RECONSTITUTION OF THE PARTIAL TAIL MODULE OF THE HUMAN MEDIATOR COMPLEX

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October 2021

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

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RNA Polymerase II (Pol II) regulates and maintains every aspect of the cell through the act of transcription of protein coding genes. Transcription of protein coding genes depends on extrinsic and intrinsic signals to the Pol II through which it generates a calibrated response. Disturbance of the calibrated response through environmental and genetic factors are the source of many disease phenotypes including cancer. These intrinsic and extrinsic signals are relayed to the Pol II through the Mediator Complex. Mediator Complex is a 30 subunit 2-MDa protein complex that transduces signals from activators and repressors to mediate Pol II generated transcriptional response through which all processes of the cell are regulated. The Mediator Complex consist of 4 modules head middle kinase and tail. Tail module of the mediator complex is a 7 protein module recruits the mediator to the upstream activating sequences through its direct interactions with activators. Therefore reconstitution of the Mediator's tail subunit is essential to mechanistically understand the Mediator's regulation of Pol II and how activators cause transcriptional responses in the cell. Through MultiBac expression system we have generated each tail subunit and a partial tail complex of the mediator. Our data indicates that Med16-Med23-Med24-Med25 forms a 4 protein subcomplex through direct interactions of each protein with the tail subunit Med16 in the absence of Med14, the architectural scaffold of the Mediator. Presence of Med14 causes Med25 to be absent from this subcomplex and a subcomplex of Med14-Med16-Med23-Med24 is generated again through the direct interaction of each subunit with Med16. The remaining 3 subunits of the tail module Med15 Med27 and Med29 found to be separate from the remaining 4 subunits as they have failed to be elucidated in any of the immunoprecipitations performed. The results overall suggest the elucidation of a partial tail module. Purification of a full tail module may require additional Mediator Complex proteins.

Key words: Mediator complex, MultiBac expression system, RNA polymerase II, Tail Module, Med16

Özet

İNSAN MEDİYATÖR KOMPLEKSİNİN KISMI KUYRUK MODULÜNÜN REKONSTİTÜSYONU

Ege Çığırgan
Moleküler Biyoloji ve Genetik Yüksek Lisans
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RNA Polimeraz II (Pol II), protein kodlayan genlerin transkripste ederek hücrenin her aktivitesini düzenler. Protein kodlayan genlerin transkripsiyonu, kalibre edilmiş bir yanıt oluşturan Pol II'ye giden dışsal ve içsel sinyallere bağlıdır. Çevresel ve genetik faktörler yoluyla kalibre edilmiş yanıtın bozulması, kanser dahil birçok hastalık fenotipinin kaynağıdır. Bu içsel ve dışsal sinyaller, Mediator Kompleksi aracılığıyla Pol II'ye iletilir. Mediatör Kompleksi, hücrenin tüm süreçlerinin düzenlendiği Pol II tarafından üretilen transkripsiyonel yanıta aracılık etmek için aktivatörlerden ve baskılayıcılardan gelen sinyalleri ileten 30 alt birimli bir 2-MDa boutundaki bir protein kompleksidir. Mediator Kompleksi, baş orta kinaz ve kuyruk adındaki 4 modülden oluşur. Mediatör kompleksinin kuyruk modülü, 7 proteinden oluşmakta olup aktivatörlerle doğrudan etkileşimleri yoluyla mediatörü akış yukarı aktive edici dizilere çağırır. Bu nedenle Mediatörün kuyruk alt biriminin yeniden yapılandırılması, Mediatörün Pol II'yi düzenlemesini ve aktivatörlerin hücrede transkripsiyonel tepkilere nasıl neden olduğunu mekanik olarak anlamak için gereklidir. MultiBac ekspresyon sistemi aracılığıyla, her bir kuyruk alt birimini ve aracının kısmi bir kuyruk kompleksini oluşturduk. Verilerimiz, Med16-Med23-Med24-Med25'in, Mediator'un mimari iskelesi olan Med14'ün yokluğunda her proteinin kuyruk alt birimi Med16 ile doğrudan etkileşimleri vasıtasıyla bir 4 proteinden oluşan bir alt kompleks oluşturduğunu göstermektedir Med14'ün varlığı, Med25'in bu alt komplekste olmamasına neden olmakta ve Med14-Med16-Med23-Med24'ten oluşan alt kompleks, her bir alt birimin Med16 ile doğrudan etkileşimi yoluyla yeniden oluşturulur. Kuyruk modülünün geri kalan 3 alt birimini Med15 Med27 ve Med29, gerçekleştirilen herhangi bir immünopresipitasyonda etkileşmedikleri için kalan 4 alt birimden ayrı olduğu gözlenmiştir. Sonuçlar genel olarak kısmi bir kuyruk modülünün kurulduğunu önermektedir. Tam bir kuyruk modülünün saflaştırılması, ek Mediatör Kompleksi proteinleri gerektirebilir.

Anahtar kelimeler: Mediatör kompleksi, MultiBac ekspresyon sistemi, RNA polimeraz II, Kuyruk Modülü, Med16

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Chapter I
Introduction

In the modern understanding of biology the information contained in the DNA is utilized by first producing RNA which has the essential information to generate proteins that are then used in a wide array of processes that are crucial to every living organism. This is called the central dogma of biology.[1] The process of producing RNA from the host DNA is named transcription. The event of transcription starts the manifestation of the genetic information in any cellular organism. In eukaryotes this process is performed mainly through RNA polymerase II. (Pol II) Due to the obvious importance of this event the process of transcription is a very calibrated process that is tightly regulated by a variety of factors. Therefore eukaryotic transcription commences with the generation of a protein complex called the pre-initiation complex (PIC). PIC contains the RNA Polymerase II which is the RNA production machinery of the cell, general transcription factors (GTFs) such as Transcription Factor IIA-H and the 30 protein Mediator Complex. In transcription the Mediator complex acts as a centralized hub and relays activator and repressor signals to the Pol II which assists in the regulation of numerous processes that include transcription and long term epigenetic silencing.[2]

1.1 Mediator Complex

Mediator of RNA polymerase II is a 25 (in yeast) or 30 (in metazoans) subunit protein complex that mediates the interaction between the activators with the general transcription machinery by recruiting the DNA binding transcription factors.[3] Regulatory signals are processed due to the ability of the Mediator to perform as a link between the two machineries and thereby promote the stable formation of the PIC. It is first identified in the 1990s as a co regulator of transcription through biochemical and genetic approaches by Kornberg and Young respectively.[4] Kornberg and colleagues isolated the Mediator Complex by utilizing crude yeast fractions that triggered activator dependent transcription in *vitro*.[5] Meanwhile Young and colleagues utilized yeast genetic screens to elucidate the first mediator genes and named them as suppressor of truncation of RNA polymerase II Carboxyl-terminal domain.[6]

As the research on the Mediator complex flourished it became apparent that the mediator isn't simply a passive adapter that simply relays the activator repressor signals to the Pol II but rather an active and pliable interpreter of signals that can discern and bring together a multitude of signals to form an

adequate output for the transcription machinery.[7] Currently it is revealed that the Mediator complex not only performs the previously the function identified during its discovery but is also involved in higher-order chromatin folding chromatin looping[8], mRNA processing and export[9], transcriptional memory[10] and re-initiation during the event of transcription[11] and activator independent basal transcription and chromatin level repression of transcription in specific contexts.[12] As the knowledge on the Mediator complex grows it is also elucidated that it is involved in many signaling cascades including p53[13], TGF-β[14], Wnt[15] and SREBP.[16]

The 30 subunit Metazoan mediator complex is around the size of 2-Mda.[3] Through yeast studies that employed both biochemical and microscopy approaches, it is determined that the Mediator has a modular structure which consist of 4 distinct modules. These are named head, middle, tail and kinase.[2] (Figure 1) These modules effectively have distinct functionalities. The head and middle modules are responsible for the interaction of the mediator with the transcriptional machinery and Pol II while tail and kinase modules are responsible for the regulation of the Mediator complex. The tail complex is a binding site for many activators while the kinase module in itself has a repressive functionality. These modules working in concert with one another determine where and when the entire complex should interact with the transcriptional machinery. The experimental observations revealed that the kinase module only reversibly associates with the rest of the mediator complex and the individual subunits of the tail module being relatively loosely associated with each other.[17,18]

The mediator complex is conserved evolutionarily between yeast to humans. However the transcriptional requirements and complexity between yeast and human is quite distinct as indicated by the difference in the number of subunits. (25 compared to 30) Due to fact that the metazoan transcription is significantly more intricate than the transcription of yeast the homology between metazoan and yeast Mediator complex is quite varied. The most conserved subunits for example Med7 and Med31 have around 50% homology between yeast and human while some of the other subunits have significantly weaker homology.[18,19] Furthermore as it can be observed from the number of subunits, 25 vs 30, there are metazoan mediator specific subunits of the Mediator. Med26 and Med30 can be given as an example for these specific subunits that are only found in the metazoan mediator and not in the yeast mediator.[20]

The structure of the mediator complex is a hot topic in research and many studies have been performed to accurately determine the structure and the architecture of the Mediator Complex in high resolution.

Due to the fact that some modules of the mediator complex are loosely associated with each other coupled with the fact that the kinase module being reversibly associated with the mediator and the mediator complex being a large protein of 30 subunits and the fact that the mediator complex is heterogenous meaning the assembly of the entire mediator isn't required for every transcriptional event and therefore not all of the subunits of the mediator is expressed in each cell,[21] determining the structure and the architecture of the mediator complex in high resolution has been a difficult process. Early electron microscopy (EM) studies revealed the architecture of Mediator complex only in low resolution as well as the Mediator-Pol II holoenzyme complex which is used in the determination of the Head Middle and Tail modules within the yeast Mediator complex.[23-25]

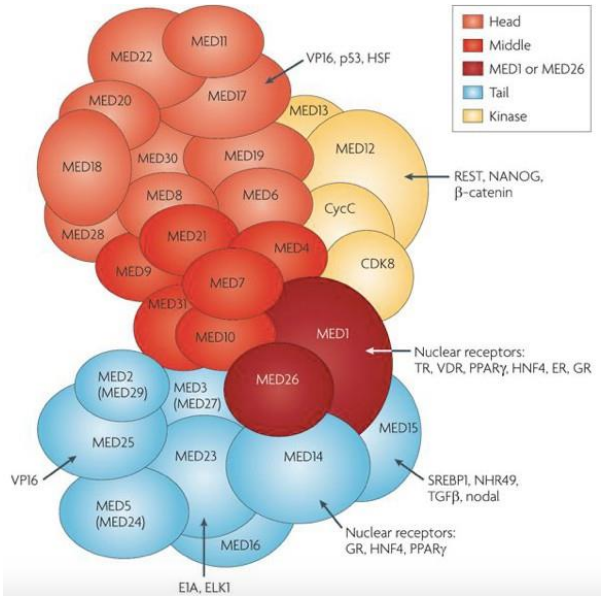


Figure 1: Mediator Complex consist of 4 distinct modules connected through various contacts within and with each other adapted from Malik et al.

1.1.1 The Head Module of the Mediator

Med6, Med8, Med11, Med17, Med18, Med20 and Med22 subunits of the mediator make up the *Saccharomyces cerevisiae* Mediator complex. These 7 subunits combined with the Mediator's middle module, is revealed to be crucial in the assembly of preinitiation complex.[21] The structure of the head module is determined through various studies initiating in 2006 in which these seven subunits of the

head module of the *Saccharomyces cerevisiae* was recombinantly expressed in insect cells which allowed the first negative stain analysis by EM.[26] The next crucial step of the research on the structure of the mediator came in 2011 as crystal structure of head module of *Saccharomyces cerevisiae* was identified at 4.3 Å resolution.[27] The overall structure of the head module and three major domains of the head are identified and named according to their resemblance and movement as neck, fixed jaw and movable jaws.[28] The overall structure had a shape similar to a wrench.[26-28] Another structural analysis this time performed on the *S. Pombe* confirmed the previously determined organization and the 3 domains of the head module.[29] The neck domain of the head module is composed of the subunits Med6, Med8, Med11, Med17 and Med22 and stabilize the head module itself.[30] One of the more critical revelations of these studies were that the head module proteins when expressed recombinantly by themselves are insoluble but they coalesce to form a soluble head module when they are co-expressed.[31-35]

The studies identifying the structure head module were a significant breakthrough in the process of the characterization of the entire mediator at high resolution. In 2014, a novel technique of cryo electron microscopy (cryo-EM) is used in two major studies on the overall shape and structure of the mediator complex which thoroughly redefined the existing knowledge of the organization of the modules of the Mediator.[24,25] Before these studies were published the general consensus of the Mediator's structure was that the head module was one side of the entire Mediator complex and middle and tail form the opposite side. These studies via cryo-EM elucidated that structure of the Mediator Complex is very distinct from the Mediator structure previously thought. Firstly the head and middle modules are revealed to be responsible for the structure that was reckoned to be the structure that is created by the middle and tail modules and the large domain on the opposite of this structure which was assumed to be the head domain was actually the tail module of the Mediator.[24,25,36] The low resolution study of the structure of the human Mediator complex further reaffirmed the architectural sameness between human and yeast Mediator even though they are known to be evolutionarily distinct, suggesting that the structure remains mostly similar due to the function that the mediator possess.[21] Additionally, with the help of these low resolution studies the metazoan specific subunits Med27, Med 28, Med29 and Med30 of the mediator complex are found to have numerous contacts with the head module while another metazoan specific subunit Med26 is found to be correlating to the middle module of the Mediator.[23] These mediator subunits were previously unassigned due to the fact that they were specific to the metazoan mediator. In an additional study on the head module of the human Mediator complex insect cells are utilized to recombinantly express the 7 head proteins and the metazoan specific subunit

Med30 to form a metazoan specific head module which consist of 8 subunits instead of the 7 which was identified in yeast studies. Additionally, this study also reconstituted a functional 15 subunit core mediator. This core mediator consists of the head and middle modules along with Med14 which stabilizes the entire structure.[17]

1.1.2 The middle module of the Mediator

Med1, Med4, Med7, Med9, Med10, Med19, Med21 and Med31 are the 8 subunits that constitute the yeast mediator. In metazoan mediator, as discussed previously, in addition to these 8 subunits, Med26 is also identified as a part of the mediator's middle module.[23] The middle module structurally was not well understood up until 2017 and the information that is known about the middle module was very limited. Only the two sub complexes of the nine protein middle module were relatively well defined. These sub complexes were Med7N/Med31[37] and Med7C/Med21[38] subcomplexes. In 2010 a heterologous co-expression strategy is utilized to elucidate a 7-subunit middle module that did not contain Med19 by Cramer et al[39] However the protein interaction map formed by this strategy at that time was restricted to the previous data[40-43] and pulldown studies[39] and therefore proved to be insufficient. Then, cross- linking experiments are performed on a six subunit middle module that lacked Med1 was co-expressed in bacteria facilitated the formation of a three-dimensional model of the middle module.[44] In 2014, insect cells were utilized to reconstitute a 5 subunit human Mediator middle module. In this reconstitution Med4, Med7, Med10, Med21 and Med31 subunits are successfully reconstituted.[17] However, this reconstitution failed to elucidate any data for the three-dimensional structure Human Mediator’s middle module. Currently available data on the structure of the middle module of human Mediator is very limited. The data that is currently obtained only revealed that N terminal domain of Med26 performs as a docking site for TFIID and ELL/EAF family containing super-elongation complexes.[45]

1.1.3 The Kinase Module of the Mediator

The kinase module consists of 4 subunits CDK8, Med12 Cyclin C and Med13. The research on the kinase module mostly indicates that this module generally has a repressive function however it is also suggested that the kinase module can also be involved in transcriptional activation.[46] The kinase module can dissociate from the rest of the Mediator complex domains.[47] This dissociative nature of the

kinase module is critical for the assembly of the PIC as it enables the mediator to unite with the PIC.[46] The available data shows no indication of the Mediator binding to the RNA polymerase II if the kinase module is present.[48] However how and why this phenomenon happens is not well understood. A high resolution analysis seems to be necessary in order to resolve the inhibitory surface that causes this lack of interaction with Pol II. Structure of the CDK8 and Cyclin C subunits of the kinase module has been well defined[49] however the structural knowledge on the Med12 and Med13 does not exist. The basic structure of the kinase module of yeast as a unit is revealed by using cryo EM analysis at 15A in 2013 which displayed Med12 as a central piece of the module that the rest of the kinase module proteins (Cyclin C, Med13 and CDK8) are connected to.[50-51]

1.1.4 The Tail Module of the Mediator

The tail module of the mediator is mainly responsible for the interaction of the entire mediator with activator and repressor proteins.[7,21,52] Due to the differences in metazoan and yeast transcriptional activation and activator proteins this module also contains the subunits that are least conserved between metazoan and yeast. In fact it can be argued that there is only a patched homology between the yeast and metazoan tail module subunits.[20,53] In yeast, the tail module of *S. cerevisiae* consists of 5 proteins Med2, Med3, Med5, Med15 and Med16 while the metazoan tail consist of 7 proteins Med15 Med16 Med23 Med24 Med25 Med27 and Med29. Due to the general lack of information about the metazoan mediator complex and its tail metazoan specific subunits Med28 and Med30 (also thought to be a part of the head module) are also proposed to be a part of the tail module.[53] Med15 and Med16 are the most conserved subunits between yeast and metazoan mediators while Med2, Med3 and Med5 in yeast tail subunit are thought to be divergent orthologs of Med29, Med27 and Med24 of the metazoan tail respectively. The relationship between Med5 and Med24 is well defined as divergent orthologs but substantial evidence that indicate the orthologous relationship between Med2-Med29 and Med3-Med27 is present even though it is not conclusive.[20] The remaining two subunits Med23 and Med25 are specific to the metazoan tail and does not have any yeast orthologs.[46]

The tail subunit however is not yet well understood in terms of architecture. Med14 for instance was previously thought to be a tail subunit due to its numerous contacts with the tail subunits but in 2014 it is revealed that Med14 is an architectural backbone of the entire mediator and has various interactions with not only the tail but also head and the middle modules of the mediator and is crucial in Mediator Complex mediated transcription since without Med14 transcription simply does not occur.[17,24,54]

Furthermore additional studies are essential to determine the interaction of the metazoan specific subunits Med28 and Med30 with the tail module. Med27, Med28, Med29 and Med30 has previously shown to make copious contacts in metazoan mediators head module while, *S.Pombe* studies have shown Med27 to have a interaction with the tail module[55] so there is much to unveil about the tail architecture along with the tail subunits as a whole.

