IDENTIFICATION OF GENERAL TRANSCRIPTION FACTOR-TWO-E AND MEDIATOR COMPLEX INTERACTIONS IN THE CONTEXT OF PRE-INITIATION COMPLEX FORMATION

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By

Onur Rojhat Karasu

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June 2018

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

IDENTIFICATION OF GENERAL TRANSCRIPTION FACTOR-TWO-E AND MEDIATOR COMPLEX INTERACTIONS IN THE CONTEXT OF PRE-INITIATION COMPLEX FORMATION

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M.Sc. in Molecular Biology and Genetics

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Transcription is a multistep process which requires the presence of many different proteins and protein complexes. The minimal protein components enabling transcription to happen are identified to execute the event *in vitro*. However, formation of the transcriptional machinery and the interactions of the proteins while forming the machinery are still not fully understood. General Transcription Factors (GTFs) are identified as the essential elements required for the transcription yet, it is still not clearly known how they are being recruited to the promoter site. Mediator Complex is known to relay the signal received from enhancer sequences to the transcription machinery which is called the Pre-Initiation Complex (PIC) right before the transcription. Here, we are aimed to characterize how Transcription Factor-Two-E is being recruited to TATA promoter by showing this recruitment is strongly corelated with Mediator complex. Besides, this recruitment of Transcription Factor-Two-E to TATA promoter may require more than one subunit of Mediator complex in addition to functional core. Also, here we confirm that Transcription Factor-Two-E co-precipitates with RNA Polymerase II (RNAP II) and TFIIH which are consistent with the existing data in literature. However, the details and the nature of these interactions are yet to be clarified. Identification of these interaction will hopefully result in insights about the sequential formation of PIC or pre-formed holoenzyme multi proteins.

Key Words: Mediator Complex, Pre-initiation Complex, General Transcription Factor-Two-E, transcription,

ÖZET

GENEL TRANSKRİPSİYON FAKTÖRÜ II-E VE MEDİATOR KOMPLEKSİNİN ETKİLEŞİMİNİN BAŞLAMA ÖNCESİ KOMPLEKSİ OLUŞUMU BAĞLAMINDA TANIMLANMASI

Onur Rojhat Karasu

Moleküler Biyoloji ve Genetik Yüksek Lisans

Tez Danışmanı: Murat Alper Cevher

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Transkripsiyon birçok proteinin ve protein kompleksinin gerekli olduğu çok basamaklı bir olaydır. Transkripsiyonu sağlayan proteinler, olayın yapay ortamda yapılmasına olanak sağlayabilecek kadar saptandı. Ancak, transkripsiyon sisteminin oluşumu ve bu oluşumu sırasında proteinlerin etkileşimlerinin neler olduğu hala tam olarak anlaşılmış değil. Genel Transkripsiyon Faktörleri (GTFler) transkripsiyon olması için temel elementler olarak tanımlandı ancak bu elementlerin de promoter bölgesine nasıl getirildiği tam olarak bilinmemekte. Mediator kompleksinin ise arttırıcı sekanslardan aldığı sinyali Başlama Öncesi Kompleksi denen transkripsiyon sistemine ilettiği biliniyor. Bu çalışmada, Transcription Factor-iki-E'nin TATA promoter bölgesine nasıl getirildiğini, bu olayın Mediator kompleksiyle güçlü bir ilgisi olduğunu göstererek karakterize etmeyi amaçladık. Ayrıca Transcription Factor-iki-E'nin Mediator TATA promoter bölgesine getirilmesi için fonksiyonel çekirdek alt birimlerine ek olarak birden fazla Mediator Kompleksi alt birimine ihtiyaç duyulabileceğini belirttik. Ayrıca, literatürdeki var olan bilgilere uygun olarak, Transcription Factor-iki-E'nin RNA Polimeraz II (RNAP II) ve TFIIH ile de birlikte çöktüğünü doğruladık. Her ne kadar bu etkileşimlerin detayı ve doğası henüz tam olarak aydınlatılamamış olsa da bu etkileşimlerin tanımlanmasının Başlama Öncesi Kompleksinin oluşumuna veya daha önceden oluşmuş holoenzim multi proteinlerine dair fikir vereceği ümit edilmektedir.

Anahtar Kelimeler: Mediator Kompleksi, Başlama Öncesi Kompleksi, Genel Transkripsiyon Faktörü-iki-E, transkripsiyon

To my dear family and my beloved sister;

I dedicate this study, for which I had to stay away from them a long time.

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Abbreviations

GTF	General Transcription Factor
Pol II	RNA Polymerase II
RNAP II	RNA Polymerase II
PIC	Pre-initiation complex
TFIIA	Transcription Factor IIA
TFIIB	Transcription Factor IIB
TFIID	Transcription Factor IID
TF-Two-E	Transcription Factor IIE
TFIIF	Transcription Factor IIF
TFIIH	Transcription Factor IIH
TAF	TBP Associated Factors
TBP	TATA Binding Proteins
Med	Mediator complex
CDK8	Cyclin Dependent Kinase 8
CCNC	Cyclin C
SEC	Super Elongation Complex
kDa	Kilo Dalton
RXN	Reaction
IPTG	Isopropyl β-D-1-thiogalactopyranoside
PC	Positive Factors
NC	Negative Factors

NAT	Negative regulator of Activated Transcription
SRB	Suppressor of RNA Polymerase B
TRAP	Thyroid Hormone Associated Protein
CRSP	Cofactor Required for Sp1 Activation
SMCC	SRB/MED Cofactor Complex
BSA	Bovine serum albumin

CHAPTER 1

INTRODUCTION

1.1. Eukaryotic Transcription System and Machinery

1.1.1. RNA Pol II Mediated Transcription and Transcriptional Steps

The central dogma is the process which converts the genetic code from DNA to RNA and then to the proteins [1]. Transcription is the process in which DNA is used as a template to make RNA in a very ordered and conserved way by the function of RNA Polymerase [2]. The RNA Polymerase was first encountered by Gladstone and Weiss in nuclei of rat liver cells in 1959 and Hurwitz and Stevens have seen the same activity in *Escherichia coli* in 1960 [3-4]. With those discoveries, the role of RNA Polymerase on transcription of DNA has been settled as a universal function. Until now, four different RNA polymerases have been discovered and named as RNA Polymerase I, II. III and IV. The RNA Polymerases I, II and III were named according to their fraction numbers while purification and they are involved in 18S and 28S rRNAs (I), mRNAs (II) and tRNAs (III) transcription processes respectively [5]. RNA Polymerase IV was discovered in last decade in plants functioning by facilitating the production of small interfering RNAs (siRNAs) [6]. Due to the distinct functions of these RNA polymerases, they require different sets of additional proteins and here we will be focusing on the transcription event mediated by RNA Polymerase II (RNAP II).

For RNAP II to start the transcription of a gene, three major steps are executed: (I) Chromosome opening and the formation of euchromatin, (II) modification of histones on the related gene for the access of activators and silencers and (III) assembly of the transcription machinery at the promoter site of the gene [7].

1.1.2. Difference of Basal and Activator Driven Transcription

Activators and repressors are the proteins required for the regulation of transcription of a specific gene or a set of genes in cells. For the activator dependent transcription to happen, it is crucial that related activator or activators sits on the corresponding Enhancer sequence sites [8]. For instance, Estrogen Receptor- α is an activator and needs to sit on to Estrogen Enhancer Sequence (EES) site to start activator dependent transcription. The specificity of an activator to its related set of genes comes from its special DNA binding domain which prevents it to bind any other enhancer sequences than its own [9]. The basal level transcription is the observed level of expression of a gene even under the suppression and executed by the general transcription machinery. The difference between activator dependent transcription and basal transcription starts from the distinct core promoter sites of these two events. Upon the binding of activator, the signal of enhancer is relayed to the transcription machinery and boost the transcription by both increasing the transcribed mRNA levels and speculatively by helping to bring the transcription machinery to the core promoter sites [10].

1.1.3. Recruitment of RNAP II and GTFs to the Promoter Site

Apart from the gene specific activators, there are some set of proteins required for the transcription to happen. Upon binding of activator, the transcription machinery is recruited to the promoter site. This transcription machinery is composed of RNAP II and some other accessory proteins which are required in the site-specific transcription initiation. The necessity of these accessory proteins was first shown by Weil in 1979. He achieved in vitro transcription by additionally putting crude subcellular extraction fractions onto the RNAP II with the native adenovirus DNA template [11]. Further examinations for identification of these accessory proteins in the extract fractions revealed different proteins eluted in increasing salt concentrations in ion exchange chromatography [11]. At the end these proteins were identified as transcription factors, named as TFIIA, TFIIB, TFIID, TF-Two-E, TFIIF and TFIIH and characterized as general transcription factors (GTFs). In the nomenclature the TFs stand for transcription factor, the roman

number represents the RNAP II dependent transcription and the letters correspond to the chromatographic fractions at which each protein was eluted [12].

Table 1.1: The elements of human transcription machinery and their functions in general transcription [13]

Factor	Protein Composition	Function
TFIIA	p35 (α), p19 (β), and p12 (γ)	Antirepressor; stabilizes TBP-TATA complex; coactivator
TFIIB	p33	Start site selection; stabilize TBP-TATA complex; pol II/TFIIF recruitment
TFIID	TBP + TAFs (TAF1-TAF14)	Core promoter-binding factor, Coactivator, Protein kinase, Ubiquitin-activating/conjugating activity, Histone acetyltransferase
TFIIE	p56 (α) and p34 (β)	Recruits TFIIH, Facilitates formation of an initiation-compentent pol II, Involved in promoter clearance
TFIIF	RAP30 and RAP74	Binds pol II and facilitates pol II recruitment to the promoter, Recruits TFIIE and TFIIH, Functions with TFIIB and pol II in start site selection, Facilitates pol II promoter escape, Enhances the efficiency of pol II elongation
TFIIH	P89/XPB, p80/XPD, p62, p52, p44, p40/CDK7, p38/Cyclin H, p34, p32/MAT1, and p8/TFB5	ATPase activity for transcription initiation and promoter clearance, Helicase activity for promoter opening, Transcription-coupled nucleotide excision repair, Kinase activity for phosphorylating pol II CTD, E3 ubiquitin ligase activity
RNAP II	RPB1-RPB12	Transcription initiation, elongation, termination, Recruitment of mRNA capping enzymes, Transcription-coupled recruitment of splicing and 3 end processing factors, CTD phosphorylation, glycosylation, and ubiquitination

Even though the components of the human general transcription machinery are known, there are two different hypotheses about how the formation of this machinery occurs. The first hypothesis suggests a sequential assembly before the initiation of transcription based on the in vitro transcription assays conducted with different chromatographic fractions of crude HeLa cell extracts [14]. According to it, after the DNA is opened for transcription, the TFIID is recruited to the TATA box and binds to the DNA

via one of its components, Tata Binding Protein (TBP) [15]. This prokaryotic σ-factor-analogous function of TBP was first discovered in *Drosophila* [16], then in mammals [17] and lastly in *S. cerevisiae* (yeast) [18]. After that, the TFIIA comes as a facilitator for TFIIA-TBP-DNA complex formation by inhibition of inhibitory elements in the environment and raises the possibility of TBP or TFIID to bind to the DNA [19]. Also, it helps the stabilization of the TBP-DNA complex by binding the complex from an opposite direction of the TFIIB binding site [20]. The other stabilizer of the TBP-TATA box complex is the TFIIB. As mentioned before, the TFIIB and TFIIA bind to the opposite sites of the TBP-DNA complex to increase the stability of the complex [20-21]. Also, the TFIIB acts as a recruiter for the RNAP II-TFIIF complex so that it marks the transcription start site [22] and helps the RNAP II-TFIIF complex to dock on the DNA [23]. The TFIIF is closely interacting with RNAP II [24] and its entry to the promoter site happens together with RNAP II. It has some distinct functions like helping RNAP II to enter to the transcription machinery by docking onto readily formed TFIIA-TBP-TFIIB-DNA complex [25], stabilizing the RNAP II on the complex by bending the DNA towards RNAP II and creating new DNA and protein interaction sites [26], enabling the Transcription Factor-Two-E -TFIIH entry to the complex [21] determining the start site of transcription along with RNAP II and TFIIB and lastly helping the RNAP II to leave the promoter site upon the transcription initiation 28]. The Transcription Factor-Two-E on the other hand interacts with several members of Pre-Initiation Complex (PIC) but it mainly recruits TFIIH to the PIC and regulates its ATPase 29], kinase [30], and helicase [31] activities. The general functions and detailed structures of Transcription Factor-Two-E will be explained in "General Transcription Factor IIE" section. The last member of PIC is the TFIIH and it is necessary for a couple of reasons. Its ATPase activity is required for promoter clearance, helicase activity is required for unrolling the DNA and kinase activity is required for CTD phosphorylation of RNAP II to stimulate transition from initiation stage [13].

The second theory is based on the existence of different subpopulations of transcription machinery containing RNAP II. According to that, the RNAP II holoenzyme is found in nucleus with different combinations of GTFs or even without the GTFs [32]. The different versions of the holoenzyme complex were eluted by using different

chromatographic conditions for purifications and separate laboratories obtained varying subsets of the holoenzyme. The representation of both pathway can be seen in the Figure 1.1 below. There are enough evidence supporting both pathways so it is important to speculate that the variations in the environment and physiological conditions determine pathway choice and formation of PIC.

