INVESTIGATING THE ROLE OF THE MEDIATOR COMPLEX IN ESTROGEN RECEPTOR ALPHA-MEDIATED TRANSCRIPTION

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By

Tuğçe Canavar

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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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M.Sc. in Molecular Biology and Genetics

Advisor: Murat Alper Cevher

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Mediator is the most important coregulator in eukaryotic transcription. It conveys the signals received from transcriptional activators on enhancer sequences to transcription machinery on promoter regions. Although Mediator itself and MED1 subunit have been implicated in the activation of estrogen receptor alpha (ERα)-mediated gene expressions and breast cancer cell proliferations, the mechanism by which Mediator interacts and brings ligand-bound ERα on its cognate element site to promoter and translates their interactions to gene activation is still unknown. We aimed to identify the interaction between ERα and Mediator to clarify this mechanism. We recombinantly generated active human core Mediator complex along with other subunits of Mediator. We interestingly saw that MEDX showed a potential interaction.

In addition to characterizing Mediator- ERα interaction, we also checked the role of the Mediator Complex in acquired tamoxifen resistance. We performed immobilized template recruitment assay to characterize the alterations in recruitment of Mediator subunits and RNA Pol II to 2XERE-TATA promoter in wild type and tamoxifen resistant MCF-7 cell lines. We tried to clarify how changes in pre-initiation complex formation causes resistance to tamoxifen in ERα-positive breast cancer patients. The increase in MEDZ subunit recruitment in resistant cells was striking and promising for further experiments. We hope that our findings will help to elucidate the detailed mechanism of ERα-dependent transcription and design alternative therapeutics.

Key Words: Mediator Complex, Pre-initiation Complex, transcription, tamoxifen resistance

ÖZET

MEDIATOR KOMPLEKSİN ÖSTROJEN RESEPTÖRÜ ALFANIN DÜZENLEDİĞİ TRANSKRİPSİYONDAKİ ROLÜNÜN ARAŞTIRILMASI

Tuğçe Canavar

Moleküler Biyoloji ve Genetik Yüksek Lisans

Tez Danışmanı: Murat Alper Cevher

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Mediator Kompleksi ökaryotik transkripsiyonunun en önemli düzenleyici proteindir. Enhancer sekanslarında bulunan transkripsiyonel aktivatörlerden aldığı sinyalleri promoter bölgelerindeki transkripsiyon sistemine iletir. Mediatorın kendisi ve MED1 alt biriminin östrojen reseptörü alfanın (ERα) aracılık ettiği gen ekspresyonlarının aktifleştirilmesinde ve meme kanseri hücrelerinin proliferasyonunda ilgisi olduğu bilinmesine rağmen Mediator'ın ligand bağlı reseptöre bağlanıp onu spesifik enhancer bölgelerinden promoter bölgelerine hangi mekanizmayla getirdiği ve etkileşimlerini nasıl gen aktivasyonuna çevirdiği hala bilinmemektedir. Biz bu mekanizmayı açıklığa kavuşturmak için Mediator ve ERα arasındaki bağlantıyı belirlemeyi amaçladık. Diğer alt birimlerle birlikte rekombinant aktif çekirdek Mediator kompleksini ürettik. İlginç bir şekilde gördük ki MEDX ile bağlanma potansiyeli göstermektedir.

Mediator ERα etkileşiminin yanı sıra Mediator kompleksinin kazanılmış tamoksifen direncindeki rolünü araştırdık. Normal ve tamoksifene dirençli hale gelmiş MCF-7 hücre hatlarında 2XERE TATA promoterına gelen RNA Pol II ve Mediator alt birimlerindeki değişimleri karakterize etmek için immobilized template recruitment assay uyguladık. Başlama Öncesi Kompleksi (PIC) denen transkripsiyon sistemindeki farklılıkların ERα-pozitif meme kanseri hastalarının tamoksifene dirençli hale gelmesindeki etkisini netleştirmeye çalıştık. Dirençli hücrelerde MEDZ alt biriminin promotera gelmesindeki artış ileriki deneyler için dikkat çekici ve umut vericiydi. Bulgularımızın ERα-aracılı

transkripsiyonun detaylı olarak aydınlatılmasında ve alternatif tedavilerin bulunmasında yardımcı olmasını umut ediyoruz.

Anahtar Kelimer: Mediator Kompleksi, Başlangıç Öncesi Kompleksi, transkripsiyon, tamoksifen direnci

To my dearest and beloved family

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Acknowledgements

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Abbreviations

ERα	Estrogen Receptor Alpha
GTF	General Transcription Factor
NR	Nuclear Receptor
Pol II	RNA Polymerase II
PIC	Pre-initiation complex
TFIIA	Transcription Factor IIA
TFIIB	Transcription Factor IIB
TFIID	Transcription Factor IID
TFIIE	Transcription Factor IIE
TFIIF	Transcription Factor IIF
TFIIH	Transcription Factor IIH
TBP	TATA Binding Proteins
Med	Mediator complex
MED	Mediator complex
SEC	Super Elongation Complex
kDa	Kilo Dalton
TRAP	Thyroid Hormone Associated Protein
CRSP	Cofactor Required for Sp1 Activation
SMCC	SRB/MED Cofactor Complex
BSA	Bovine serum albumin
CTD	C-terminal domain

NTD	N-terminal domain
ChIP	Chromatin Immunoprecipitation
ITRA	Immobilized Template Recruitment Assay
IP	Immunoprecipitation
MAPK	Mitogen-activated protein kinase
PI3K	Phosphoinositide 3-kinase

CHAPTER 1

INTRODUCTION

Eukaryotic mRNA transcription requires (i) the assembly of a set of evolutionarily conserved general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH to build the accessory proteins on the core regions of most RNA PolII-dependent gene promoters for particular binding and proper transcription initiation [71]; and (ii) the multiprotein Mediator Complex, which is the most critical coactivator that can act as a physical bridge to help the communication between especially gene-specific transcription factors bound at enhancers and Pol II along with GTFs at the promoter region forming the pre-initiation complex (PIC) [1].

1.1. Mediator Complex

Mediator was first purified from *Saccharomyces cerevisiae* as a 25 subunit complex protein arranged into four modules called `head` and `middle`, which constitute active core Mediator; `tail` and `kinase`[72]. Crystal structures are available for head module and some subunits of middle and kinase modules. The human Mediator complex subunits were purified and initially designated as TRAP (thyroid hormone receptor-associated proteins) because they were identified in a protein-complex which is pulled down together with the ligand-bound receptor when it is isolated from cell extracts [65, 66]. Roeder and colleagues then identified this 2-MDa metazoan Mediator composed of 30-subunits by using biochemical techniques and mass spectrometry. By using Multibac baculovirus expression system, they also reconstituted a functional 15 subunit human core Mediator Complex [3]. Following, researchers successfully produce homogenous recombinant 15-subunit yeast core Mediator by using Med14 subunit as a scaffold [2]. The structure of Mediator is conserved from yeast to humans, besides the metazoan complex has specific

subunits some of which are Med26 and Med30 [3]. Like yeast complex, human Mediator has also a modular arrangement and its subunits are organized into four subcomplexes. The composition and structure of Mediator complex is, however, variable and dynamic in the cellular contexts [46]. Its composition might change in different cell types at different stages of growth and development. The stable core complex is composed of head and middle modules, which are tightly bound to each other, and reversibly associates with kinase module including CDK8-Cyclin C pair along with Med12 and Med13 subunits. The small Mediator which lacks 600 kDa CDK8-kinase module is called PC2 [73] and its composition generally includes metazoan specific subunit, Med26. Kinase module and Med26 are generally found in the composition of Mediator in a mutually exclusive manner [74]. Further, the association of variable metazoan specific subunits with the core complex can result in additional gene-specific variants [4].

Mediator is generally considered as a co-activator because of being an important target for DNA-bound transcription factors which recruits Mediator into regulatory sites throughout the genome via possible interaction with its subunits and ,thereby, causing a DNA-loop formation. However, studies in yeast and human demonstrated that Mediator has also role in activator-independent transcriptional regulations. The isolated RNA Pol II holoenzyme from both human and yeast has contained Mediator subunits. Further studies including ChIP-on-ChIP technology has revealed that Mediator also localizes upstream and coding regions of many genes on a genome-wide scale [5]. A model of RNA Pol II- Mediator complex identified in yeast by electron microscopy revealed that several subunits of Pol II such as Rpb4 and Rpb7 might interact with the head and middle module of Mediator [75]. Additionally, functional assays involving 15-subunit active human core complex reconstituted by Cevher et al, also showed that the reconstituted bimodular complex containing head and middle modules requires the Med14 incorporation into the assembly as an architectural backbone. More importantly, the existence of Med14 in the complex is necessary for basal transcription and facilitated Pol II interaction [3]. Overall studies have suggested that Mediator regulates both basal and activator-dependent transcription and PIC formation by facilitating the recruitment of Pol II and GTFs to the target gene promoters and stabilizes these multiprotein complexes at the promoter.

1.1.1. Activator-Dependent Transcriptional Regulation of Mediator Complex

In eukaryotes, RNA polymerase II initiates transcription at specific sites on the genome. It is recruited to transcription start site via auxiliary protein complex, called pre-initiation complex (PIC), which is composed of transcription factor II A (TFIIA), TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and Mediator Complex [6]. Mediator stabilizes PIC formation by serving as a central scaffold for assembly of other PIC components. Its large size enables this function as different subunits can interact with different components of the PIC. Importantly, the recruitment of Pol II to the transcription site requires Mediator that interacts with C-terminal domain (CTD) of RNA Poll II Rpb1 subunit [7, 8,].

Mediator Complex also enables the communication between gene-specific, enhancer-bound transcription factors (TFs) and RNA Pol II together with the rest of PIC [9]. It serves as a molecular bridge to direct signals from a DNA-binding TF to Pol II enzyme to control enhancer-specific transcription. Different TFs might interact with distinct Mediator subunits to mediate expressions of their target genes in cell-type specific context as well.

1.1.1.1. Structural Dynamics of Mediator Complex upon Transcription Factor Binding

The function of Mediator Complex correlates with the dynamic change in its structure upon binding of different TFs. As compositional change in the complex might abolish the TF-bound activity in the transcription, structural shift is also equally important for the Pol II activity. For example, whereas Med23 knockout does not affect other TF-Mediator regulated transcriptions, ELK1-Med23 mediated target genes are not activated in MED23-knockout murine embryonic stem cells [76]. On the other hand, the activation of Pol II enzyme on the promoter of early growth response factor 1 (Egr1) associates with the structural change in phosphorylation-dependent ELK1-Med23 interaction [10]. The structural shift spanning over entire Mediator complex after binding of a TF to a single site is first observed when SREBP-1α (sterol regulatory element-binding protein 1) [77] and VP16 (a transcription factor of herpes simplex virus, HSV), activation domains bind to Mediator and creates different structural organization without affecting its subunit composition. Moreover, EM (electron microscopy)-based experiments showed that these

TFs interact with different subunits of Mediator complex. As SREBP-1α associates with Med14 and Med15, VP16 relates to Med25 and Med17. VP16-targeted in vitro transcription is ceased when Med25-Mediator complex is depleted from HeLa cell nuclear extracts by using anti-Med25 antibody. Also, RNAi-knockdown of Med25 from human cells leads to the inhibition of VP16-activated gene expressions [11, 12, 13, 14]. Undergoing different structural alterations upon binding to distinct domains of TFs affects the transcriptional state by controlling the recruitment of Pol II and defines the participation of additional coactivators to the pre-initiation complex (PIC). For instance, Taatjes and Meyer groups revealed that p53 interacts with Mediator via its either p53AD (activation domain) or p53CTD (C-terminal domain) by binding to Med17 and Med1 subunits respectively. Interaction with activation domain creates a conformational change in Mediator such that it exposes a large domain causing the transition of Pol II to elongation state from an inactive state. However, binding to p53CTD results in a distinct conformational alteration leading to inactivation of Pol II-complex [15]. A general conclusion from these studies is that TFs that binds to different interaction surfaces on Mediator might lead to distinct structural shifts and coactivator recruitment, resulted in separate regulations in the PIC formation.

1.1.1.2 The Role of Mediator Complex in Gene Looping

Activation of gene specific TF-targeted gene expressions is regulated by DNA-loop formations integrating enhancer-bound TFs with RNA Pol II-complex at the promoter regions. The participation of Mediator complex in both enhancers and core promoters during active gene transcription is first identified in murine embryonic stem cells [67]. Chromosome conformation capture (3C) experiments showed that in the cell-type specific gene transcriptions, Mediator works with cohesin and cohesin loading factor Nipb1 to bring enhancer regions to promoters to form proper DNA-looping [16]. Normally, long-distance enhancer-promoter bridges are not observed in the yeast gene transcriptions, however, mutant yeast ortholog of Med16 enables upstream regulatory regions to activate transcription [17]. This is also consistent with the ability of Mediator to interact with Pol II CTD. In this way, it can stabilize the promoter-enhancer gene looping. Further, the length of human RNA Pol II CTD is far longer than yeast's, which is correlated with the increase in the length of promoter-enhancer interactions in human [18].

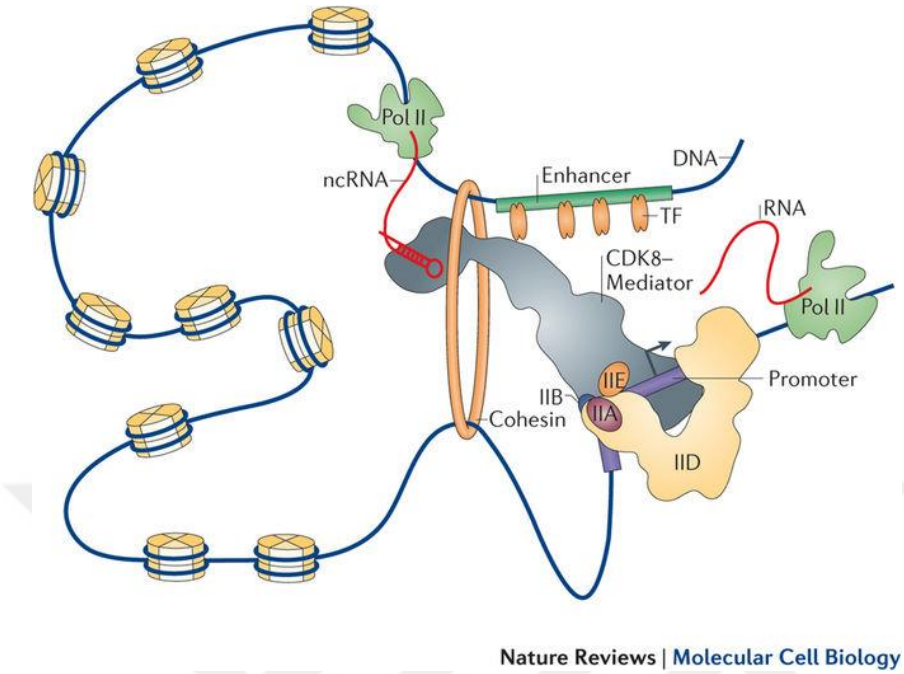

Figure 1.1. Mediator enables communication between enhancer-bound TFs and transcriptional complex at the promoter [62].