The tail module interacts with the upstream activator sequences and are involved in the regulation of transcription through altering the structure and function of the Mediator.[7,21] One study performed in S. Cerevisiae revealed a rotation in the tail module of the mediator when Gcn4, a transcription factor, is present.[24] Therefore it can be said that transcription factors have an important ability of facilitating changes in the conformation of the mediator through the tail subunit to induce transcription of its target genes.[7]

Calling up the mediator to the upstream activator sequences is dependent on the tail module only. Most activator mediator associations uncovered so far have been defined via the tail module.[2,21] It has been experimentally observed that the tail module increases the number of mediators in activator sequences to which it binds. In addition, the Tail module is the first target of transcription factors and it has been experimentally shown that it is brought to human mediator enhancer regions by interacting directly with transcription factors.[52] The tail module binds to transcription factors and activators, combining many signals and functional data, allowing the mediator to generate a specific and properly calibrated transcription signal.[2,56]

However, how these regulatory signals are transmitted is not yet clear. One of the most important reasons for this uncertainty is that the structure of the human mediator is not fully understood in fact the structure of the tail module is still remains fully unresolved. The reason for this is that the mediator tail module has a very dynamic and flexible structure, and the mediator is conformationally very heterogeneous.[2] Tissue-specific mediator expressions have also been observed experimentally.[57] Many tail module proteins have been observed to interact with the core mediator by forming small subcomplexes.[2] However there have been some developments in the elucidation of the structure of the tail complex. In 2018 the initial structure of the Med23 the biggest subunit of the tail module at 130kDa is completely elucidated and characterized. The crystal structure of mediator with activators at 2.8 Å resolution allowed a better understanding of the relationship between activators and the mediator.[58] C terminus of Med14 the architectural backbone of the mediator is found to be intimately interacting with

the mediators tail since deletion of its C terminus is resulted in the loss of the tail subunits of Mediator experimentally.[24,54] However the complete structure of the tail module and the function of tail proteins have not been fully elucidated to date. However some stable subcomplexes such as the Med16, Med23 and Med24 in metazoans[59,60] and Med2, Med3 and Med15 in yeast[24] are identified.

Many studies revealed that the tail module of the Mediator interacting with transcription factors.[61] Mediator tail subunits are found to be contacting with transactivation domains of various transcription factors through their activator binding domains with the assistance of nuclear magnetic resonance (NMR) such as but not limited to VP16/Med25[62,63] sterol regulatory element protein /Med15,[64] Gcn4/Med15,[65,66] ATF6α/Med25,[67] ELK1/Med23[68] Nuclear Hormone Receptor 49/Med15[69] and TGF-β/Med15[70] Other studies also revealed that activators target the tail subunits individually. Because of the sheer number of activator-tail complex interactions along with the fact that many of these activators being essential for the growth differentiation and homeostasis of the cell such as Med15's interaction with SREBP1 which is crucial for lipid metabolism[64] and Med23's interaction with ELK1 an important protein of the MAP kinase pathway[68] highlights the importance of the Mediator tail and the necessity of a detailed structural architecture of the mediator to be resolved in order to further elucidate the extent of the role of the mediator complex in activator mediated transcription. The elucidation of the structure of the mediator tail and the mediator tail subunits themselves would have significant consequences in biology research and therapeutics of many diseases including cancer.

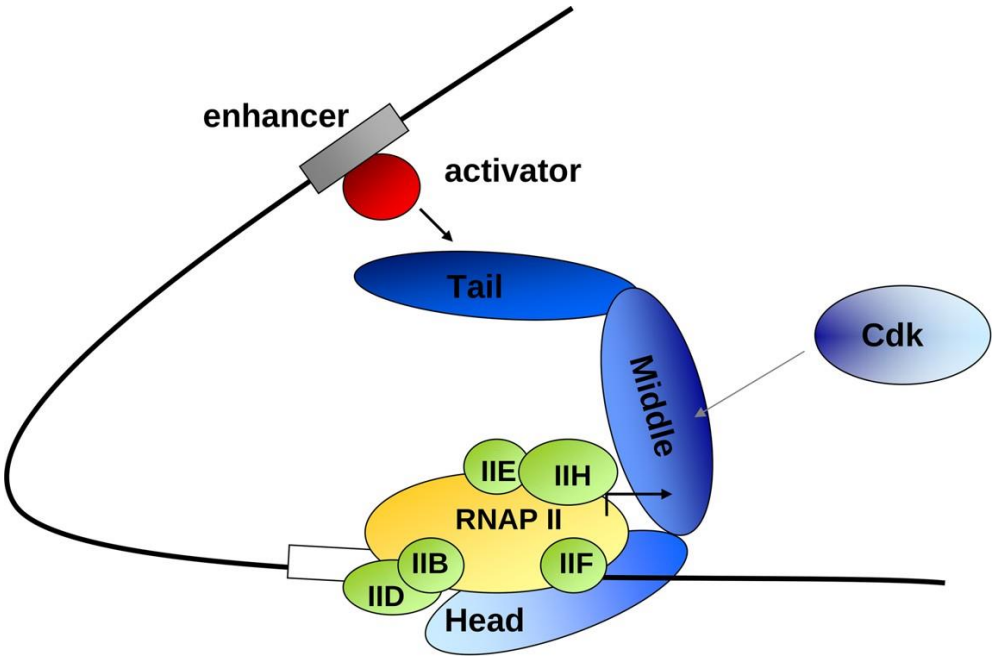


Figure 2: The activators interacts solely with the Tail Module of the Mediator during the initiation of transcription adapted from Toth-Petroczy et al.

1.2 The Mediator Complex in Disease

Regulation of transcription caused by intrinsic and extrinsic signals is essential for the maintenance and regulation of each and every cell. Therefore, spatiotemporal control of the RNA Polymerase II is critical for the wellbeing of the cell and the organism.[71] The disturbance of the RNA polymerase II dependent processes through genetic or environmental causes can be responsible for various diseases including cancer. Consequently, the Mediator complex is essential for the maintenance of the cell's homeostasis, growth, development, communication and differentiation due to its regulatory effects on the RNA Polymerase II and transcription.[2,71] Abnormal expression or generation of Mediator complex and its subunits are involved in many diseases including cancer neurodevelopmental and cardiovascular diseases.[71] Mutations or misfunctioning of the mediator subunits are the cause of various neurodevelopmental diseases. A missense mutation of L371P in MED17 head module gene which was identified as a result of screening study of 79 individuals of Caucasus Jewish origin is associated with infantile cerebral and cerebellar atrophy.[72] 2 missense mutations of R961W and N1007S in Med12 kinase module gene results in X-linked mental retardation syndromes such as Optiz Kaveggia and Lujan syndromes.[73,74] Mutations of the Mediator subunits are a direct cause of cardiovascular diseases

as well. The most common birth defect in humans is the congenital heart disease. Transposition of the great arteries (TGA) is the most prevalent cyanotic heart disease in infants.[75] Med13L, a Med13 paralog was found to be relevant in this disease as it was identified in a patient with TGA and further screening of 97 patients exhibiting the disease revealed 3 missense mutations of MED13L, E251G, R1972H and D2023G.[76]

Mediator complex has various roles in cell signaling and is a part of copious signaling pathways that control growth, differentiation and development. Because of this wide array of function many of its subunits are connected to different cancer phenotypes. Most of these involvements are found as the aberrant expression of the mediator's subunits individually.

Med1 subunit was one of the initial subunits to have a direct link established with a cancer phenotype through various studies. Med1 is found to be intricately connected to breast and prostate cancer. Through studies performed in animals, estrogen hormone (17-β-estradiol; E2) is found to be promoting and inducing breast cancer which caused research on drugs that counteracts these effects of estrogen or ablates estrogen directly.[77,78] E2 and its receptors ERα and ERβ are valuable targets for breast cancer therapy. ERα especially is found to be recruiting the mediator complex in order to initiate the transcription of ERα target genes. The recent research indicates that this recruitment happens with the interaction of ERα with the Med1 subunit and Med1 is a critical co activator of ERα.[79-82] A research conducted on mutant mice that is defective Med1-ERα- binding showed mammary gland development defects and diminished expression of the genes targeted by ERα.[83] Another study performed by Zhu and his colleagues further confirmed the role of Med1 in ERα positive breast tumors and found that Med1 mRNA was overexpressed in over 50% of the breast tumors linking Med1 with ER positive breast cancer.[84] Breast cancer is not the only cancer type that Med1 is indicated to be involved in. Another study determined that Med1 is overexpressed in around 50% prostate cancer cases, suggesting a function for Med1 in the progression of prostate cancer.[85]

Another mediator subunit Med28 has also been revealed to be involved in breast cancer phenotypes. Breast cancer patients are screened for Med28 and this screening revealed that Med28 protein level is increased in ductal and invasive ductal carcinoma of the breast. Additionally, the expression level of Med28 was also found to be relevant to the disease outcome in which higher expression of Med28 signifies a higher probability of death in early as well as late stage breast cancer[86] highlighting a role for Med28 as a prognostic marker for breast cancer.

Med17 a head module subunit is found have a very significant relationship with cancer due to the fact that The N-terminal activation domain of p53 (p53AD) interacts specifically with Med17. A disruption of this interaction through mutation studies suggests that this interaction is critical for the tumor suppressive function of Med17 since this mutation prevents expression of most p53 target genes and leads to tumor formation.[87] Med17 is also found to be upregulated in prostate cancer although the current effect of Med17 on prostate cancer is currently has not been well defined.[88]

Mediator complex and its subunits are directly linked with many of the signaling pathways of the cell including Wnt/-catenin pathway. This pathway is responsible for the phenotype of the intestinal crypt progenitor cells. In colon cancer the intestinal crypts are the location where the intestinal tumors are produced by the progenitor derived epithelial cells start differentiating.[89-92] Therefore Wnt/β-catenin pathway is very much involved in the formation of the tumors of the colon. In cancer phenotypes the pathway becomes constitutively active, and therefore causing the intestinal epithelial cells to proliferate in a way that leads to colon cancer. The Mediator Complex's kinase module more specifically Med12 was believed to be a part of Wnt/β-catenin signaling as the C terminal transactivation domain of β-catenin interacts with Med12 to initiate transcription.[93]

Med12 is directly linked to the Cdk8 and Cyclin C the other proteins of the mediator kinase module according to the architecture of the kinase module and depletion studies. The mediator architecture suggests that the kinase module Cdk8 Cyclin C subcomplex assemble around Med12[94] while depletion of Med12 is found to be leading to reduced levels of CDK8/CyclinC inclusion to the Mediator complex[95] underlining the importance of Med12 on the kinase module. Biochemical assays indicate that Med12 is also involved in the activation of the Cdk8 kinase.[96] Loss of function screens performed by Firestein et al so as to determine kinases and phosphatases essential for the transactivation of β-catenin and the proliferation of colon cancer cells found 9 candidate genes that were crucial for both aspects. Cdk8 was not only one of them but also set apart from the remaining 8 by being the only gene that was revealed to have a copy number gain mutation in a significant percentage of colon cancer phenotypes. The study also elucidated that Cdk8 is essential for proliferation of colon cancer, transcription of genes mediated by β-catenin and cell transformation.[97] Even though Cdk8 seems to be the main subunit that seems to be significantly involved in Wnt/β-catenin pathway mediated colon cancer, It is known that β- catenin binds directly to Med12.[93] Therefore it can be said that not only

Cdk8 but also Med12 is intimately involved in colon cancer because of their significant role in β-catenin signal transduction.

Cdk8 is also identified to be involved in progression of melanoma, a deadly skin cancer according to a study performed by Kapoor et al. The study identified Cdk8 as a mH2A repressed gene by utilizing marcoH2A knockdown cells, suggesting that Cdk8 could be a possible indicator of malignancy in melanoma cases.[98]

Med14 the architectural scaffold of the core complex is found to be involved in breast cancer[99] and in depletion studies revealed to be crucial in the proliferation of prostate cancer.[100]

Med30 a metazoan specific subunit of the mediator is upregulated in breast cancer and ovarian cystadenocarcinoma (OV). Med10, Med21 and Med26 is also upregulated in OV phenotypes. Med10 is also upregulated in lung cancer while Med7 is upregulated in kidney renal cell carcinoma and Med4 the middle module subunit gene is upregulated in prostate cancer and downregulated in cervical cancer.[101] Furthermore a deletion of the MED4 is associated with a milder phenotypic expression of retinoblastoma in genotype phenotype studies.[102]

Even though a direct link between these proteins and many of the cancer phenotypes are not yet delineated to its fullest extent aberrant expression of these proteins in various cancer types indicate that abnormal functioning of mediator proteins and cancer are intertwined with each other.

1.2.1 Mediator's Tail Module in Disease

Since the Mediator Tail has distinct regulatory functions on the entire mediator complex and therefore the Pol II mediated transcription due to its relationship with activator and repressor proteins the aberrances that are observed in the subunits of the mediator tail is also involved in a significant amount of disease phenotypes whether as the direct cause of these phenotypes or as being differentially expressed. Because of the significance of the mediator complex many of the deletions or the mutations of the head and middle domains of the mediator is usually lethal. Tail mutations on the other hand does not end up being lethal due to the fact that they can be disposable to some extent during transcriptional processes. In fact deletion of none of tail subunits the affect the viability of the cell. Even if all tail proteins are deleted in the same cell line, the cell can survive. Obviously this has a catastrophic effect

on the transcriptional profile of the cell as many RNAs were found to be upregulated and downregulated in this scenario.[7] This makes the mediator tail to be linked to disease a great deal more than the other modules however it also makes them a much more viable therapeutic targets.