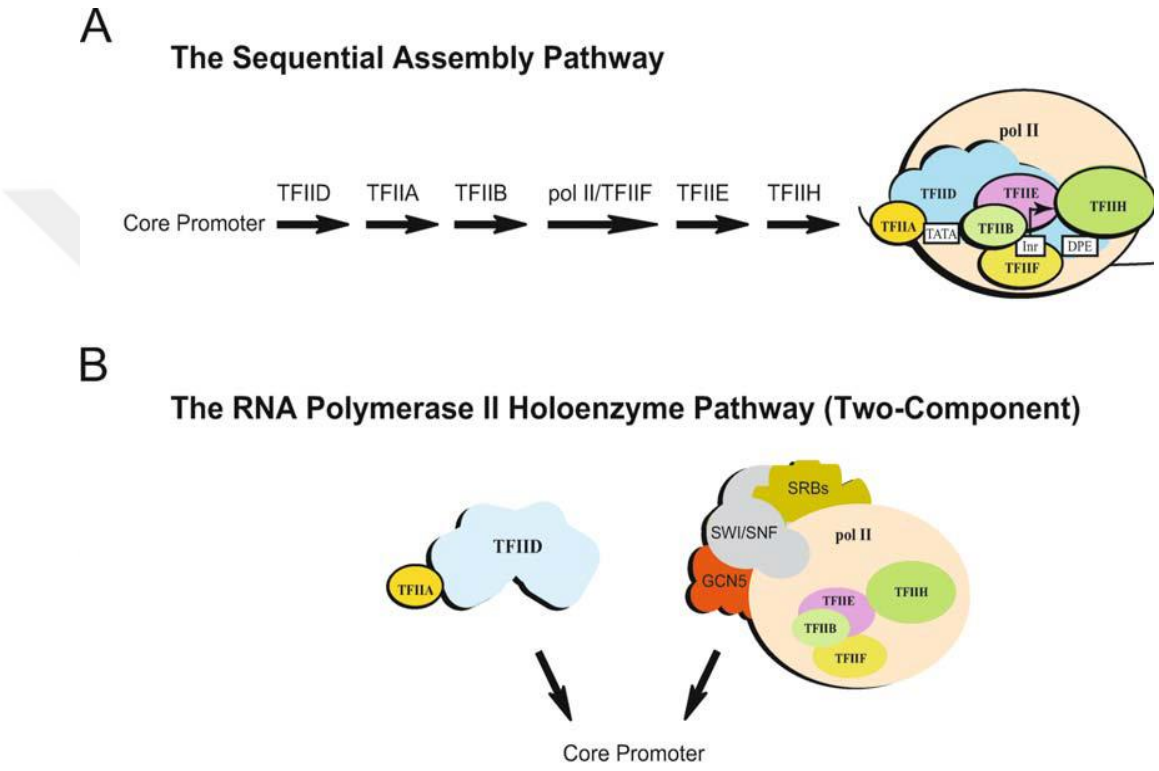


Figure 1.1: The panel A show the sequential assembly of PIC in the described order and panel B shows the Holoenzyme model in two different subpopulations [13].

1.1.4. TAFs, PCs and NCs

Further in vitro transcription assays for revealing the core elements of transcription machinery for activator dependent transcription showed some controversial results about the requirements of some GTFs. Separate laboratories identified different elements so it can be speculated that the variations in purification steps can result in the elution of some distinct factors enabling the transcription. However, it was shown that TBP, which is a

subunit of TFIID, is not sufficient on its own for the transcription [33]. This finding then followed by the finding of TBP Associated Factors (TAFs) which are also identified as the subunits of TFIID and characterized as activator dependent transcription regulators that are transmitting the signal from activator to the machinery [33]. Thus, the usage of TAFs with TBP which is the TFIID itself or presence of only TBP determines activated or basal level transcription respectively. Later, the biochemical studies identified driver regulators other than TAFs from the cell extracts which are called as Upstream Stimulatory Activity (USA) [34] and these activities involved both activation and repression so they were divided into one Negative Cofactor (NCs) and four Positive Cofactors (PC1-4) which are working as a coactivator in the presence of the activators and working as a repressor to stop basal transcription in the absence [35], so their function depends on the presence and absence of activators. Although there were some matching elements in these PC fractions, the PC2 is important especially since it is composed of many subunits which are later identified as belonging to Mediator Complex [36] that is also a general cofactor relaying the gene specific signals from activators or repressors to the transcription complex [37].

1.2. Human Mediator Complex

At the beginning, Mediator Complex was found in *S. cerevisiae* composing of 11 essential and 9 accessory subunits which makes a 20-subunit protein complex [38]. The human mediator complex was first purified as human Thyroid Hormone-α Receptor Associated Protein (TRAP) Complex. This complex was eluted together with human Thyroid Hormone Receptor-α (hTR-α) and enables in vitro transcription when a DNA template that includes T3 Response elements or TREs is used [39]. Later, many subunits of the Mediator complex were also found in independently purified protein complexes like SRB/MED-containing cofactor complex (SMCC) which contains TRAP220 TRAP170 and TRAP 100 in common with TRAP complex [40], activator-recruited complex (ARC) [41], vitamin D receptor interacting protein complex (DRIP) [42], cofactor required for Sp1 activation (CRSP) which also shares some subunits with TRAP complex [43], Positive Cofactor 2 (PC2) [35] and negative regulator of activated transcription (NAT)

which has indeed a repressive role in transcription due to the repressor function of CDK8 [44].

1.2.1. Presence of Different Forms of Mediator Complex in Cell and The Assembly Question

As mentioned before, there are many Mediator-like subpopulations in the cell. Those subpopulations contain some subunits which are identified as Mediator complex subunits. Other subunits of those subpopulations represent a very high homology with Mediator complex elements. These findings show us that Mediator Complex can be found in cells in different versions which are composed of varying subunits and that creates a high heterogeneity for the Mediator Complex. Mediator complex is present in a very low amount in cell and has that high heterogeneity. The complex is also very dynamic and flexible which makes the complex very hard to study [45]. Some defined subpopulations of Mediator Complex were MED13(TRAP240), MED12(TRAP230), Cyclin C, and CDK8 subcomplex [46] and MED23(hSUR2), MED24(TRAP100) and MED16(TRAP95) subcomplex [47]. Many other studies based on a subunit of Mediator Complex also describes that heterogeneity. For example, the Zhang, X. et. al., (2005) [45] mentions the versions of Mediator complex with and without MED1/TRAP220 by pointing out the presence of MED1/TRAP220 subunit only in some subpopulations [45]. Due to this dynamic structure and high heterogeneity, the human Mediator Complex is defined into modules which are the Head, Middle Tail and CDK8 containing Kinase module [48]. The first question here to be asked is that how such a complex is being formed in a cell. It requires further examinations and analysis to determine if those subpopulations act as pre-formed structures to be assembled after being induced by a regulator or a sequential de novo assembly happens for the whole Mediator formation.

1.2.2. Function, Structure and Architecture of Human Mediator Complex

The multi-subunit human Mediator Complex is a 30-subunit complex in humans. As mentioned before, it has the Head, Middle, Tail and Kinase modules even though the

composition of these modules differ in separate papers from independent laboratories. The first functional identification of human Mediator Core Complex was done in 2015 which shows that the Head, Middle, Med14 and Med26 forms the transcriptionally active core of the complex [49]. It is the function of Mediator to receive signals from regulatory pathways in a cell and to turn it into a transcriptional response [50]. Mediator Complex is the hub for many distinct pathways where the signal is converted and transmitted to transcriptional machinery. Since the Head and Middle modules along with Med14 gives the functionality to the complex, it is important to speculate that these distinctive subunits of each pathway, that are resulted from physiological conditions of a cell, rest in the Tail and Kinase subunits. The representation of some identified subunits for separate pathways can be seen in Figure1.2 below.

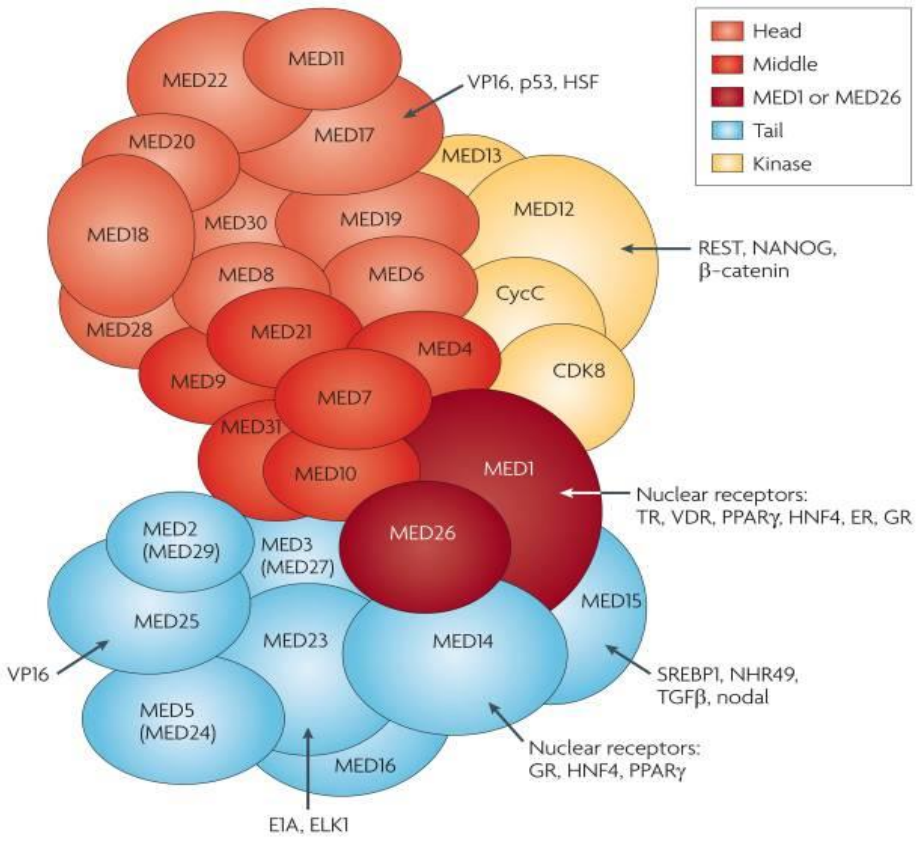


Figure 1.2: Representation of modular structure of Mediator Complex and some distinct subunits identified for regulatory pathways [50].

1.2.3. Effects of Mediator's Architecture and Modular Structure on Its Function and Regulations of Mediator Complex in Transcription and Pre-initiation Complex Formation and Function

As it was mentioned before, the mammalian Mediator Complex has 30 subunits and 4 of these subunits are of Kinase module [48]. Due to the distinct functions and dynamic interactions with Mediator Complex, CDK8 containing Kinase module sometimes counts as separate and Mediator Complex is told to have 26 subunits [46]. Since the Transcription Factors (TFs) have separate and different binding domains from each other, Mediator Complex can also bind multiple TFs instead of interacting them individually. Also, due to its interactions with the PIC, it can be said that the Mediator Complex also has a high number of interactions with RNAP II even though the exact mechanism of Mediator Complex - RNAP II interaction is not known yet.

Since the composition of Mediator also changes much even within the same cell, this ability of Mediator to detach from its subunits according to the executed function makes it mechanistically important for the transcription. Some studies also found and purified Mediator complexes that are lacking different numbers of Mediator subunits apart from the Kinase Module (e.g. PC2) [51-52]. Since these Mediator Complexes cannot transmit the signal coming from the TFs that bind to the lost subunits, this creates a direction in the selection of the transcribed gene. In such cases, it is shown that the Med1 binds to the thyroid hormone receptor(TR) and its knock out stopped the TR induced transcription [39] and Med23 is shown to bind ELK1 [53]. Again, the knockout of Med23 causes an inhibition on the ELK1 activated gene expression [53]. It is important to add that these knockouts do not stop the transcription regulated by other gene specific TFs. [39-53] Thus it can be concluded that the Mediator Complexes having fewer subunits than whole Mediator Complex should have a more specified function.

Another structural tool of Mediator, the Kinase module, gives very distinct properties to the complex. It is known that the Kinase module has a reversible interaction

with the complex which is also very dynamic and this interaction creates a different sub-population of complex often called as CDK8-Mediator Complex [54-55]. When this subpopulation is further examined, it is seen that the Med26 subunit is not notably present in that subcomplex resulting in a 29 subunit Mediator. [56] Even though the exact mechanism underlying the association and dissociation of Kinase module is not fully figured, some studies suggest the ubiquitination and phosphorylation of Med13 may have link to that event [57]. In that aspect, it is hypothesized that the PARP1 protein which normally ribosylates proteins [58] and FBW7, which is a ubiquitin ligase, stimulates the dissociation of Kinase module from the Mediator Complex [57-58]. Also, it is being thought that the structural changes upon the binding of Kinase module to the Mediator complex prevents the RNAP II-Mediator interaction [59]. The RNAP II Carboxyl Terminal Domain (CTD) associated Mediator complex has failed to show a presence of kinase module subunits [59] and consistently the Kinase module bound Mediator did not pull down any RNAP II subunits in purifications [60]. Yet, when the initiation is completed, the re-association of Kinase module to the Mediator somehow starts the elongation step. [61] Besides, Kinase module incorporated Mediator Complex fails to bind to TFIIH which results in the inhibition of RNAP II - TFIIH interaction and prevents promoter escape. By this way, re-binding of new RNAP II to the same promoter is also prevented since RNAP II cannot leave the PIC [62].

The Mediator is thought to form many interactions with RNAP II [63-64] and this interaction may create a scaffold for the PIC formation since the Mediator Complex transmits the signals from TFs to RNAP II and PIC which is called as the bridge model [65-66]. Some studies suggest that the recruitment of RNAP II via Mediator Complex happens due to its association with RPB1 subunit [61]. Also, it is thought that the Mediator Complex regulates the RNAP II entry to the PIC with the involvement of GDOWN1 which is a factor that binds to RNAP II when it is not Mediator bound and thus inhibits transcription by disrupting the TFIIF-RNAP II interaction [67-68].

Studies about the transcriptional elongation showed that the Med26 may interact with the Super Elongation Complex (SEC) and may help the initiation of elongation [69]. Interestingly, the same studies show Med26 to bind TFIID as well and suggest that the facilitative role for Med26 in PIC formation and then a regulatory function in elongation upon a regulatory signal due to its interaction with SEC [69]. These data also bring a proposition for that integrated roles of Mediator, GDOWN1 and SEC by stating a configurational remodeling or switching system. Additionally, Med26 has been shown to colocalize with the heterochromatin in contrast with other Mediator Complex subunits which can bring an explanation to the antagonistic function of Mediator and heterochromatin regions in humans [70].

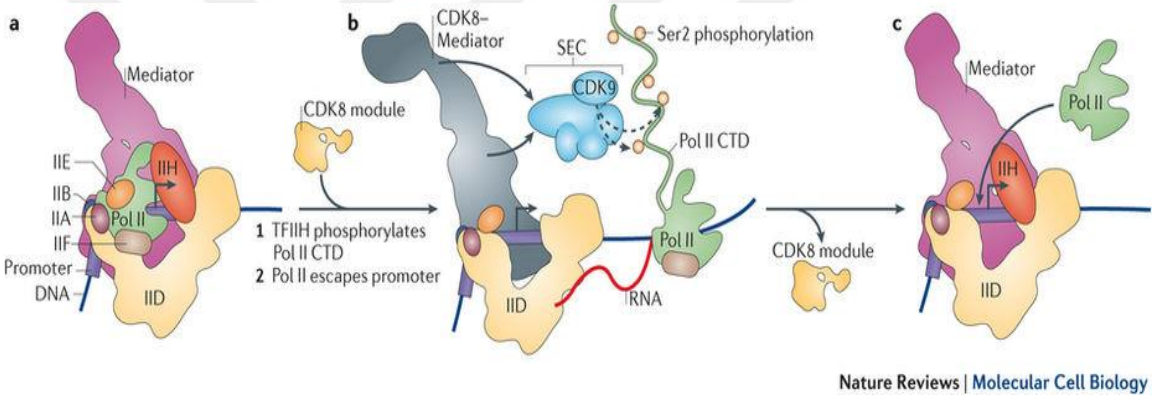


Figure 1.3: Representation of Mediator-dependent Transcription in PIC formation, initiation and elongation [71].