1.1.1.3 Mediator Complex & Nuclear Receptor Functions

Nuclear Receptors (NRs) are transcription factors which shows generally ligand-dependent activation to regulate sequence-specific gene expressions involved in development, homeostasis and cell differentiation. During translocating signals received from a cognate ligand to the promoter region of a target gene, NRs needs transcriptional coregulators that are mainly classified into two main categories namely coactivators and corepressors. Unlike corepressors operating with antagonist-bound NRs and block expression, coactivators help in the activation of gene expression. Many coactivator complexes are involved in chromatin remodeling, enzymatic reactions and the recruitment of transcriptional machinery. Steroid hormone receptors associate with steroid receptor coactivator (SRC) family members of the p160 class coregulators to control assembly of histone-modifying factors [19]. CREB-binding protein (CBP)-p300 is another coactivator that has histone acetyltransferase (HAT) activity regulating chromatin relaxation at the promoter region [20].

Mediator Complex acts directly on the RNA Pol II-complex and PIC formation. It is a general cofactor for ligand-dependent NRs and after purification process, many isolates of NRs are pulled down with Mediator. Med1, one of the subunits of Mediator, is most associated with nuclear receptors. It contains two leucine-rich amino acid motif "LXXLL" so-called "NR-box", which is frequently present in almost every nuclear receptor coactivators [21]. This motif is necessary for Mediator complex to provide direct interactions with NRs and regulate Med1-dependent activation of NRs such as estrogen receptor (ER), glucocorticoid receptor (GR), vitamin D receptor (VDR), peroxisome proliferator-activated receptor (PPAR), retinoic acid receptor (RAR), and retinoid X receptor (RXR). Furthermore, the importance of Med1 in the NR-dependent gene expressions is also confirmed with in vivo studies. For example, Med1 deletion in mouse embryonic fibroblasts (MEF) forms a deficiency in PPAR-mediated adipogenesis without affecting other NR-mediated developmental pathways [22, 23, 24].

Med25 is another subunit that contains LXXLL motif in its C-terminus and recently has been associated with the regulation of HNF4-mediated gene expressions. Direct binding of Med25 LXXLL motif to HNF4 organizes the assembly of the coactivators and induces the expressions of genes that are functional in lipid and drug metabolism [25].

Conclusion from these studies is that the Mediator Complex is a critical coactivator having a broad impact on NR-mediated transcription via its ability to interact with many proteins involved in transcriptional complex under favour of its multi-subunit structure.

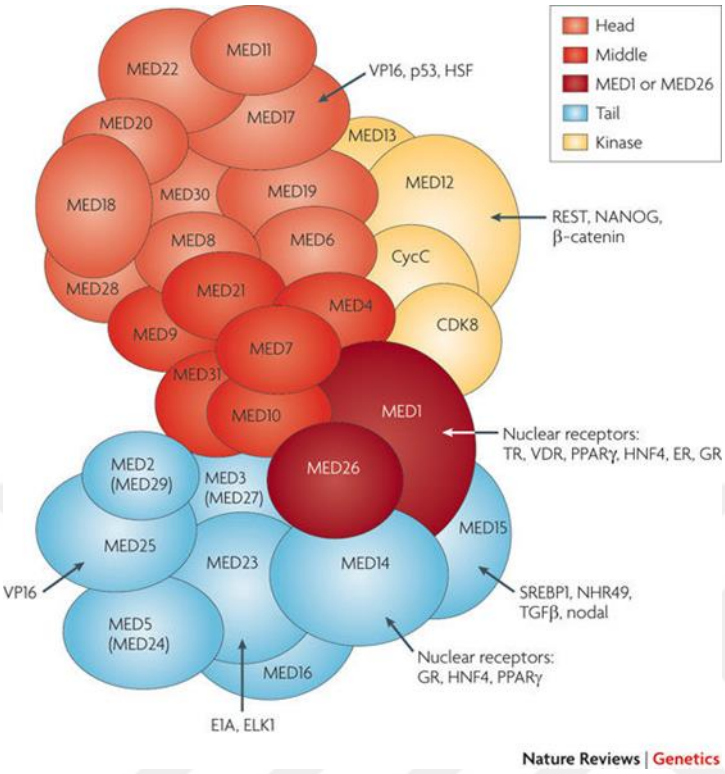

Figure 1.2. Modular structure of Mediator Complex and its relation with transcription factors [63].

1.2 Breast Cancer and Estrogen Receptor (ERα)

Breast Cancer is highly diagnosed cancer type after lung cancer in women worldwide. The incidence ratio observed in women to men is 125 to 1 [26]. This is because it depends on hormonal factors, especially steroid hormones whose endogenous level determines the risk of developing the disease. One of these steroid hormones is estrogen that is the immediate stimulus to mammary development coordinating the formation of ductal structures and enhancement of lobules from normal epithelium [27, 28]. Estrogen is synthesized from cholesterol mainly in ovaries, placenta and corpus luteum and stimulates estrogen receptor alpha (ERα).

ERα is a member of nuclear receptor family and its presence in a tumor is diagnostic marker. The variation in its expression and distribution is related to age and reproductive history [29]. Since breast cancer is a heterogeneous disease with respect to clinical-

pathological features and the status of receptor expressions, it is classified into distinct subtypes with different morphologies and clinical outcomes which are luminal A, luminal B, HER2 over-expression, basal and normal-like tumors. The expression of ERα in a tumor is accepted as a favorable prognostic biomarker and the large majority of ERα-positive breast tumors take place under luminal A and luminal B subtypes [30, 31]. 80% of breast cancer patients are ERα positive, which makes the receptor a good prognostic target for drug responsiveness [32].

1.2.1 Estrogen Receptor (ERα) Structure

Human ERα is located on the chromosome 6 and shares the common structure with all other nuclear receptors. It is composed of variety of regions named A/B, C, D, and E/F [78]. These regions forms different functional domains that are involved in transcriptional regulations. A/B region called the N terminal activation function domain (AF-1) and E/F region (AF-2) involved in ligand binding domain (LBD) works synergistically in the regulation of ligand-dependent transcriptional activity by recruiting and binding to a number of coactivators [79]. These two functional domains mostly work in a synergistic manner but AF-1 can also be activated in the absence of a ligand in certain cell and promoter specific contexts, depending on the phosphorylation status of ERα. For example, the phosphorylation site, Ser118 located on AF-1 region is an important target for MAPK signaling pathway involved in the ligand-independent transcriptional activation of the receptor. AF-2 domain consist of 12 α-helix forming a hydrophobic cleft which is masked until a ligand/ coactivator occupation and [80], is important for the interaction with Src-family of coactivators. Therefore, AF-2 is essential to regulate ligand-dependent activation by activating ERα through conformational changes. ERα binds to the ERE-sequence on DNA through its two zinc-finger domains which were designated as DNA-binding motifs. These protein structures organized by zinc ions are located in the DNA binding domain of the receptor (DBD, C region) [81, 82]. D region called hinge domain, on the other hand, has a role in binding to chaperone heat-shock proteins in the inactive state of ERα and in coordination of dimerization upon ligand stimulation [33, 34].

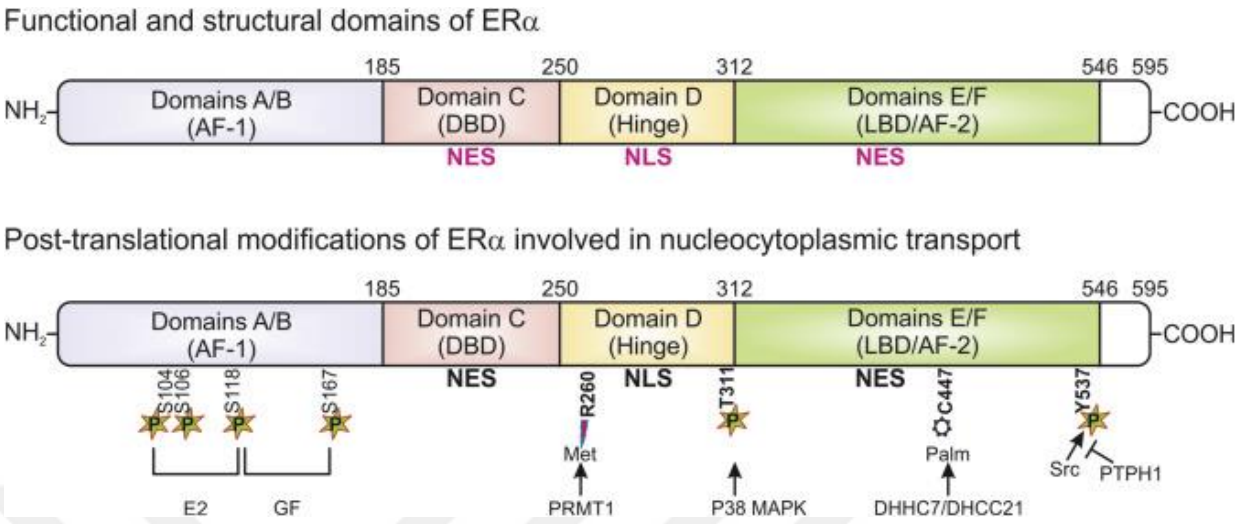

Figure 1.3. Functional and structural domains of ERα [64].

1.2.2 Estrogen Receptor-Mediated Transcription

The ERα-dependent transcriptional activities are divided into two distinct categories, depending on how the receptor initiates the formation of basal transcription machinery on the promoter of target genes. These two different signaling are called `genomic` and `non-genomic` pathways. Non-genomic signaling is appeared through rapid effect of estrogen binding to the membrane associated ERs (mER). mER regulates this activity either via cross-talk with other membrane-linked receptors or activation of a diversity of signaling cascades such as Raf/Ras/MAPKs, PKCs, PI3K/AKT, and cAMP protein kinase A (PKA). For example, ERK and PI3K/AKT activities increase when E2-bound ER activates EGF receptor. However, it is important to note that ER signaling through other receptors is cell type-specific. In addition to non-classical signaling, nuclear ERα regulates the genomic transcriptional activity in a canonical model which involves direct binding of the receptor to the specific sequences on promoter regions. This classical model of transcription mediated by nuclear ERα is the subject of this thesis and is elucidated more in detail in the following sections [34, 35, 36].

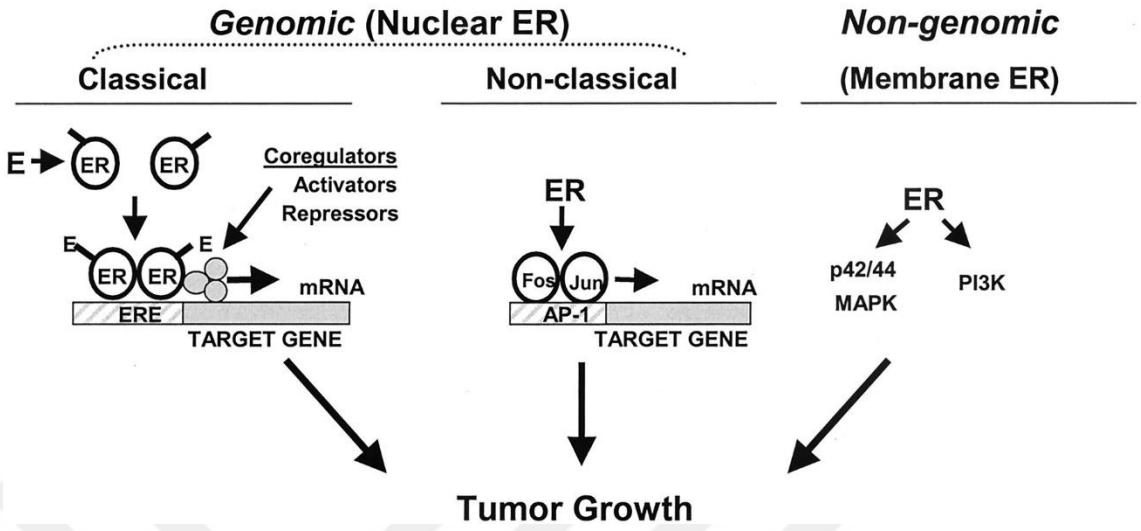

Figure 1.4. Estrogen and ERα-mediated transcription pathways [68].

1.2.3 Genomic Transcriptional Activity of Estrogen Receptor (ERα)

The ultimate goal of ERα, as a nuclear steroid hormone receptor, is to elicit the assembly of RNA Pol II transcriptional complex to the promoter regions of estrogen-responsive genes. In the canonical signaling pathway, the regulation of ERα-target gene expressions involves the direct binding of the ligand-bound receptor to the specific enhancer sequences on DNA. ERα transactivates as dimers, however, unliganded ERα is located in the cytoplasm as a monomer, bound to a heat shock (Hsp) chaperone protein complex. This chaperone Hsp competes with estrogenic ligands by interacting with the ligand binding domain (LBD) of ERα and keeps the receptor inactive state by preventing it from binding to DNA [37]. Upon binding of estrogen, ERα dissociates from chaperone protein and undergoes conformational changes leading to dimerization and activation of its transactivation domains. Active dimeric ERα translocates into the nucleus and binds to specific DNA sequences called estrogen response elements (EREs). This estrogen-responsive motif was first identified in the promoter of *Xenopus laevis* vitellogenin and shows properties of an enhancer such as acting in distance and orientation-independent manner. ERα binds directly to this cis-acting enhancers which are inverted six nucleotide palindromic repeats separated by a three nucleotide spacer [38]. Binding of dimeric ERα to ERE sequence causes allosteric effects on its structure unmasking interaction sites for

the recruitment of coactivators, thereby, the assembly of transcriptional machinery on the promoter regions of ERα-target genes. Therefore, in order to reveal the transcriptional regulations of estrogen-responsive genes, it is critical to elucidate intermediary coactivator proteins that bridges dimeric receptor in the enhancer region to the components of RNA PolII complex on the promoter.

1.3 Proposed Relations between Mediator Complex and ERα

Roeder group demonstrated the activity of Mediator Complex on ERα-mediated in vitro transcription by using ERE-containing promoter templates, purified transcription factors, purified Mediator and estrogen receptor expressed with baculovirus system. The cell-free system reveals that the presence of Mediator improves the ERα-dependent basal transcription [39, 40]. Additionally, Jiang group showed impaired development in the mammary gland by generating mutation in the LXXL motif on MED1 subunit in mice, suggesting the importance of MED1-interaction with the transcription factors [69].

1.3.1 Kinase Module and Breast Cancer & ERα

The kinase module of Mediator Complex that is composed of four subunits which are MED13, MED12, and CDK8-Cyclin C pairs binds to the core Mediator complex reversibly via its MED13 subunit. Therefore, Mediator purifications derived from nuclear extracts via different biochemical techniques were further distinguished according to whether it includes kinase module or not. Based on early biochemical studies, kinase module has been generally associated with the repression of transcription [41]. As purified Mediator containing kinase module restrained the activator-dependent transcription, the core Mediator lacking this module activated. EM-based studies on yeast Mediator explained this observation by the finding that when kinase module binds to the core Mediator, resulting conformation blocks the interaction surface between Pol II and core Mediator [42]. However, the notion that kinase module has only repressive role in transcription is inconsistent with new findings and it has been understood that kinase module can both repress and activate transcription depending on the cell-context.