Hereditary motor and sensory neuropathy more commonly known as Charcot Marie tooth disease is an inherited disease that is associated with the tail module. The most probable cause of the disease is a A335V mutation in Med25 gene of the tail subunit. This missense mutation is found to be disrupting the interaction of the Med25 with the rest of the Mediator complex which cripples the transcriptional regulation of the genes responsible for the functionality of the peripheral nervous system.[71]

DiGeorge syndrome which is observed in approximately every 3000 live births and is one of the most commonly observed multiple congenital anomaly syndrome.[103] The syndrome not only causes physical symptoms of cardiac defects, dysfunction of the immune system, palatal anomalies, facial dysmorphism, and hypocalcemia but also mental symptoms of psychoses and schizophrenia.[104] The main cause of disease is the deletion of around 3Mb region on chromosome 22. This 3Mb region also contains the gene for the tail module subunit Med15[105] Med15 is critically involved in SREBP1α and TGFβ mediated SMAD2/3 signaling pathway which is involved in development and lipid metabolism.[14] Reduced expression of Med15 caused by the deletion of the Med15 gene may be the leading cause for the DiGeorge syndrome.[71]

Tail mediator subunits are also involved with various cancer phenotypes. The mediator tail subunit Med23 is revealed to be binding to ER through two LXXLL motifs on the N end and Med23 through binding to ER is involved in the regulation of transcriptional activities of ER-dependent genes.[106] A study conducted by Lin et al using small hairpin RNAs to silence Med23 expression in HER2 positive subtype of breast cancer indicated that Med23 has an important role in drug resistance, invasion, and metastasis of breast cancer.[107]

Another tail subunit that is involved in cancer is Med27. Med27 is highly expressed in osteosarcoma (OS) which is common malignant primary bone tumor that is known for responding having a poor response to chemotherapy.[108] Through micro-RNA targeting studies of the osteosarcoma cells performed on mice Ding et al revealed that decreasing Med27 expression causes suppression of tumor growth and a decrease in Ki-67, a osteosarcoma biomarker, positive cells.[109]

Med16 is also involved in a cancer phenotype and Med16 is found to be down regulated in papillary thyroid cancer.[110] Thyroid cancer has been the most prevalent endocrine malignancy over the past few years, with the fastest growing incidence globally, among which papillary thyroid cancer (PTC) is the most common histologic type and accounts for approximately 80% of all cases.[111] The study performed by Gao et al revealed that further inhibition of Med16 results in enhanced cell migration, epithelial-mesenchymal transition (EMT), and RAI resistance. Additionally the same study revealed that knockdown of this tail subunit causes the activation of transforming growth factor β (TGF-β).[110]

Med29 is another protein that is revealed to be significant in pancreatic cancer, one of the deadliest and most rapid cancer types. Kuuselo et al revealed that Med29 expression is significantly overexpressed in pancreatic cancer however the mechanism is not well known since an overexpression study performed after the lentiviral based overexpression data is observed by Kuuselo et al revealed a significant decrease in pancreatic tumor formation.[112]

As it can be observed Mediator and Its proteins are intimately involved in many disease phenotypes and cancer types. Further investigations are required to fully understand the role of individual Mediator subunits in order to develop therapeutic options to treat wide range of human diseases.

1.3 RNA Polymerase

Transcription is crucial for any living organism. The organisms that do not have their own transcription machinery such as viruses are not considered alive and the presence of the transcriptional machinery is one of the most important elements that is used to classify organisms as living. Therefore, RNA polymerase is extremely fundamental to any living organism to survive. RNA polymerase and its activities are first discovered by Sam Weiss in 1959. The research conducted demonstrated the incorporation of all four-nucleoside triphosphate into a nucleotide in rat liver.[113] Jacob and Monod published the landmark study on lac operon that demonstrated both the mechanism and regulation of transcription in 1961.[114] RNA polymerase became one of the initial hot topics in molecular biology in the late 1960s and In 1969 Richard Burgess and his colleagues purified RNA polymerase homogenously for the first time in 1969. Richard Burgess also elucidated that the start of transcription is controlled through the dissociable sigma subunit.[115] Again in 1969 with the discovery of three separate classes of RNA polymerases by Roeder et al the biggest milestone in understanding the eukaryotic mechanism of transcription is achieved.[116]

1.3.1 RNA Polymerase I

RNA polymerase I regulates the levels of ribosomal components and cell growth. it is mainly involved transcription of ribosomal RNA (rRNA) genes. Due to the fact that it transcribes many copies of rRNA genes it synthesizes almost 60% of all the total volume of RNA generated in the cells. The transcriptional output of Pol I is massive in fact microscopic studies show that rRNA transcription by Pol I resembles a "Christmas tree" since each rRNA gene is transcribed by many Pol I enzymes. [117] Pol I consists of fourteen subunits and is 590kDa in size.[118] The structure of *S. Cerevisiae* Pol I is revealed in 2013 by Christoph et al. The structure consists of a Pol I dimer lacking the mobile A49 WH domain and some other surface loops.[119] The core structure of Pol I contains 10 subunits; A190, A135, AC40, AC19, A12.2, Rpb5, Rpb6, Rpb8, Rpb10, and Rpb12.[120] The purified Pol I was functional in both DNA templated RNA extension and cleavage.[119]

The main transcriptional limitations of Pol I, II and III is conserved functionally as well as structurally. Structural studies revealed that RNA polymerase I and III contains a sub-complex similar to TFIIF which is important in the stabilization of Pol II. Pol I and II also utilize TFIIB like factors and contain domains related to TFIIE.[120]

Due to its importance in the generation of proteins by translating the ribosomal genes deregulation of the RNA Pol I results in a multitude of diseases including cancer.

1.3.2 RNA Polymerase II

The RNA polymerase II (Pol II) is one of the most crucial machinery in any living organism. While RNA Polymerase I and III transcribe ribosomal genes and other non coding regions of the DNA Pol II alone is responsible for the transcription of the protein coding genes. The protein coding genes perform many processes such as cell differentiation, maintenance and homeostasis. Pol II is directly responsible for the regulation of the transcription of these genes and therefore these processes. Protein coding genes are regulated through intrinsic and extrinsic signals these signals all coalesce in Pol II as Pol II is the machinery that transcribes the mRNA required for protein expression once the multitude of signals are interpreted. Therefore the initiation of transcription which is performed by Pol II is the most significant point of the regulatory processes.

Pol II is a twelve subunit protein complex. These proteins are named as RNA Binding Protein (Rpb) 1-12. Rpb1 and Rpb2 the two largest protein subunits of the Pol II.[121] Rpb1 contains the carboxy terminal domain heptapeptide repeats that are essential for polymerase activity. Heptapeptide repeats that consist of serine and threonine residues are phosphorylated at different stages of transcription to initiate elongate and terminate the transcription process[122] while Rpb2 the second largest protein of the complex is thought to be responsible for the elongation of the mRNA since deletion of this subunit causes a significant decrease in the rate of elongation.[123] Through studies it is also revealed that Rpb2 interacts with Rpb3, Rpb5, Rpb8 and Rpb11.[124] The other subunits Rpb3-12 are not yet well understood which highlights the importance of the generation of the recombinant proteins and reconstitution of this complex.

In order to understand Pol II and its transcriptional regulatory effect clearly it is necessary to understand the structure of Pol II initiation complex which includes Pol II, general transcription factors TFIIB, TFIID, TFIIE, TFIIF, and TFIIH, and the interactions between the mediator complex and Pol II during the formation of the pre-initiation complex (PIC).[125]

The structural studies of the PIC started 20 years ago through the analysis of the TATA binding protein (TBP) in DNA bound[126] and unbound[127,128] states. The analyses showed that TBP is a saddle shaped protein that attaches to DNA's minor groove. Once bound, it twists the DNA by 90 degrees. Further studies elucidated that TFIIA and TFIIB general transcription factors are located on the sides of the TBP when it is bound to the DNA.[129,130]

In 2009 the total structure of the PolII when bound by TFIIB is revealed which showed that TFIIB is located on the wall of Pol II. Subsequently Pol II-TFIIB-TBP-DNA complex model in an open and closed promoter DNA conformation was established.[131] An electron microscopy study in 2013 the locations of TFIIE, TFIIF, and TFIIH were visualized in PIC which contained Pol II TFIIB, TBP, DNA and TFIIA. This study placed TFIIE on Pol II's clamp domain.[132]

PIC assembly initiates with the specific binding of TBP to the TATA box region. TATA box sequence in humans is located around 30 base upstream of the transcription start site (TSS).[133] Even though TATA box seems to be crucial for transcription since it is conserved from yeast to humans,[134] there are some promoter regions which do not have the TATA box region. These regions are named as TATA-less

promoters. However the evidence shows that PIC assembly seems to be very similar between TATA box containing and TATA-less genes since studies performed on yeast show that TBP is bound throughout the genome whether TATA box is present or not indicating similarity in PIC assembly. Like the TATA box, the binding of TBP is also conserved between yeast and humans which is shown by the similarity of the structures formed by TBP when it is bound to DNA.[135,136,137,138]

General transcription factor TFIIA has a is not essential for basal transcription but it has a role in stabilizing the complex formed with the binding of TBP to DNA.[139] It enables constitutive and activator driven transcription of genes.[140] TFIIA forms a heterodimer that binds under the TBP and upstream of TATA box. TFIIA binds to these regions with the help of its two conserved domains which are the 4-helix bundle and 12 stranded β-barrel.[141] This method of binding does not alter the complex formed by TBP DNA interaction.[130]

TFIIB which was located on the flank of the TBP-DNA complex along with TFIIA is essential for the recruitment of Pol II to the promoter region.[142,143] It not only initiates the RNA synthesis but also stabilizes the initiation complex. TFIIB through its N terminal domain recruits Pol II[144] and TFIIB is also responsible for the TBP-DNA interaction and the subsequent bending of the DNA which is necessary for transcription through its C terminal domain.[50,145] In archaeal transcription the only requirements for transcription to occur are the presence of TBP and TFB which is the homolog of TFIIB.[146] Structural studies reveal that Pol II and TFIIB interacts with one another to form B-reader and B-linker helix domains. The B-linker opens up DNA and maintains the transcription bubble[131] during the process while the B-reader binds to the template DNA strand and assists in recognizing the initiator sequence.[147,148]

Another transcription factor that is found to be interacting with Pol II is TFIIF.[149] TFIIF is a heterodimer that consists of 2 subunits TFIIFα and TFIIFβ.[150] In yeast cells 50% of Pol II is located coupling with TFIIF homologs Tfg1 and Tgf2.[151] TFIIF has a role in stabilizing the PIC[152] and the transcription bubble,[153] prevents nonspecific DNA Pol II interactions,[154] selection of transcription start site,[155] prevention of Pol II pausing.[96] It also facilitates phosphodiester bond formation and initial transcription of RNA.[156] As it can be understood from its multifunctional role in transcription it is essential in transcription. The absence of TFIIF prevents the cell from starting transcription in vitro.[153] The TFIIF heterodimer has two structural domains that are named as the arm and the winged helix. The arm is formed at the N terminus of the heterodimer while the winged helix domain is located at the C

terminus.[157,158] The winged helix domains bind to a dimerization module. This dimerization module is revealed to be the anchor that binds TFIIF to the Pol II.[159,160,161] The structural data on the Pol II and TFIIF is not clear due to the EM model and the data obtained from other sources not correlating with each other.[162] The Pol II-TFIIF complex still remains a mystery.

TFIIE transcription factor is another transcription factor that is a heterodimer. It is made up of two subunits TFIIEα and TFIIEβ.[163] The N terminus of TFIIEα contains winged helix domain and a zinc finger domain in its N terminus. These two motifs are important in its interaction with its counterpart TFIIEβ. This interaction assists in transcription.[164] TFIIEβ is located at the upstream of the transcription start site according to the crosslinking data obtained.[165] Just like its archaeal homolog TFE TFIIE is bound to Pol II's clamp domain.[161,166] Another important function of the TFIIE is the recruitment of TFIIH to the transcription machine. TFIIE acts similar to a bridge that connects TFIIH to Pol II.[167,168]

TFIIH is a ten subunit transcription factor that consist of a 7 subunit core. The core consists of XPD, XPB, p62, p52, p44, p34 and TTD. The other the proteins CDK7, MAT1, and cyclin H is linked to this core through XPD. These three proteins form a subcomplex named cyclin activating kinase-subcomplex[169] TFIIH is an essential transcription factor for the beginning of transcription because it contains ATPase and helicase activity from its two proteins XPD and XPB which is important in the creation of the transcription bubble.[170,171] The mechanism of the helicase activity is as follows: TFIIH rotates the DNA from a fixed protein complex at the TATA box. It acts similar to a wrench. The movement of the wrench generates the torque required to melt the DNA.[172]

The final transcription factor TFIID is important in recognition of the promoter, PIC assembly and chromatin remodeling[173] It is a protein complex around 1.2MDa that contains TBP which is a crucial protein in transcription as explained previously and its associated factors. There are 14 TBP associated factors (denoted as TAFs)[174] coupled with the heterogeneity of the subunit and low amount of material made structural studies of TFIID very difficult and only recently core TFIID structure is elucidated at 12A resolution.[175] 13 of the 14 proteins of the TFIID complex are conserved throughout yeast and humans highlighting their importance in transcription.[174] Yeast studies further delineated on the importance of TFIID as they revealed that it contributes to most of the Pol II transcribed genes.[175] However the most important role of TFIID is the recognition of the promoter region. TBP and other

TAFs of TFIID are responsible for identifying many promoter regions which include TATA box, downstream promoter elements and motif ten element.[176-179]

The Pol II due its function is a very sophisticated RNA polymerase which requires a significant amount of regulation to perform adequately. This makes it challenging to fully grasp the mechanism of mRNA transcription and there is still much unravel about the structure and the mechanism. This makes Pol II and its regulators a prime candidate for disease research.

1.3.3 RNA Polymerase III

The RNA polymerase III (Pol III) is responsible for transcription of transfer RNAs (tRNAs) and various other noncoding RNA that are involved in ribosomes and protein synthesis.[180] Pol III similar to other RNA polymerases is a protein complex. It overall consists of 17 subunits and has a core region much like the other polymerases of 10 subunits. Pol III mediated transcription of tRNA genes requires transcription factor TFIIIB and TFIIIC. TFIIIB consists of 3 subunits TBP and Brf1 (a paralog of TFIIB) and Bdp1. TFIIIC consist of 6 subunits. Pol III overall is recruited to a stably assembled TFIIIB upstream of TSS while TFIIIC connects to the intragenic A and B block promoter elements.[181] In ribosomal RNA transcription TFIIIC is usually guided to this position by another transcription factor TFIIIA. In metazoans the transcription process of the RNA Pol III can be different from the canonical assembly due to the presence of a different type of Pol III promoters. These promoters are not reliant of intragenic sequence elements. This difference in the promoter sequence changes the proteins required for the assembly process of the PIC. TFIIIA and TFIIIC become irrelevant as SNAPc protein takes their place and Brf1 subunit of TFIIIB is replaced by Brf2 which is a different paralog of TFIIB.[182,183]

Ribosome and tRNA synthesis and therefore the Pol III mediated transcription research usually focused due to the ability of yeast cells to respond to nutrient level through altering their synthesis of said proteins.[184,185] This research on yeast revealed a master regulator protein for the synthesis of ribosomal and transfer RNAs named MAF1.[186,187] MAF1 knockout experiments performed in yeast cells showed that when MAF1 is eliminated from the cells the ability of the cells to suppress Pol III mediated gene transcription was significantly perturbed. The control of MAF1 over the Pol III mediated transcription is also confirmed in mammalian cells as MAF1 is revealed to be crucial for Pol III mediated transcription repression in mammalian cells when treated with DNA damaging agents, serum starvation and TORC1 inhibitors.[188,189]

Perturbation of Pol III transcription machinery just like other polymerases is responsible for cancer and many complex diseases. Microarray studies reveal that breast cancer and myeloma cells have elevated tRNA levels and its iso-acceptors. Since tRNAs are produced by Pol III this observation indicates that Pol III activity is significant in cancer phenotypes.[190,191]

Since RNA polymerases are the essential machinery for generating all RNA that is required by the cells, they are ubiquitously involved in vital processes within the cells therefore elucidating the structure and the function of RNA polymerases is essential for a better understanding of many diseases and many biological processes.