As it can be seen from Figure-3 above, in Panel A, Mediator Complex first helps the formation of the PIC by bringing RNAP II and other TFs to the promoter site [72] At the beginning, The CTD of RNAP II is not phosphorylated much but with the actions of TFIIH, it gets phosphorylated and initiate the transcription which is called the promoter escape (at serine 2 and serine 5 residues of the heptad repeats) [73]. After nearly transcribing 50 to 60 nucleotides, RNAP II stalls at some genes [74]. Although it is not known whether Mediator complex stays interacting with stalled RNAP II, Panel B represents the situation after the Mediator Complex and RNAP II bonds are seized and Kinase module entry to Mediator Complex happens since they have an exclusive behavior when binding to Mediator Complex. At this stage, it is shown that the CDK8 associates with SEC to stimulate the stalled RNAP II to go on with elongation [61-75]. It is also

predicted that the Med26 can have a role at this stage by dissociating from complex upon binding of Kinase module. The Panel C shows the recruitment of a new RNAP II by the remaining PIC which is called as re-initiation [76].

1.3. General Transcription Factor IIE (GTF-TWO-E\TF-TWO-E)

1.3.1. Structure of GTF-TWO-E

As it was explained in Figure-1, in the sequential assembly of PIC, Transcription Factor-Two-E enters the complex after the RNAP II-TFIIF recruitment. The Transcription Factor-Two-E consists of two subunits which are named as Transcription Factor-Two-E - α and Transcription Factor-Two-E -β [77]. (Although at first it was thought that this protein works in a heterodimer structure with two α and two β subunits [77], later studies showed that the protein works as a α+β heterodimer form [78].) The α subunit is a 439-amino acid long polypeptide with a molecular mass of 56 kDa and the β subunit is a 291-amino acid long and 34 kDa polypeptide [77]. The predicted interaction sites and of Transcription Factor-Two-E subunits on the represented aminoacids sequences are shown in the Figure-4 below.

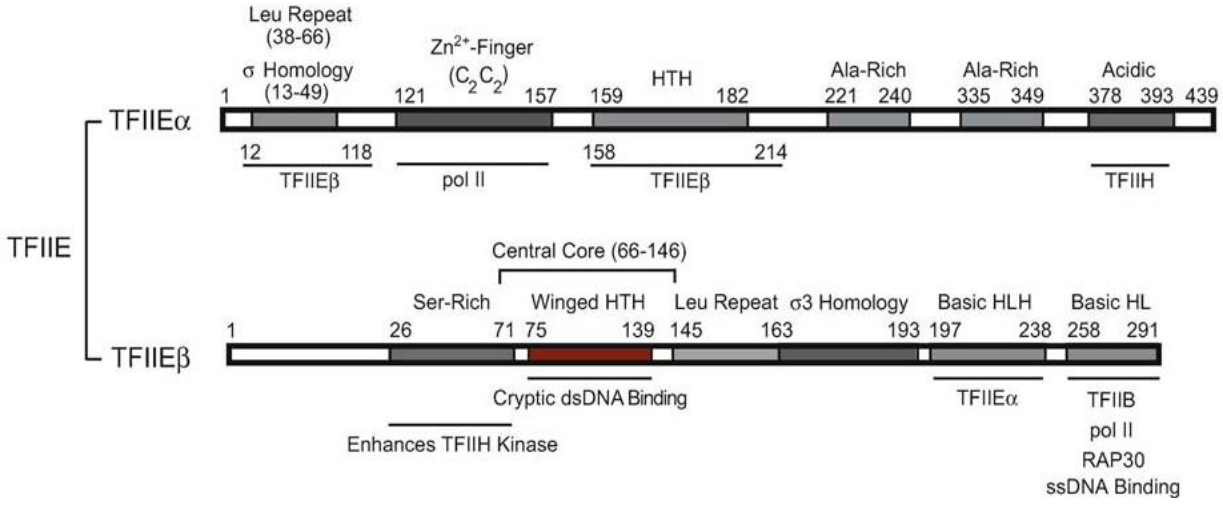


Figure 1.4: Represented amino acid sequences of both subunits of Transcription Factor-Two-E and their predicted interaction sites in corresponding domains [13].

The N-terminal domain of α subunit which is between 12th and 118th amino acids appears to interact with the Transcription Factor-Two-E -β by the region between 197th-238th amino acids. The C-terminal domain on the other hand appears to be the interaction site of α subunit and TFIIH [13]. Since it is known that the Transcription Factor-Two-E helps the recruitment of TFIIH to the PIC [79], it is quite logical to see a direct interaction between Transcription Factor-Two-E and TFIIH. Upon looking the Figure 1.4, there are some distinct regions in the subunits. For example, the α subunit has a homology with the bacterial σ factor at the residues between 13th and 49th amino acids, there is a leucine repeated part from 38th to 66th residues, the zinc finger domain is between 121st and 151st one Helix-Turn-Helix (HTH) motif domain between 159th and 182nd aminoacids. There are two different alanine rich domains following the HTH domain and close to the C-terminus, there is an acidic domain formed mainly with aspartic and glutamic acids between 378th and 398th aminoacids [13]. Due to the nature of zinc finger motifs, their function is mainly to bind the DNA. Generally, a zinc finger domain is composed of 3 or 4 beta strands as it is seen in TFIIB, TFIIS and RPB9 orthologs but the zinc finger domain of Transcription Factor-Two-E differs from others by having one alpha helix and two beta sheets in an ββαβββ order. The Zn^{+2} ion is binding to Cysteine amino acids at 129, 132, 154 and 157 locations [80]. The HTH domain of α subunit has a homology between archaeal TFE which also has a HTH motif and helps the transcription initiation by facilitating the TATA box recognition [81] This is the only predicted function for the HTH domain of human Transcription Factor-Two-E -α subunit.

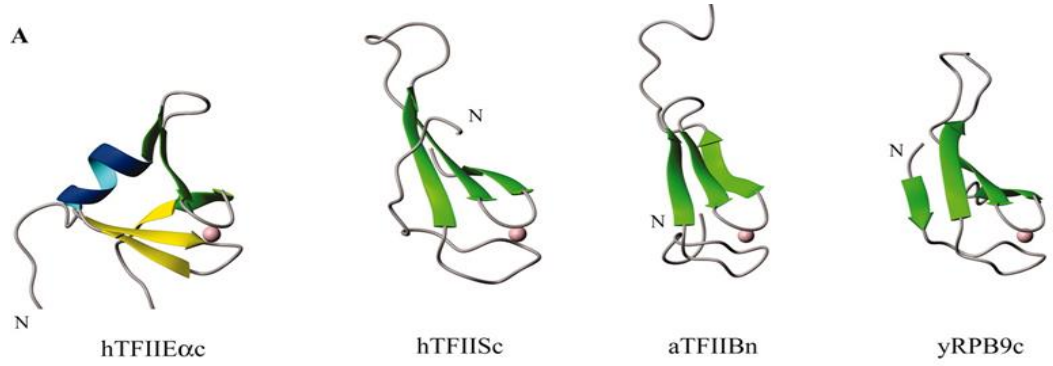


Figure 1.5: The cartoon representation of human Transcription Factor-Two-E α, human TFIIS, archaeal TFIIB and yeast RPB9 [80].

On the other hand, the β subunit also has some structurally distinct sites. There is a serine rich site between 26th and 71st amino acids and a winged HTH site in 75th and 139th residues. Then comes a leucine rich site between 145th and 163th residues and a bacterial σ factor subdomain 3 homology site between 163rd and 193rd amino acids [13]. The C-terminus contains two basic Loop-Helix-Loop and Helix-Loop domains. The homology predictions state a resemblance with the Krüppel TF of *Drosophila* which has a DNA binding function. Also, the Winged HTH region has a homology with the RAP30 from its RNAP II binding domain. Even though these interactions are predicted, more experiments are required to define the certain interaction sites [13-79].

1.3.2. Functions of GTF-TWO-E

In the literature, there are three different functions that are assigned to GTF-TWO-E. As it can be seen from the Table-1.1, these functions are to recruit the TFIIH to the PIC, to help the assembly of PIC and to help the promoter clearance. The indicated functions of Transcription Factor-Two-E domains and their sites can be seen in the Figure-6 below [78].

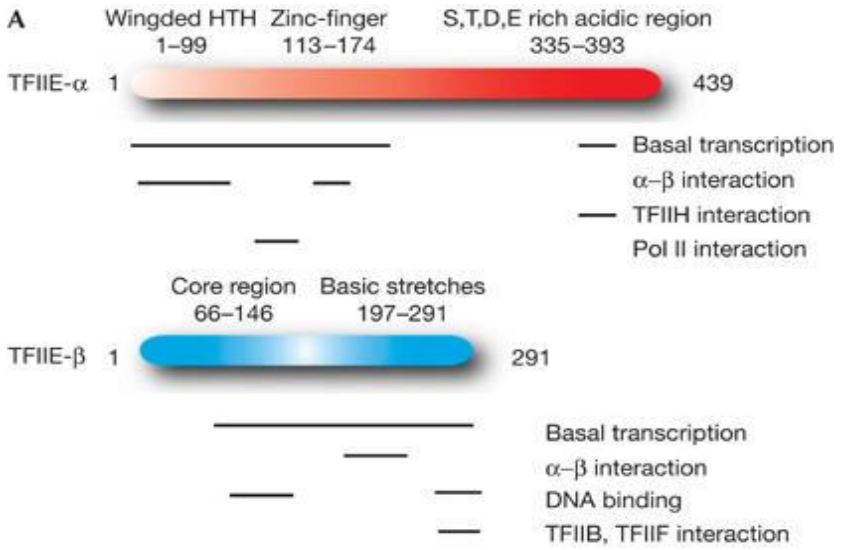


Figure 1.6: The representation of indicated functions of Transcription Factor-Two-E subdomains according to the computer based studies and predictions [78].

Even though it is not clear yet how the Transcription Factor-Two-E is being recruited to the promoter site at the beginning, there are some suggested candidates like Krüppel protein [82], Antennapedia Abd-B homeodomain proteins which are shown to bind Transcription Factor-Two-E *in vivo* [83] however, it is not clear if they also involve in the recruitment of Transcription Factor-Two-E to the promoter region and to the PIC. Due to its location in PIC, the Transcription Factor-Two-E is thought to be interacting with TFIIF, TFIIB, RNAP II and TFIIH [13].

The interaction of Transcription Factor-Two-E and TFIIH have been shown in many different studies. This interaction enables TFIIH to be recruited to the PIC at the promoter site. The absence of TFIIF prevented the TFIIH entry to the PIC [84]. The defined interaction of Transcription Factor-Two-E and TFIIH is on the C-terminus domain of Transcription Factor-Two-E -α subunit. This interaction enables Transcription Factor-Two-E to regulate the kinase and ATPase activities of TFIIH so that it phosphorylates the CTD domain of RNAP II and starts the elongation [79]. Also, the promoter region where Transcription Factor-Two-E sits is found as -10 where the Transcription Factor-Two-E also helps TFIIH helicase activity for promoter clearance at +1 and DNA melting [85]. It is also shown that the Transcription Factor-Two-E and TFIIH are not required for in vitro transcription from a pre-melted template using adenovirus promoter (AdE4) [85]. Thus, it has been concluded that the Transcription Factor-Two-E does not only recruit TFIIH but also act as a mechanistical check point from initiation to elongation stages [84] The co-occupancy between Transcription Factor-Two-E and TFIIB was also reported by glycerol sedimentation analysis [86]. The same method was also used to show the co-occupancy of RNAP II and Transcription Factor-Two-E without defining any specific subunits. This method only gives the conclusion that these two proteins (Transcription Factor-Two-E -TFIIB and Transcription Factor-Two-E -RNAP II) co-exist together and elute together [86]. It is also shown that Transcription Factor-Two-E co-purifies the non-phosphorylated RNAP II specifically [84] which is also logical since the aim of recruitment of TFIIH via Transcription Factor-Two-E is to phosphorylate the CTD and to start elongation.

1.4. Baculovirus Expression System

Baculovirus expression system has been designed to overcome some important obstacles emerged due to the nature of bacterial expression system. More important of these obstacles can be said as lacking eukaryotic post translational modifications and inability of producing large proteins. Also, the stability of the vector produced mRNAs in bacterial system is quite low and thus the assembly of the protein complexes produced under the same promoter, does not fully happen in the system [87].

The Baculovirus expression system for multi-protein complexes has two different expression cassettes with T7 and Cre/Lox translocation sites. These vectors, which are pFBDM and pUCDM respectively, contain polyhedron (polh) and p10 viral promoters with two different multiple cloning sites (MCSs) to allow cloning of genes of interest via digestion with a bunch of restriction enzymes. For the first selection, to distinguish the gene inserted vectors, pUCDM and pFBDM contain chloramphenicol and ampicillin resistance markers. To sub clone multiple genes, both vectors have a multiplication module which contain BstZ17I and SpeI restriction sites at the beginning and PmeI and AvrII restriction sites at the end of the cloning region [88]. After the genes are cloned to the vectors the procedure follows as described below in Figure-7

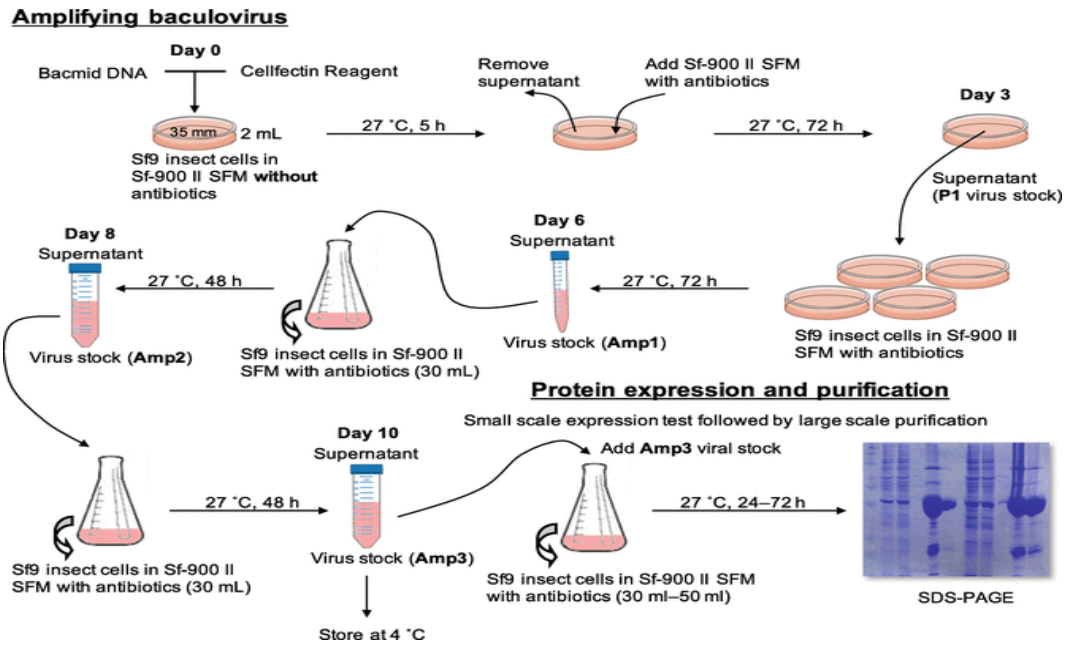


Figure 1.7: Summary of Baculovirus expression system for virus production and protein expression [89].