To date, some of kinase module subunits are implicated in oncogenic alterations in many cancer types like colon cancer, melanoma, endometrial cancer (CDK8), osteosarcoma, T-ALL (CCNC) and prostate cancer (MED12). Recently, they are also implicated in the key role of gene transcriptions involving in the proliferation of breast cancer cells. It is revealed that kinase subunits are part of the enriched coregulators on the estrogen response element (ERE) site. By using streptavidin beads and biotinylated ERE-E4 templates, O'Malley group determined the enriched coregulators from nuclear extracts on E2 liganded ERα. Then mass spectrometry and immunoblotting analyses identified these 17 enriched proteins and revealed that kinase subunits; MED13, MED12, CDK8, and CCNC are some of them [43]. Consistent with the previous studies showing that high expressions of CDK8 and CCNC inversely correlates with shorter recurrence-free survival in breast cancer, CDK8 inhibitor (Senexin A) blocks the E2-induced mRNA transcription of ERα-responsive genes such as TFF1 and GREB1 in MCF7 cells. Also, both shRNA-based knockdown and CRSPR-Cas9-mediated knockout of CDK8 showed the same inhibitory effect on estrogen-stimulated transcription of GREB1 gene [44, 45]. All these studies suggest that kinase module of Mediator Complex might have a key role in the transcriptional regulation of estrogen-dependent genes in ER-positive breast cancer.

1.3.2 The Role of Mediator Complex in Tamoxifen Resistance

Tamoxifen is a selective ER modulator (SERM) that has been used for 30 years at all stages of breast cancer. Like other SERMs, its agonist or antagonist activity might be altered by coregulators recruited to the promoter in a cell-context manner. Whereas tamoxifen acts as an agonist in the uterus, it acts as an antagonist in the breast by competing with estrogen for ligand binding domain (LBD) of estrogen receptor and preventing transcriptional activity [47, 48, 49]. Although it has been an effective chemotherapeutic reagent, 50% ER positive breast cancer patients acquired resistance to tamoxifen.

Tamoxifen resistance is categorized into two groups which are de novo resistance that is unresponsive to tamoxifen treatment since the very beginning and acquired resistance. De novo resistance is modeled by the transfection of Her2 gene into MCF7 cell line that is then causing tumor growth in xenograft mice even in the presence of tamoxifen. On the

other hand, acquired resistant cell lines are obtained from initially sensitive cells after a long term therapy.

Although the molecular mechanism that causes tamoxifen resistance has not been fully understood, MED1 subunit of Mediator Complex is suggested to be the regulator of Her2-mediated tamoxifen resistance. Studies support that phosphorylation of MED1 at specific sites is important for its function and this is regulated by ERK activity mediated by HER2 signaling in tamoxifen resistant cells. Further, it is shown that impairment in its phosphorylation leads to recruitment of corepressors NCOR and SMRT to the ERα-target gene promoter, otherwise they are not assembled together with phosphorylated form of MED1. As in vivo analyses demonstrate that higher expression of MED1 in breast cancer patients is correlated with the decrease in recurrence-free survival, knockdown of MED1 in ERα positive cell lines results in the inhibition of ERα-mediated transcriptional activity [50, 51].

1.4 Baculovirus Expression System for Recombinant Protein Production in Insect Cells

In eukaryotic organisms, many proteins are not singular entity but multi-subunit complexes which are even part of a bigger cellular network. Therefore, it is very essential to reveal their structure, functional roles and interactions within the complex or with other protein assemblies in order to understand their necessities in many cellular events [52]. To investigate the role of these complexes at the molecular level, high amount and properly folded proteins are required to be used in structural studies like imaging of protein interactions in atomic resolution or biochemical techniques such as immunoprecipitation and DNA-pull down assays. Unfortunately, endogenous proteins are often synthesized in low amounts in their cell environment or hard to delete as some of them are essential [53]. Thus, recombinant protein production has been mandatory to obtain sufficient amount of functional proteins for experimental needs.

Prokaryotic expression system, especially the use of E.coli as an expression host, is one of the well-established methods in laboratories to produce recombinant proteins in cheap and easy way [54]. However, bacterial system does not allow the adjustment of expression

ratios in co-expressing of individual subunits when the vector containing multiple genes is delivered. Besides, eukaryotic protein complexes require post-translational modifications and proper folding which are not provided by a prokaryotic host to the all extend [55].

Baculovirus expression system has gained increased interest to obtain recombinant proteins in high yields and proper eukaryotic protein processing conditions. Baculovirus is a double stranded circular DNA that invades arthropods, especially insects. The most worked and manipulated baculovirus for the generation of recombinant expression vector is *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV). The reason why AcMNPV is a good tool for more than 100 mg recombinant protein production per 1 liter of insect cells is because of to produce large amounts of polyhedrin production which appears in nuclei of insect cells during AcMNPV infection. This is turned into an advantage by using polyhedrin promoter (polh) to control the expression of a DNA sequence encoding a protein of interest. The generation of recombinant viral DNAs was first achieved in insect cells co-transfected with transfer plasmid containing gene of interest under the control of polh and purified AcMNPV genomic DNA. Homologous recombination (double crossover) provides the replacement of foreign DNA in transfer plasmid with polyhedrin gene in the baculovirus genome [56]. However, the low frequency of homologous recombination (estimated as 0.1%) and isolating low amounts of recombinant baculovirus vectors from co-transfected cells were required for a new modification. This problem was solved by using linearized baculovirus genome, which increases the frequency of homologous recombination up to 20% and decreases replication of non-recombinant viral genome replication [57]. Later, researchers improved another approach in which E.coli strain is generated to produce a plasmid (bacmid) that integrates with the gene of interest in transfer vector. When transfer vector is transformed into E.coli containing the viral bacmid, the gene of interest in transfer plasmid is integrated into the polyhedrin region of the bacmid via Tn7 transposition and deletes LacZ gene in the bacmid by permitting the blue/white selection. This approach enables scientists who are not familiar with virology but bacterial methods to make a viral progeny in insect cells in easier way.

Although modifications enabled researchers to obtain high amounts of recombinant individual proteins, the isolation of recombinant multi-subunit complexes from host cells co-infected with viruses expressing only one subunit was still an obstacle. Due to the difficulties in controlling expression ratios of individual subunits in infected-cells, resulting from incapability of titer-arrangements of viruses, co-infection was not an effective method for the complex formation. Therefore, it was more logical to eliminate co-infection and rather to use one baculovirus expressing all genes of interest. To achieve this, researchers created Multibac system by modifying transfer vectors allowing the integration of multiple genes in nonsequential way. They generated two vectors which are PFBDM and PUCDM. The expression of genes placed either side of the multiplication module via proper restriction sites in the expression cassettes is under the control of polh or p10 viral promoters. The multiplication module contains PmeI&AvrI and BstZ171&SpeI or Nru&SpeI restriction sites. Since these sites are compatible, the expression cassette containing two genes of a transfer vector can be cleaved and combined with another one and finally in this way, desired amounts of genes can be inserted into a baculovirus expression vector [58].

Multibac expression system has been widely used by scientists to purify functional and stoichiometric protein complexes in insect cells. Sf9 and High Five are two generally used cell lines. Since they can grow in either adherent or suspension, to be able to grow them in spinner flasks or stirred tanks provides researchers a chance to produce their proteins in an amount as large as they want. The largest protein complex that was produced by Dr. Cevher is active human core Mediator Complex. He used this multibac expression system to jointly express Mediator subunits and reconstituted functional 15-subunit Mediator.

1.5 Aim of the Study

The ultimate goal of our study is to find the estrogen receptor alpha (ERα) interactive form of Mediator Complex to prevent ERα-mediated tumorigenesis of breast cancer. By blocking ERα-Mediator interaction, we aimed to stop tumor formation specifically at transcriptional regulation by disrupting the expressions of only ERα-responsive genes but not the others in cell type specific manner. Our hypothesis is that Mediator Complex directs the signal transmission from enhancer-bound ERα to transcription machinery on promoters of ERα-responsive genes. To carry out investigation of our hypothesis, we first tried to identify the possible interaction subunits and recruited subunits on the estrogen response element site (ERE); then as a second study, aimed to clarify the role of Mediator on tamoxifen resistance in breast cancer by working on wild type and MCF-7 cells line acquired resistance to tamoxifen.

CHAPTER 2

Materials, Solutions & Buffers

2.1. Cell Culture Media, Supplements, Reagents, Buffers and Equipment

Product Name	**Brand & Catalog No**
Grace`s Insect Media (TNM-FH)	Lonza Biowhittaker #04-649F
Gentamicin	Thermo Fisher #15750060
Poloxamer	Sigma-Aldrich #16758
Fetal Bovine Serum (FBS)	Biowest #181H-500
DMEM low glucose, without phenol red	Thermo Scientific #11880028
Penicillin/Streptomycin	Gibco #15140-122
Nonessential amino acid (NEAA)	Lonza #BE13-114E
Insulin solution from bovine pancreas	Sigma #I0516
Cellfectin II Reagent	Invitrogen #10362-100
β-Estradiol	Sigma-Aldrich #E8875
Tamoxifen	Sigma-Aldrich # T5648-1G
100mm and 6 well plates	Corning Coster
10 ml, 250 ml and 500 ml spinner flasks	
10X PBS pH 7.4	Home Made

Table 2.1 Solutions, reagents and equipment required for cell culture

2.1.1. Wild-type and Tamoxifen-resistant MCF7 Cell Culture Condition

- DMEM low glucose, without phenol red
- 10% FBS-heat inactivated and filtered
- 1X Pen/Strep (100 units/ml penicillin and 100µg/ml streptomycin)
- 1X NEAA

- 10 µg/ml insulin

2.1.2 Sf9 Insect Cell Culture Condition

- Grace`s Insect Media
- 10% FBS-heat inactivated
- 50 µg/ml gentamicin
- 1X Poloxamer

2.2 SDS-PAGE, Western Blot and Coomassie Blue Staining Buffers

Acrylamide/Bisacrylamide Solution (30%)	292g/L Acrylamide, 8g/L bisacrylamide
10% Ammonium Persulfate (APS)	100g/L APS
1X SDS-PAGE Running Buffer	25mM Tris, 192mM Glycine, 0.1%SDS
1X Transfer Buffer	25mM Tris, 192mM Glycine, 20% methanol
1X PBS-T	8mM Na_2HPO_4, 137mM NaCl, 2.7mM KCl, 2mM KH_2PO_4, 0.05% Tween20
Stripping Buffer	62.5mM Tris-HCl (pH 6.8), 2% SDS
4X SDS-PAGE sample loading buffer	240mM Tris-HCl (pH 6.8), 40% glycerol (v/v), 8% SDS (w/v), 0.04% bromophenol blue, 5% beta-mercaptoethanol
Coomassie Brilliant Blue Solution	40% H_2O, 10% glacial acetic acid, 50% methanol, 0.1% CBB R-250 (w/v)
Destaining Solution	50% H_2O, 40% methanol, 10% glacial acetic acid

Table 2.2 SDS-PAGE, Western Blot and Coomassie Blue Staining Buffers

2.3 Immobilized Template Recruitment Assay (ITRA)

Dynabeads M-280 Streptavidin	Invitrogen Thermo Fisher Scientific #11205D
Blocking Buffer	10X Assay Mix, 5mg/ml BSA, 12.5mM DTT, 1%NP40, 5mg/ml PVP
10X Assay Mix	0.2M HEPES-KOH (pH 8.2), 50mM $MgCl_2$
Wash Buffer	40mM HEPES, 4mM $MgCl_2$, 100mM KCl, 4mM DTT, 0.1% NP40
2X B&W Buffer	10mM Tris-HCl (pH 7.5), 1mM EDTA, 2M NaCl

Table 2.3 Solutions required for ITRA using Streptavidin Dynabeads

2.4 Protein Extraction from SF9 Insect Cells and Immunoprecipitation (IP)

Anti-flag M2 Affinity Agarose Beads	**Sigma-Aldrich #A4596**
Flag Peptide	Sigma #F3290
BC1000* (*salt concentration)	**BC0* (*salt concentration)**
20mM Tris-HCl (pH: 7.9 at 4 ^oC)	20mM Tris-HCl (pH: 7.9 at 4 ^oC)
20% Glycerol	20% Glycerol
0.1 mM EDTA	0.1 mM EDTA
0.5 mM PMSF	0.5 mM PMSF
0.5 mM DTT	0.5 mM DTT
1M KCL	

Table 2.4 Buffers for protein extractions from insect cells and IP

2.4.1 *Antibodies Used in Immunoblotting and Immunoprecipitation*

Product Name	**Brand & Catalog No**	**Dilutions**
Estrogen Receptor α (D8H8) rabbit mAb	Cell Signaling Tech. #8644	1:1000
Med14 rabbit pAb	Abcam #ab170605	1:1000

Med15 rabbit pAb	Proteintech #115661AP	1:1000
Med16 rabbit pAb	Santa Cruz Biotech.	1:1000
Med25 (A-7) mouse mAb	Santa Cruz Biotech. #SC393759	1:1000
MEDX	Home Made Rockefeller University –R.G.R Lab	1:1000
MEDY rabbit pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
Med6 rabbit pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
Med12 rabbit pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
Med26 rabbit pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
MEDZ rabbit pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
Med30 rabbit pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
Rpb1 (8WG16) mouse pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
Rpb5 rabbit pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
Rpb6 rabbit pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
P62 rabbit pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
P53 rabbit pAb	Home Made Rockefeller University –R.G.R Lab	1:1000
Anti-mouse IgG, HRP-linked Antibody	Cell Signaling #7076S	1:10000

Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling #7074S	1:10000

Table 2.4.1 Antibodies used in Western Blot and IP

CHAPTER 3

Methods

3.1. Plasmid Construction

3.1.1. Primer Design

The DNA coding for flag tagged full-length human estrogen receptor alpha (hERα) was amplified by using the primers 5′- GC GAATTC ATG GAC TAC AAA GAC GAT GAC GAC AAG ACC ATG ACC CTC CAC-3′ as forward and 5′-GC GTCGAC TCA GAC CGT GGC AGG-3′ as reverse. EcoRI cleavage sequence was added to the forward primer and SalI cleavage sequence was inserted into the reverse primer. cDNA was used as a template in the PCR of full-length flag tagged hERα. The PCR product was cleaved with EcoRI and SalI, purified, and cloned into the PFBDM vector by ligation. The full-length hERα was fragmented according to its domains. The primers for residues 1-185 (N terminal region, transactivation domain), 185-355 (DNA binding domain and hinge) and 301-595 (ligand binding domain) of hERα are 5′- GC CTCGAG ATG CATCATCATCATCATCATCAT AAA ACC ATG ACC CTC CAC-3′, 5′-GC GCTAGC TCA ACA GTA GCG AGT CTC CTT-3′, 5′-GC CTCGAG ATG CATCATCATCATCATCATCAT AAA GCA GTG TGC AAT GAC-3′, 5′-GC GCTAGC TCA GGC CGT CAG GGA-3′, 5′-GC GAATTC ATG CATCATCATCATCATCATCAT AAA GAC CAG ATG GTC AGT-3′, 5′-GC GCGGCCGC TCA GAC CGT GGC AGG-3′ respectively. The hERα fragments were tagged with histidine residues. The PFBDM plasmid containing full-length flag tagged hERα was used as a template for the PCR

amplifications of fragments. Their PCR products were restricted with the enzymes corresponding to cleavage sites in their sequence, purified and inserted into PFBDM vector by restriction and ligation protocols as described below.

3.1.2 cDNA Synthesis

cDNA was synthesized from total mRNA of Hela cells using the protocol of Thermo Scientific Revert Aid First Strand cDNA synthesis kit #K1622, provided by the manufacturer.