1.4 Baculovirus Expression System

Baculovirus, also known as the nucleopolyhedrovirus (NPV) are viruses that generate inclusions in the nucleus of the cells they infect. In molecular biology they are used to generate recombinant proteins through viral infections and are named as baculovirus expression vector system (BEVS). The tool is developed in 1980s by two laboratories by cotransfection of Sf21 with the viral DNA together with a vector to replace *Autographa californica nucleopolyhedrovirus*' (AcMNPV) polyhedrin gene to generate recombinant viruses.[192,193]

In the 40 years after its inception the technology of the baculovirus expression systems have grown significantly as a tool to generate a system that can be used to produce a high quantity of different proteins in eukaryotic systems. One of the more important landmarks of the BEVS research is achieved with the development of a vector that contained the entire genome of AcMNPV that is also suitable for propagation with *E.Coli*[194] This allowed for the researchers to generate recombinant protein generating viruses easier than the previous methods. This new system is named as the Bac-to-Bac® and became commercial by Invitrogen. The system works by combining a vector that has the gene of interest for the recombinant protein which is now called a shuttle vector into the genome of AcMNPV (will now be referred as bacmid) which can now be generated as a vector. The shuttle vector can be transposed into the genome vector of the bacmid through the Tn7 sites that are found in both vectors. These Tn7 sites recognize one another and with the help of Tn7 recombinase can be transposed. This technique allowed for the easier selection and isolation and also allowed for a higher yield of recombinant viruses.[195] However, there were still certain issues with this system, this recombination system did not allow for

multiple proteins to be expressed through one virus and infections with multiple viruses was necessary to express more than one protein and because multiple viral infections were difficult to control the simultaneous generation of multiple recombinant proteins was not assured which made it an inefficient for many areas of research.[196,197]

This problem was eliminated with the creation of different shuttle vectors such as Invitrogen's pFastBacDual which allowed for the expression of two genes and therefore two proteins and can also be ligated to one another through conventional restriction digestion methods to insert the MCS and promoters of one vector to the other one and the MultiBac system, an optimized bacmid vector which allows for the expression of multi-subunit protein complexes.[198]

As the baculovirus expression system research started to flourish other systems have also emerged. One system flashBAC™ combines the bacmid technology and insect homologous recombination for baculovirus production.[199] Another system which is called OmniBac as its name implies is a vector that can use both homologous recombination and bacmid technology to generate baculoviruses.[200] Bac-2-the-Future system greatly reduces the time and effort required for virus generation[201], biGBac system on the other hand uses Gibson assembly to assemble many DNA fragments for the production of baculovirus.[202]

Expression profiles of the recombinant proteins can be manipulated with the help of enhancers promoters and cis regulatory elements. Thus, the promoters that are used in this system is critical to how the recombinant proteins are produced in baculovirus expression systems. In baculovirus expression the most commonly used promoter regions were polyhedrin (polh) and p10. These two promoters are used mainly for their high rate of transcription and late generation of proteins allowing for an increased viral yield.[203] Recently Vp19 or pSeL is started to be used in combination with polh to increase the protein yield.[204,205]

Bacculovirus expression of proteins is also quite dependent on the insect cell lines that are used to produce viruses and proteins. 3 insect cell types are most commonly used for this purpose. IPLB-SF21-AE (known as Sf21) which is derived from the pupal ovarian tissue of the insect *Spodoptera frugiperda*[206] and its subclone Sf9. BT1-Tn5B1-4 (known as Hi5) is the final insect cell line which is frequently used. It is derived from *Trichoplusia ni* insects' adult ovarian tissue .[207] All 3 cell types can be grown as monolayers or suspensions in 27C without the need for external CO2 addition and can be

used with serum free media that many mammalian cells require.[208] These 3 commonly used cells are used for different purposes due to their differing intrinsic qualities. Sf21 and its subclone Sf9 is used for the production of a high titer of virus due to their weakness against viral infections while Hi5 is mostly used for protein production since they are more resistant to stress caused by lack of nutrients and they are larger compared to the Sf21 and Sf9 which allows them to produce more recombinant proteins when infected with baculovirus. One caveat of the Hi5 cells compared to Sf9 and Sf21 is that they produce proteases up to 3 times more than the other two which may cause the recombinant protein to degrade.[209] Sf21 cells compared to its subclone Sf9 is more vulnerable to sheer stress, changes in pH and osmotic changes and can also grow faster and denser.[210]

BEVS research is still continuing very rapidly as new improvements are still being made to produce better cell lines to improve the quality and the quantity of recombinant proteins generated by this method and combination of genome editing techniques such as CRISPR-Cas9 and the baculovirus vector research is thought to be able to improve the expression of recombinant proteins through induction of specific mutations, deletions and insertions that Crispr-Cas9 system provides.[211]

1.5 The Aim of The Study

The primary aim of the study was to recombinantly express each protein of the mediator's tail and reconstitute the Tail subunit of the Mediator complex in order to identify the structure of the mediator complex and the interactions between each protein through biochemical assays. Furthermore reconstitution of the Mediator's Tail subunit will allow for the better examination of the activator tail interactions and can be utilized in reconstitution of the entire Mediator complex. Through the usage of MultiBac baculovirus expression system is used to express each tail protein and the reconstitution through coinfection experiments. As a result interactions between many subunits but not all of the mediator tail have been identified which demonstrates the complexity of the Mediator's tail module.

Chapter 2

Materials

2.1 Materials for Molecular Cloning

Product Name	Manufacturer	Catalogue Number
GeneJET™ Gel Extraction Kit	Thermo Fischer Scientific	**K0691**
GeneJET™ Plasmid Miniprep Kit	Thermo Fischer Scientific	**K0503**
NEB Gibson Assembly Kit	New England Biolabs	**E5510S**
Phusion High-Fidelity PCR Master Mix with HF Buffer	Thermo Fischer Scientific	**F531L**
Fast Digest Value Pack	Thermo Fischer Scientific	**K1991**
Fast Digest DpnI	Thermo Fischer Scientific	**FD1703**
Quick Dephosphorylation Kit	New England Biolabs	**M0525**
Taq DNA Polymerase, recombinant (5 U/µL)	Thermo Fischer Scientific	**EP0406**
GeneJET PCR Purification Kit	Thermo Fischer Scientific	**K0701**
TaKaRa DNA Ligation Kit LONG	TaKaRaBio	**6024**
RevertAid First Strand cDNA Synthesis Kit	Thermo Fischer Scientific	**K1621**
Tango Buffer 10X	Thermofischer Scientific	**BY5**

Table 1: List of materials used for Molecular Cloning

2.2 Materials for Cell Culture, Buffers, Reagents And Glassware

Product Name	Manufacturer	Catalogue Number
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**
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**
Wheaton Spinner Flasks	Wheaton	

Table 2: List of products utilized for insect cell culture transfection and infection studies.

2.3 Buffers for Protein Extraction and Purification.

BC0	BC1000
40mM Hepes pH:7.6	40mM Hepes pH:7.6
10% Glycerol	10% Glycerol
4mM MgCl2	4mM MgCl2
0.4mM EDTA pH: 8.0	0.4mM EDTA pH: 8.0
0M KCL	1M KCl
0.5mM PMSF	0.5mM PMSF
0.5mM DTT	0.5mM DTT

Table 3: List and contents of buffers used for extration of recombinant proteins from insect cells.

2.4 Materials Used for Immunoprecipitation and Purification.

Product Name	Manufacturer	Catalogue Number
Anti-flag M2 Affinity Agarose beads	Sigma-Aldrich	A4596
Flag Peptide	Sigma-Aldrich	F3290

Table 4: Products used for purification of coinfections, recombinant proteins and immunoprecipitation experiments

2.5 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: List and Contents of the buffers and materials used for SDS-PAGE, Western blot analysis and Coomassie staining analysis.

2.6 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
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
Med 15	Proteintech	11566-I-AP	1:1000
Med16	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
Med27 (B-7)	Santa Cruz Biotechnology	sc-390296	1:1000
Med29 (B-1)	Santa Cruz Biotechnology	sc-393800	1:1000

Table 6: The antibodies used in immunoprecipitation and western blot analysis.

Chapter 3

Methods

3.1 Construction of Plasmids

The plasmids were constructed mostly through restriction cloning. The coding DNA of the desired protein is amplified through polymerase chain reaction by using forward and reverse primers that contain required restriction sites for the proper digestion of both the amplicon and the vector. The cloning is then continued with the restriction digestion of the vector and the insert and the CIP treatment of the vector and NEB's T4 ligase and Takara Long Ligase ligation protocol.

3.1.1 Primer Design for Polymerase Chain Reaction

In order to perform the projects many plasmids were needed to be generated therefore many primers were created for the polymerase chain reaction which is the initial step for the generation of the plasmids. The full list of primers can be seen below:

Primer Names	Primer Sequence
His:MED15 Forward Primer BamHI	5'-GCGGATCCATGCACCACCATCACCATCACGCTGACGTTTCCGGGCAAGAG-3'
Med15 Reverse Primer XbaI:	5'-GCTCTAGACTAGGCGGCTGAGAGGCA-3'
Med16 Forward Primer XhoI:	5'-GCCTCGAGATGTGTGATTTGCGGCGGCCAG-3'
Flag:Med16 Forward Primer XhoI:	5'-GC ATGGACTACAAAGACGATGACGACAAGGCT TGTGATTTGCGGCGGCCAG-3'
Med16 Reverse Primer NheI:	5'-GCGCTAGCTCACGGACGGTCCTCTGG-3'
Forward Med 24 NheI	5'-GCGCTAGCATGAAGGTGGTCAACCTG -3'

Reverse Med24 KpnI	5’-GCGGTACCTCAGAGTGCAGCAATGGC-3’
HA:MED23 Forward Primer NheI	5’GCGCTAGCATGTACCCATACGATGTTCCAGATTACGCTGCTGAGACGCAACTGCAGAGC-3’
Flag:Med23 Forward Primer NheI	5’GCGCTAGCATGGACTACAAAGACGATGACGACAAGGCTTGAGACGCAACTGCAGAGC-3’
MED23 Reverse Primer KpnI	5’-GCGGTACCTCACTGAGTTACTGGTAAAG-3’
MED25 Forward Primer XhoI	5’-GCCTCGAGATGGTCCCCGGGTCCGAGGGC-3’
MED25 Reverse Primer NheI	5’-GCGCTAGCTCAGATGAGATCCATGAG -3’
MED27 Forward Primer EcoRI	5’-GCGAATTCATGGCGGACGTGATAAATG-3’
MED27 Reverse Primer XbaI	5’-GCTCTAGACTACTGCCGGCAGGTGTC-3’
MED29 Forward Primer EcoRI	5’-GCATGGAATTCGCTGCATCCCAGCAG-3’
MED29 Reverse Primer XbaI	5’-GCTCTAGATCACAGAGTGCCCCCAGG-3’
HA:RPB3 Forward Primer NotI	5’-GCGCGGCCGCATGTACCCATACGATGTTCCAGATTACGCTGCACCGTACGCCAACCAG-3’
RPB3 Reverse Primer XbaI	5’-GCTCTAGATTAATTTATGGTTAG-3’
HA:RPB4 Forward Primer NotI	5’-GCGCGGCCGCATGTACCCATACGATGTTCCAGATTACGCTGCAGCGGCGGGTGGCAGC-3’
RPB4 Reverse Primer XbaI	5’-GCTCTAGATTAATACTGAAAGCT-3’
HA:RPB5 Forward Primer EcoRI	5’-GCGAATTCATGTACCCATACGATGTTCCAGATTACGCTGCAGACGACGAGGAGGAG-3’
RPB5 Reverse Primer XbaI	5’- GCGCTAGCCTACTGCACCAGCCG-3’
HA:RPB6 Forward Primer EcoRI	5’-GCGAATTCATGTACCCATACGATGTTCCAGATTACGCTGCATCAGACAACGAGGAC-3’
RPB6 Reverse Primer XbaI	5’-GCGCTAGCTCAGTCGGTGATGAT-3’
HA:RPB7 Forward Primer EcoRI	5’-

	GCGAATTCATGTACCCATACGATGTTCCAG ATTACGCTGCATTCTACCATATCTCC-3’
RPB7 Reverse Primer XbaI	5’-GCTCTAGATCAGCTTACAAGCCCCAA-3’
HA:RPB8 Forward Primer EcoRI	5’- GCGAATTCATGTACCCATACGATGTTCCAG ATTACGCTGCAGCGGGCATCCTGTTTGAG- 3’
RPB8 Reverse Primer HindIII	5’- GCAAGCTTTCAGGCGAGGTTCAGAAGGC T-3’
HA:RPB9 Forward Primer EcoRI	5’- GCGAATTCATGTACCCATACGATGTTCCAG ATTACGCTGCAGAGCCCGACGGGACTTAC -3’
RPB9 Reverse Primer	5’- GCGCTAGCTCACTCGGTCCAGCGGTGGCC -3’
HA:RPB10 Forward Primer EcoRI	5’- GCGAATTCATGTACCCATACGATGTTCCAG ATTACGCTGCAATCATCCCTGTACGCTGC- 3’
RPB10 Reverse Primer HindIII	5’- GCAAGCTTTCACTTCTCCAGGGGTGCATA- 3’
HA:RPB11 Forward Primer EcoRI	5’- GCGAATTCATGTACCCATACGATGTTCCAG ATTACGCTGCAAACGCCCCTCCAGCCTTC- 3’
RPB11 Reverse Primer HindIII	5’- GCAAGCTTCTACTCAATTCCTTCCTGCTT- 3’
HA:RPB12 Forward Primer EcoRI	5’- GCGAATTCATGTACCCATACGATGTTCCAG ATTACGCTGCAGACACCCAGAAGGACGTT -3’
RPB12 Reverse Primer	5’- GCAAGCTTTCATCGAGCATCAAAAACGAC -3’

Table 7: The primer sequences for the molecular cloning of the coding sequences of Mediator’s Tail Subunit and Pol II subunits.

3.1.2 cDNA Synthesis

The cDNA synthesis for is performed by using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Cat no: K1622) according to the instructions of the manufacturer. The entire mRNA of MDA-MB-231, HEK293T, SKBRT, BT-434 and MCF7 is synthesized into cDNA.

3.1.3 Protocol for Polymerase Chain Reaction (PCR)

Using Phusion High Fidelity PCR Master Mix (Thermo Fischer Scientific, Catalog No: F-5315) and primers described in 3.1.1 the protein coding DNA of each protein is amplified. The PCR reaction mixes are created as follows:

Phusion High Fidelity PCR Master Mix	25µl
Forward Primer	2.5µl
Reverse Primer	2.5µl
DMSO	1-3µl (Depending on the reaction)
DNA	15ng for plasmid DNA / 5µl for cDNA
ddH20	Up to 50µl

Table 8: The reaction mixtures for Phusion PCR for the amplification of coding sequences.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	33
Annealing	variable	30 sec	
Extension	72°C	Variable (30sec per kb)	
Final Extension	72°C	7 mins	1

Table 9: The PCR conditions for Phusion PCR for the amplification of coding sequences.

In order to confirm the reaction and collect the DNA the PCR products were ran on Agarose Gel. The percentage of the agarose in the gel on each reaction varied between 0,6% for longer products and 1.2% for smaller products. Tris Acetic Acid EDTA (TAE) buffer is used to both dissolve agarose and to fill the tank. Through UV visualization with the help of UV transilluminator and GeneRuler 1kb+ or 200bp+ ladders (depending on the size of the product) the products were confirmed. The products were collected from the gel for the next steps of the cloning procedure with the GeneJET™ Gel Extraction Kit.

3.1.4 Digestion of Vector and PCR Products

pFBDM Vector and the PCR products were digested using Thermofischer Scientific's FastDigest value pack kit which consist of the most common restriction enzymes used and the 10X Fast Digest Buffer. NEB's EcoRI and XbaI restriction enzymes and Tango buffer (Thermo Fischer Scientific Catalog No: BY5) are also used for some digestions that are performed.

The reaction set up can be found below:

10X Fast Digest Buffer / 10X Cutsmart Buffer	3µl
DNA Sample	600ng for insert, 2µg for vector
Restriction Enzyme I	1.5µl
Restriction Enzyme II	1.5µl
ddH20	Up to 30µl

Table 10: The reaction mixtures employed for the restriction digestion of the vector pFBDM and the inserts.

Once the reactions are set up, the reactions that utilized Fast Digest buffers and enzymes were incubated at 37ºC for 30 minutes. The other reactions which is performed by using NEB's EcoRI and XbaI restriction enzymes and the Tango Buffer were incubated for 2 hours at 37ºC in order to achieve complete digestion of the restriction sites.

The digestion products were ran on Agarose Gel just like the PCR products and purified with GeneJET™ Gel Extraction Kit as explained in 3.1.3 section.

The double digested vector was dephosphorylated using Quick CIP (NEB cat no: M0525S) as shown in table 11. The sample was incubated at 37ºC for 20 minutes. After dephosphorylation, the sample was purified using GeneJET™ PCR Purification Kit.

10X Cutsmart Buffer	2µl
DNA Sample	1pmol
Quick CIP	1µl
ddH20	Up to 20µl

Table 11: The contents of the reaction mixture for Quick CIP treatment.

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 for 16 hours using thermocycler. 40ng of vector and the appropriate amount of insert is used to perform the procedure.

3.1.6 Ligation with Long Ligase

Double digested inserts and pFBDM vector were ligated at 1:1 using Takara Long Ligase and its associated buffer at 65C for 3 minutes and 16oC for 16 hours using thermocycler.