After the genes are cloned into vectors, the plasmids are transformed into the bacteria that has the Bacmid. Through Cre-Lox recombination and Tn7transposition, the plasmids are added to the Bacmids. The Bacmid DNA carries the LacZ gene for blue/white selection in DH10Bac cells. When the gene cloned plasmid is translocated into the Bacmid successfully, the Bacmid DNA is isolated and used for transfections with SF9 cells to generate the first generation of viruses called the P0 viruses. Then the amplification of the viruses can be made to create P1 and P2 and P3 generations and to increase the virus titers. Then the created viruses can be used for the expression of multi-subunit proteins in high quantities [88].

CHAPTER 2

MATERIALS and METHODS

2.1. MATERIALS

2.1.1. Tables for Required Materials, Buffers and Solutions

Table 2.1: Immobilized template recruitment assay using streptavidin Dynabeads buffers

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

Table 2.2: Buffers for protein extractions from SF9 insect cells

BC1000	BC0
20mM Tris-HCl (pH: 7.9 at 4 ^oC)	20mM Tris-HCl (pH: 7.9 at 4 ^oC)
20% Glycerol	20% Glycerol
0.1 mM EDTA	0.1 mM EDTA
0.5 mM PMSF	0.5 mM PMSF
0.5 mM DTT	0.5 mM DTT
1M KCL	

Table 2.3: Solutions for nuclear extract from HeLa cells

Solution I	Solution II
40 mM HEPES	40 mM HEPES
1.5 mM $MgCl_2$	1.5 mM $MgCl_2$
10 mM KCl	0.2 mM EDTA
0.5 mM DTT	0.5 mM DTT
0.5 mM PMSF	0.5 mM PMSF
	0.3 M NaCl
	25% sterile Glycerol

Table 2.4: Materials for preparation of SDS-PAGE resolution gels

Resolution Gel Percentage	7%	10%	12%	15%
ddH_2O	7.4ml	6.25 ml	5.20 ml	3.65 ml
Tris (1M pH:8.8)	3.45 ml	3.45 ml	3.45 ml	3.45 ml
5% SDS	300 ul	300 ul	300 ul	300 ul
30% Acylamide	3.75 ml	5 ml	6 ml	7.5 ml
10% APS	100 ul	100 ul	100 ul	100 ul
TEMED	10 ul	10 ul	10 ul	10 ul

Table 2.5: Materials for preparation of SDS-PAGE stacking gel

ddH_2O	3 ml
Tris (0.5 M pH:6.8)	1.25 ml
5% SDS	100 ul
30% Acylamide	670 ul
10% APS	50 ul
TEMED	5 ul

Table 2.6: Buffers required for western blotting

1 X SDS-PAGE Running Buffer	**1 X SDS-PAGE Transfer Buffer**
25 mM Tris	25 mM Tris
192 mM Glycine	192 mM Glycine
0.1 % SDS	10% Methanol
Membrane Stripping Buffer	**Coomassie Brilliant Blue Staining**
62.5 mM Tris-HCl (pH: 6.7)	0.1% Coomassie Brilliant Blue R20 (w/v)
2% SDS	50% Methanol (v/v)
100 mM Betamercaptoethanol	10% Acetic Acid (v/v)
	40% ddH_2O

Table 2.7: Other materials and solutions for western blotting

1X PBS-Tween	8 mM Na_2HPO_4, 150 mM NaCl, 2 mM KH_2PO_4, 3 mM KCl, 0.1% Tween20
Destaining Solution	50% H_2O, 40% Methanol, 10% Acetic Acid
30% Acrylamide (v/v)	292,2 gr/L Acrylamide, 7.8 gr/L Bisacrylamide
10% Ammonium Persuşphate (APS)	10 ml ddH_2O, 1 gr Ammonium Persulphate
4X SDS Loading Buffer	240 mM Tris-HCl (pH:6.8), 8% SDS (w/v), 40% Glycerol (v/v), 0.04% Bromophenolblue, 5% Betamercaptoethanol

Table 2.8: Used Antibodies in Western Blots

Med 12	Home made *	Med27	Santa Cruz Cat. No: Sc-390296
Med13	Home made *	Med29	Santa Cruz Cat. No: SC 393800
Med14	Abcam Cat No: ab170605	Med30	Home made *
Med15	Proteintech Cat. No: 115661AP	RPB1	Home made *
Med16	Santa Cruz	RPP62	Home made *
Med17	Home made *	CDK7	Cell Signaling
Med19	Home made *	CDK8	Home made *
Med23	Home made *	CCNC	Home made *
Med24	Home made *	XPB	Cell Signaling
Med25	Santa Cruz Cat. No: SC393759	Anti-Histidine	Cell Signaling
Med26	Home made *	Anti-Flag	Sigma Cat. No: F7425
GDOWN1	Home made *	TF-Two-E-α	Home made *

*Home made antibodies are produced at Rockefeller University, USA.

Table 2.9: Mediums required for GTF IIB, IIE and IIF production in BL21 Bacteria Cells

LB-Agar Plates	LB Medium	SOC Medium
10 gr Tryptone	10 gr Tryptone	2% Tryptone
5 gr Yeast Extract	5 gr Yeast Extract	0.5% Yeast Extract
10 gr NaCl	10 gr NaCl	0.4% Glucose
15 gr Agar	950 ml ddH2O	10 mM NaCl
951 ml ddH2O		2.5 mM KCl
		5mM MgSO4
		5mM MgCl2

Table 2.10: Other used materials and Kits

Anti-flag M2 Agarose Affinity Agarose Beads	Sigma Cat. No: A4596
Flag Peptide	Sigma Cat. No: F3290
Streptavidin Dynebeads M-280	Invitrogen Thermo Fisher Cat. No: 11205D
BCA Protein Kit Assay Kit	Thermo Fisher Pierce BCA Kit Cat. No: 23227
Restriction Value Pack	Thermo Fisher Fastdigest Restriction Set Cat. No. SM1553
Gel Extraction Kit	Thermo Fisher Gel Extraction Kit Cat. No: K0691
Plasmid Isolation Kit	Thermo Fisher Gel Extraction Kit Cat. No: K0502
Lyophilized Lysozyme enzyme	Sigma Aldrich Cat. No:
cDNA Synthesis RevertAid RT Reverse Transcription Kit	Thermo Scientific Cat. No: K1691

2.2. METHODS

2.2.1. Production and Purification of Mediator Complex Modules

The Head (H), Middle (M), Head+Middle (H+M), Head+Middle+14 (H+M+14) and Head+Middle+14+26 (H+M+14+26) complexes were readily purified as it is stated in Cevher et al. 2014 [49]. The Mediator complex subunits were cloned to the pFBDM and pUCDM Baculovirus expression system vectors with different tags like Flag, 6xHis and HA. Mediator Head module (Med6-8-11-18-19-20-22-30), Middle module (Med4-7-9-10-21-26-31) Med 17 and Med 14 were inserted into the vectors with tags of HA:Med7, His:Med10 and Flag:Med14. Then the Plasmids were integrated into the Bacmid DNA and amplified. After the purification of Bacmid DNA and transfection of Bacmid into the SF9 cells, first generation P0 viruses were created. Then the amplification of the P0 viruses were also made to the second and third generations P1 and P2 with a higher titer of viruses. So as to obtain the proteins, the infections of Head module virus and Middle module virus and co-infections of H+M, H+M+14, H+M+14+26 were done by infecting nearly 100ml 100 million Hi5 cells for 5 to 6 days. The titrations were determined according to the stoichiometric production of the complexes. To collect the cells, the suspension grown Hi5 cells were centrifuged at 1500 rpm for 10 minutes and supernatant was discarded. The pellet was re-suspended in 4 ml BC500 (5 ml BC1000 + 5ml BC0, 5mmDTT, 5mmPMSF) and cells were homogenized by douncing 3times. After centrifugation of the lysate at 14000 rpm for 15 minutes, pellet was discarded and supernatant was diluted to 300mM salt concentration by addition of BC0 in a drop by drop manner. The extract then incubated with the M2 and followed by HA agarose beads at 4^{o}C overnight to pull down the modules from the tagged subunits. After washing the beads with BC300+0.1%NP-40 five times, complexes were eluted with of 0.5mg/ml Flag or HA peptides to the corresponding beads. Elution step was repeated 3 times at 4^{o}C for 45 minutes in rotation and checked with Coomassie brilliant blue staining and Western blots.

2.2.1. Depletion of Mediator Complex from HeLa Nuclear Extracts

Depletion of Mediator complex from HeLa cell nuclear extract was done according to the procedure stated in Cevher et al., 2014 [49]. The depletion was checked again with Western blot.

2.2.2. Immobilized Template Recruitment Assay Using Streptavidin Dynabeads

2.2.2.1. Production of Template

In order to produce the TATA containing template DNA fragments, conventional PCR was used. Biotinylated primers were used to allow the binding of templates to the streptavidin beads. The PCR was conducted with the program shown below and the products were extracted from agarose gel with Gel Extraction Kit (Table 2.10).

Table 2.11: PCR program for TATA template multiplication

Reagents	10x Taq Buffer (5U/ul)	dNTP (10mM)	Forward Primer (10mM)	Reverse Primer (10mM)	TATA Template (10 ng/ul)	$MgCl_2$ (25mM)	ddH_2O
Added Volume (ul)	5	1	1,5	1,5	1	4	36

PCR cycle temperatures (°C)	95	95	68	72	72	4
Time (minutes)	4	0,5	1	1	7	Till taken
		34 cycles				

2.2.2.2. Fixing the Template to the Streptavidin Dynabeads

Dynabeads (10 ul/rxn) were shaken to homogenize the slurry and 10 ul for each reaction was taken into eppendorf tubes. Beads were washed with 1XB&W buffer (Table 2.1) for 5 times by putting the tubes on the magnetic rack each time for 90 seconds. Then beads were resuspended in 150 ul 2XB&W buffer. Nearly 8 ug template was added on to the beads to immobilize and volume was completed to 300 ul with H_2O addition. The beads were incubated at room temperature for 15 minutes by inverting the eppendorf tubes

in every 5 minutes to prevent aggregation and heterogenous distribution of the template. Then the tubes were put onto the magnetic rack again and the liquid buffer was taken out but not discarded since the amount of bound template can be checked by finding out the final concentration of the DNA in the buffer. The Dynebeads were then washed with 300ul of 1XB&W buffer with 0.5mg/ml BSA and 0.003% NP-40 for two times. After that the beads were blocked by addition of 100 ul of Blocking buffer and incubation at room temperature for 15 minutes. Finally, the beads were washed with 500 ul washing buffer two times and stored at +4 ^{o}C in 150 ul Wash buffer.

2.2.2.3. Immobilized Template Recruitment Assay

6 reactions were planned with HeLa nuclear extract (NE) and Mediator depleted nuclear extract (ΔMediator NE) by the addition Head (H), Head+Middle (H+M), Head+Middle+14 (H+M+14), Head+Middle+14+26 (H+M+14+26) recombinantly and one negative group with no recombinant Mediator modules added. Then the prepared beads were washed 4 times with 1X assay mix including 0.025% NP-40 and 0.25 mg/ml BSA. Then 300 ul 2X assay mix including 60 ug/ml *E. coli* genomic DNA was put onto the beads and beads were divided into 6 tubes since 6 reactions were planned. After that, stated amount of recombinant proteins were added onto the beads as it is shown in Table 13 below. The reaction was incubated at 30 ^{o}C for 50 minutes by mixing in every 10 minutes. Following the incubation, beads were washed with 50mM NaCl and 16 ul 1X SDS loading buffer was added into each reaction tube to further check by western blot.

Table 2.12: Immobilized template recruitment assay plan with added NE and protein amounts

HeLa NE (Full NE)	ΔMediator NE					ΔMediator NE + recombinant proteins
	Recombinantly added Mediator Modules					
	H	M	14	26	N. Cont.	(ΔMediator NE only)
+	+	+	+	+		H+M+14+26
	+	+	+			H+M+14
	+	+				H+M
	+					H

For the Full NE, 180ug is added while for the H, H+M, H+M+14 and H+M+14+26 4500ug, 5940ug, 5400ug and 6300ug were used respectively

2.2.3. Protein Extractions from SF9 Insect Cells

Table 2.13: Produced subunits, their modules and usage amount of viruses

Modules	Produced Mediator Subunits	Amount of infected Sf9 cells	Amount of used virus
Kinase Module	Med12	25ml (10^6 cells/ml)	1ml
	Med13	25ml (10^6 cells/ml)	1ml
	CDK8	25ml (10^6 cells/ml)	1ml
	CCNC	25ml (10^6 cells/ml)	1ml
Tail Module	Med15	25ml (10^6 cells/ml)	1ml
	MEd16	25ml (10^6 cells/ml)	1ml
	Med23	25ml (10^6 cells/ml)	1ml
	Med24	25ml (10^6 cells/ml)	1ml
	Med25	25ml (10^6 cells/ml)	1ml
	Med27	25ml (10^6 cells/ml)	1ml
	Med29	25ml (10^6 cells/ml)	1ml
Head Module	Med19	50ml (10^6 cells/ml)	1.5ml

The readily prepared P1 and P2 generation viruses were produced for the Tail and Kinase subunits as it is described in the production of Mediator modules section. After the infections, the cells were centrifuged at 1500 rpm for 10 minutes and supernatant was discarded. The pellet was re-suspended in 4 ml BC500 (5 ml BC1000 + 5ml BC0, 5mmDTT, 5mmPMSF) and cells were lysed by douncing 3times with glass douncers. After centrifugation of the lysate at 14000 rpm for 15 minutes, pellet was discarded and supernatant was diluted to 300mM salt concentration by addition of BC0 in a drop by drop manner. The extract then incubated with the M2 and HA agarose beads at 4^{o}C overnight to pull down the modules from the tagged subunits. After washing the beads with

BC300+0.1%NP-40 five times, complexes were eluted by addition of 0.5mg/ml Flag of HA peptides to the corresponding beads. Elution step was repeated 3 times at 4ºC for 45 minutes in rotation and checked with Coomassie brilliant blue staining and Western blots.