3.1.3 Polymerase Chain Reaction (PCR) Protocol

Thermo Scientific Phusion High-Fidelity PCR Master Mix #F-5315 was used in reactions for the amplification of coding DNA of interests.

2x HF Phusion Master Mix	25 µl
Forward Primer (10 µM)	1.5 µl
Reverse Primer (10 µM)	1.5 µl
cDNA (template DNA)	5 µl
ddH_2O	17 µl
Total	50 µl

Cycle Step	Time	Temperature	Cycle
Initial Denaturation	30 sec	98°C	1 cycle
Denaturation	10 sec	98°C	35 cycles
Annealing	30 sec	**	

Extension	*	72°C	
Final Extension	7 min	72°C	1 cycle
*changes according to the length of amplified DNA **changes according to the melting temperatures of primers			

Table 3.1 Protocol and condition for PCR using Phusion High-Fidelity PCR Master Mix

0.8 % or 1% agarose gel was used to run PCR products with different sizes. Products were excised and purified by using Thermo Scientific GeneJet Gel Extraction Kit #K0691.

3.1.4 Digestion of PCR products with Restriction Enzymes

PFBDM vector and all PCR products were cleaved with restriction enzymes corresponding cleavage sites included in their primers. Thermo Scientific FastDigest Value Pack kit (#K1991) was used in all reactions.

Double digestion of PCR products

10X Fast Digest Buffer 2µl
Enzyme I 1µl
Enzyme II 1µl
PCR product 16µl

Double digestion of Pfbdm vector

10X Fast Digest Buffer 2µl
Enzyme I 1µl
Enzyme II 1µl
PFBDM vector (300 ng/ul) 8µl
ddh20 8µl

The reactions were incubated 2 hours @37°C in water bath. In the case of vector restriction, 1 µl of (Thermo Scientific FastAP Thermosensitive Alkaline Phosphatase #EF0652) alkaline phosphatase was added after 1hour incubation. Restricted products extracted and purified with GeneJet Extraction kit #K0691.

3.1.5 Ligation of inserts and PFBDM vector

Ligation reactions were set up based on the guidance of NEBligation calculator and occurred @16°C overnight.

3.2 DH5 α Competent Cells Preparation

50 µl of DH5α cells were grown in 2 ml of LB medium for 12-16 hours, then transferred into 100 ml of fresh LB medium. The DH5 α cells were incubated for 2-2.5 hours in 37°C shaker until the OD reached 0.6A. The cells were collected into a 50 ml conical tube and chilled on ice for 10 min. The cells were centrifuged at 2000 rpm for 10 min at 4°C and the supernatant was discarded. The pellet is dissolved in 10 ml of ice cold 0.1 M $CaCl_2$. After centrifugation at 2000 rpm for 10 min at 4 °C, the supernatant was removed and the pellet was dissolved in ice cold 2 ml of 0.1M $CaCl_2$. The suspension was incubated in cold room overnight. 1 ml of 50% glycerol was mixed and 100 µl of bacteria was aliquoted into centrifuge tubes. They were frozen in liquid nitrogen and stored at -80°C.

3.3 Transformation of ligation products to Dh5α competant cells

30 µl of Dh5α competant cells were mixed with the 20 µl of ligation product and incubated on ice for 30 min. They were heat shocked at 42°C in water bath for 45 sec and incubated for 2 min on ice. 800 µl of fresh LB medium without any antibiotic was added into the reaction mixture followed by the incubation for 2-3 hours at 37°C in shaker. The reaction tubes were centrifuged at 3000-4000 rpm for 5 min and excessive LB medium was discarded. The pellet was dissolved in 100 µl LB and spread on agar plate containing 100 µg/ml ampicillin. Bacteria colonies were grown at 37°C incubator overnight. Selected single colonies were inoculated in 2 ml of LB medium containing 100 µg/ml ampicillin

for bacteria amplification to purify large amount of plasmids containing inserts. They were incubated overnight at 37°C in shaker followed by plasmid extraction by using Thermo Scientific GeneJet Plasmid Miniprep Kit #K0503. The cloned product was checked by PCR.

3.4 Transformation of PFBDM vectors with the insert into DH10Bac

Same transformation protocol mentioned above was applied to DH10Bac, an E.coli strain that includes a baculovirus shuttle vector (Bacmid) and a helper plasmid. After transformation, a recombinant Bacmid was generated as a result of transposition of PFBDM expression construct. The agar plate contained 50 µg/ml kanamycin, 100 µg/ml ampicillin and 10 µg/ml tetracycline, 100 µg/ml X-Gal and 0.17mM IPTG for blue/white selection. Single colonies appeared after 36 hours and a white colony was selected inoculated in 4 ml of SOC medium containing three antibiotics for 14-16 hours and the Bacmid isolation was performed as mentioned below.

3.5 Isolation of Recombinant Bacmid DNA from DH10Bac Transformants

A single transformed DH10Bac colony was grown in 4 ml of SOC medium containing 50 µg/ml kanamycin, 100 µg/ml ampicillin and 10 µg/ml tetracycline in a 37°C shaking incubator for 13-14 hours. 1.5 ml of bacterial culture was centrifuged at 14000 g for 1 min to get cell pellets. The supernatant was removed and the pellet was resuspended in 300 µl of Solution I, cell suspension buffer. After adding 300 µl of Solution II, cell lysis buffer, the mix was incubated for 5 min at room temperature until the suspension changed from turbid to translucent. 300 µl of 3M potassium acetate, pH 5.5, neutralization solution was added slowly and the suspension was kept on ice for 7 min. It was centrifuged for 10 min at 14000g in cold room. The supernatant was transferred into the tubes containing 800 µl of 100% isopropanol and was incubated for 7 min on ice. The sample was centrifuged at 13000 rpm for 15 min at room temperature. Without disturbing translucent DNA pellet at the bottom of the centrifuge tube, the supernatant was removed and 500 µl of 70% ethanol was added to wash the pellet. The sample was centrifuged at 13000 rpm for 5 min. This

washing step was repeated twice, then the centrifuge tube was kept in room temperature for 5-7 min to allow drying of ethanol. Finally, the Bacmid DNA was dissolved in 40 µl of 1X TE buffer, pH 8.0. Its concentration was measured by NanoDrop spectrophotometer and stored at +4°C to be used in transfection of Sf9 cells.

Solution I (cell lysis buffer):	50mM Tris-HCl (pH 8.0), 10mM EDTA, containing RNAse A at 0.2 mg/ml
Solution II (neutralization solution):	200mM NaOH, 1% SDS
TE Buffer:	10mM Tris-HCl (pH 8.0), 0.1mM EDTA

Table 3.2 Solutions used in Bacmid isolation

3.6 Cationic Liposome-mediated Transfection of Sf9 cells with Bacmid DNA

Approximately $8x10^5$ to $1x10^6$ cells per well was seeded into the 6 well plates. 6 µg of Bacmid DNA and 4 µ**l** of Cellfectin Transfection Reagent were mixed with 200 µl of serum free Grace`s Insect Medium and incubated at room temperature for 15 min. The media on cells was aspirated and the Bacmid DNA-lipid mixture was added dropwise on top of cells. After a short incubation time, 800 µl of serum free Grace`s Insect media was added to make a final volume of 1 ml. After 5 hours post-transfection at 27°C, the transfection mixture was removed and 2 ml of full growth medium (Grace`s Insect medium, supplemented and 10% heat inactivated FBS) was added. The cells were incubated at 27°C for 5-7 days or until the morphology of Sf9 cells changed as a sign of viral infection. When the cells seemed enlarged and started to detach from the plate, they were collected into centrifuge tubes by scrapping. The media containing cells was centrifuged at 2000 rpm for 5 min. The supernatant media contained first generation of recombinant baculovirus particles and was stored in a cryovial at +4 °C as PO virus. To

check the existence of the recombinant protein, the cell pellet was dissolved in ddH_20 and mixed with SDS-loading dye to be analyzed by Western Blotting.

Amplifying the Virus Stock: To amplify the virus titer, 100 µl of PO virus was added into 50 ml of Sf9 insect cells cultured in Grace`s insect media supplemented with 5% FBS and incubated for 5-6 days. Secreted viruses present in media were collected as P1 virus. By using 1 ml of P1 virus, same protocol was applied for the production of P2 virus stock.

3.7 Purification of Recombinant Flag-ERα Proteins from SF9 cells Infected with Flag-ERα-P2 virus stock

After incubation for 3 days with 1ml of P2 virus, $50x10^6$ of Sf9 cells in spinner flask was transferred into a 50 ml falcon tube and centrifuged at 1500 rpm for 5 min. The cell pellet was dissolved in 4 ml of BC500 solution containing 0.5mM PMSF and 0.5mM DTT. The suspension was lysed three times with 10 minutes intervals on ice by using a dounce homogenizer and centrifuged at 12000 rpm for 25 min at 4°C in centrifuge. 66 µl of 10%NP40 and 2.6 ml of BC0 solution were added slowly by using an injector into the supernatant containing soluble recombinant proteins in order to make the salt-concentration of the solution 300mM and 0.1% NP40. The protein solution was mixed with 100 µl of anti-flag M2 agarose beads washed with 1 ml of BC300 solution containing 0.5mM PMSF, 0.5mM DTT, and 0.1% NP40 for five times. Beads are conjugated with Anti-flag M2 antibody that can recognize and bind to flag tagged ERα proteins. The mixture was incubated overnight and centrifuged at 1500 rpm for 3 min. Beads were washed with 1 ml of BC300 solution containing 0.5mM PMSF, 0.5mM DTT, and 0.05% NP40. By adding 75 µl of BC200 solution containing 0.5mM PMSF, 0.5mM DTT, and 0.05% NP40, the pulled-down proteins were then eluted from M2 agarose beads using flag peptide for 45 min (0.05 mg/ml). 10 µl of eluate was loaded into 7% of acrylamide gel to perform SDS page gel electrophoresis and the gel was then stained with Coomassie brilliant blue solution (50% methanol, 10% glacial acetic acid, 40% dH_20) to check the recombinant protein.

BC0* solution:	40mM Hepes pH 7.5, 4mM $MgCl_2$, 0.4mM EDTA, 15% Glycerol, 0.5mM DTT, 0.5mM PMSF
BC1000* solution:	1M KCl, 40mM Hepes (pH 7.5), 4mM $MgCl_2$, 0.4mM EDTA, 15% Glycerol, 0.5mM DTT, 0.5mM PMSF

Table 3.3 Salt Buffers used in recombinant protein extraction from insect cells

* The number near 'BC' refers to the salt (KCl) concentration in milimolar unit and concentrations were arranged by mixing both solutions in proper amounts. (e.g.: BC300 contains 300mM salt)

3.8 Immunoprecipitation Assay

M2 agarose beads (20 µl of the 50% slurry of beads (10 µl of packed beads) was used for each reaction) were washed with1 ml BC300 solution containing 0.1% NP40 for 5 times and centrifuged at 3000 rpm for 1 min. Recombinant flag-ERα nuclear extract in BC300 solution was added onto the M2 agarose beads with or without 30nM β-estradiol (Sigma-Aldrich, #E8875) or 4hydroxytamoxifen (4-OHT). The mixture was incubated for 3 hours at rotator in cold room. The nuclear extract was discarded after centrifuging at 3000 rpm, 1 min. The beads were washed with 1 ml of BC300 solution containing 0.1% NP40. The tubes were spinned quickly to make sure that any of excess buffer does not remain 200 µl of recombinant nuclear extracts in BC300 solution containing 0.1% NP40 (Med12, MEDZ, MEDX, MEDY, Med25) were added onto the beads separately. The mixture was incubated for 3 hours at rotator in cold room and, then, centrifuged at 3000 rpm for 1 min at +4°C to discard supernatant. The pull-down sample on beads was washed 5 times with 1ml of BC150 including 0.05% NP40. After removing all excess washing buffer from beads, 15 µl of SDS loading dye was added to be ready for Western Blotting.

3.9 Cell Culture

MCF7 cell line showing acquired resistance to tamoxifen (MCF7-TamR) was gifted from Sahin Lab (Bilkent University, Ankara). Both wild type and Tam-R MCF7 cells were

grown in low glucose DMEM without phenol red, supplemented with 10% heat-inactivated FBS, 1X non-essential amino acids, 1X Penicillin/Streptomycin and 10 µg/ml insulin. They were passaged by washing with 1X PBS (pH 7.4) followed by trypsinization for 3 min in 37°C incubator.

Sf9 insect cells were grown in Grace`s insect media supplemented with 10% heat-inactivated FBS and 50 µg/ml gentamicin. They were maintained in stirred flasks stably at 26-27°C room temperature.

3.10 Nuclear Extract (NE) Preparation from MCF7 and MCF7-TamR cells

After discarding their medium, cells were washed twice with ice cold 1X PBS and detached in 10 ml of 1X PBS by using a scraper. To collect cell pellet, cells in 1X PBS were centrifuged at 1500 rpm for 10 min. The pellet was resuspended in 4 ml of 40mM Hepes pH 7.5, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT (dithiothreitol), and 0.5mM PMSF (phenylmethylsulfonyl fluoride). The suspension was lysed three times with 10 min intervals in ice by using a douncer. The lysate was, then, centrifuged at 6000g for 10 min. The supernatant was kept in 25% glycerol at -80°C as a cytoplasmic fraction. Nuclear pellets were dissolved in an appropriate amount of 40mM Hepes pH 7.5, 1.5mM $MgCl_2$,25% glycerol, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF, and 0.3M NaCl. The suspension was incubated for 30 min in rotator at 4°C and centrifuged for 15 min at 10000g. The supernatant (NE) was snap-frozen in liquid nitrogen and stored at -80°C until usage. Protein concentrations were determined by Pierce BCA Protein Assay Kit #23227.

3.11 Immobilized Template Recruitment Assay

Beads-immobilized DNA preparation

2XERE-ptata fragment (generously provided by Prof. Dr. Mesut Muyan) with length of ~400 bp was obtained by PCR using primers biotinylated at their 5` ends and pERE as a template. 100 µl of Dynabeads M280 Streptavidin (Invitrogen) # 11205-D were washed twice with 300 µl of 1X binding and washing (B&W) buffer (5mM Tris-HCl pH 7.5, 0.5mM EDTA, 1M NaCl) containing 0.5 mg/ml BSA followed by three wash with 300 µl of 1X B&W buffer. 6 µg biotinylated 2XERE DNA was bound to beads in 1X B&W

buffer by incubation for 15 min at room temperature. Bead-immobilized DNAs were washed three times with 300 µl of 1X B&W buffer containing 0.5 mg/ml BSA followed by two 1X PBS washings and blocked with 200 µl of Blocking Buffer (10x Assay mix, 5 mg/ml BSA, 5mg/ml PVP, 12.5mM DTT, 1% NP40) at room temperature for 15 min. After two washings with 150 µl of Wash Buffer (40mM HEPES, 4mM MgCL2, 4mM DTT, 100mM KCl, 0.1% NP40), beads-DNAs immobilized on the beads were kept in 100 µl of wash buffer at 4°C until further use.