3.2 Gibson Assembly

The plasmid of the coding sequence of Rpb1 required Gibson Assembly to generate. Therefore Gibson Assembly is performed using NEB's Gibson Assembly Kit and the method based on the manufacturer's instructions. The Gibson assembly is performed through restriction digestion. Restriction digestion is performed in order to linearize the plasmid due to the inefficacy of PCR based linearization of the vector compared to the restriction digestion based linearization.

3.2.1 Primer Design for Gibson Assembly

The primers designed for Rpb1 coding sequence can be seen below. These primers allowed for 30 base overlaps of the insert with the vector on each side in order to facilitate the assembly more efficiently. The primers also contained a histidine tag which is not only used for detection of the protein but also purification studies which were outside the scope of this project and therefore will not be addressed further.

Rpb1 Forward Primer	5'-CAAAGGCCTACGTCGACGAGCTCACTAGTCGCATGCATCATCACCATCACCACGCTCACGGGGGTGGCCCCCCCTCGGGGGACAGC-3'
Rpb1 Reverse Primer	5'-CTGATTATGATCCTCTAGTACTTCTCGACATCAGTTCTCCTCGTCACTGTCATCCGGGCTG-3'

Table 12: Sequences of Primers utilized for the Phusion mediated PCR of Rpb1's coding sequence for Gibson Assembly.

3.2.2 Protocol for Polymerase Chain Reaction for Gibson Assembly

The polymerase chain reaction for the Gibson assembly is performed using Phusion High Fidelity PCR Master Mix (Thermo Fischer Scientific, Catalog No: F-5315) and primers described in 3.2.1 the protein coding DNA of each protein is amplified. The PCR reaction mixes are created as follows:

Phusion High Fidelity PCR Master Mix	25µl
Forward Primer	2.5µl
Reverse Primer	2.5µl
DMSO	1-3µl (Depending on the reaction)
DNA	15ng for Rpb1 in pcDNA3.1 (Addgene Cat No:35175)
ddH20	Up to 50µl

Table 13: The reaction mixtures for Phusion PCR for the amplification of coding sequence for Gibson Assembly.

Step	Temperature	Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	33
Annealing	variable	30 sec	
Extension	72°C	Variable (30sec per kb)	
Final Extension	72°C	7 mins	1

Table 14: The reaction conditions PCR conditions for Phusion PCR for the amplification of coding sequence for Gibson Assembly.

The reactions are confirmed and the DNA is collected utilizing 0.6% Agarose Gel. Tris Acetic Acid EDTA (TAE) buffer is used to both dissolve agarose and to fill the tank. Through UV visualization with the help of UV transilluminator and GeneRuler 1kb+ ladder the products were confirmed. The products were collected from the gel for the next steps of the cloning procedure with the GeneJET™ Gel Extraction Kit according to the manufacturer's instructions.

3.2.3 DpnI Digestion of the PCR Product

DpnI digestion is performed by utilizing DpnI and the 10X Fast Digest Buffer of Thermo Scientific's Fast Digest Kit.

10X Fast Digest Buffer	3µl
DNA Sample	20µl
DpnI	1.5µl
ddH20	Up to 30µl

Table 15: The contents of the reaction mixture for DpnI treatment

The reactions were set up according to the table 15 (above) , and incubated at 37ºC for 2hours in order to achieve total elimination of the vector that is used for the PCR reaction.

After the digestion is complete, DpnI digestion products were ran on Agarose Gel just like the PCR products and purified with GeneJET™ Gel Extraction Kit as explained in 3.1.3 section.

3.2.4 Digestion of the pFBDM Plasmid

pFBDM Vector were digested using Thermofischer Scientific's FastDigest NotI and XbaI restriction enzymes and the 10X Fast Digest Buffer as follows:

10X Fast Digest Buffer	3µl
pFBDM Vector	2µg
XbaI	1.5µl
NotI	1.5µl
ddH20	Up to 30µl

Table 16: Digestion of the pFBDM plasmid for Gibson Assembly

Once the reactions are set up, the reactions were incubated at 37ºC for 2 hours so as to achieve complete digestion of the vector products. The digestion products were ran on Agarose Gel and purified with GeneJET™ Gel Extraction Kit as explained in 3.1.3 section.

The double digested vector was dephosphorylated using Quick CIP (NEB cat no: M0525S) as shown in table 17. The sample was incubated at 37ºC for 20 minutes. After dephosphorylation, the sample was purified using GeneJET™ PCR Purification Kit.

10X Cutsmart Buffer	2µl
DNA Sample	1pmol
Quick CIP	1µl
ddH20	Up to 20µl

Table 17: The contents of the reaction mixture for Quick CIP treatment.

3.2.5 Assembly of the Construct

The construct is assembled in 3 insert to one vector ratio. 60Ng of vector is used with 216.6ng insert to perform the procedure and the reaction mixture is set up as follows:

2X Gibson assembly Master Mix	5µl
Vector pFBDM	60ng
His-Rpb1 insert	216.6ng (3:1 ratio)
ddH20	Up to 10µl

Table 18: The contents of the reaction mixture for Gibson Assembly

The assembly is performed by incubating the reaction mix at 60C for 8 minutes and 50C for 1 hours in a thermocycler according to the protocol created by Birla et al which revealed that this methodology yields better efficiency of assembly compared to the methodology provided by the manufacturer.[212]

3.3 Preparation of Competent DH5α Cells

Competent cells are prepared using a method based on Inoue H. Et al.[213] NEB-5-alpha cells (Catalog No: C2987H) were first streaked on an LB plate that contained no antibiotics and single colonies were observed after 16 hour incubation. The next day one of the colonies were selected and used to inoculate 25ml LB broth on a shaker for 8 hours at 37ºC. The shaking procedure was used to aerate the cells to increase the efficiency of growth. After 8 hours the 4ml of the culture is used to inoculate 100ml of LB broth at 20-21C overnight with vigorous shaking of around 200rpm . Once the cells reached an OD595 of 0.55, the culture is placed on ice for 10 minutes. The cells are then put in falcon tubes and centrifuged for 10 minutes at 4000rpm. The supernatant is collected and 16 ml of ITB which is a buffer consisting of, 10mM PIPES, 250mM KCl, 55mM MnCl2 and 15mM CaCl2 and a pH of 6.7, is added to the pellet. The pellet is then resuspended in ITB buffer gently through shaking and swirling. Then the cells are centrifuged again for 10 minutes at 4000rpm and the supernatant is discarded. 4ml of ITB Buffer is again added to the pellet and gently resuspended. Finally 0.3ml of DMSO is added to the resuspension and the cells are dispensed into 50µl aliquots.

3.4 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 OD600 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 CaCl2. 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 CaCl2. 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 50µl cell suspension.

3.5 Transformation of Competent Cells (DH5α and DH10Bac)

50ng of plasmid or 5µl of the ligation product is used to transform 50µl DH5a cells. Once the plasmid or the ligation product is added the cells are placed on ice for 30 minutes and heat shock is applied for 45 seconds at 42C using a water bath. After the heat shock is complete the cells are put on ice for 2 minutes and 800µl of LB broth is added. The resulting mixture is placed on a shaking incubator which shook the cells vigorously at 200rpm and 37ºC for one hour. Once the incubation is complete the mixture is centrifuged at 4000rpm for 4 minutes and 600µl of the supernatant is removed. The pellet which contained the cells is then resuspended in the remaining 200µl of the LB broth and 100-150µl of the mixture is used to spread the cells on LB plates that contained 100µg/ml Ampicillin and put on incubator at 37ºC overnight.

For DH10Bac cell transformation 100ng of plasmid is used to transform 50µl of cells. After the plasmid is added to the cells the cells are placed on ice for 30 minutes similar to the DH5a transformation. Then just like the Dh5a transformation heat shock is performed on the cells using a water bath at 42C for 45 seconds and placed on ice for 2 minutes. Then 800µl of LB broth is added and the cells are placed on a non shaking incubator at 37ºC for 8 hours. After the incubation is complete the cells are centrifuged at 4000rpm for 4 minutes and 600µl of the supernatant is removed. The remaining 200µl LB broth and the pellet is mixed through resuspension and 30µl of the mix is added to 70µl of LB broth for dilution and the resultant 100µl mixture is spread onto a, 100µg/ml Ampicillin, 10µg/ml tetracycline, 100 µg/ml kanamycin, 0.17mM IPTG and 100µg/ml X-Gal plate for blue white screening. The plates are then put

into a 37C incubator for 36-40 hours until blue and white colonies are observed and can be differentiated from one another.

All transformations are controlled using Colony PCR and Agarose gel based size. Colony PCR is performed with Taq Polymerase and its associated buffers and materials which can be seen below:

10X Taq Polymerase Buffer	25µl
Forward Primer	2.5µl
Reverse Primer	2.5µl
DMSO	1-3µl (Depending on the reaction)
MgCl2	4µl
DNA	5µl
ddH20	Up to 50µl

Table 19: The reaction mixtures for Taq PCR for the Colony PCR to observe the presence of the insert in the ligated and transformed plasmid.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	5 mins	1
Denaturation	95°C	30 sec	33
Annealing	variable	30 sec	
Extension	72°C	Variable (1minute per kb)	
Final Extension	72°C	7 mins	1

Table 20: The PCR conditions for Taq PCR for the Colony PCR to observe the presence of the insert in the ligated and transformed plasmid.

3.6 Recombinant Bacmid Isolation from Transformed DH10b Cells

As discussed in the introduction DH10Bac vectors contain a shuttle vector called BacMid which is essential for the recombinant protein generation in insect cells and the DNA required for the generation of the recombinant protein is inserted to the BacMid vector through transposition of the Tn7 sites. Due to the position of the BacMid Tn7 site any occurrence of transposition causes the enzymatic reaction to x-

gal to cease and colonies to appear white. After the DH10Bac are transformed and incubated white colonies are selected since they appear to have transposed Tn7 site and therefore the coding DNA for the recombinant protein. The selected colonies are grown in 5ml of LB Media supplemented with 100µg/ml Ampicillin overnight by shaking at 37ºC

3ml of the cells are then removed and centrifuged at 5000rpm for 5minutes to collect the cell pellet and the supernatant is discarded. The cell pellet is then resuspended with 300µl of Buffer 1. (15mM Tris-HCl pH 8.0, 10mM EDTA and 100µg/ml RNaseA) Immediately after 300µl of Buffer 2 (0.2N NaOH and 1% SDS) is added and mixed by inverting the tube 8-10 times. The resultant mixture is then incubated at room temperature for 5 minutes. After the incubation is complete 30µl of 3M of Potassium Acetate pH:5.5 and mixed by inversion until a significant amount of white precipitate is formed. The resultant suspension is then placed on ice and incubated for 10 minutes before being centrifuged at 14000 rpm for 15 minutes. Then 700µl of the clear supernatant is carefully collected avoiding the white precipitate and put into 700µl of 100% isopropanol, mixed by inversion and placed on ice for 10 minute incubation. Once the incubation is complete the mixture is spun at 14000 rpm for 10 minutes. The supernatant is removed carefully so as not to disturb the lentil sized pellet and the pellet is washed with 500µl of 70% ethanol. The ethanol is then collected and the pellet is left to air dry for 3-5minutes until the pellet starts to become slightly transparent. Finally 30µl of ultra pure water is added and the pellet is allowed to dissolve through flicking. Due to the ephemeral nature of the BacMid plasmids the collected plasmids are measured using NanoDrop and used in the transfection of Sf9 cells immediately.

3.7 Transfection of Sf9 Cells with Recombinant BacMids

Sf9 cells were seeded in a 6 well plate with each well containing 1.0-1.2x10^6 cells and incubated at 27ºC without any light source for 40 minutes-2hours to ensure that the cells are attached to the well. 6µg of the BacMid plasmid containing the coding DNA for the recombinant protein is measured and added to 200µl of serum free media along with 4-5µl of Cellfectin II reagent. The mixture is homogenized by flicking and incubated at room temperature for 20 minutes. Then the media on top of the now attached cells is removed via aspiration. The DNA and Cellfectin containing serum free media is added on top of the cells dropwise so as to ensure a homogenous transfection of the cells and 1.5ml of serum free grace media is added to ensure the cells are supplemented with adequate amount of media. After 5 hours of incubation at 27ºC in dark, the media used for transfection is aspirated and 2ml of full grace media

(Grace media supplemented with 10% FBS, 1% poloxamer and 50µg/ml Gentamycin) is added and the cells are left at 27ºC in a dark environment for 5-7 days depending on the rate of transfection and the subsequent infection. Once the incubation is complete the cells and the media are removed from the cell and centrifuged to separate the cells from the media at 400rpm for 5 minutes. The supernatant is collected as P0 virus and the pellet is resuspended with 2X SDS Gel loading buffer for SDS-Page and Western Blot analysis for recombinant protein expression. After the expression of the recombinant protein is confirmed via Western Blot, P0 is used to amplify the virus through infection of 50 million Sf9 cells with 100µl of the P0 virus to create P1 virus and incubated for 5-7 days in order to achieve a significant amount of virus that can be used for further recombinant protein expression studies and co-infection studies.

3.8 Recombinant Production of Proteins

The recombinant proteins are produced by utilizing the Hi5 insect cells which has a better potential of generating recombinant protein compared to the Sf9 cells that are used to produce the virus. 10 million Hi5 cells are plated at 1 million per ml ratio on a 10cm plate along with Express Five SFM media which contained 18mM L-glutamine, 1% poloaxamer and 50µg/ml Gentamycin and incubated in dark at 27C to allow for the cells to get accustomed to the environment and adhere to the plate. Once the cells adhere to the plate 100µl of P1 virus is added dropwise directly to the plate and slightly shaken by hand to ensure homogeneity of infection. Then the cells are incubated at 27ºC in dark for 2 days and collected via pipetting. The cells are centrifuged to separate them from the media and the media is discarded while the pellet which contained only the cells are kept for lysis. The pellet is lysed in 500µl of BC300 (made by appropriate mixing of BC0 and BC1000 buffers) per ml of pellet (1ml=500µl, 2ml=1000µl etc.) by a douncing homogenizer. The pellet is dounced 10 times and left for incubation for 10 minutes on ice three times. The resultant mixture is collected and centrifuged at 13000rpm for 15 minutes. The supernatant is collected on eppendorfs and 15µl of the supernatant directly used for SDS PAGE and Western Blot analysis with the rest snap frozen in liquid nitrogen and stored in -80C for future use.

3.9 Purification of Recombinant Proteins Using Anti-Flag M2 Agarose Beads

For generation of the recombinant proteins the procedure that is utilized on section 3.9 is performed on 100 million Hi5 cells that are incubated on a spinner flask. The amount of virus used were between 100µl-500µl so as to achieve the optimal expression of each recombinant protein that will be purified.

After the homogenization is completed, the supernatant is collected in a 15ml falcon tube. During this time 100µl of anti-flag M2 agarose beads were washed 5 times with BC300 buffer that contained 0.1% NP-40. After the wash is complete the beads are placed to the 15ml falcon tube that contained the protein extract and incubated in a rotator at 4ºC for 16 hours. After the incubation is complete the beads were pooled via centrifugation at 1500rpm at 4ºC, the supernatant is removed. Then the beads were washed once with 4ml of BC300 with 0.1% NP-40 and four times with 1ml of BC300 with 0.1% NP-40. Then 100µl BC100 (made with appropriate mixing of BC0 and BC1000) containing 0.02% NP-40 was added to the beads along with 50µg/ml flag peptide and incubated on a rotator for 45 minutes at 4ºC . After the incubation the beads were centrifuged and the supernatant is collected. 10µl of the supernatant is used for analysis via SDS PAGE and Coomassie Staining while 2µl of the protein is used along with 5µl of the protein extract before purification in Western Blot analysis to confirm the purification.

3.10 SDS-PAGE Analysis, Western Blot Analysis, Coomassie Staining

The SDS-PAGE analysis is performed with hand made Tris-Glycine gels. The acrylamide/bisacrylamide percentage of the gels that are used depended on the size (in kilodaltons) of the protein that is analyzed. Depending on the experiment different ladders such as PageRuler of thermo scientific or Prestained Protein Marker of Genaxxon is used. The gels were ran at first 70V and after the ladder bands started to separate the voltage is increased to 110V and the gel is ran until the dye ran out. This is done to ensure adequate and even separation of the proteins.

Coomassie staining is performed by rinsing the fully ran SDS-PAGE gel in dH20 first and then placing it in the Coomassie stain solution and incubating it for 20-35 minutes on RT on a shaker until bands could be observed. After the incubation the Coomassie stain is removed and the gel is rinsed with dH2O one more time to remove the remaining stain and placed on the Coomassie destaining solution for 2 hours on a shaker in order to accentuate the bands.