2.2.4. Production of GTF IIB, IIE and IIF in BL21 Bacteria Cells

TFIIB, TF-Two-E (IIE-αand IIE-β) and TFIIF (Rap30 and Rap74) were readily cloned into Flag and His tagged pET11d and pET15b bacterial expression vectors, respectively. The cloned expression vectors then transformed into the BL21 bacterial cells for protein production. Firstly, a 4ml starter culture was made for each TF with 100ug/ml ampicillin addition. Overnight incubation of the cells allows the production of a very concentrated bacterial culture. Then this starter culture is used before any precipitation happened and 300ml of SOC media was inoculated with that starter culture for each TFs again with the addition of 100ug/ml ampicillin addition. When the OD_{600} reaches to 0.4, IPTG was added to induce the production of the proteins with a final concentration of 0.8 mM. In order to allow the protein expression, the culture was incubated at 36 ºC for 3 hours and then cells were collected by centrifugation at 4500 rpm for 10 minutes. After this point, pellet can be frozen with liquid nitrogen for further use or the lysis procedure can be followed to extract the produced proteins.

The lysis step was conducted with Lysozyme enzyme. A lysozyme stock was prepared with 100 mg/ml concentration. Then the pellet was resuspended in Lysis buffer which is described below. 100 ul of lysozyme stock was added into 10 ml resuspended cells (final concentration of Lysozyme: 1mg/ml). After adding 5 mM of PMSF to the resuspended cells, the cells were rocked for 1 hour at +4 ºC.

Table 2.14: Contents of bacterial cell lysis buffer for protein extraction

Lysis buffer (500mM salt)		Lysis buffer (0mM salt)	
Material	Final Concentration	Material	Final Concentration
HEPES (pH:7.9)	40 mM	HEPES (pH:7.9)	40 mM
KCl	0.5 M	KCl	-
NP-40	0.10%	NP-40	0.10%

Glycerol	10%	Glycerol	10%
DTT	2 mM	DTT	2 mM

After 1hour rotation, the mixture was centrifuged at 1800 rpm at +4 ^{o}C and the salt concentration was diluted into 300mM salt concentration by addition of lysis buffer prepared without KCl (ddH_2O was added instead). Finally, the extracted TF proteins were stored at -80 ^{o}C by freezing the diluted, 300 mM salt containing supernatant with liquid nitrogen.

2.2.5. Immunoprecipitation Using M2 Agarose and Sepharose Beads

For each reaction 10 ul of M2 agarose beads were used. The extracts of TF-Two-E -α and –β subunits were mixed to allow dimerization of the protein *in vitro.* Firstly, the beads were washed with BC300 containing 5mM PMSF, 5mM DTT and 0.1%NP-40 5 times (with 1 ml each time). Then, the flag tagged TF-Two-E α and β extract mixture was put onto the beads and incubated on rotator for 3 hours at +4 ^{o}C. Secondly, the TF-Two-E extract was taken out and the beads were washed 2 times with BC150 containing same amount of PMSF, DTT and NP-40. Then 500 ul of Mediator Complex subunit extracts were put onto the beads and incubated again on rotator for 3 hours at +4 ^{o}C. After the incubation, the extract was taken out and the beads were washed 4 times with BC150 containing 5mM PMSF, 5mM DTT and 0.05% NP-40 (with 200 ul of BC150 and by inverting the tube 4 times in each wash) Lastly, 16 ul of 2X SDS loading buffer was added onto the beads and protein content was further analyzed by Western blot.

The sepharose beads were used to conduct a reverse IP since the mediator subunits do not contain a Flag or His tag. The procedure has only one additional step of attaching the relevant antibody to the sepharose beads. For that the beads again washed 5 times with the BC300 containing 5mM PMSF, 5mM DTT and 0.1%NP-40. Then 2 ul of antibody was added onto 100 ul of BC300 and the beads. The mixture was incubated on rotator for

3 hours at 4+ ^{o}C. Then the procedure follows as in M2 beads for attachment of first protein and second protein's addition.

2.2.6. Cloning of His-tagged RNA Polymerase II Subunits

Table 2.15: Primers used for clonings of RNAP II subunits with 6xHis tags

His-RPB8 EcoRI Forward	GCGAATTCATGCATCATCATCATCATCATGC GGGCATCCTGTTTGAG
RPB8 Hind3 Reverse	GCAAGCTTTCAGGCGAGGTTCAGAAGGCT
His-RPB9 EcoRI Forward	GCGAATTCATGCATCATCATCATCATCATGA GCCCGACGGGACTTAC
RPB9 Hind3 Reverse	GCAAGCTTTCACTCGGTCCAGCGGTGGCC
His-RPB10 EcoRI Forward	GCGAATTCATGCATCATCATCATCATCATAT CATCCCTGTACGCTGC
RPB10 Hind3 Reverse	GCAAGCTTTCACTTCTCCAGGGGTGCATA
His-RPB11 EcoRI Forward	GCGAATTCATGCATCATCATCATCATCATAA CGCCCCTCCAGCCTTC
RPB11 Hind3 Reverse	GCAAGCTTCTACTCAATTCCTTCCTGCTT
His-RPB12 EcoRI Forward	GCGAATTCATGCATCATCATCATCATCATGA CACCCAGAAGGACGTT
RPB12 Hind3 Reverse	GCAAGCTTTCATCGAGCATCAAAAACGAC

In order to attach the required His tag to the subunits of RNAP II, readily extracted whole mRNAs were used to produce the cDNAs of the related RNAP II subunit. By using the oligodT primes, the cDNAs and following that the dsDNAs of each subunit were produced. The dsDNAs were then digested with the restriction enzymes which are EcoRI and Hind3 for each subunit. by following the instructions of the manufacturer, dsDNAs

and pFBDM baculovirus expression vector was digested and ligated. After the ligations, the constructs were transformed to *E. coli* DH5α cells. Formed colonies were checked again with PCR and positive colonies were grown for miniprep to obtain the insert having plasmids. The PCR programs for each subunit can be found below.

Table 2.16: Program for Template Production with His Tagged Primers

Reagents	10x Taq Buffer (5U/ul)	dNTP (10mM)	Forward Primer (10mM)	Reverse Primer (10mM)	Template (10 ng/ul)	$MgCl_2$ (25mM)	ddH_2O
Added Volume (ul)	5	1	1,5	1,5	1	4	36

	Initial Denaturation	Denaturation	Annealing	Elongation	Final Extension	Storage
PCR cycle temperature (^oC)	95	95	*	72	72	4
Time (minutes)	4	0,5	1	1	7	
		34 cycles				

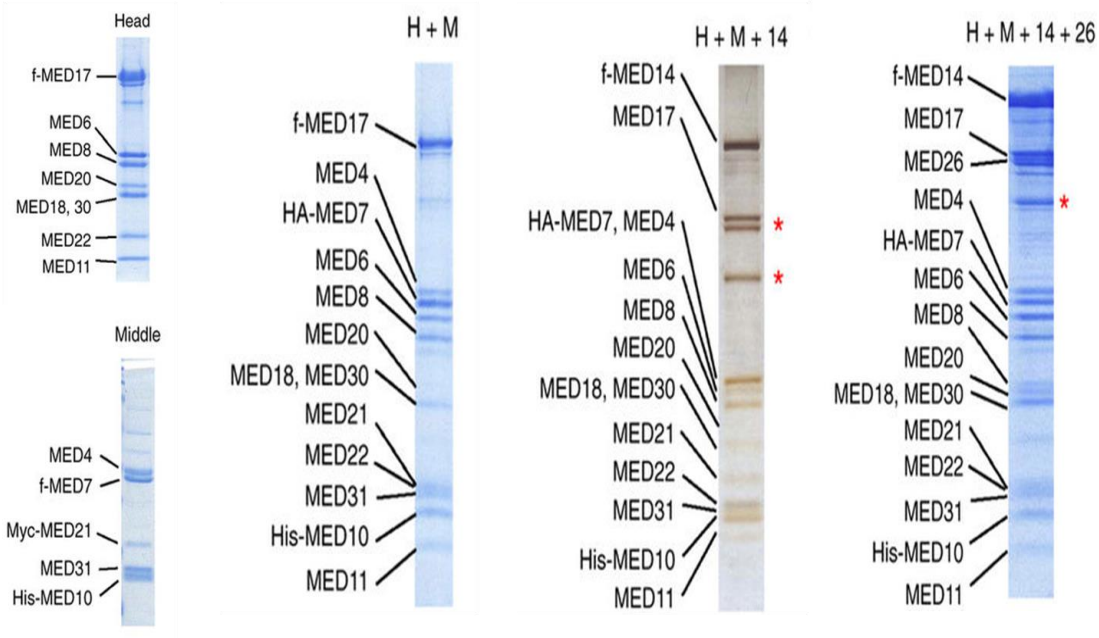
*Annealing temperature for the RPB8-9-10-11 and 12 were set as 68, 66, 65, 63 and 58^{o}C respectively.

CHAPTER 3

RESULTS

3.1. Purification of Functional Human Core Mediator Complex with Baculovirus Expression System

As mentioned before, Mediator complex has a high heterogeneity and a very dynamic structure which is in a reciprocal relation with the presence of the subcomplexes. Since the presence of endogenous Mediator complex is very low, the studies on these subcomplexes require the recombinant productions of the subcomplexes [48] the Baculovirus expression system provides the needs of a recombinant multisubunit protein production procedure [87]. As explained in Figure-1.7, the Multi-Bac system was used to produce the human Mediator functional core complex with Head, Middle, and Head+Middle combination by Cevher et al. (2014). The Med14 and Med26 were also added to the core due to their roles in the complex to give functionality [49].



(This figure is taken from Cevher et al., 2014 [49].)

Figure-3.1: Coomassie staining result of recombinantly purified Head (H), Middle (M), H+M, H+M+14 and H+M+14+26 core Mediator complex produced by Multi-

Bac expression system. *H, M, H+M and H+M+14+26 viruses were used to infect Hi5 insect cells and cells were harvested 72 hours after the infection. Then the extracts were incubated with M2 agarose beads and subcomplexes were eluted with 0.5 mg/ml Flag peptide. The Coomassie stain also shows that the subcomplex subunits have an approximate stoichiometry. The corresponding subunits were shown with lines and stars indicate the impurities.*

Figure 3.1 shows the produced H, M, H+M, H+M+14 and H+M+14+26 subcomplexes stained with Coomassie brilliant blue. Each protein is shown with the corresponding subunit names and impurities are stated with stars (*). Impurities does not correspond any subunits of those subcomplexes.

1.2. Dependency of PIC Elements to Mediator Complex to be Recruited to the Promoter Site

Since the Mediator complex has the function of helping the formation of PIC at the promoter site [66], immobilized template recruitment assay was performed to see which elements depend on Mediator Complex to be brought to the PIC. Immobilized template recruitment assay is a method in which promoter containing DNA fragments were attached to Dynabeads which are magnetic. Addition of nuclear extracts onto those DNA fragments allows us to detect proteins that are located to promoter region of DNA fragment. Here, TATA promoter was used downstream of p53 enhancer sequence. Two different systems were designed with Mediator containing HeLa nuclear extract (NE) and Mediator depleted HeLa nuclear extract (denoted as ΔMediator NE) to see the changes in the protein composition in promoter region upon Mediator depletion. The H, H+M, H+M+26 and H+M+14+26 subcomplexes were additionally used to recover the recruitments.

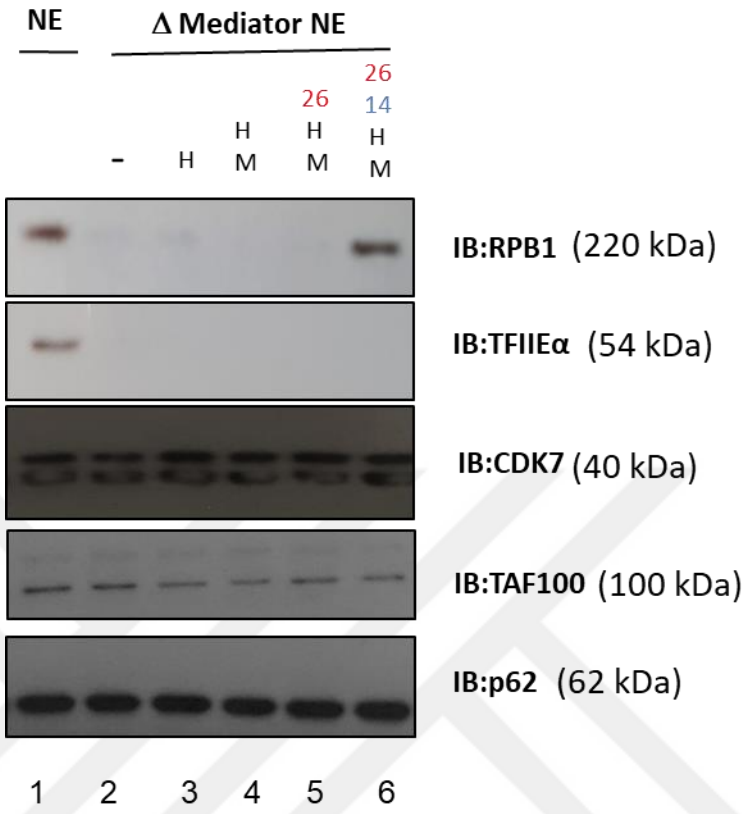


Figure-3.2: Immobilized template recruitment assay executed with and without mediator presence. The H, H+M, H+M+26 and H+M+14+26 (functional core) subcomplexes were added separately to see the difference in the recruitment. *NE represents the full HeLa nuclear extract and the ΔMediator NE is the Mediator depleted HeLa nuclear extract. The system was designed to show the difference in recruitment with one positive control (lane1), one negative control (lane 2) and four different subcomplexes which are H, H+M, H+M+26 and H+M+14+26 in lanes 3, 4, 5 and 6 respectively. RPB1 is a subunit of RNAP II, TAF100 is a subunit of TFIID, CDK7 and p62 are subunits of TFIIH and* TF-Two-E *-α is a subunit of* TF-Two-E. **(This figure was done by Ms. Yasemin Barış)**

In Figure 3.2, lane 1 shows the detected proteins which were recruited to the TATA promoter site. The negative control (which contains no additional Mediator subcomplexes), lane 2, showed no presence of RPB1 and TF-Two-E -α when Mediator is fully depleted. Additions of H, H+M, H+M+26 did not recover the recruitment of RPB1

and TF-Two-E -α (lanes-3, 4, 5). When functional core (H+M+26+14) was added (lane 6) RPB1 recruitment was again recovered as in lane 1. However, TF-Two-E -α was still absent when whole Mediator is not present. This result showed that the RNAP II and TF-Two-E require the assistance of Mediator complex to be recruited to the PIC on promoter site. Addition of functional Mediator core recovered the RNAP II recruitment yet it was not sufficient to recover TF-Two-E recruitment (lane 6). The TAF100, CDK7 and p62 can be used as the loading control.