ERE-Coregulators Pull-down Assay

10 µl of bead-immobilized DNA was used for each reaction and washed three times with 1X assay mix (containing 0.25 mg/ml BSA and 0,025 %NP40) followed by incubation in 750 µg of 4-OHT sensitive and resistant MCF7 nuclear extracts at 30°C water bath for 50 min. Also 50 mg/ml BSA was added for each reaction to avoid nonspecific recruitments of coregulators on 2XERE fragments. After incubation, beads were washed three times with 300 µl of 1X assay mix. Supernatant was completely removed and beads were resuspended in 15 µl of 2X SDS loading dye. After 5 min boiling, samples were loaded into 7-10% acrylamide gel for SDS-page gel electrophoresis followed by immunoblotting.

3.12 Immunoblotting

Samples were transferred from SDS-Page polyacrylamide gel to the PVDF membrane activated with 100% methanol in 1X Tris-glycine and 20% methanol for 2.5-3 hours at 300mA on ice. The membrane in which the proteins are stuck was blocked at room temperature for 2 hours in 5% nonfat milk-1X PBS. The blocked membrane was cut into strips of different size ranges followed by addition of primary antibody and incubated at 4°C overnight. After six washings with 1X PBS-0.1% Tween, the membrane was incubated in secondary antibody-HRP conjugates (horse anti-mouse #7076S, goat anti-rabbit #7074S Cell Signaling Technology) in 0.1% nonfat milk-1XPBS for 2 hours at RT, followed by 1X PBS-0.1% Tween washings. Signal development was performed either on X-ray film or with the Amersham Imager 680 by using Pierce ECL Western Blotting, after an appropriate exposure time.

All primary and secondary antibodies were prepared in 1:1000 ratio and 1:10000 ratio respectively.

3.13 Co-Immunoprecipitation (Co-IP) Analysis

MCf7 cells growing in regular low glucose DMEM with 10% heat inactivated FBS (#S181H, Biowest) were passaged into low glucose DMEM without phenol red with 10% charcoal stripped FBS (#S181F, Biowest) for at least 2 days before performing the experiment to avoid possible estrogenic activity of phenol red and to elucidate the effect of estrogen. Cells were treated for 45 min with 100nM β-estradiol (Sigma-Aldrich, #E8875) in DMSO. After treatment, the cells were cross-linked on the plate with 1% formaldehyde in 1X PBS for 10 min at room temperature. Cell pellets were collected in 100mM Tris-HCl pH 9.4 and 10mM DTT and washed with Buffer I and Buffer II, which are listed below, respectively followed by 1 min centrifugation at 7000 rpm.

Buffer I:	10mM EDTA, 0.5mM EGTA, 10mM Hepes pH 6.5, and 0.25% Triton-X
Buffer II:	200mM NaCl, 1mM EDTA, 0.5mM EGTA, and 10mM Hepes pH 6.5

Table 3.4 Buffers used in Co-IP experiment

The pellets were then resuspended in 300 µl of lysis buffer (50mM Tris-HCl pH 8.0, 10mM EDTA, 0.2 mg/ml Leupeptin, and 1% SDS) and sonicated 3 times for 30 seconds at maximum settings. The suspension was centrifuged at 3000 rpm for 10 min followed by addition of 150mM NaCl, 1% Triton-X and 10% glycerol to the supernatant. This protein suspension with fragmented chromatin was stored at -80°C until usage. 20 µl of protein A-sepharose bead slurry for one reaction was washed with 1 ml of BC300 solution including 0.05% NP40 for 5 times and was then mixed with 2 µl of Estrogen receptor alpha monoclonal antibody (Cell Signaling Tech, #8644) in BC300 solution. The suspension was incubated in a rotator overnight followed by discarding the antibody and the addition of protein-fragmented chromatin mixture. After 3 hours incubation, protein-bound beads were washed 5 times with BC150 solution. After discarding the solution, 15

µl of 2X SDS-loading dye was added to the beads. Samples were analyzed by Western Blotting to check the proteins that are pulled down with ERα. Differently from other immunoblottings, Clean-Blot IP Detection Kit (HRP) (Thermo Fisher #21232) was used as described in the manufacturer`s protocol not to detect igG proteins in the beads.

CHAPTER 4

RESULTS

4.1. Purification of Human Mediator Complex Modules and Recombinant Flag-ERα Using Baculovirus Expression System

As previously suggested, it is important to dissect human Mediator Complex into partial assemblies to understand underlying interactions behind its functional roles in the activator-dependent transcription. Therefore, it was necessary to purify human Mediator modules and subunits by using MultiBac baculovirus expression system mentioned before. Separate transfer vectors (PFBDM) are constructed to jointly express an array of subunits included in Head and Middle modules. Bacmid DNAs are synthesized in modified DH10MultiBac E.coli and transfected to Sf9 cells to generate and amplified expression viruses with high-titer. The titer of viruses are arranged to obtain stoichiometric amounts of all the subunits in resulting partial complexes followed by infection of Hi5 insect cells with Head (H), Middle (M), H+M, H+M+MED14+MED26+MED1 viruses. Since each module constructs includes one subunit with a flag tag, cell lysates containing partial complexes are incubated with anti-flag M2 agarose beads followed by the elution with the flag peptide. Figure 4.1A shows the Coomassie staining of purified modules and multi-module assemblies (from Cevher et al. 2014) [3]. As in vitro transcription analyses indicated, the reconstituted core Mediator complex (H+M+14+26) promotes RNA Pol II-mediated transcription with purified Pol II and general transcription factors from insect cells or corresponding stable Hela cell lines. Therefore, they can be used in further functional analyses [3].

The Mediator complex, especially MED1 subunit and kinase module, was implicated as a key regulator in the mammary gland development and the transcription of ERα-responsive

genes in the previous studies [39, 40, 43]. To further characterize the role of Mediator Complex on ERα-mediated transcription, I produced recombinant flag-tagged ERα by using baculovirus expression system with the purification technique mentioned above. Since the estrogen receptor is a small protein unlike Mediator as a multi-subunit complex, DH10Bac strain of E.coli is used for Bacmid formation rather than using MultiBac expression system. Figure 4.1B shows the Coomassie staining of purified recombinant 66kDa-ERα.

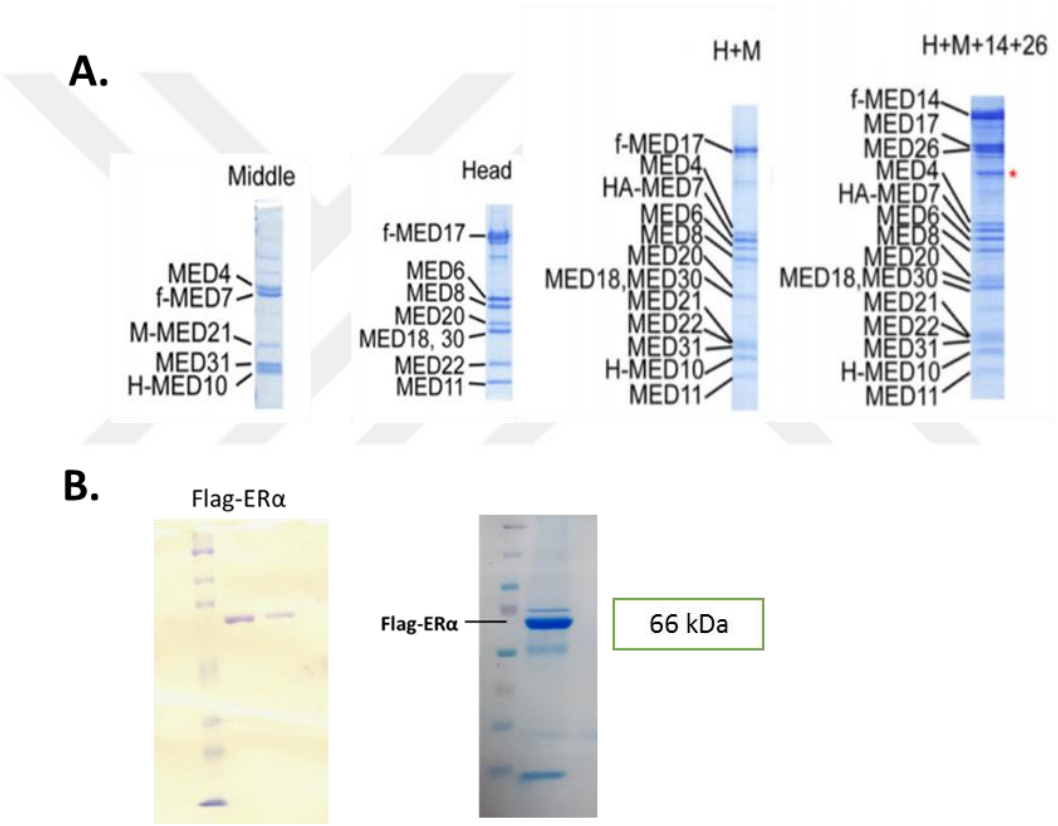

Figure 4.1. Reconstituted human Mediator sub-complexes and Flag-ERα using MultiBac Baculovirus Expression System. ***A)*** *Coomassie staining of purified Head, Middle, head+middle, and core complex (Head+middle+MED26+MED14). Hi5 cells were infected with corresponding viruses and after 3 days-incubation followed by affinity pull-down of the complexes from cell lysates by using anti-flag M2 agarose beads and their elution with 0.5 mg/ml flag peptide [3].* ***B)*** *SDS-PAGE analysis (Coomassie-blue staining) of purified Flag-ERα from Sf9 cells infected with the corresponding virus and undergone affinity purification after 3 days. Cell lysates were incubated with anti-flag M2*

agarose beads and recombinant protein was eluted from beads via addition of 0.05 mg/ml flag peptide.

Due to its multi-subunit structure, Mediator Complex present many interaction surfaces to enhancer-bound nuclear receptors in order to form DNA-looping and to direct signals from ligand bound-receptors to the transcription assembly on promoter region. It is known from previous studies that when ERα-bound beads are incubated with Hela nuclear extracts, Mediator subunits are pulled down and purified Mediator enhances ERα-mediated transcription in in vitro transcription assays upon E2 treatment [39, 40]. Although it is claimed that Med1 is the subunit that facilitates the interaction between Mediator and ERα, recombinant core Mediator complex along with Med1 subunit was not be able to interact with ERα as strong as purified Mediator did. (According to IP experiments performed by another graduate student in our lab). Therefore, which compositional form of Mediator complex activates the ERα-mediated transcription or which subunit/subunits bind to ERα remained to be elucidated.

4.2 Immunoprecipitation analysis suggests that MEDX might interact with ERα

RNA-Seq data (generated by our collaborator Dr. Suhail Akhtar Ansari) was our starting point. The data was showing that MEDZ copy number is dramatically high in breast cancer patients. Additionally, MEDZ and ERα has an inverse correlation. So, we started to check if there is an interaction between ERα and Mediator subunits. Immunoprecipitation assay was performed by using Flag-ERα, MEDX, MEDZ, and MEDY nuclear protein extracts isolated from Sf9 insect cells via baculovirus expression system in order to understand if there is a direct interaction between any Mediator subunits and ERα. Flag ERα was bound to anti-flag M2 agarose beads with or without 30nM E2 and with or without 100nM Tamoxifen followed by incubation with nuclear extracts of kinase subunits separately in 300mM salt concentration. After stringent washes at 150nM salt concentration along with detergent, proteins interacted with bead-bound ERα were checked by Western Blot. We did not need to equilibrate the amounts of non-tagged proteins (MEDX, MEDZ, MEDY) since we focused on investigating the presence/absence of any interaction. As seen in

Figure 4.2 we observed a possible strong interaction between MEDX and ERα which also seemed to decrease upon E2 treatment. To be able to see the same outcome without observing unspecific bands resulting from washing conditions or excess amount of the extract, the same experiment was repeated with less amount of nuclear proteins (Figure 4.2B**)**. When proteins are getting large, their possible interaction surfaces also increase. This might suggest that it is easier for MEDY to bind to Flag-ERα nonspecifically (Figure 4.2A lane 7 and 8). Since MEDX is a small protein, we speculate that the interaction of MEDX with ERα can be specific.

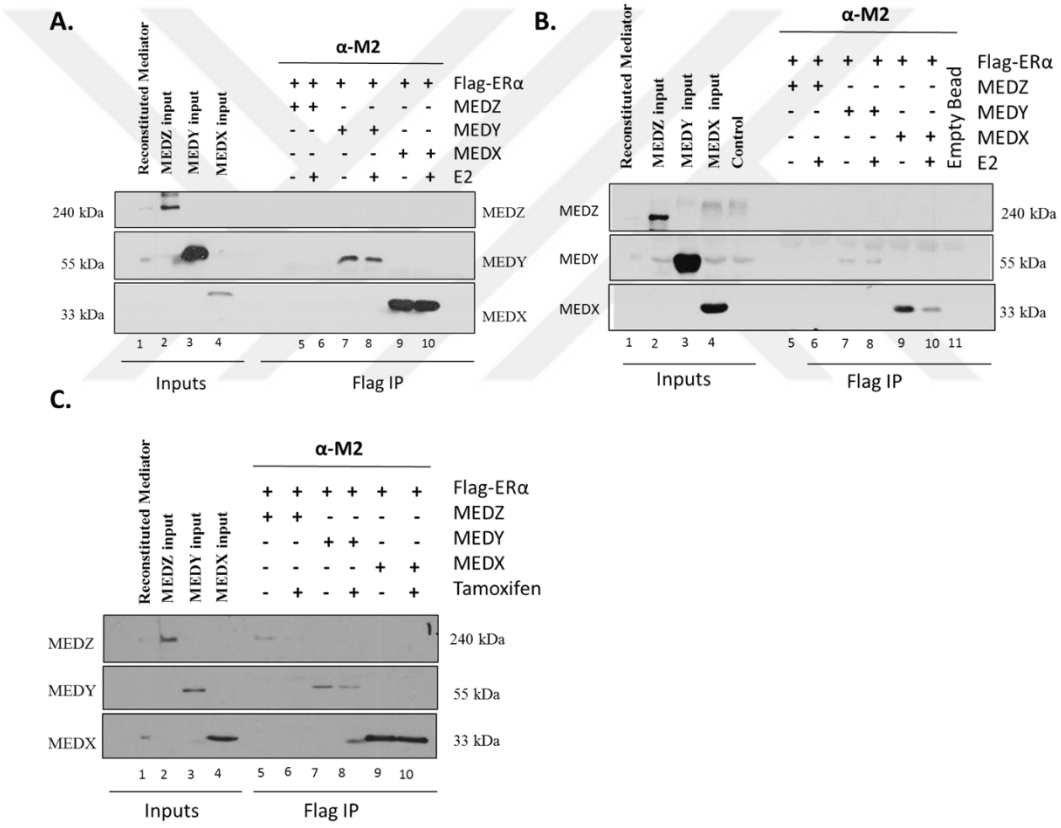

Figure 4.2 Immunoprecipitation of ERα with MEDX, MEDY and MEDZ subunits with or without E2 and tamoxifen. ***A, B)*** *Western Blot analysis of kinase subunits pulled down with ERα-bound anti-flag M2 agarose beads. Flag-ERα nuclear extract was incubated with anti-flag M2 agarose beads in BC300 solution with or without 30nM E2* ***C)*** *and with or without 100nM tamoxifen for 3 hours at rotator. After washing with BC300 solution to remove unbound proteins, nuclear extracts of MEDX, MEDY and MEDZ subunits in BC300 solution were added into separate ERα-bound M2 agarose beads.*

4.2.1 IP experiment to check the possibility of nonspecific interaction between MEDX and anti-flag M2 agarose beads

To clarify that the MEDX binds to ERα specifically, we checked the binding capacity of MEDX to empty anti-flag M2 agarose beads and the binding capacity of Flag-ERα to other control proteins; one is from kinase subunits MED12, and the other is from tail subunits MED25. Two immunoprecipitation assays were performed for each subunit to eliminate the possibility of their binding to empty beads. The beads were incubated either with BC300 solution containing Flag-ERα as a control or only with BC300 solution followed by the incubation with MEDX, MED25 and MED12 containing nuclear extracts. As seen in Figure 4.2.1A, MEDX interacts only with ERα-bound beads but not the bead itself. The interaction of MEDX (Figure 4.2.1B, lane 9) is slightly higher than those of MED12 (Figure 4.2.1B, lane 4) and MED25 (Figure 4.2.1B, lane 6). The anti-MED25 antibody might be recognizing unspecificly as we see similar quantity of bands on lanes 6, 4 & 5.