Western Blotting is performed by transferring the proteins from the fully ran SDS-PAGE gel to the PVDF membrane. The transfer process is performed at a current of 330mA for 2 hours 15 minutes. After the transfer is complete blocking is performed with either 5% Milk in PBS or blocking buffer from Clean-Blot IP detection Kit depending on the contents of the SDS-PAGE. Primary Antibody incubation is performed at 4ºC on a shaker overnight and the next day the membrane is washed three times with PBS-T solution and placed on secondary antibody incubation for 1.5 hours at room temperature. Finally, the

membrane is washed again three times with PBST and developed using Amersham Imager 600 with Pierce ECL plus or ECL substrates.

3.11 Coinfection of the Tail Subunits

Coinfection of the tail subunits were performed infecting 100 million Hi5 cells that are placed on a spinner in 1 million per ml density with different amount of tail subunit viruses. For Med23 coinfection, His:Med15, Med16, Flag:Med23, Med24, Med25+Med27 and Med29 viruses were used for one setup and His:Med15, Flag:Med16, HA:Med23, Med24, Med25+Med27 and Med29 viruses were used for the other setup. For Med14 coinfection, Flag:Med14, His:Med15, Med16, Med24, Med25+Med27 and Med29 viruses were used. For Tail coinfections with Med14, Flag:Med14, His:Med15, Med16, HA:Med23 Med24, Med25+Med27 and Med29 were utilized. The expression of the recombinant proteins in this system is first measured by creating the protein extract from the infection according to the protocol explained in 3.9 and using western blotting for visualization. Once the results and the amount of virus to be used for simultaneous expression of each protein is achieved the infection is performed and the protein extract is collected again. After the collection of the protein extract the purification protocol for recombinant proteins explained in 3.10 is utilized to observe which proteins of the tail subunit pulls together and forms a stable protein complex with the flag tagged protein at each reaction. The results were observed via SDS PAGE and Coomassie Staining and Western Blot analysis made with 2µl of the purification protein being compared to the 5µl of the protein extract before purification.

3.12 Immunoprecipitation (IP) Using Anti-Flag M2 Agarose Beads

Immunoprecipitation is performed via Anti-flag M2 agarose beads and 12µl of beads were used in each single reaction. First the beads were carefully measured in order to ensure the same volume of beads were used in each IP reaction and then washed with 1ml of BC300 buffer + 0.1%NP-40 at room temperature 5 times. After the wash is complete the solution is discarded and 150µl of BC150 buffer is added to the beads along with 0.375µl of NP-40 (0.025%) and 2-4µl of purified flag tagged proteins Med14, Med16 and Med23 (depending on the amount of purified protein observed in Coomassie Staining analysis) and incubated on a rotator for 3 hours at 4ºC. The beads were then centrifuged to separate the beads and the supernatant was discarded. The beads were then washed 4 times with 1ml BC200 + 0.1% NP-40 and 50µl of unpurified recombinant protein extract, 50µl of BC0 and 50µl of BC150 along with 0.375µl (0.025%)

of NP-40 were added to the beads to achieve an incubation at 150mM of salt concentration. The samples were incubated again at 4ºC in a rotator for 3 hours. After the incubation, the beads were centrifuged and the supernatant is removed. The beads were then washed with BC150+0.1% NP-40 4 times. After removing all the supernatant very carefully 20µl of 2X loading dye is added on top of the beads and beads are stored at -20C for western blot analysis.

3.13 Cell Culture

Sf9 insect cells were grown and maintained using grace insect cell media with 10% FBS, 50 µg/ml gentamicin and 1% Poloxamer in a spinner flask. Hi5 cells on the other hand were grown using Express five SFM media supplemented with 18mM L-glutamine, 1% Poloxamer and 50 µg/ml gentamicin and the grace insect cell media (described above for Sf9 cells) at a 3:1 ratio respectively.

Chapter 4
Results

4.1 Generation of Mediator Tail Proteins Through Baculovirus Expression System

The tail module of the Mediator of the RNA polymerase is an essential part of the mediator complex as it integrated relays a multitude of internal and external signals to the RNA transcription machinery to allow a calibrated transcriptional response from the cell.[7] Previously Cevher et al successfully reconstituted a core mediator complex that contains many head module and middle module proteins along with Med14 and Med26. The core mediator can be described as the collection of the essential proteins that are required for Pol II recruitment and Pol II mediated transcription.[17] Even though the core mediator successfully initiates transcription in vitro, it has limited interaction with the activator and repressor proteins since tail subunits of the mediator is directly responsible for these interactions and with these interactions the tail module controls the transcriptionally active core mediator to generate a transcriptional response.[46] Therefore, reconstitution of the Mediator's tail module is essential to understand the relationship of the mediator complex with activator and repressor proteins transcription factors and the PIC in vitro and will assist in the understanding of many disease phenotypes.

The Mediator tail consist of 7 proteins Med15, Med16 Med23, Med24, Med25, Med27 and Med29. These proteins are recombinantly expressed through baculovirus expression system. (Figure 3) In order to achieve the recombinant expression of the protein of interest the BacMids are generated from scratch. First the coding sequence of each protein is placed in a pFBDM vector through restriction digestion mediated molecular cloning and then placed in BacMids through Tn7 transposition and subsequent blue white screening. (Figure 3) These recombinantly expressed proteins were utilized in immunoprecipitation studies and the virus that was generated for the expression of these proteins were used in coinfection studies in order to reconstitute the tail module of the mediator with baculovirus expression system and Hi5 insect cells.

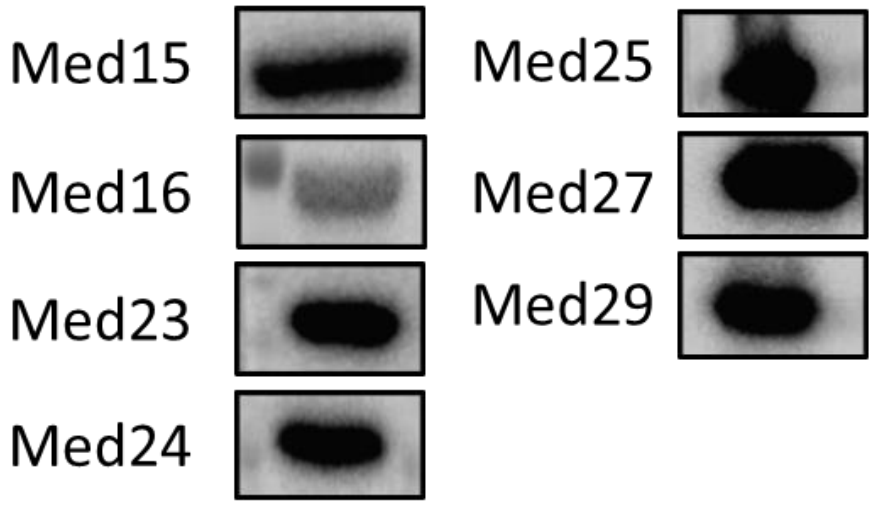


Figure 3: Recombinant generation of each tail subunit protein. SDS-PAGE analysis followed by Western Blotting for each tail subunit individually using baculovirus expression system

4.2 Coinfection of All Subunits of The Tail Module Fails to Yield a Complete Tail Module

In order to reconstitute the tail subunit of the Mediator the virus generated by the baculovirus expression system of each tail subunit is utilized to coinfect Hi5 cells. 6 different viruses were used to infect Hi5 cells to recombinantly express the 7 subunits of the Mediator Complex his:Med15, Med16, Flag:Med23, Med24 Med25, HA:Med27 and Med29. Once the expression of each protein is confirmed and calibrated through SDS PAGE and Western Blot studies the tail subunit of the Mediator is purified through Flag:Med23 as it is the largest protein of the entire tail module at 130kDa. The resulting purification is tested to observe the subunits that come together alongside with the Flag:Med23 via Coomassie Staining and Western Blot. (Figure 4A) This purification failed to yield the entire tail module of the mediator as his:Med15, HA:Med27 and Med29 failed to come together with the rest of the tail subunit. However, Flag:Med23 brought Med16, Med24 and Med25 alongside to form a 4 subunit tail module as evidenced by both the Coomassie staining and the Western Blot. This finding is in line with the previous research made on the Mediator complex which suggests that Med23-Med24 and Med16 forms a subcomplex and intimately interacts with each other. Other studies also revealed that Med25 extensively interacts with Med24 and Med16 which is also observed in this pulldown as Med25 came together with Med23, Med24 and Med16

4.3 Presence of Med14 Does Not Have A Significant Effect On The Binding Of Med15, Med27 And Med29 To The Tail Mediator

Med14 is one of the most fundamental subunits of the tail complex as it is revealed to be the architectural scaffold of the entire Mediator and elucidated to be crucial in Mediator mediated Pol II transcription by Cevher et al.[17] Previously thought as a part of the tail module due to its extensive interactions with the tail module subunits through its CTD domain[214] Med14 is also recombinantly expressed as well in order to elucidate more information about the tail module and achieve a successful reconstitution of the entire tail module. The virus essential for the recombinant expression of Med14 were already generated by Cevher et al previously so molecular cloning is not utilized for this procedure. (Figure 4B) Med14 is also selected for this purpose due to studies establishing Med14 and Med15 to be interacting with one another. In order to observe whether Med14 can interact with the proteins that failed to come together with the Flag:Med23 purification of the mediator tail Flag:Med14 is coinfected with his:Med15, HA:Med27 and Med29 subunits of the tail module. Med14 in this coinfection failed to pull together any of the 3 subunits as indicated by both western blot and Coomassie staining results. (Figure 4C) Mediator has an ability to access different conformational states. This ability is essential for the function of the mediator complex and its interactions with the other elements of the PIC and transcription.[55] Therefore, we have decided to coinfect the entire mediator along with Med14 as a method to bring together the entire tail module in a coinfection study. Flag:Med14 virus along with the viruses 7 subunits of the tail module his:Med15, Med16, HA:Med23, Med24 Med25, HA:Med27 and Med29 are used to coinfect Hi5 insect cells and purified with the assistance of M2 flag beads through Flag:Med14. The purified protein is then ran on SDS PAGE and Coomassie Staining and Western Blot analyses is performed. These studies revealed that addition of Med14 to the tail subunit failed to create a complete tail subunit as Med14 pulldown did not bring Med15 Med27 and Med29 alongside which indicates that Med14 appears to be inconsequential for the binding of Med15 Med27 and Med29 to the remaining tail subunits.

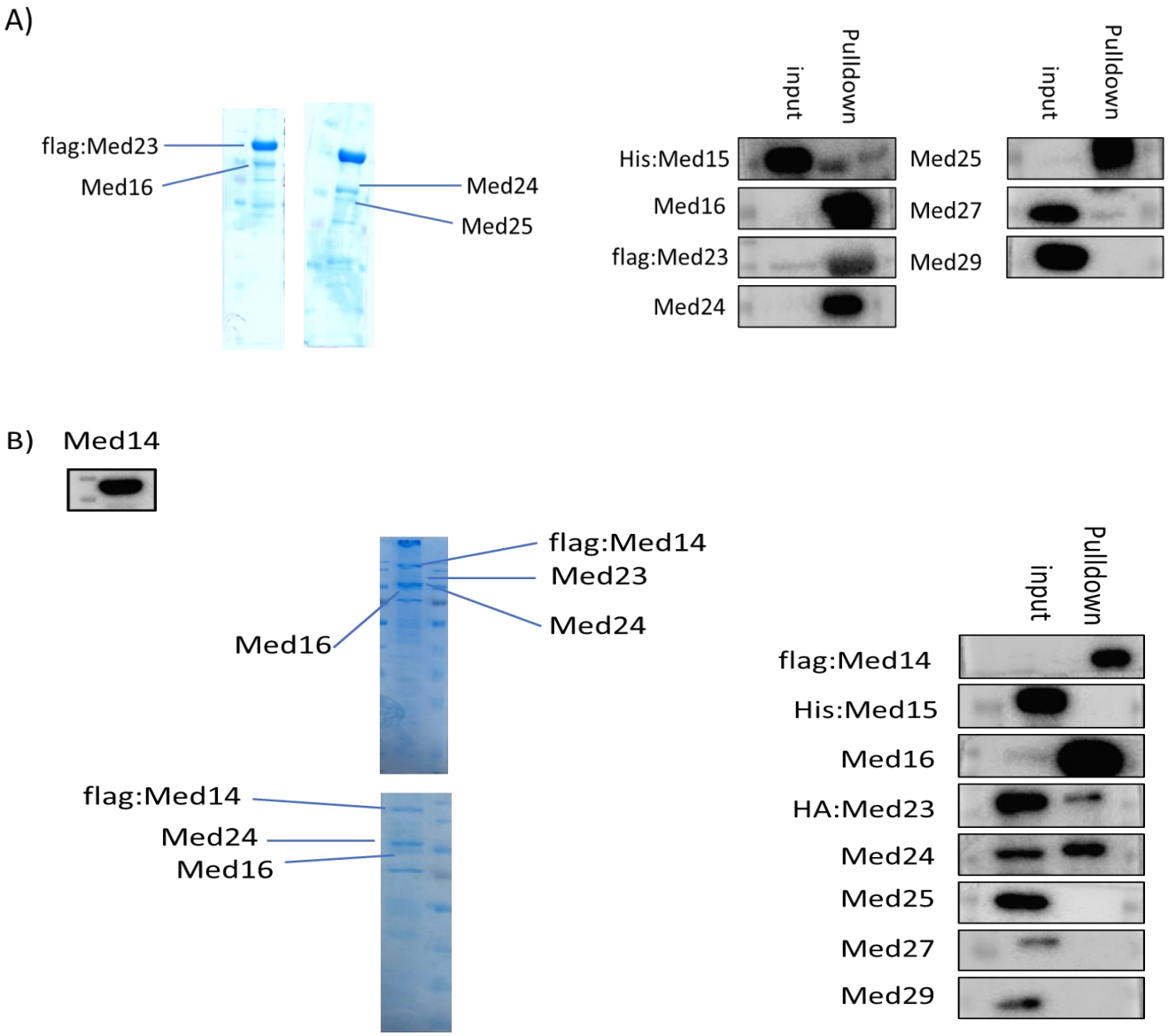


Figure 4: Coinfection of tail subunits and purification of the tail module A) SDS PAGE analysis followed up with coomassie staining and western blotting of coinfection tail subunits purified through Flag:Med23 with M2 Flag beads without the inclusion of Med14. B)SDS PAGE analysis followed up with coomassie staining and western blotting of coinfection tail subunits and Med14 purified through Flag:Med14 with M2 Flag beads. C) SDS PAGE analysis followed up with western blotting of coinfection tail subunits Med14 Med15 Med27 and Med29 purified through Flag:Med14 with M2 Flag beads

4.4 Presence of Med14 Disallows the Binding of Med25 to the Med23 Med24 Med16 Complex

The Med14 pulldown of the coinfection also revealed an interesting phenomenon. The pulldown through Med14 brought Med16 Med24 and Med23 that are shown to be interacting with each other in the previous tail pulldown but not Med25 which is also a protein that is revealed to be connected in the tail module pulldown (Figure 4A) even though Med25 was stably expressing in the system. (Figure 4B) creating a partial tail structure that consist of 4 proteins. Med14, Med16, Med23 and Med24 with Med25 being excluded.

4.5 Med16 is the Focal Point of Interaction with Many of the Tail Subunits

Due to the phenomenon occurring between Med23 and Med25 along with the fact that Med15 Med27 and Med29 failed to come together with the rest of the tail subunits we have decided to perform IP studies to observe which proteins are directly interacting with each other. Med14 is first selected due to its interactions with the tail and its general importance to the mediator itself.[7,17,214] Flag:Med14 is recombinantly expressed and purified using flag M2 beads (Figure 5A) while the remaining proteins are recombinantly expressed and stored as protein extracts. Immunoprecipitation is performed by precipitating purified Flag.Med14 with the extracts of each tail protein. The immunoprecipitation studies revealed that Med14 only directly binds to Med16 (Figure 5B) and does not bind to any other protein of the tail module directly. These findings contrast many MS and microscopy studies which suggest Med14 interacting with many of the tail subunits including Med15.[7,24,53,54] The yeast studies also suggested that Med2 the yeast counterpart of Med29 is also found to be interacting with Med14 but the IP studies also suggest that there is no direct interaction between the metazoan counterparts of the two proteins.