1.3. Immuno-precipitation Result of Kinase Module- TF-Two-E Association

As it is said, Mediator complex has four modules which are Head, Middle, Kinase and Tail [51]. The Head Middle modules form the functional core and it is not sufficient to rescue the recruitment of TF-Two-E to the promoter site. So, it can be speculated that the association of Mediator complex- TF-Two-E is via the subunits apart from the functional core subunits which are Tail and Kinase module subunits.

After the determination that TF-Two-E may possibly associate to the Mediator Kinase and tail subunits, the recombinant production of each subunit was done by using the Baculovirus expression system as it is explained in Methods chapter. Med12, 13, CCNC and CDK8 are the subunits that form the Kinase module. They were cloned to the Baculovirus expression plasmid separately instead of a multi-subunit clone in one plasmid. Then, produced viruses were used to infect SF9 cells and the cells were collected nearly 5 days subsequently to the infection. Since they do not contain any tags, the proteins were used as cell lysates instead of purified proteins. For each subunit 3.33 ml of extract obtained in 300mM salt solution. Since the aim is to check a sign of interaction or association, inputs of each proteins show difference due to the fact that there was no strict necessity for protein amount equilibration for the first screening by immunoprecipitation (IP) experiments.

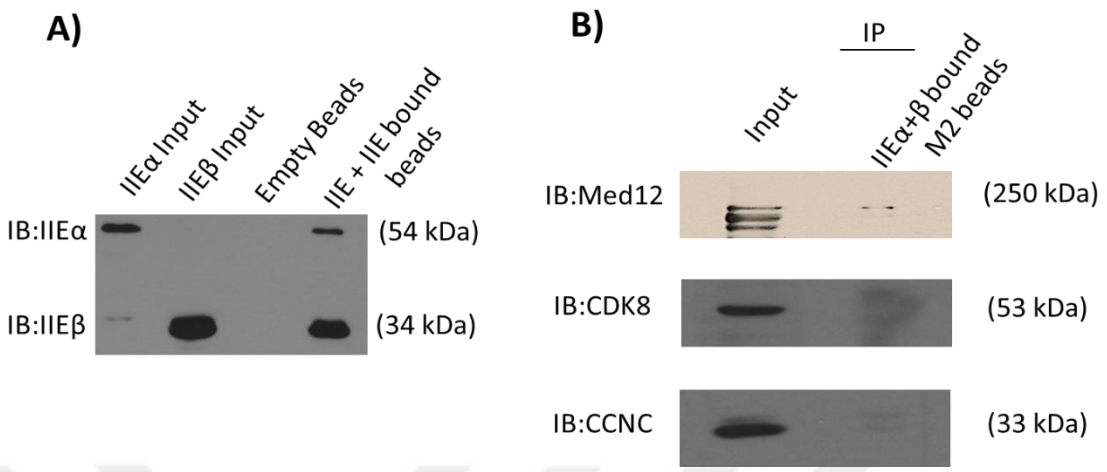


Figure-3.3 Western blot result that shows the Kinase module subunits which are pulled down by flag-tagged TF-Two-E bound M2 agarose beads. ***A)*** *The inputs of two flag tagged TF-Two-E subunits and bead bound TF-Two-E subunits are shown. M2 agarose beads without TF-Two-E subunit attachment were also loaded to eliminate the possible bands of impurities that may result from the beads.* ***B)*** *The expressed Kinase module subunits were checked on the flag-tagged TF-Two-E α&β bound M2 agarose beads which contains cross-linked flag antibodies on it. Cross-linked anti-Flag antibodies do not dissociate from beads so flag input is not necessary. Med13 extract was also prepared but the input showed no presence of Med13 so it is excluded from the result.*

In figure 3.3, first the Flag tagged TF-Two-E subunits were attached to M2 agarose beads (3.3- panel A). M2 agarose beads has cross-linked anti-Flag antibodies on itself and can bind to Flag tagged proteins so when flag tagged TF-Two-E extracts added onto M2 beads, they are TF-Two-E subunits bind to M2 beads. Panel B shows IP of kinase module subunits with TF-Two-E bound M2 agarose beads. Here, the band of Med12 on IP lane in panel B shows that Med 12 has a potential to have an association with TF-Two-E since CCNC and CDK8 subunits showed no presence on the TF-Two-E bound beads after the beads were washed with 150mM salt solution. The smear like band that occurred in Med12 input may be a result of proteolytic degradation happened during extraction procedure or after the extract was stored. Also, the pulled down Med12 showed the same pattern with the Med12 input. Upon these patterns, it can be concluded that the TF-Two-E association

site of Med12, if it ever exits, could be step wise lost due to the degradation which in return first decreases and then completely seizes the interaction.

After seeing the possible interaction with Med12 and TF-Two-E, the TF-Two-E subunits were this time separately bound to the M2 agarose beads to figure out which subunit has the major, if not complete, role in this association. Figure-3.4 shows the IP of Med12 with separate TF-Two-E subunits.

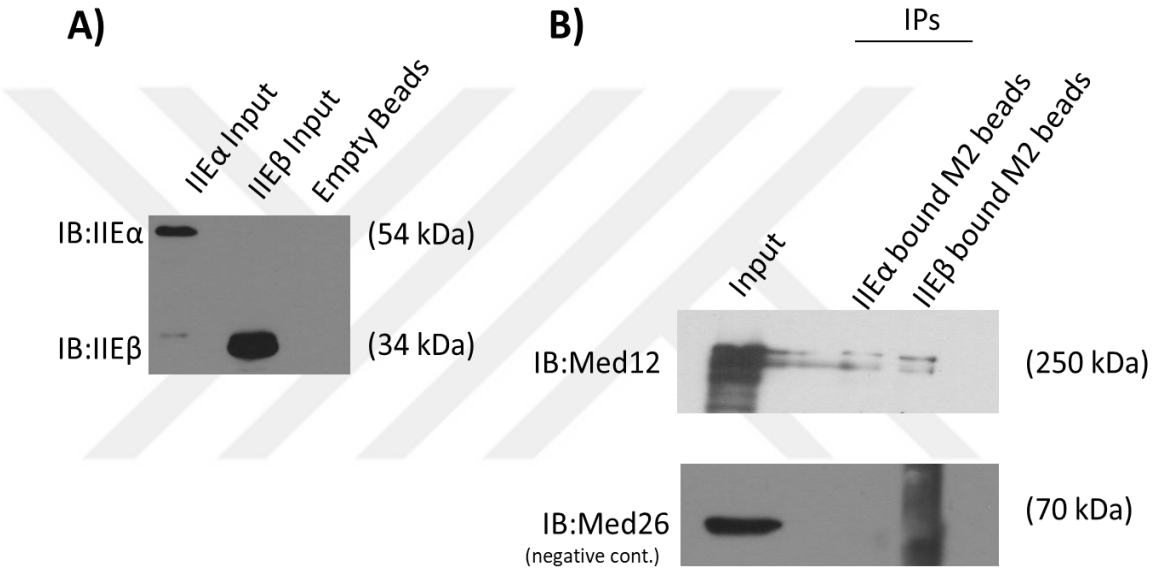


Figure-3.4: Western blot result showing the pulled down Med12 subunit on TF-Two-E -α and TF-Two-E -β bound M2 beads. ***A)*** *The immunoblots of TF-Two-E subunits shown as separate inputs. Empty beads were also loaded to eliminate the possible bands of impurities that may result from the beads.* ***B)*** *Med12 and Med26 immunoblots on input lane and IP lanes with TF-Two-E -α and TF-Two-E -β bound beads are seen. Med26 was used as a negative control and shoed no specific bands with two subunits.*

Figure 3.4 shows two panels A and B. Panel A shows the immunoblots of both subunits of TF-Two-E with empty bead negative control. When Med12 is pulled down with TF-Two-E -α and TF-Two-E -β bound M2 agarose beads (lanes of IP with IIEα and IIEβ in figure 3.4 panel B), both lanes showed some quantities of Med12 present on both the flag-tagged TF-Two-E -α and TF-Two-E -β bound M2 agarose beads The well after the input was left empty to avoid any leak from the input loaded well into the other wells which may create a deceiving false positive result. The negative control was Med26 which

was a member of core Mediator so it was known that they do not interact at the TF-Two-E recruitment to promoter event. The smear on TF-Two-E -β bound beads lane may be due to the proteomic degradation happened during the procedure even TF-Two-E α and β bound beads were treated in the same way and experiment was conducted at the same time for both subunit.

In order to have a conclusive inference about TF-Two-E -Med12 association, it was necessary primarily to check the ability of Med12 to bind the TF-Two-E absent M2 agarose beads and secondarily to carry out a reverse IP experiment at which the Med12 was bound onto the beads and presence of TF-Two-E was checked by western blot. Thus, the reverse IP was done as it can be seen in Figure-3.5 below.

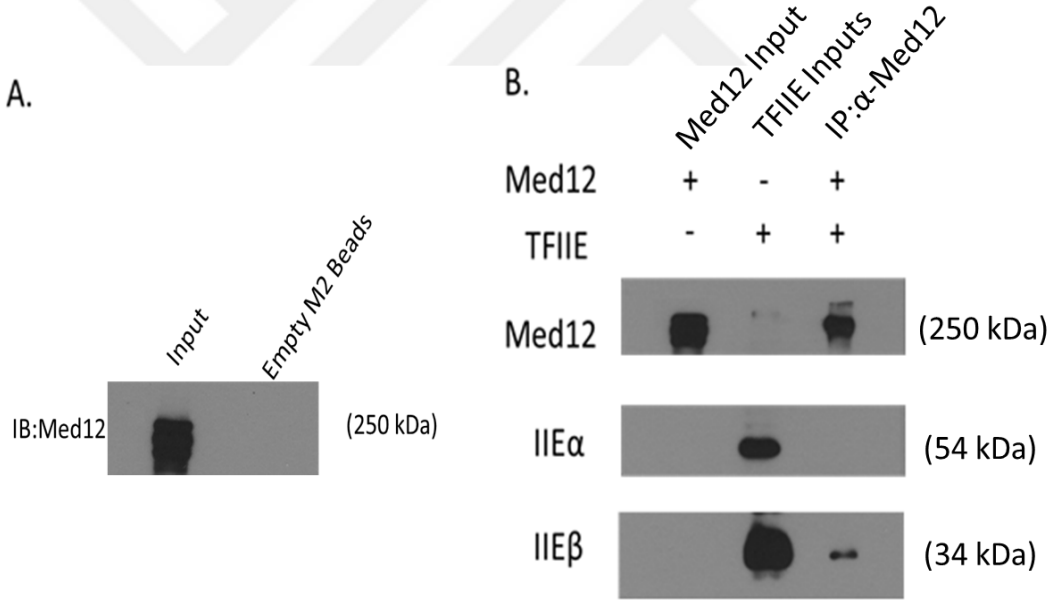


Figure-3.5: Western blot results of Med12 pull down with empty beads and reverse IP with Med12 bound Sepharose beads. ***A)*** *The presence of Med12 is shown both in the extract and on the empty beads.* ***B)*** *Western blot result showing the pulled down* TF-Two-E *-α and TF-Two-E -β subunits on Med12 bound Sepharose beads. The presence of Flag-tagged TF-Two-E -α & β subunits on Med12 bound Sepharose beads were checked by Anti-flag antibody. TF-Two-E -α showed no bands even though TF-Two-E -β showed a band.*

In figure 3.5 panel A, the IP of Med12 with empty M2 agarose beads was done to eliminate the probability of Med12 to bind the beads nonspecifically and since beads without TF-Two-E showed no Med12 binding, reverse IP was conducted. Panel B shows input of Med12, inputs of TF-Two-E subunits and IP of TF-Two-E with Med12 bound sepharose beads. In the IP lane, it was checked if Flag tagged TF-Two-E subunits were pulled down with Med12 bound sepharose beads. there is only a band of TF-Two-E -β. The reverse IP which shows that the TF-Two-E -α has no ability to pull down Med12 even though TF-Two-E -β may have a possibility for the interaction. However, TF-Two-E alpha and beta inputs are not same and TF-Two-E beta input is more than TF-Two-E alpha. It is highly likely that the excess amount of TF-Two-E beta present in the extract is the reason of the band in spite of the washing step that was done with 150 mM salt solution. According to that result, it can be speculated that the TF-Two-E band may be a result of excess presence in the extract compared to TF-Two-E -α but the possibility of an interaction still remains to be cleared out in further.

1.4. Immunoprecipitation Result of Tail Module- TF-Two-E Association with Additional Check of Med19

After the Kinase module results, the screening was continued with Tail module subunits. Previously prepared viruses for each single subunit of Tail module were used to infect Sf9 cells and the cells were collected after nearly 5 days after the infection. The cell lysates were prepared as it is explained in Methods chapter. Again, they did not have any tags so the lysates were used directly for the immune-precipitation experiment. Just to screen the associations, the TF-Two-E subunits were bound to beads again as a heterodimer so each bead has both subunits attached to itself. Figure-3.6 shows the western blot result of pulled down Tail subunits with flag-tagged TF-Two-E bound M2 agarose beads.