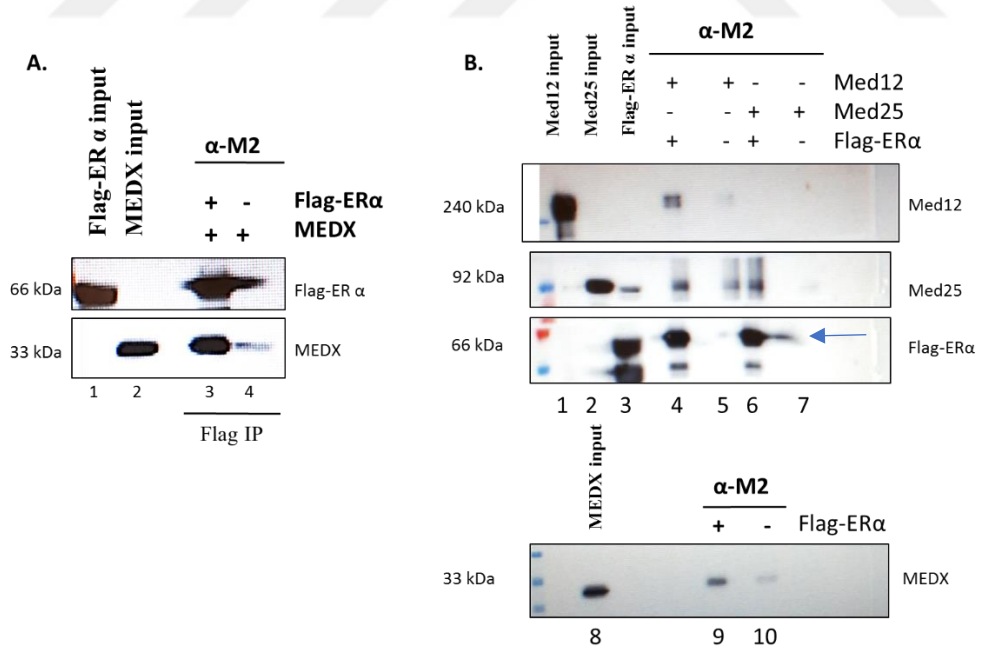

Figure 4.2.1 IP experiments show a possible interaction between ERα and MEDX.

A, B) *Western Blot analysis of ERα-interaction assay. Anti-Flag M2 agarose beads were incubated with either BC300 solution or BC300 containing Flag-ERα for 3 hours at*

rotator. After washing BC300 solution once to remove unbound ERα proteins, MED12, MEDX and MED25 nuclear extracts in BC300 were added and incubated for 3 hours. Protein-bound beads were washed with BC150 five times. Pull-down of proteins with ERα was validated by indicated antibodies.

4.2.2 Co-immunoprecipitation analysis with Flag-MEDX and endogenous ERα in MCF7 nuclear extracts

To understand if there is indeed an interaction between MEDX and ERα, we decided to perform reverse IP. Since we do not have recombinant ERα without a flag-tag, recombinant Flag-MEDX was purified from Hi5 cells and bound to anti-flag M2 agarose beads followed by incubation with MCF7 nuclear extracts previously treated with/without E2 on ice for 30 min. As seen in Figure 4.2.2A, the endogenous ERα interacts with MEDX contrary to androgen receptor (AR) which shows similar patterns in both ERα-bound and empty beads. Although the decrease in the interaction upon E2 treatment is consistent with previous direct IP, the pull-down of ERα with MEDX is very weak compared to input concentration, which might suggest that it can be an unspecific interaction. The interaction on the other hand might be prevented from side interactions of endogenous proteins in MCF7 nuclear extract with either MEDX or ERα. As a result, we cannot draw a conclusion yet about whether there is a direct interaction between MEDX and ERα without performing further IPs with purified recombinant ERα without a tag or its purified truncated forms and or without showing the interaction in the context of Mediator Complex.

Figure 4.2.2B shows the pull-down of endogenous MEDX with endogenous ERα-bound protein A sepharose beads. We saw the interaction between MEDX and ERα when we used total protein extracts from MCF7 cells.

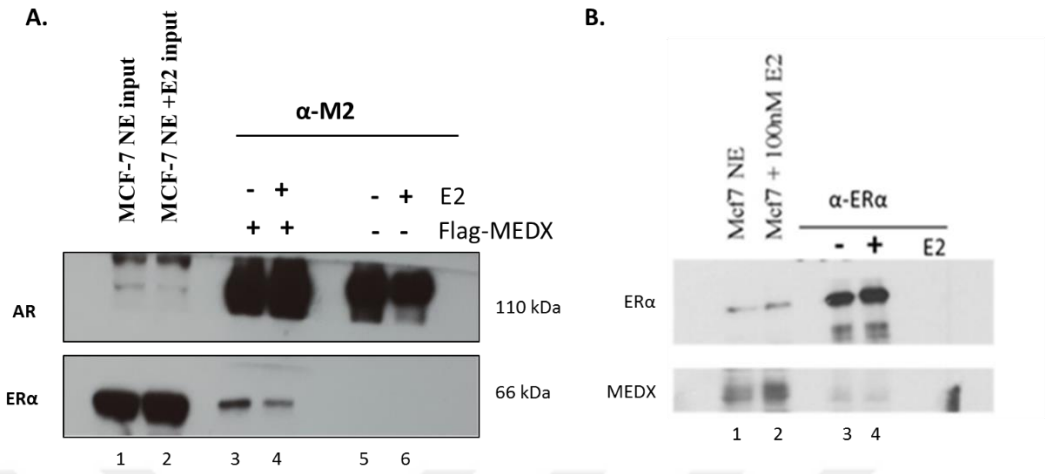

Figure 4.2.2 Co-immunoprecipitation analysis with Flag-MEDX, endogenous MEDX and ERα. ***A)*** *Western Blot analysis shows a possible interaction of ERα. Co-IP experiment was performed by using recombinant Flag-MEDX and anti-flag M2 agarose beads. MCF-7 nuclear extracts incubated with/without 100nM E2 on ice for 30 min were used as an endogenous ERα source.* ***B)*** *MCF-7 cells treated with/without 100nM E2 for 45 min were fixed with 1% formaldehyde for 10 min and whole cell protein extracts were obtained and incubated with protein A sepharose beads previously bound to anti-ERα.*

4.3 Characterization of Mediator-subunit expression levels in wild-type (WT) and tamoxifen-resistant (TamR) MCF-7 cells

Tamoxifen which is a selective estrogen receptor modulator (SERM) has been widely used for breast cancer treatment so far. Its antagonist or agonist activity depends on target tissue and target estrogen receptors (alpha or beta) expressed in those tissues. This dependency is actually partially resulted from the difference in coregulator recruited by tamoxifen-bound estrogen receptors in those tissues to promoter regions. Therefore, tamoxifen resistance should be investigated at the stage of transcriptional regulations of ERα target genes involved in tumor progression; such as oncogenes, growth factors or kinases. The activation of these genes are controlled by two main steps in transcription. First step is the recruitment of chromatin modifying coactivators (AIB1, CBP, GRIP1, PCAF, p300, and SRC-1) by ligand-bound ERα on ERE site to break chromatin barrier and allow gene

regulation [60, 61]. Second step is the PIC formation composed of general transcription factors, Pol II and Mediator Complex. How Mediator Complex regulates the events in the second step and how these regulations are changed when resistance to tamoxifen is acquired are not known and should be elucidated in order to reveal the underlying mechanisms of tamoxifen resistance.

To do this, we first identified the Mediator subunits whose protein expression levels differ in wild type MCF-7 and Tamoxifen-resistant (TamR or MCF-7 4-OH) MCF-7 cell lines (kindly gifted by Sahin Lab, Bilkent University). As seen in Figure 4.3, MEDZ increases in resistant cell line, which is accordance in the RNA-seq data.

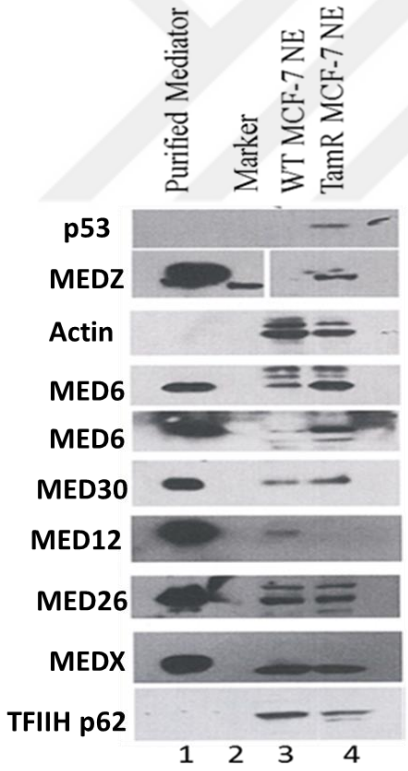

Figure 4.3 Comparison of protein expression levels of Mediator subunits in wild-type and tamoxifen resistant (TamR) MCF-7 cell lines. *Western Blot analysis of Mediator subunits expressed in WT MCF-7 and TamR MCF-7 cell lines. Purified Mediator Complex from HeLa cell line stably expressing f: Nut2 was loaded as a reference for corresponding*

molecular weights of Mediator subunits. 25 µg nuclear extracts from both lines were loaded. Actin expression was checked as a loading control.

4.4 Immobilized Template Recruitment Assay (ITRA) shows the difference in levels of Mediator subunits and RNA Pol II recruitment to promoter DNA

As we focus our work at PIC stage, we conduct our experiments on naked DNA and pass the chromatin barrier. To identify endogenous Mediator subunits bound to ERα on its cognate response element (ERE) in both wild type and tamoxifen resistant MCF-7 cell lines, we performed ITRA by using magnetic streptavidin-coupled Dynabeads. A plasmid containing 2X ERE site and TATA promoter (generously gifted by Prof. Dr. Mesut Muyan, METU) used as a template to amplify biotinylated 2ERE-TATA promoter DNAs. Then, these DNAs were immobilized to streptavidin Dynabeads under favor of strong streptavidin-biotin affinity. Immobilized beads were incubated with nuclear extracts (NEs) from both cell lines in the presence/absence of E2 or tamoxifen. We saw that MEDZ and MEDY recruitment levels increased in resistant cells whereas RNA Pol II level decreased, consistent with the fact that kinase module binds to human core Mediator via its MEDZ subunit reversibly and possibly blocked Mediator-dependent RNA Pol II recruitment. As a control, MED14 recruitment to promoter did not change in both cell lines (Figure 4.4C).

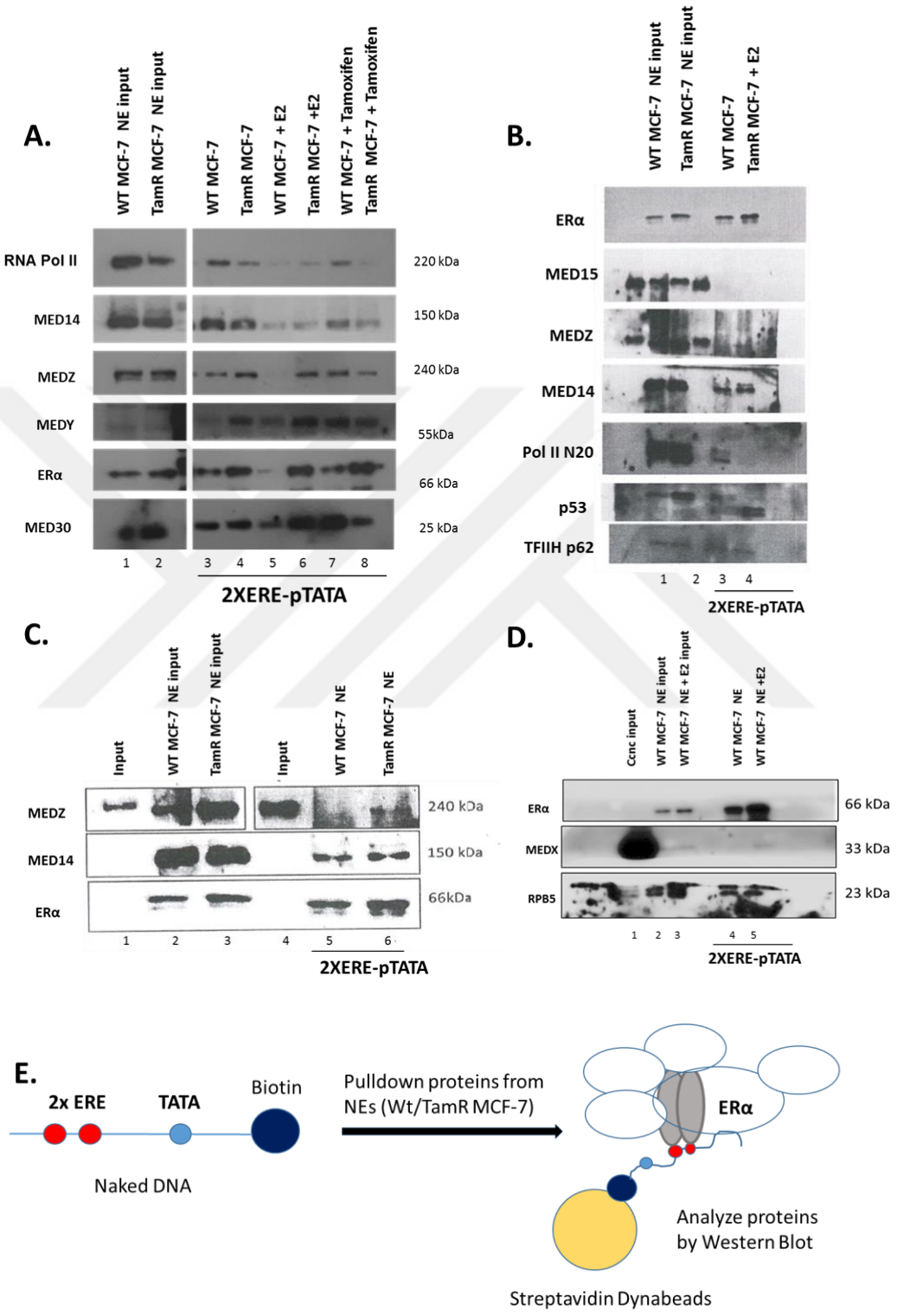

Figure 4.4 Mediator subunits and RNA Pol II recruitments on ERα-bound 2XERE-TATA promoter region. ***A)*** *Nuclear extracts from both cell lines were treated with*

100nM 17β-estradiol (E2) or 7.5μM tamoxifen on ice for 30 min followed by incubation with 2XERE-TATA promoter template-fused magnetic beads for 50 min at 30°C. After washes, bound proteins were checked by immunoblotting. Pulldown proteins bound to ERα are seen in lanes 3-8. ***B)*** *Nuclear extracts from both cell lines were treated with 100nM 17β-estradiol (E2) on ice for 30 min followed by incubation with 2XERE-TATA promoter template-fused magnetic beads for 50 min at 30°C. After washes, bound proteins are checked by immunoblotting. Pulldown proteins bound to ERα were seen in lanes 3-4* ***C)*** *Nuclear extracts from both cell lines were incubated with 2XERE-TATA promoter template-fused magnetic beads for 50 min at 30°C. After washes, bound proteins were checked by immunoblotting. Pulldown proteins bound to ERα are seen in lanes 5-6.* ***D)*** *Nuclear Extract of wild type MCF7 treated with/without 100nM E2 were incubated. . Pulldown proteins bound to ERα are seen in lanes 4-5.* ***E)*** *Schematic representation of ITRA for detection of Mediator-ERα complexes on EREs. 2XERE-TATA promoters were immobilized to magnetic streptavidin Dynabeads and incubated with NEs. TATA promoter containing 2ERE site was amplified by PCR by using biotinylated primers and immobilized to magnetic beads (the method in section 3.11).*

4.5 Mediator-depleted nuclear extracts does not show decrease in ERα protein level

As Mediator interacts with ERα, to observe the degree of interaction and to see if there is a possible decrease in ERα level after the depletion of Mediator from Hela nuclear extracts, we compared ERα protein levels in Mediator-depleted and non-depleted Hela NEs by Western Blot. We did not observe any change. This was expected since the interaction between kinase module and ERα, which we expect, must be dynamic and not stable. Therefore, ERα protein level did not reduce in Mediator-depleted NE.