Because Med14 only seemed to be interacting with Med16, we have decided use Med23 the largest subunit of the Mediator to elucidate more about the interaction of mediator tail subunits with each other. (Figure 6A) The IP reactions of Med23 with the other subunits of the tail complex revealed that like Med14, Med23 directly interacts with Med16 only. (Figure 6B) Even though many EM and cross linking coupled with mass spectrometry research shows Med23 having many interactions with the rest of the tail subunits the IP study indicates that the only interaction of Med23 is with Med16.[7,24,53,54] The coinfection of the tail subunits indicated that tail subunits Med14, Med23 Med24 Med16 and Med25 to be interacting with each other depending on the presence of Med14 in the system. (Figures

3,4) However, the IP reactions of Med23 and Med14 revealed that these two proteins to be directly interacting with Med16 and none of the other proteins suggested by coinfection studies. This emerged Med16 as a candidate protein for an IP study to understand the interactions of tail module proteins with each other. Therefore a recombinant flag:Med16 protein is generated and purified using M2 Flag beads (Figure 7A) and the purified protein is used in IP reactions. The IP reactions revealed that Med16 to be interacting with both Med25 and Med24. (Figure 7B) Taken in accordance with the previous findings of Med16 being directly in contact with Med23 and Med14, Med16 is directly in contact with many of the tail subunits by itself and can be considered as a focal point of the tail module of the mediator. This result obtained from the IP reactions also indicates that the Med23-Med16-Med24 subcomplex of the Mediator Tail is formed by two proteins binding directly to Med16.

Tail coinfection studies suggested that in the presence of Med14 Med25 cannot bind to the Med14-Med23-Med16-Med24 subcomplex of the mediator. The IP reactions of Med16 also revealed that Tail subunit Med25 is found to be directly interacting with Med16, suggesting that Med25 is capable of binding to the other tail proteins through Med16 but this binding is hindered in the presence of Med14 and Med25 cannot bind to Med16 efficiently when Med14 is present.
As it can be seen from IP studies, proteins that failed to be pulled together with the rest of the tail subunits during the coinfection studies namely Med15, Med27 and Med29 also did not come together with any one of Med14 Med16 and Med23 which suggests that the interaction of these proteins to the rest of the tail module proteins is not obstructed by external or internal factors and the reason for the lack of interaction is not caused by the coinfection system but rather a distinct reason that is currently unknown.

The IP reactions as a whole argues that many of the interactions provided by EM and crosslinking coupled with mass spectrometry data is potentially observed by the close proximity of the subunits rather than a complex interaction of tail subunits with each other. The IP reactions reveal that most of the tail subunits coalesce and bind to the focal protein of Med16. Further evidence for this phenomenon comes from the fact that the absence of Med16 in a coinfection procedure usually results in only the protein with the flag tag to be pulled by itself (data not shown). Hence, we can conclude that Med16 is a critical protein for the structure of the tail module.

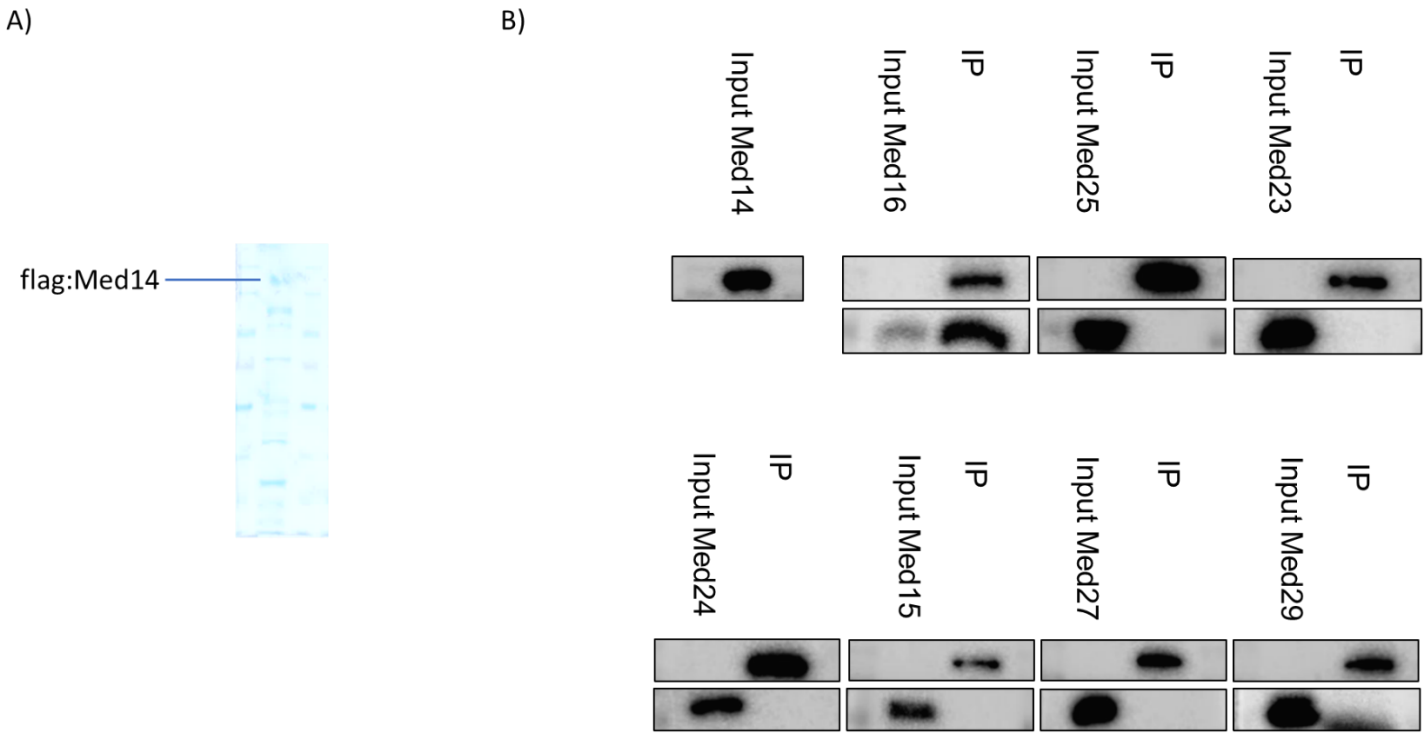


Figure 5: Immunoprecipitation of Med14 with the tail subunits. A) Flag:Med14 is recombinantly expressed and purified using M2 Flag beads. **B)** Western Blot of the IP between flag Med14 and the tail subunits.

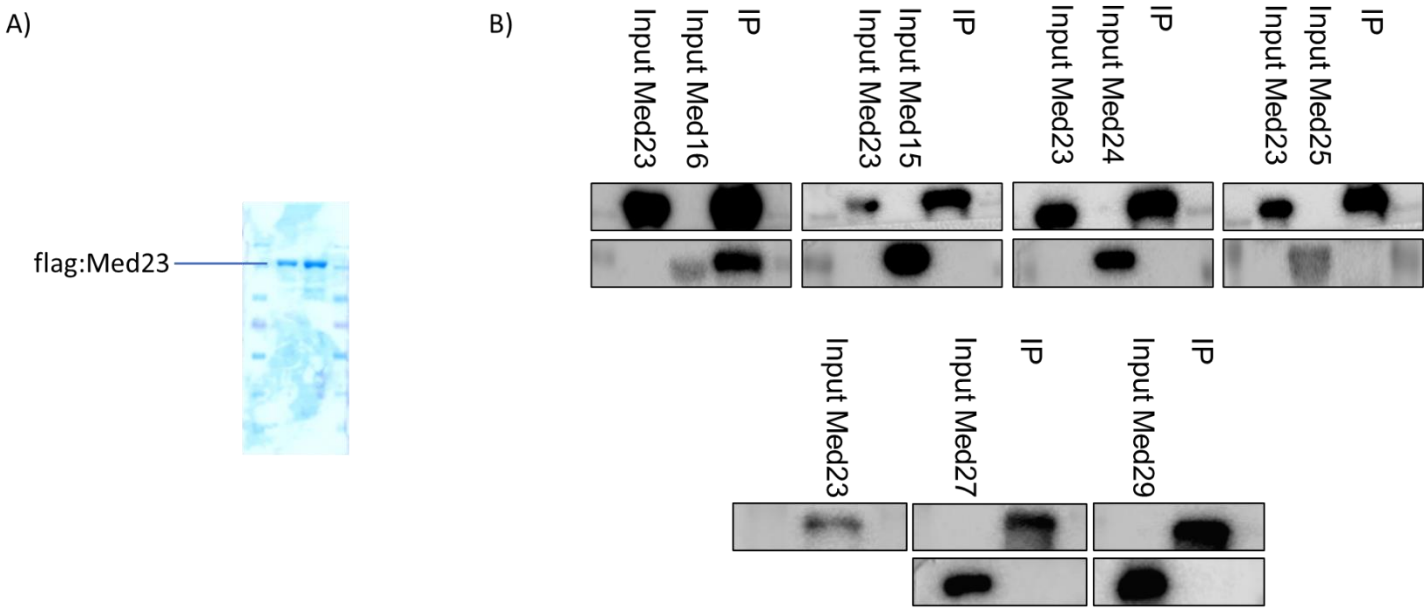


Figure 6: Immunoprecipitation of Med23 with the tail subunits. A) Flag:Med23 is recombinantly expressed and purified using M2 Flag beads. **B)** Western Blot of the IP between flag Med23 and the tail subunits.

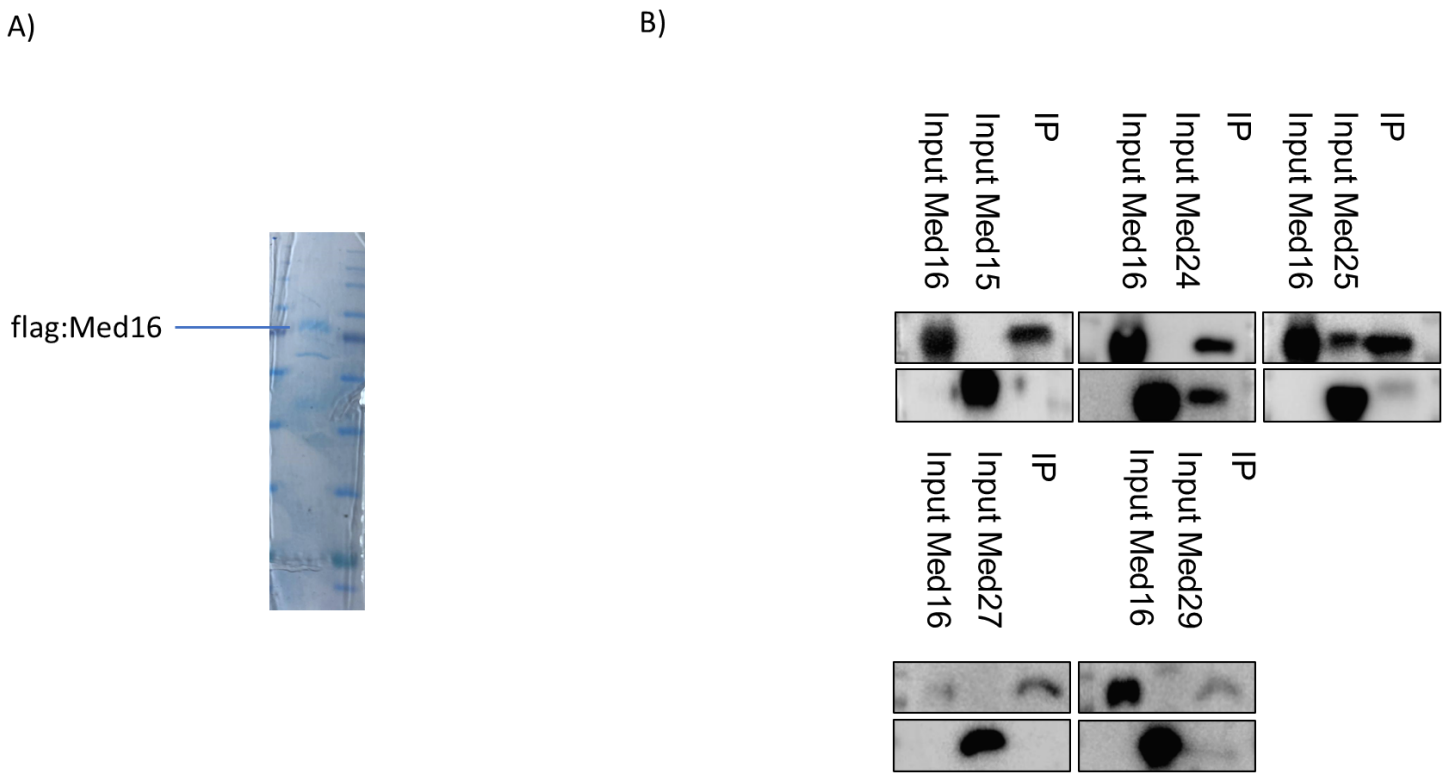


Figure 7: Immunoprecipitation of Med16 with the tail subunits. A) Flag:Med16 is recombinantly expressed and purified using M2 Flag beads. **B)** Western Blot of the IP between flag Med16 and the tail subunits except Med23

4.6 Generation of RNA Polymerase Proteins Through Baculovirus Expression System

Once activator and repressor dependent recruitment of the Mediator Complex occurs, it recruits Pol II along with transcription factors in order to facilitate the transcription of the RNA.[2] Because the mediator of the RNA Polymerase as its name implies is dependent on Pol II generation of the 12 proteins of Pol II is crucial to understand transcription and the Mediator complex as a whole. Therefore these 12 subunits of the mediator complex are recombinantly expressed through baculovirus expression system. The BacMid vectors for these proteins were generated from scratch through molecular cloning procedures and the recombinant protein expressing viruses are created. (Figure 8) These proteins were and will be utilized in Pol II reconstitution projects involving core mediator Pol II interactions that are outside the scope of this report.

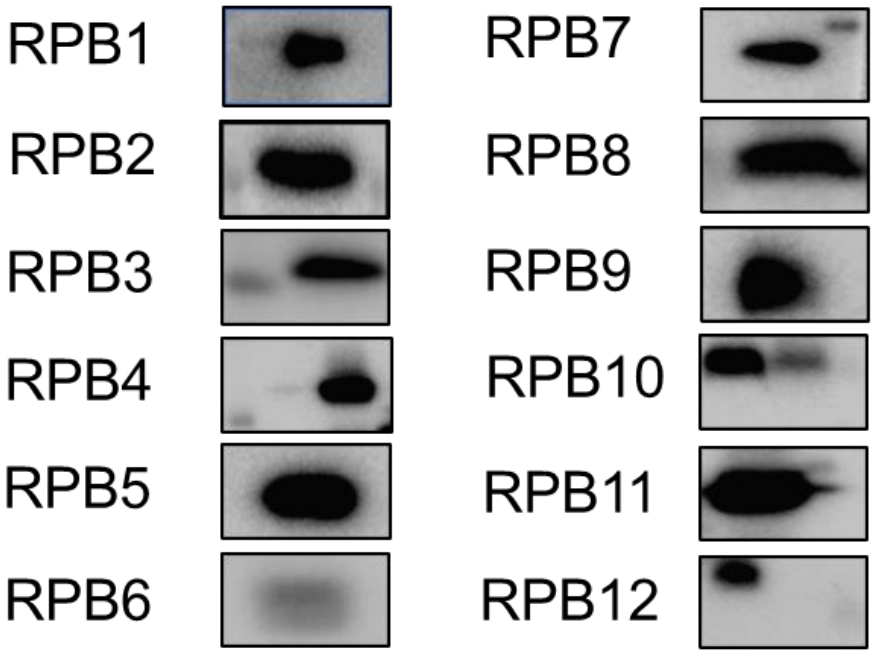
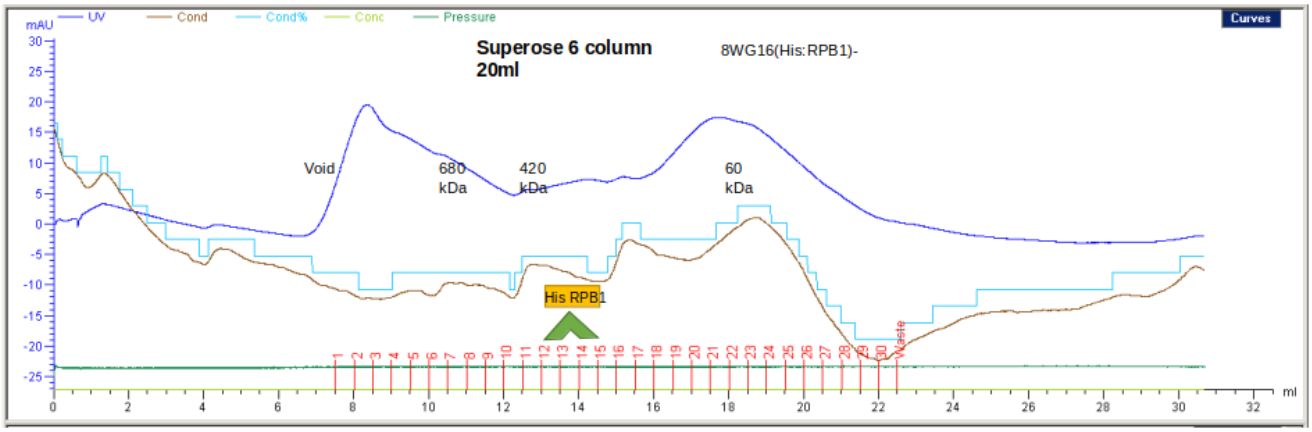