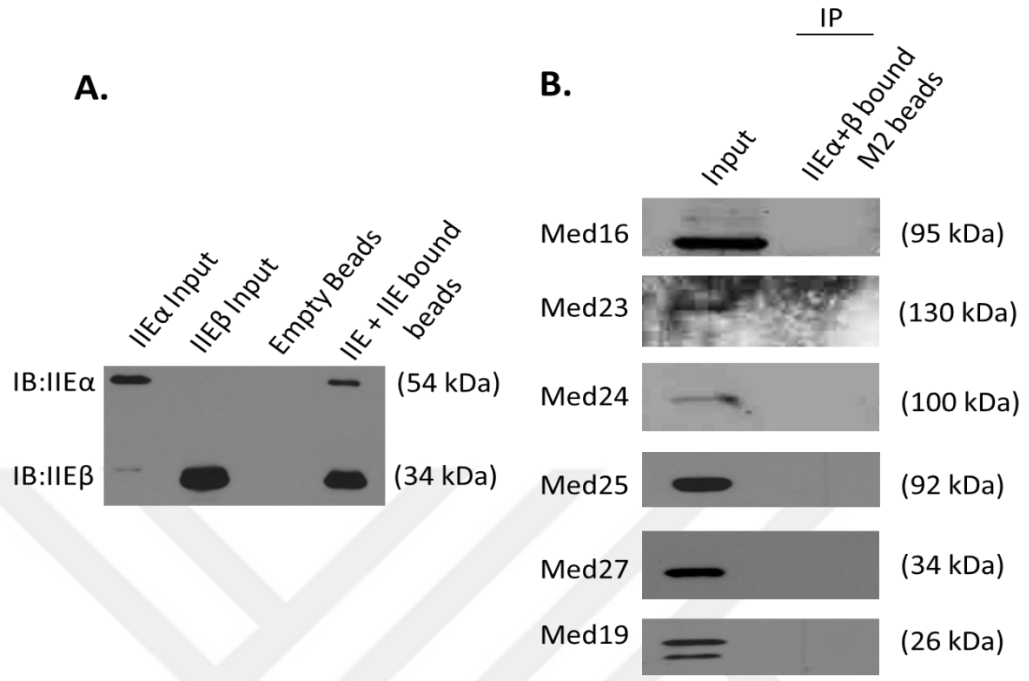


Figure-3.6: Western blot result that shows the Tail module subunits which are pulled down by flag-tagged TF-Two-E bound M2 agarose beads ***A)*** *The inputs of TF-Two-E subunits and bead bound TF-Two-E subunits are shown. Empty beads were also loaded to eliminate the possible bands of impurities that may result from the beads.* ***B)*** *The expressed Tail module subunits were checked on the flag-tagged TF-Two-E bound M2 agarose beads. Med15 extract was also prepared but the input showed no presence of Med15 so it is excluded from the result.*

In Figure 3.6, first the TF-two-E subunits were bound to the M2 agarose beads and their presence on beads were checked with immunoblotting as in panel A. When the beads were attached to TF-two-E subunits, Tail module subunit extracts were put on those beads separately and Panel B shows the pulled down Tail subunits with TF-two-E bound M2 agarose beads. Even though some subunits showed a very weak band on the input lane as in panel B, Med23 input, all the expressed Tail subunits has failed to be pulled down by TF-Two-E bound beads. Also, Med19, which is a head module subunit, was checked because of the fact that it acts separately when forming the functional core and is not pulled down together with the core after it is assembled. However, it also has failed to show an interaction or association with TF-Two-E subunits.

1.5. Comparison of Mediator Depleted Nuclear Extract and Full Nuclear Extracts in the Manner of TF-Two-E -Mediator Complex Association

After the immunoprecipitation experiments conducted with one by one Kinase and Tail module subunits showed no certain interaction with TF-Two-E, the possibility of requirement of more than one subunit for the TF-Two-E association has risen. To check such kind of an association, it seemed a nice method to check the Mediator depleted and full HeLa nuclear extracts in the context of TF-Two-E interaction.

It is known that the RNAP II interacts with Mediator Complex directly and thus, depletion of Mediator Complex causes a relative decrease in the amount of RNAP II so, in order to check the nature of TF-Two-E -Mediator complex interaction, comparing nuclear extracts with and without Mediator Complex, was planned as the next step. If the interaction between TF-Two-E -Mediator Complex is as direct as RNAP II-Mediator Complex, it is logically expected to see a decrease in the TF-Two-E amount when Mediator complex is pulled out of the nuclear extract like the case of RNAP II. In order to do that, Mediator depleted Hela nuclear extract and normal HeLa nuclear extract were checked by Western blot as it can be seen in Figure-3.7 below.

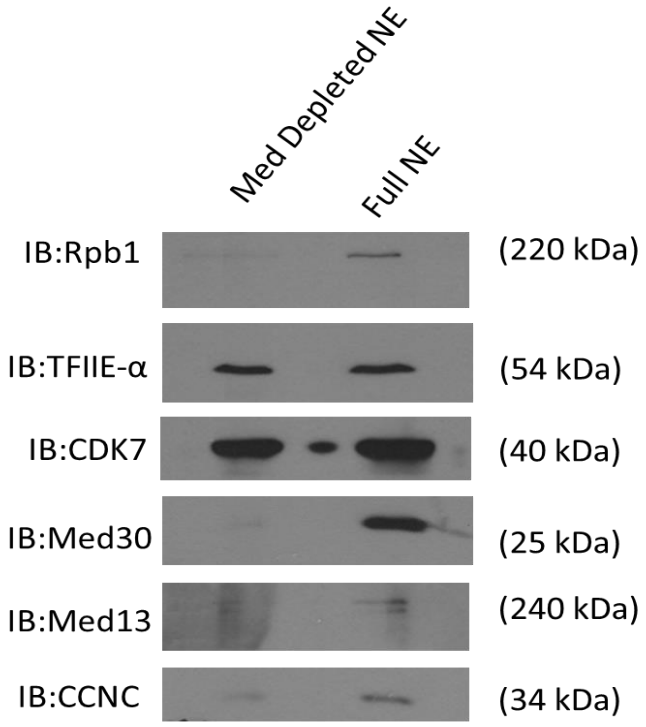


Figure-3.7: Western blot check of nuclear extracts with (full NE) and without (Med Depleted NE) Mediator Complex. *Shown bands of TF-Two-E are the TF-Two-E -α subunit bands. RPB1, which is the biggest subunit of RNAP II, was checked as a positive control because of its direct interaction with Mediator Complex. Med30, Med13 and CCNC were checked as the controls for Mediator Complex depletion and CDK7, which is a subunit of TFIIH, was used as the equal loading control.*

The Figure-3.7 showed that the depletion was done successfully since the levels of Med30, Med13 and CCNC was nearly diminished. Also, the positive control worked well too as it can be seen from the decreasing RPB1 levels upon Mediator Complex depletion. However, there was no change in the levels of TF-Two-E -α subunit representing the TF-Two-E amount in the extracts like the case of TFIIH subunit CDK7. Upon this result, it can be speculated that the Mediator Complex and TF-Two-E association is not likewise the RNAP II and Mediator complex interaction.

1.6. Association of TF-Two-E with PIC Elements

In order to check which elements of PIC interact with the TF-Two-E, the proteins pulled down by the Flag-tagged TF-Two-E bound M2 agarose beads were incubated with HeLa nuclear extract. After the washing step which was done with 150 mM salt solution, beads were separated from all attached and pulled down proteins by addition of 2XSDS loading buffer and heating at 95 ^{o}C for 4 minutes.

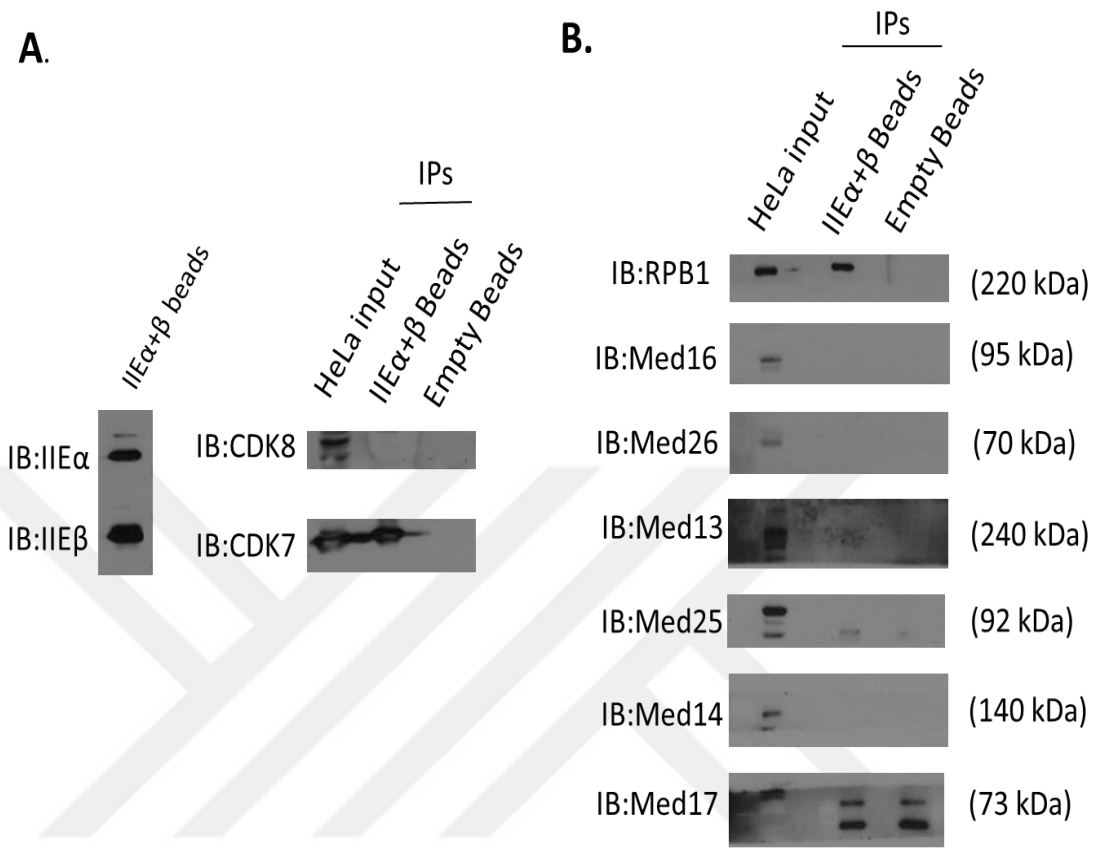


Figure-3.8: Checked proteins on the Flag-tagged TF-Two-E bound M2 agarose beads after the incubation with 10 ug/ul concentrated HeLa nuclear extracts. *Mediator Complex (Med 13, 14, 16, 17, 25, 26 and CDK8), RNAP II (RPB1) and TFIIH (CDK7) were checked.* ***Panel A and Panel B*** *shows different gel percentages of the same IP experiment (A: 10%, B:7%)*

According to Figure 3.8, TF-Two-E bound M2 agarose beads could pull down CDK7 which is a subunit of TFIIH and RPB1 which is a subunit of RNAP II. However, no Mediator subunits were pulled down with TF-Two-E bound M2 beads. In order to figure out which TF-Two-E subunit is responsible for the pull downs, separate TF-Two-E subunits were bound to M2 beads and IP experiment was repeated.

1.7. Association of RNAP II and TFIIH with Separate TF-Two-E Subunits

According to the Figure-3.8, it can be said that RNAP II and TFIIH have clear interactions with TF-Two-E unlike Mediator Complex. The bands appeared on the Med17 membranes are not matching with the input and also present on the empty lane as well. Thus, it can be said that these bands are either a result of nonspecific binding of proteins in the nuclear extracts or they are impurities resulted from the beads themselves. After these data, another IP was designed to see which TF-Two-E subunits bind to RNAP II and TFIIH. As in the case of Med12, again, TF-Two-E subunits were attached to the beads separately and TF-Two-E -α and TF-Two-E -β bound M2 agarose beads were again put into the HeLa nuclear extract.

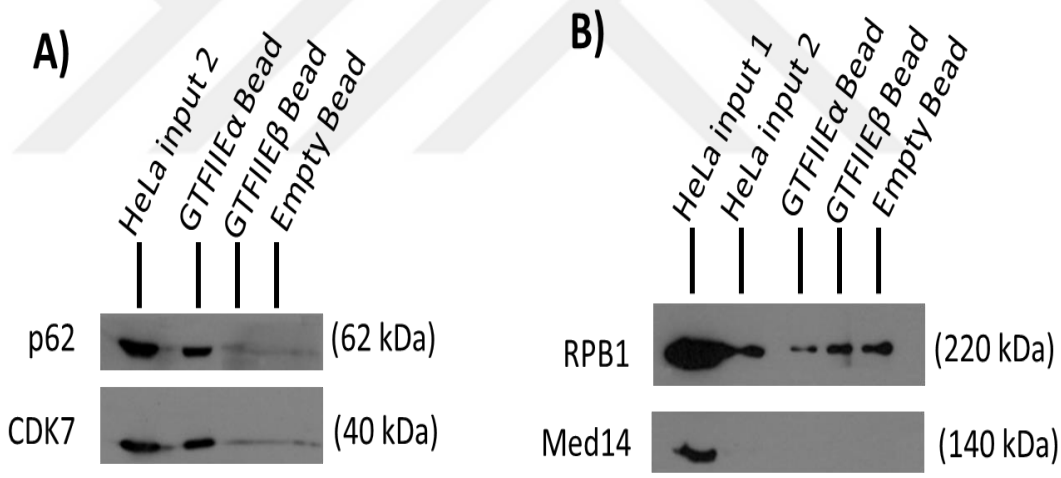


Figure-3.9: Western blot analysis to check which subunit of TF-Two-E binds to RNAP II and TFIIH. A) IP of XPB and p62 which are subunits of TFIIH. **B)** IP of RNAP II subunit RPB1 and Med14. *Two HeLa inputs with different concentrations (HeLa input 1:10ug/ul and 10ul loaded to SDS-PAGE gel. HeLa input 2: 9.5ug/ul and 4ul loaded to SDS-PAGE gel since we run out of HeLa nuclear extract 2) were loaded as the inputs and Med14 was checked as the negative control since TF-Two-E does not directly bind or interact with it.*

The result in Figure 3.9 shows a thicker band in RPB1 in the empty bead lanes indicating that the visible interactions are not specific even though the conditions of IP

experiment were not on purposely changed (again washed with 150 mM salt solution). Also, the XPB membrane shows bands in all lanes with a relatively decrease in TF-Two-E -β and empty bead lanes. However, the Med14, which was the negative control, has no bands in TF-Two-E bound and empty bead lanes. Besides, p62 and CDK7 showed clear interactions with only TF-Two-E -α subunits. Thus, there is the possibility that a harsher washing condition can eliminate the nonspecific bands in RPB1 and XPB membranes if they are really not specific. For the inputs, in panel B an additional HeLa input was used. After the IPs of TFIIH, RPB1 and Med14 subunits were done with HeLa nuclear extract 2, there remained a very little amount (4 ul) of HeLa nuclear extract 2 to be used as input and we used HeLa nuclear extract 1 not to miss inputs in that experiment. It is important to say that all IPs were done with HeLa nuclear extract 2 and HeLa nuclear extract 1 was only used as an input.