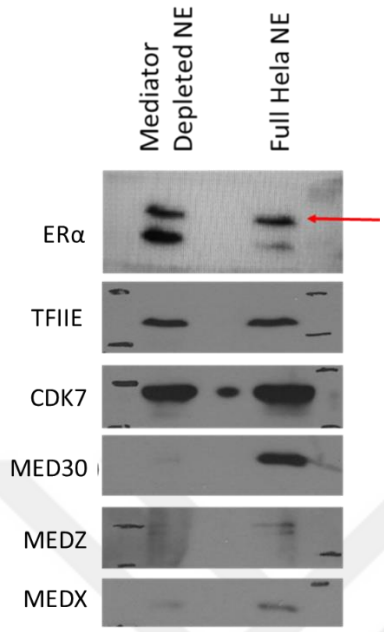

Figure 4.5 Comparison of ERα protein level in Mediator-depleted and full HeLa Nuclear Extracts. *Mediator was depleted from HeLa NEs by using Med30 antibody-bound beads. Med30, MEDZ and MEDX levels were detected to control Mediator depletion. TFIIE and CDK7 (TFIIH subunit) were positive controls as general transcription factors.*

4.6 MEDY inhibitor increases the recruitment of MEDY in TamR cells

It was shown by Roninson group that MEDY inhibition attenuates estrogen induced transcription. They showed that MEDY inhibitor caused the dose-dependent decrease in luciferase activity in T47D-ER/Luc cells, expressing firefly luciferase under control of an ER-dependent ERE containing promoter. In addition, Treatment MCF7 cells with both inhibitor and E2 prevented the expression of ER-target genes GREB1, TFF1 and CXCL12. To see the effect of MEDY inhibitor in recruitment of Mediator subunits to ERE containing promoter in both WT and TamR cell lines, we performed ITRA.

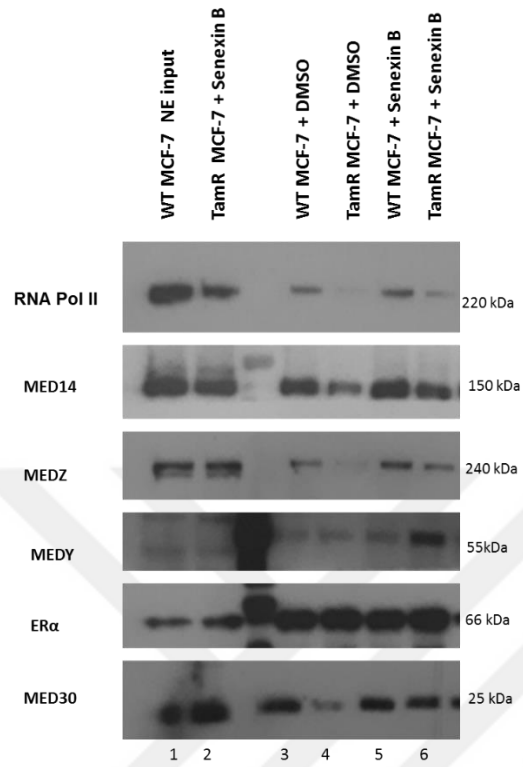

Figure 4.6 Mediator subunits recruited to ERE containing promoter upon treatment of MEDY inhibitor.

Nuclear extracts from both cell lines were treated with 5 µM MEDY inhibitor on ice for 30 min followed by incubation with 2XERE-TATA promoter template-fused magnetic beads for 50 min at 30°C. After washes, bound proteins were checked by immunoblotting. Pulldown protein bound to ERα are seen in lanes 3-6.

We observed that MEDY recruitment increased in TamR cells under the control of MEDY inhibitor whereas it did not change in wild type cells. ChIP experiment performed by Roninson group showed that MEDY was strongly recruited to GREB1 promoter/enhancer regions upon the addition of E2, however, this recruitment was not significantly changed by MEDY inhibitor [44]. While there was not significant change in MEDY recruitment between wild-type treated with DMSO as a control and wild type treated with the inhibitor, it was interesting that there was increase in MEDY recruitment in TamR cells. Therefore, we speculated that the drug might prevent ERα-dependent transcription by recruiting more MEDY to the promoter since MEDY-module has an inhibitory role in the transcription.

CHAPTER 5

DISCUSSION

As earlier studies have shown the association of Mediator Complex with ERα upon E2 stimulation, we tried to understand which subunit does this interaction. Therefore we used our well-constructed in vitro methods to elucidate this association. We also tried to understand the Mediator composition that is recruited to ERE sites. Since Mediator has compositional heterogeneity in different tissues, changes in composition affects sequential factor recruitment during PIC formation. Therefore, it is highly important to determine the composition of Mediator Complex that regulates ERα target genes on ERE templates. To achieve this, it is necessary to dissect multi-subunit Mediator Complex which is composed of 30 subunits into modular pieces and obtain a chance to add or remove individual subunits. Our Principal Investigator, Asst. Prof. Murat Alper Cevher has accomplished the reconstitution of 15-subunit functional human Mediator by using Baculovirus expression system. Therefore, we have the power of using these reconstituted variants in our functional interaction assays by adding onto the recombinant core (Figure 4.1B).

It has been shown that MED1 mediates the association of Mediator with ERα via its NR-box motifs, however, it is still not known that how signals from ligand-bound ERα are conveyed to recruitment of RNA Pol II complex. So, another graduate student Yasemin Barış first checked if there is any interaction between ERα and head or middle modules or functional core Mediator containing MED1 (Head+middle+MED14+MED26+MED1). The interaction observed was weak as compared to the full Mediator- ERα. This suggests us that tail or kinase modules must be important to facilitate the role of Mediator in the receptor recruitment. Moreover, preliminary data from our collaborator Dr. Özgür Şahin showed that RNA-seq data demonstrated that there was increase in MEDZ copy number

in breast cancer patients. Since reconstitution of tail and kinase modules have not been completed yet, we searched the possibility of interactions between the receptor and individual subunits whose viruses already present for infection to insect cells. We showed a possible interaction between MEDX and ERα in IP experiments (Figure 4.2 and 4.2.1). We used empty beads as a negative control showing no band detection in the absence of ERα. MED12 and MED25 were used as a negative control to see if they showed same bands with MEDX to understand whether the interaction with MEDX is specific. We observed a weak band corresponding to MED12 in ERα-bound beads but we speculate that MED12 might be a sticky protein since it has been also bound to TFIIE and to AR in IP experiments (data not shown). Then, we checked the reverse interaction by fusing Flag-MEDX which we purified by using baculovirus expression to anti-flag M2 beads and incubating with MCF-7 nuclear extracts. Although there was no ERα interaction with empty beads at all and consistency with previous IP experiments in terms of decrease in interaction of MEDX with ERα in the presence of E2, the interaction of endogenous ERα was weak (Figure 4.2.2). According to results, we speculate that MEDX might be a binding partner of ERα, which keeps the receptor inactive state. After E2 stimulation, kinase module should be dissociated from active ERα since it has inhibitory role in transcription. So, we decided to further verify the interaction (in chapter 6, discussed in detail). It is also possible that ERα recruitment might rely on more than one interaction interface between ERα and kinase-tail modules, which can be solved by the integration of reconstituted kinase and tail modules to further experiments. Figure 5.1 indicates proposed mechanism for Mediator-facilitated communication of liganded ERα on its cognate response element with transcription machinery on promoter region.

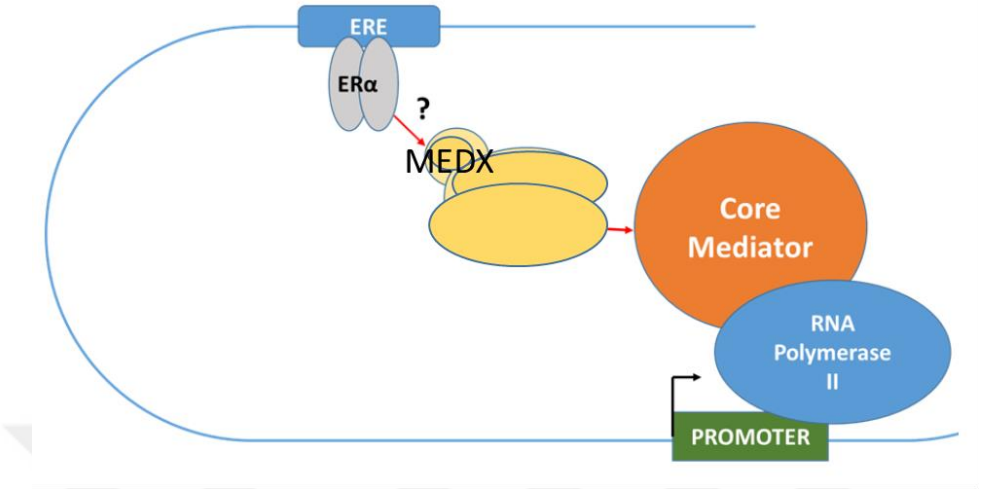

Figure 5.1 Proposed model for Mediator-ERα interaction.

MEDX and MEDY are binding partners. MEDX can be found separate from the rest of the kinase module. We speculate that MEDX regulates the recruitment of ligand-bound ERα on ERE site to promoter regions of target genes. This regulation must be dynamic since MEDY-module dissociates from Mediator to allow RNA Pol II-Mediator binding.

As a second aim, we searched the alterations in the expression levels of Mediator subunits in wild-type and Tamoxifen resistance MCF-7 cell lines and how these alterations affect the recruitments of other subunits and Pol II to ERα-dependent promoter regions. We know that Mediator has a dynamic composition and heterogeneity in its composition in different cell types. Therefore, we wanted to check the possibilities that antagonist effect of tamoxifen might be regulated by Mediator. To understand the role of Mediator in tamoxifen resistant breast cancer, we checked the expression levels of Mediator subunits in wild type and tamoxifen-resistant MCF-7 cell lines (kindly given by collaborator Asst. Prof. Özgür Şahin, Bilkent University). We saw that nuclear MEDZ level is higher in resistance cell lines as opposed to other subunits. Then we focused on changes in recruitment of Mediator subunits into promoter containing estrogen response element sites. To skip earlier stages in the transcription as it is not necessary, we worked on naked DNA template. We performed immobilized template recruitment assays by using biotinylated 2XERE-TATA promoter DNAs fused to magnetic beads (plasmid used as a

template was generously gifted by Prof. Mesut Muyan, METU) and observed that MEDZ was recruited more to promoter region in resistant cell line, consistent with the RNA-seq data. Also recruited ERα level increases in resistant cell line, this suggested that recruitment of MEDZ was ERα-dependent. Co-administration of MEDZ knockdown and tamoxifen treatment (performed by our collaborator) increased the protein expression of ERα and caused the cell growth inhibition in MCF7 and T47-D cells.

Eukaryotic transcription activators regulates the expression of target genes through recruitment of co-activators. Several diseases are resulted from abnormal activity of transcription activators. However therapeutic targeting efforts have been prevented by a lack of detailed molecular knowledge of the mechanism. If we solve this mechanism, we can design therapeutic drugs that block specifically the interaction between transcription activator (ERα) and co-activator (possibly kinase module). For example, Nishikawa group found that the ortholog of Med15 (Gal11) in fungi and human pathogen *Candida glabrata* associates with pleiotropic drug resistance transcription factor (Pdr1) orthologues, which are key regulators of the multidrug resistance pathway. KIX domain in Gal11facilitated the interaction. They identified a small-molecule inhibitor to block the interaction between Pdr1 activation domain and Gal11 KIX domain. The molecule inhibited Pdr1-dependent gene activation and re-sensitizes drug resistance [70].

Based on our collaborator's experiments, we think that kinase module, particularly MEDZ, might regulate tamoxifen resistance in ER+ positive breast cancer through Her2 pathway. Our purpose is to find the detailed mechanism and block this interaction. To date, many therapeutics against ER+ breast cancer target ER by blocking the binding of ER to estrogen. However, the expression of ER-target genes depends on the downstream regulations including the interaction between ERα and co-activators. Since Mediator is the most important co-activator, blocking its interaction with ERα is the best approach to stop the expressions of proliferative genes in tumorigenesis. As well as an alternative therapeutic approach, it is more advantageous than other drugs like tamoxifen.

CHAPTER 6

FUTURE PERSPECTIVES

To date, studies have emphasized the significance of MED1 subunit ERα-mediated transcription by creating mutations on its phosphorylation sites or its LXXL motif (which is binding domain for activators) and by showing its knockdown effects. Interestingly, in our hands, we saw that we need more subunits of the Mediator to get full Mediator-ERα response. Therefore, in the future, we plan to expend IP experiments and include kinase to the core and check their interaction with ERα and see if Mediator will be recruited to ERα target gene promoter. Additionally, we revealed a possible interaction between MEDX and ERα by using recombinant proteins (Figure 4.3, Figure 4.3.1 and Figure 4.3.2). To be sure about this interaction and find exact domain of ERα interacting with MEDX (if it exists), we truncated ERα into three fragments with residues 1-185 (N terminal region, transactivation domain), 185-355 (DNA binding domain and hinge) and 301-595 (ligand binding domain). 6XHis-tagged fragments were cloned into PFBDM vector. Figure 6.1 shows PCR results done for verifying the presence of inserts into the PFBDM constructs after ligation.