Figure 8: Recombinant generation of each RNA Polymerase II subunit protein. SDS-PAGE analysis followed by Western Blotting for each tail subunit individually using baculovirus expression system

4.7 Rpb1/POLR2A is Cloned Generated and Purified for the Very First Time

Rpb1 is one of the most crucial proteins of the Pol II since it contains the CTD heptapeptide repeats which is essential for the transcription to occur, continue and terminate,[122] is the largest protein subunit of the Pol II at with a size of around 220kDa of size[121]. Due to its size and the structure of its coding sequence which has copious guanine and cytosine repeats it proved extremely difficult for the researchers to generate a recombinant clone of the Rpb1 protein. In fact the most common method of generating a full length Rpb1 protein was through pulldown experiments of mammalian cells. In this study Gibson assembly coupled with baculovirus expression system is utilized to clone and generate the His Rpb1 virus. Gibson assembly method by Birla et al[213] is utilized due to the size of the coding sequence and the coding sequence containing many of the restriction digestion sites available and baculovirus expression system is used to generate the recombinant protein with His tag. The recombinant protein is then purified through Sepharose 6 beads and ACTA purifier. (Figure 9)

A)



B)

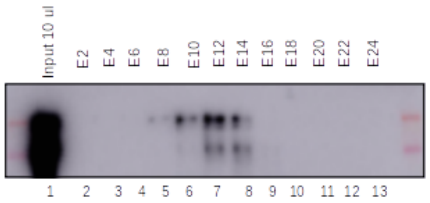


Figure 9: Purification of the recombinant His:Rpb1 Protein: A) Size exclusion chromatography of recombinant His-RPB1 subunit of Pol II via 20 ml Superose 6 column using ÄKTA system. The numbers corresponds to the 500 ul fractions collected at a given time point. B) Western blot analysis of Superose 6 purified His-RPB1. The numbers indicate collected 500ul fractions during the procedure.

Chapter 5
Discussion

This study is performed as a progression of Dr Cevher's published work in 2014 as him and his colleagues successfully elucidated a mediator core complex that can successfully interact with Pol II and perform transcription. The tail complex of the mediator will be an essential addition to the core mediator due to its role in integrating activator and repressor signals to create a specific transcriptional output. Reconstitution of the mediator tail and its subsequent interaction with the core mediator complex would assist in the further delineation of the function of the mediator's tail and its importance in mediator complex mediated transcription and interactions of mediator's tail with both the core mediator and activator repressor proteins. It would allow a further understanding of the mediator itself and many disease phenotypes since Mediator's tail module is involved in many disease phenotypes including many cancer subtypes.[71-112] This study tries reconstitute the mediators tail subunit as well as trying to discern the mediator tail and its structure through biochemical methods.

In order to reconstitute the tail module of the mediator MultiBac expression system and molecular cloning techniques were needed to be utilized as many of the tail proteins did not exist as plasmids or viruses and due to the mediator subunits importance to the viability of the cells and its many points of interaction with the other subunits of the mediator purification of the tail through eukaryotic cells were impractical. Many studies tried to elucidate the structure of the tail from immunoaffinity purifications of eukaryotic cells and cryo EM approaches which resulted in a suboptimal differentiation of the tail subunit alone. These studies resulted in some of the tail subunits to remain structurally unresolved. For instance one of the more recent studies dated March 2021 that allowed for the structure of many tail proteins to be revealed through cryo EM approach resulted in Med15 subunit of the Mediator to be structurally unresolved.[53] This study allowed the tail subunits to be observed separately from the mediators other subunits.

The study resulted in an incomplete reconstitution of the tail as only interactions of Med23-Med16-Med24 subcomplex is successfully generated and Med15 Med27 and Med29 did not bind in any of the coinfections generated and any of the single IP experiments performed. Med25 is also previously revealed to be interacting significantly with the 3 protein subcomplex as well. In this study we have

found out that this is partially true that as long as Med14 is not introduced, Med25 persistently interacts with the Med23-Med26-Med24 through Med16 but presence of Med14 forbids Med25 from binding to the Med23-Med16-Med24 subcomplex. The exact reason for this phenomenon is unknown but one of the reasons could be that Med14 binding to the Med16 causes a conformational change in the Med23-Med16-Med24 subcomplex that may cause the occlusion of the zone Med25 binds to in in vitro settings sue to the absence of other proteins of the mediator complex. In a cryo-EM study, El Khattabi et al indicated that in purified *Mus Musculus* mediator complex Med25 is present in sub-stoichiometric levels[7] which may indicate the phenomena that we observed regarding binding of Med25 in the presence of Med14.

The recombinant Med15 one of the more important proteins of the tail module is found to be easily degraded in coinfection studies. Many proteins of the tail subunit generated through infections remains stable in both coinfections and single infections for up to 72 hours (data not shown) however Med15 protein remained stable for less than 48 hours as after 46 hours the size of the protein band degrades to around 95kDa.(data not shown) The exact reason for this phenomenon still remains unknown but it resulted in the coinfections studies to be performed at around 45-46 hours after the start of infection and prohibited longer coinfection studies which may have resulted in a better yield of proteins and a better probability of interactions between the proteins of the tail during the incubation.

Med27 Med29 and Med15 proteins of the tail subunit did not purify with any of the coinfection studies or the IP studies made with Med14 Med16 and Med23 and currently seem separate from the rest of the tail that has certain interactions with each other and Med14. In yeast studies Med27, Med29 and Med15 form a 3 protein subcomplex with each other that interacts with Med14.[24] The interactions between these 3 proteins of the tail were not observed in both coinfections and IP studies. Med15's disassociation from the other tail subunits is especially perplexing since many studies involving both yeast and human mediator indicate that Med15 is interacting with CTD domain of Med14.[24,214] However, this was not confirmed in the IP studies performed. Additionally, these studies suggests that a dimer of Med27 and Med29 interacting with the Med16 subunit of the mediator's tail module[53] however this interaction is not present in both coinfection and IP studies performed. There might be many reasons for the dissociation of these three subunits from the rest of the tail module. One of the more significant reasons for this disassociation may be because of the inherent heterology of the tail and the understanding that the tail module of the mediator is variable and loosely associated[7,24,46] and

presence of certain activator or repressor proteins may be required to assemble the mediator's tail in full. Another reason could be that the mediator tail may require additional proteins that are not currently associated with either the head middle tail or the kinase module. Med28 and Med30 are 2 distinct proteins that are not clearly identified as tail module proteins or a part of the other modules of mediator that are found out to be distinctly associating with tail module subunits. Recent cryo EM studies by El Khattabi et al and Zhao et al suggest Med28 and Med30 to be tail module proteins[7,53] and identifies their interaction with each other to form a dimer and together interacting with a dimer formed by Med27 and Med29 while previous studies by Tsai et al and Cevher et al indicate Med30 as a head module subunit.[17,24] Med1 another protein that is identified as a part of the middle module but was not essential in the formation of the core mediator[17] is another protein that may have an importance. Med1 is revealed to be interacting with Med24[215] and dissociation of Med1 Med24 interaction causes a significant change in the conformation of the Mediator as revealed by cryo-EM studies performed on metazoan cells by Zhao et al at 4A of resolution.[53] Therefore expression of other proteins such as Med28 Med30 and Med1 in a coinfection study may assist in the elucidation of a full tail complex.

The IP experiments revealed that Med14 Med23 Med24 and Med25 all interact with Med16 directly and do not interact with each other. These findings further solidify the idea that is suggested in cryo EM studies that Med16 is a structural focal point of the Tail module[24,53,216] as the IP studies clarify that all other tail proteins bind to Med16 and not with each other even though the cryo EM studies suggest many points of interaction between tail subunits.[46,53] A possibility of false contacts due to close proximity of the subunits with each other may be the reason for the suggested interactions not being present in biochemical assays.

Overall both IP and the coinfection experiments revealed a partial tail module that is organized around Med16 in which the binding of Med23 or Med25 in the presence of Med14 depending on an unknown mechanism. (Figure 10)

In this study we have cloned and generated the Rpb1 subunit of Pol II in full length and recombinantly expressed said protein with His tag. This is done for the very first time in this study as no other study reported a successfully cloned Rpb1 and Rpb1 is usually isolated through pulldowns from mammalian cell types. This crucial finding has already started to be crucial in determining various aspects of the Pol II and the Mediator. It is already used to reveal that the Mediator core directly interacts with a

single protein of the polymerase Rpb1 even though EM studies suggested numerous interactions between the core mediator and the Pol II proteins. The other Pol II proteins Rpb2-12 generated through molecular cloning and subsequent MultiBac expression system is a crucial tool for both Pol II and mediator research as well. Furthermore, these proteins could be used in coinfection studies to recombinantly purify a Pol II protein that could be used in many distinct studies related to Mediator complex Pol II and disease.

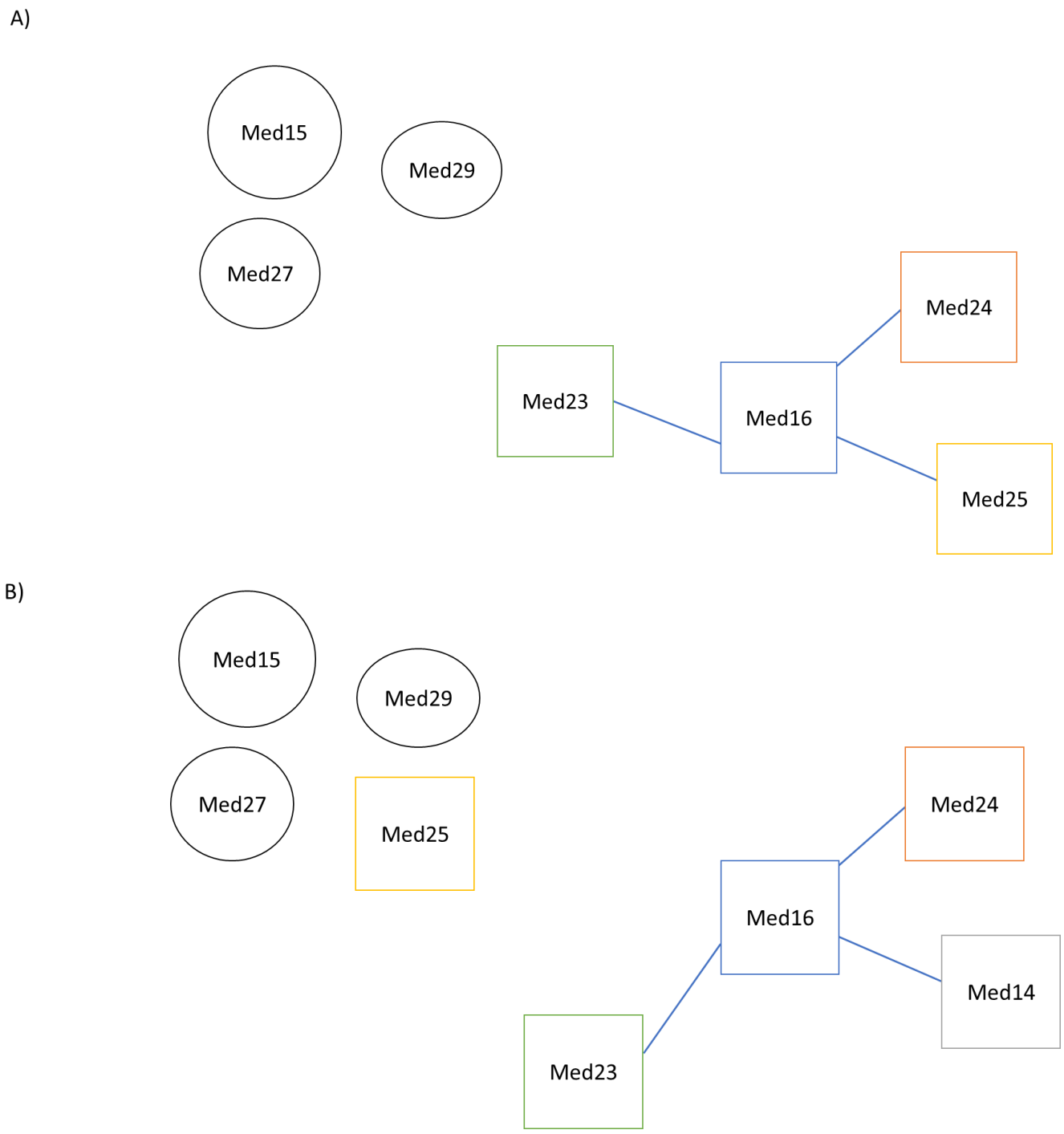


Figure 10: The partial tail complex assembles around Med16. A) In the absence of Med14 Med23 Med24 and Med25 directly binds to Med16 to partially form the tail complex as Med15 Med27 and Med29 did not interact with the rest of the tail proteins **B))** In the presence of Med14, Med23 Med24 and Med14 directly binds to Med16 to partially form the tail complex while Med25 which can interact with Med16, is excluded. Med15 Med27 and Med29 did not interact with the rest of the tail proteins

Chapter 6
Future Perspectives

The tail reconstitution and the IP experiments showed that many tail subunits assemble around and directly interact with Med16 to form the tail module of the mediator and generates two structures depending on the presence of Med14. These structures are Med14-Med16-Med23-Med24 and Med16-Med23-Med24-Med25. These structures are mostly in line with the findings of Cryo-EM studies performed by other researchers.[7,53] However, the experiments failed to produce a complete tail subunit since 3 subunits of the Mediator's tail module did not interact with these structures at all during the IP and coinfection. Therefore, further studies need to be performed to elucidate the complete tail module along with interactions of each subunit with each other. In order to achieve this other presumed subunits of the tail Med28 and Med30 needs to be generated through MultiBac expression system along with Med1 which is not a part of the core Mediator and the Tail module but has significant interactions with Med24. Presence of these proteins in coinfection studies and IP studies may elucidate further information about the tail complex and allow us to generate a complete tail module. To this end Med1 and Flag:Med1 is generated (data not shown) in order to observe the interactions of Med1 with the rest of the tail This protein will be used to perform single IP and coinfection studies with the rest of the tail complex.

Additionally due to its lack of interaction with the rest of the tail proteins, Med27 Med15 and Med29 and their interactions with each other along with other mediator subunits remained poorly understood. Therefore, these interactions along with the interactions of these proteins with the remaining tail proteins Med24 and Med25 should also be examined in order to further understand the tail complex. Another study that could be performed that could further enhance the understanding of the tail module would be performing a coinfection study with Med29 Med27 and Med15 only which will assist our understanding of the Mediator since data obtained from yeast[24] and the cryo-EM data of the mammalian mediator by Zhao et al[53] suggests that these proteins especially Med27 and Med29 have a strong probability of direct interaction.

The phenomenon regarding the interactions Med25 with Med16 with regards to coinfection studies should be examined through coinfection studies through performing competition assay between Med14 and Med25 with Med16 protein.

The novel recombinant cloning of the Rpb1 protein will be a crucial tool to understand the Pol II and the Mediator complex. As the largest and the most crucial subunit of the Pol II through its many domains including the CTD domain which is phosphorylated during both the initiation elongation and the termination of the transcription. This newly generated Rpb1 could be used in many different areas of RNA and RNA polymerase research.

Finally, the other generated proteins of the Pol II protein should be utilized in coinfection studies and IP studies to elucidate a reconstituted Pol II and understand the binding of Pol II subunits with each other.

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