CHAPTER 4

DISCUSSION

The aim of this study started as to characterize the Pre-initiation complex elements which depended on Mediator to be recruited to the promoter sites and to understand the corresponding Mediator subunits that played roles in those recruitments. With that aim, we identified a high correlation between Mediator complex presence at the promoter and the recruitment of TF-Two-E. Due to the very large size, dynamic structure and heterogeneity of the Mediator complex, it was hard to understand the recruitment mechanism of GTFs (focused on TF-Two-E) to promoters. Therefore, we decided to divide Mediator into its modules as well as its subunits to dissect the interactions or associations formed with TF-Two-E.

As its function, Mediator Complex is a multi-subunit transcriptional regulator that has a role in different stages of transcription such as chromatin remodeling, Pre-initiation complex formation, promoter opening and elongation [13-71]. Therefore, Mediator Complex is also an essential element for the transcription. As we are focusing on the PIC, we skipped characterization of other earlier stages. Thus, here we are underscoring the characterization of Mediator-dependent PIC elements for transcription on naked DNA template. To do that, immobilized template recruitment assay (ITRA) was performed with one varying condition: presence and absence of Mediator complex as it can be seen in Figure-4.1 below.

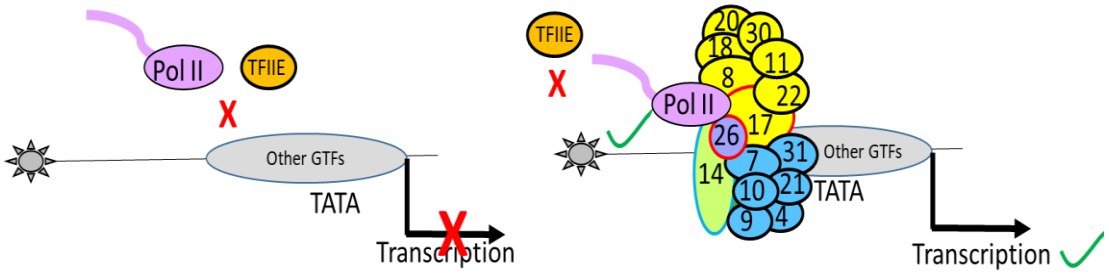


Figure 4.1: Visual representation of designed ITRA experiment which shows two different systems without Mediator and with functional Mediator core complex. *The left side represents the Mediator depleted system in which the DNA is bound to Streptavidin Dynabeads where recruitment of RNAP II and TF-Two-E is prevented. The right side shows the system which contains the functional Mediator core complex and the successful recruitment of RNAPII to the TATA promoter site. Interestingly, TF-Two-E is still not brought to the promoter due to the lack of full Mediator complex.*

Comparison of the elements recruited to the used TATA promoter site in the presence and absence of Mediator Complex enabled us to see that RNAP II and TF-Two-E requires Mediator to enter the PIC on promoter region. In order to identify the major subunits having a role in these recruitments, a strategy of addition of recombinantly produced Mediator complex modules was followed to catch a rescue function with the additions. As it can be seen in Figure-3.2, addition of H+M+14+26, which is called as the functional Mediator core, to the Mediator lacking ITRA system rescued the RNAP II recruitment however, the same Mediator core that is also transcriptionally active (data not shown) failed to recover the recruitment of TF-Two-E. Since the presence of the full Mediator complex successfully enabled TF-Two-E entry to the PIC, we speculated that the responsible subunit of Mediator enabling this recruitment should reside in Kinase or Tail modules which have the subunits other than the functional Mediator core. Here, we specifically tried to characterize the Mediator Complex- TF-Two-E interaction by expressing the subunits of the Mediator one by one with Baculovirus expression system using insect cells and performed immunoprecipitation experiments.

According to the results of Kinase module immunoprecipitation experiments, Med12 showed a potential to be the main subunit for TF-Two-E recruitment. However, due to the negative result of reverse immunoprecipitation experiment in Figure-3.5 we decided to move on with the Tail module subunits for further analysis. The Tail subunits were also expressed in Baculovirus expression system one by one. Even though protein levels in the inputs are not the same for all subunits, protein equilibration was not necessary to have an idea about which subunits of Mediator interacts with TF-Two-E. As

Figure-3.6 shows the Tail subunits were also failed to present an interaction with TF-Two-E.

Although the immunoprecipitation experiments did not show any possible direct interactions between Mediator Complex subunits and TF-Two-E, it is also known that Mediator complex was a crucial necessity for TF-Two-E recruitment, yet the functional core is not sufficient to rescue this interaction. There may be two possible reasons for this result. One reason may be that the TF-Two-E recruitment rely on more than one Mediator subunits at the same time (e.g. a module). Thus, one to one immunoprecipitation may have fail due to the lack of other subunits. The other reason may be that the TF-Two-E and Mediator complex may not directly interact with each other. Another protein, proteins or cascades may enable Mediator to mediate the TF-Two-E entry to the PIC. Apart from the Mediator complex link, in gene specific transcription, Krüppel, Antenna and Abd-homeodomain proteins have been shown to associate with TF-Two-E for its recruitment to promoter site in *Drosophila* [13]. However, these proteins work as a TF for the gene specific and activator dependent transcription and for the basal transcription, TF-Two-E is also shown to be required when promoter is not melted yet. Thus, it can be speculated that possibility of having other proteins between Mediator and TF-Two-E is relatively higher for now.

Also, it is known that Mediator complex interacts with RNAP II [13]. By starting from this data, it was speculated that depletion of Mediator complex should also decrease the amount of RNAP II in extracts. By taking this assumption as a control, it was speculated that depletion of Mediator complex should also decrease the amount of TF-Two-E in the extract if the nature of the interaction between TF-Two-E and Mediator complex is similar with RNAP II-Mediator interaction. According to the result in Figure 3.7, the RNAP II (RPB1) amount did indeed decrease with depletion of Mediator complex. However, amount of TF-Two-E did not change. This shows that the nature of TF-Two-E -Mediator complex interaction is different from RNAP II-Mediator interaction. The reason behind this can be the presence of other proteins or cascades between Mediator complex and TF-Two-E. Somehow, interaction or association of TF-Two-E -Mediator complex may not be as direct as in RNAP II interaction yet Mediator complex still manages the

TF-Two-E entry to the PIC as found from Figure 3.2. From this aspect, the way of Mediator complex to mediate the TF-Two-E recruitment must be further investigated.

Also, to identify the interactions of TF-Two-E with PIC elements, analysis of proteins pulled down with TF-Two-E beads from HeLa nuclear extracts confirmed that TF-Two-E interacts with RNAP II and TFIIH. According to Figure3.8 and 3.9, TF-Two-E interacts with RNAP II when there is both subunits of TF-Two-E and TFIIH (apart from XPB) binds majorly to the TF-Two-E -α subunit. Even though the interaction between TF-Two-E and RNAP II was found before [13], the corresponding subunits of TF-Two-E and RNAP II were not clearly shown by biochemical assays especially from the aspect of RNAP II. Also, TFIIH interaction site of TF-Two-E was figured out by truncation of TF-Two-E [79] but the corresponding TFIIH subunit and the subpopulation of TFIIH to which TF-Two-E binds among all TFIIH subpopulations, remains to be clarified.

CHAPTER 5

FUTURE PERSPECTIVES

What it is known now about the Mediator Complex and TF-Two-E relation is that Mediator complex is involved in the recruitment of TF-Two-E to promoter regions and facilitate TF-Two-E entry to the PIC. However, it is also known that although the Mediator Complex is essential for this recruitment, functional core Mediator is not sufficient to rescue this recruitment. So, we predicted that the TF-Two-E and Mediator interaction or association may be via the remaining Mediator subunits which make the Tail and Kinase modules of the complex. However, the immunoprecipitation experiments conducted with Tail and Kinase subunits one by one ended up with no interaction. Thus, we speculated that TF-Two-E requires a combination of subunits instead of a one by one interaction, to form a sustainable link which will carry it to the promoter. To test that, Mediator Tail and Mediator Kinase modules must be reconstituted with the same baculovirus system used to produce the functional Mediator core. After the reconstitution, these modules should be integrated to the functional core since their simple addition onto the extract will not form a working subcomplex [49]. Then, it can be checked if multiple subunits are required for the TF-Two-E interaction.

In order to specifically clarify the interaction sites of TF-Two-E with the RNAP II and TFIIH, primarily, it is important to express each subunits of these multi-proteins and then try to pull them down with TF-Two-E bound beads. In order to do that, we first started cloning of RNAP II subunits as can be seen in the Figure-5.1 below. Since produced TF-Two-E contains Flag-tag in each subunit, here the subunits of the RNAP II were cloned with a 6xHis tag attached to the N-terminal regions to ease the procedure of further IP experiments.

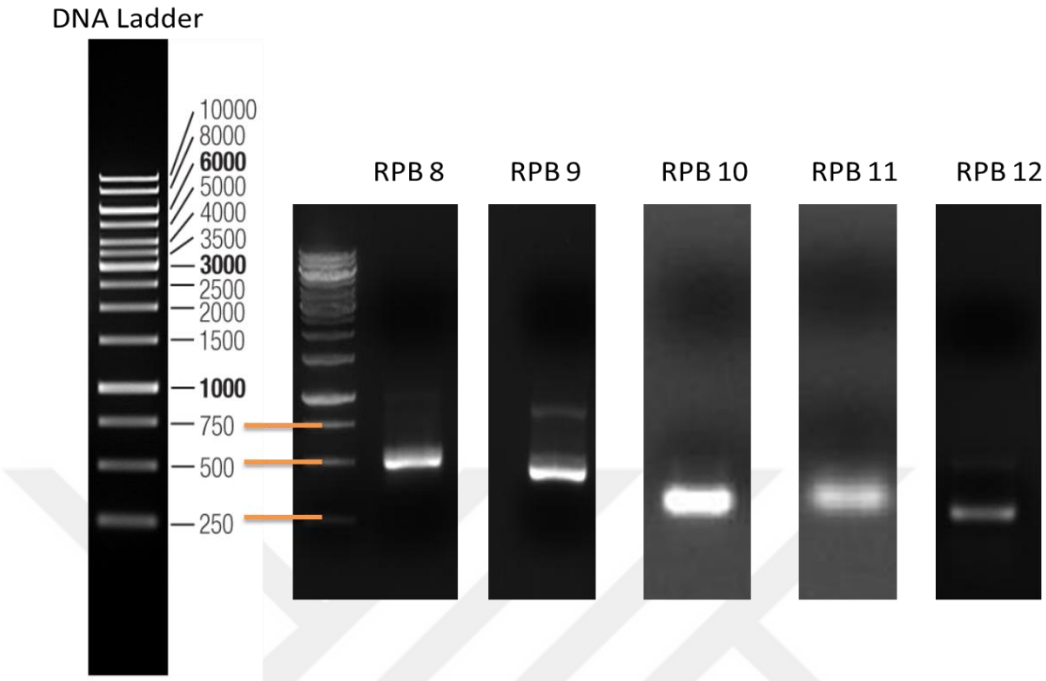


Figure 5.1: The agarose gel electrophoresis images of cloned RPB subunits into pFBDM vector. *The RPB subunits were cloned into pFBDM vector and then the plasmids of positive colonies were isolated. This figure contains the results of PCR reactions in which the isolated plasmids were used as a template to verify the clonings.*

After all the 12 subunits are cloned, they can be used in baculovirus expression system to stably express and extract the proteins separately as it has been done for Mediator complex subunits [89]. RNAP II subunit or subunits responsible for TF-Two-E interaction can be found this way. Revealing such an interaction will also help to clarify the assembly of RNAP II and PIC on the promoter site.

To further dissect the sequential assembly and holoenzyme pathways, procedure that has been applied to TF-Two-E can also be applied to other GTFs. For that purpose, TFIIB and TFIIF which are also expressed in the bacterial expression system due to their small size, can be used as the next step. Figure-5.2 shows the purified TFIIB and produced TFIIF subunits (Rap30 and Rap74).

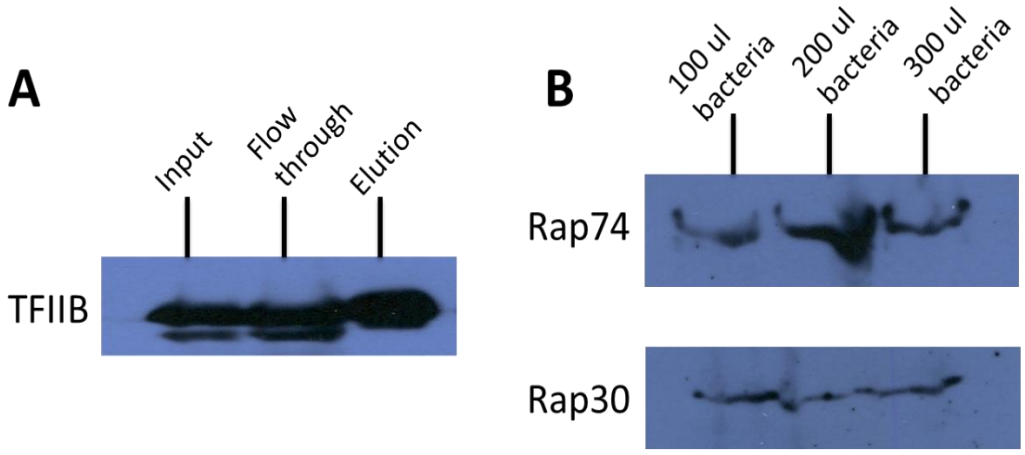


Figure-5.2: Western blot check of purified TFIIB and produced TFIIF subunits. *A) Flag-tagged TFIIB (35kDa)* present in *pET11D bacterial expression plasmid produced by IPTG induction. Then it was purified by incubation with M2 agarose beads. B) 6xHis tagged Rap30 and Rap74* present in *pET15b bacterial expression vector produced by IPTG induction. Smear bans in Panel B are due to the whole bacterial extracts.*

When the TF-Two-E -Mediator, TF-Two-E -RNAP II and TF-Two-E -TFIIH interactions are fully identified, then the same procedure may be followed for TFIIB and TFIIF to clarify the PIC formation and role of Mediator complex in that pathway.

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