism by which DNA is repaired. Moreover, it would be interesting to investigate if there are any intrinsic differences in the Mediator complex upon single strand break versus double strand break. This can be done via comparing UV treatment and drugs that induce double strand break such as cisplatin.

The Mediator complex has also been implicated with several other diseases including breast cancer. In an effort to understand the mechanism of tamoxifen resistance in breast cancer, we aimed to screen the Mediator complex subunits to identify if it has a particular role in it. Western blot analysis revealed that expression of majority of the Mediator complex subunits does not change when MCF-7 cells acquire resistance to tamoxifen or upon tamoxifen treatment. However, many subunits increase in expression such as Med1, Med12, Med13 and Med15 when the cells are subjected to tamoxifen treatment (both wild type and tamoxifen resistant cells) implicating that these subunits play a role in the pathway. Immobilized template recruitment assay also demonstrated that Med13 get recruited to ERE more strongly as compared to non-ERE template which signifies that it can be utilised for therapeutics for ER positive breast cancer. Apart from

that, variable expression of other Mediator subunit is seen upon acquiring tamoxifen resistance. This should be further investigated using RNA-Seq analysis to see if it supplements. Moreover, resistant to other drugs should be explored further in connection with Mediator complex as the tail module interacts with a vast amount of activators.

CHAPTER 6

Future Perspectives

The binding assays showed that recombinant His-RPB1 interacts with head+middle+14+17 subcomplex of human Mediator complex. This interaction lies in the NTD Med14 region which is approximately 84 kDa. The study by Robinson et al identified Mediator-Polymerase PIC structure through cross linking coupled with mass spectrometry and cryo-EM that showed the binding region between Pol II and Mediator complex[213]. However, the study did not encompass all the possible interaction between Mediator and Pol II. In fact, it did not show the most critical interaction of Pol II with Med14 which is coherent with previous studies showing that Med14 is critical for human Mediator interaction with Pol II but it is not essential for yeast Mediator Pol II interaction[10]. To get a deeper insight into the interacting surface between RPB1 and core Mediator(-Med26), large quantity of purified head+middle+Med14+Med17+His-RPB1 was produced as shown in figure 12.

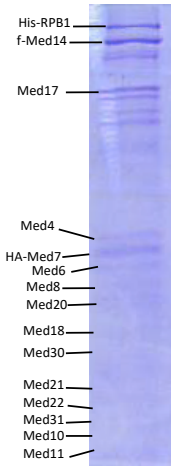

Figure 12: Purification of His-RPB1-core Mediator sub complex. A) SDS-PAGE followed by Coomassie staining of anti-flag pull down of His-RPB1+head+middle+14.

The aim is to get the protein complex crosslinked so that the interacting region can be narrowed down to individual amino acids. The protein complex (head+middle+14+His-

RPB1) was concentrated using Dyna beads for His-tag isolation (data not shown). To elute the proteins from dyna beads, 300mM imidazole was used. In order to perform the crosslink coupled with mass spectrometry, a collaboration with Albert J. R. Heck's lab has been formed. Disuccinimidyl sulfoxide (DSSO) was a generous gift from Heck lab and will be potentially used to perform the crosslinking of head+middle+14+RPB1 protein complex. As a trial, crosslinking using DSSO was performed that showed that imidazole interferes with crosslinking and increasing the crosslinker to 1mM could not result in crosslinking of the head+middle+14+RPB1 protein complex (data not shown). Whereas, the protein crosslinked efficiently in the absence of imidazole (data not shown). This shows a potential that the reconstituted protein complex can be crosslinked and examined using mass spectrometry to elucidate the interacting surface between RPB1 and Med14 of core Mediator complex. Consequently, cryo-EM can be performed to further comprehend the structure responsible for Pol II mediated gene transcription.

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