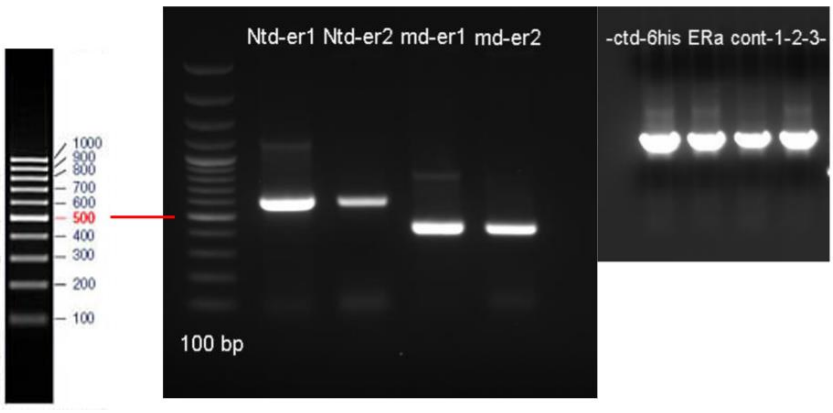

Figure 6.1 Gel Electrophoresis results of truncated ERα inserts in PFBDM constructs. *By using plasmid containing Flag-ERα insert as a template, 6XHis-tag truncated ERα (Ntd: N terminal domain, md: DNA binding domain and ctd: ligand*

binding domain) were amplified and ligated with PFBDM vector. The image shows the gel electrophoresis result of PCRs done to control the presence of our inserts in plasmids we constructed. Ntd-ERα (transactivation domain): 1-185 residues, 555bp; md-ERα (DNA binding domain): 185-355 residues, 510bp; ctd-ERα (ligand binding domain): 301-595 residues, 882bp.

These constructs were then transformed into DH10Bac E.coli strain to produce bacmid DNA followed by transfection of these bacmid DNAs into Sf9 insect cells for the generation of virus expressing our proteins of interest. Figure 6.2 shows the recombinant truncated ERα proteins in cell pellets collected from Sf9 cells infected with corresponding viruses.

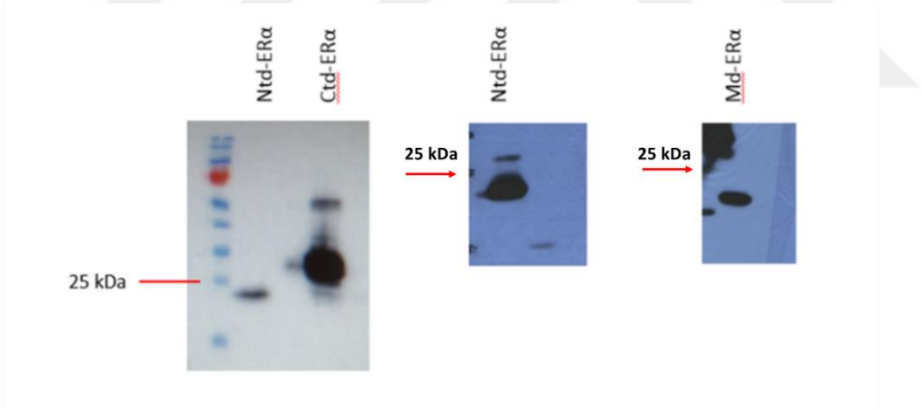

Figure 6.2 Western Blot analysis of isolated truncated ERα proteins from Sf9 cells infected with corresponding P0 viruses. *Sf9 cells were infected with P0 viruses of truncated proteins. After 3 day incubation, cell pellets were collected and used for SDS-Page Gel Electrophoresis. His-tag monoclonal antibody was used for immunoblotting of truncated proteins with 6XHis-tag.*

Figure 6.2 shows the truncated proteins in Sf9 cell pellets. When we tried to extract them by using our nuclear protein extraction protocol for soluble proteins, we saw that they were stuck in nuclear pellet. Therefore, optimization of protocol for insoluble proteins

should be the next step. We already have the virus of Flag-MEDX ready for protein production in insect cells.

As a result, the interaction of Mediator Complex (MEDX as a strong candidate) with ERα will further be investigated via biochemical assays like IP and ITRA by using these truncated ERα proteins and Flag-MEDX. The 3D structure model of possible interaction between ERα and MEDX is shown in Figure 6.3.

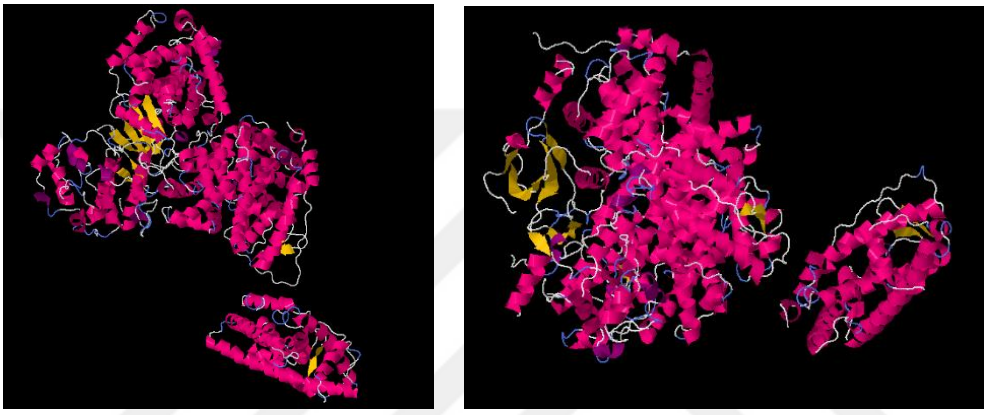

Figure 6.3 Structure model showing possible interaction surfaces between ERα and MEDX.

In the project on MEDZ-tamoxifen resistance, we will perform ITRA experiments in TATA promoter that does not contain ERE sequence to check the ER-dependency of Mediator-subunit recruitment in wild type and resistant cell lines. We will also use natural ER-target gene promoters. Our collaborator has already done in vivo experiments and he showed that synergistic effect of tamoxifen and MEDZ knockdown decreased the tumor volume in the mice inoculated with MCF7 tamoxifen resistant cells. We can check the effect of MEDY inhibitor as well.

BIBLIOGRAPHY

1. Conaway, Ronald C., et al. “The Mammalian Mediator Complex and Its Role in Transcriptional Regulation.” *Trends in Biochemical Sciences*, vol. 30, no. 5, 2005, pp. 250–255. doi:10.1016/j.tibs.2005.03.002.
2. Plaschka, C., Larivière, L., Wenzeck, L., Seizl, M., Hemann, M., & Tegunov, D. et al. (2015). Architecture of the RNA polymerase II–Mediator core initiation complex. *Nature*, *518*(7539), 376-380. doi: 10.1038/nature14229
3. Cevher, M., Shi, Y., Li, D., Chait, B., Malik, S., & Roeder, R. (2014). Reconstitution of active human core Mediator complex reveals a critical role of the MED14 subunit. *Nature Structural & Molecular Biology*, *21*(12), 1028-1034. doi: 10.1038/nsmb.2914
4. Ries, D., & Meisterernst, M. (2011). Control of gene transcription by Mediator in chromatin. *Seminars In Cell & Developmental Biology*, *22*(7), 735-740. doi: 10.1016/j.semcdb.2011.08.004
5. Borggrefe, T., & Yue, X. (2011). Interactions between subunits of the Mediator complex with gene-specific transcription factors. *Seminars In Cell & Developmental Biology*, *22*(7), 759-768. doi: 10.1016/j.semcdb.2011.07.022
6. Grünberg, Sebastian, and Steven Hahn. "Structural Insights Into Transcription Initiation By RNA Polymerase II". *Trends In Biochemical Sciences*, vol 38, no. 12, 2013, pp. 603-611. *Elsevier BV*, doi:10.1016/j.tibs.2013.09.002.
7. Kim, Young-Joon et al. "A Multiprotein Mediator Of Transcriptional Activation And Its Interaction With The C-Terminal Repeat Domain Of RNA Polymerase II". *Cell*, vol 77, no. 4, 1994, pp. 599-608. *Elsevier BV*, doi:10.1016/0092-8674(94)90221-6.
8. Thompson, Craig M. et al. "A Multisubunit Complex Associated With The RNA Polymerase II CTD And TATA-Binding Protein In Yeast". *Cell*, vol 73, no. 7, 1993, pp. 1361-1375. *Elsevier BV*, doi:10.1016/0092-8674(93)90362-t.
9. Myers, L. C. et al. "Mediator Protein Mutations That Selectively Abolish Activated Transcription". *Proceedings Of The National Academy Of Sciences*, vol 96, no. 1,

1999, pp. 67-72. *Proceedings Of The National Academy Of Sciences*, doi:10.1073/pnas.96.1.67.

10. Wang G, Balamotis MA, Stevens JL, Yamaguchi Y, Handa H, Berk AJ. Mediator requirement for both recruitment and postrecruitment steps in transcription initiation. Mol Cell 2005;17:683–94.
11. Taatjes, D. J. "Structure, Function, And Activator-Induced Conformations Of The CRSP Coactivator". *Science*, vol 295, no. 5557, 2002, pp. 1058-1062. *American Association For The Advancement Of Science (AAAS)*, doi:10.1126/science.1065249.
12. Ebmeier, C. C., and D. J. Taatjes. "Activator-Mediator Binding Regulates Mediator-Cofactor Interactions". *Proceedings Of The National Academy Of Sciences*, vol 107, no. 25, 2010, pp. 11283-11288. *Proceedings Of The National Academy Of Sciences*, doi:10.1073/pnas.0914215107.
13. Mittler, G. "A Novel Docking Site On Mediator Is Critical For Activation By VP16 In Mammalian Cells". *The EMBO Journal*, vol 22, no. 24, 2003, pp. 6494-6504. *Wiley*, doi:10.1093/emboj/cdg619.
14. Yang F, DeBeaumont R, Zhou S, Naar AM. The activator-recruited cofactor/Mediator coactivator subunit ARC92 is a functionally important target of the VP16 transcriptional activator. Proc Natl Acad Sci USA 2004;101:2339–44.
15. Meyer KD, Lin SC, Bernecky C, Gao Y, Taatjes DJ. p53 activates transcription by directing structural shifts in Mediator. Nat Struct Mol Biol 2010;17:753–60.
16. Kagey MH, Newman JJ, Bilodeau S, Zhan Y, Orlando DA, van Berkum NL, et al. Mediator and cohesin connect gene expression and chromatin architecture. Nature 2010;467:430–5.
17. Dobi, K. C. & Winston, F. Analysis of transcriptional activation at a distance in *Saccharomyces cerevisiae. Mol. Cell. Biol.* **27**, 5575–5586 (2007).
18. Bartolomei, M. S., Halden, N. F., Cullen, C. R. & Corden, J. L. Genetic analysis of the repetitive carboxyl-terminal domain of the largest subunit of mouse RNA polymerase II. *Mol. Cell. Biol.* **8**, 330–339 (1988).
19. York, Brian, and Bert W. O'Malley. "Steroid Receptor Coactivator (SRC) Family: Masters Of Systems Biology". *Journal Of Biological Chemistry*, vol 285, no. 50,

2010, pp. 38743-38750. *American Society For Biochemistry & Molecular Biology (ASBMB)*, doi:10.1074/jbc.r110.193367.

20. Vo, Ngan, and Richard H. Goodman. "CREB-Binding Protein And P300 In Transcriptional Regulation". *Journal Of Biological Chemistry*, vol 276, no. 17, 2001, pp. 13505-13508. *American Society For Biochemistry & Molecular Biology (ASBMB)*, doi:10.1074/jbc.r000025200.
21. Bulynko, Yaroslava A., and Bert W. O'Malley. "Nuclear Receptor Coactivators: Structural And Functional Biochemistry". *Biochemistry*, vol 50, no. 3, 2011, pp. 313-328. *American Chemical Society (ACS)*, doi:10.1021/bi101762x.
22. C.X. Yuan, M. Ito, J.D. Fondell, Z.Y. Fu, R.G. RoederThe TRAP220 component of a thyroid hormone receptor- associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion Proceedings of the National Academy of Sciences of the United States of America, 95 (1998), pp. 7939-7944
23. K. Ge, M. Guermah, C.X. Yuan, M. Ito, A.E. Wallberg, B.M. Spiegelman, *et al.*Transcription coactivator TRAP220 is required for PPAR gamma 2-stimulated adipogenesis Nature, 417 (2002), pp. 563-567
24. Q. Wang, D. Sharma, Y. Ren, J.D. FondellA coregulatory role for the TRAP-mediator complex in androgen receptor-mediated gene expression The Journal of Biological Chemistry, 277 (2002), pp. 42852-42858
25. R. Rana, S. Surapureddi, W. Kam, S. Ferguson, J.A. GoldsteinMed25 is required for RNA polymerase II recruitment to specific promoters, thus regulating xenobiotic and lipid metabolism in human liver Molecular and Cellular Biology, 31 (2011), pp. 466-481
26. Ly, Diana et al. "An International Comparison Of Male And Female Breast Cancer Incidence Rates". *International Journal Of Cancer*, vol 132, no. 8, 2012, pp. 1918-1926. *Wiley*, doi:10.1002/ijc.27841.
27. Shyamala, G. "Roles Of Estrogen And Progesterone In Normal Mammary Gland Development". *Trends In Endocrinology & Metabolism*, vol 8, no. 1, 1997, pp. 34-39. *Elsevier BV*, doi:10.1016/s1043-2760(96)00207-x.

28. "Influence Of Estrogen Plus Progestin On Breast Cancer And Mammography In Healthy Postmenopausal Women". Vol 102, no. 4, 2003, p. 869. *Ovid Technologies (Wolters Kluwer Health)*, doi:10.1097/00006250-200310000-00032.
29. Gabrielson, Marike et al. "Association Of Reproductive History With Breast Tissue Characteristics And Receptor Status In The Normal Breast". *Breast Cancer Research And Treatment*, 2018. *Springer Nature*, doi:10.1007/s10549-018-4768-0.
30. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Lonning PE, Borresen-Dale AL. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98:10869–10874.
31. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, E LP, Børresen-Dale AL, Brown PO, Botstein D. Molecular portraits of human breast tumors. Nature. 2000;406:747–752.
32. Brufsky, Adam M. "Long-Term Management Of Patients With Hormone Receptor-Positive Metastatic Breast Cancer: Concepts For Sequential And Combination Endocrine-Based Therapies". *Cancer Treatment Reviews*, vol 59, 2017, pp. 22-32. *Elsevier BV*, doi:10.1016/j.ctrv.2017.06.004.
33. Hilton, H., Clarke, C., & Graham, J. (2018). Estrogen and progesterone signalling in the normal breast and its implications for cancer development. *Molecular And Cellular Endocrinology*, *466*, 2-14. doi: 10.1016/j.mce.2017.08.011
34. Marino, M., Galluzzo, P., & Ascenzi, P. (2006). Estrogen Signaling Multiple Pathways to Impact Gene Transcription. *Current Genomics*, *7*(8), 497-508. doi: 10.2174/138920206779315737
35. Cui, J., Shen, Y., & Li, R. (2013). Estrogen synthesis and signaling pathways during aging: from periphery to brain. *Trends In Molecular Medicine*, *19*(3), 197-209. doi: 10.1016/j.molmed.2012.12.007

36. Hall, J., Couse, J., & Korach, K. (2001). The Multifaceted Mechanisms of Estradiol and Estrogen Receptor Signaling. *Journal Of Biological Chemistry*, *276*(40), 36869-36872. doi: 10.1074/jbc.r100029200
37. Dhamad, A., Zhou, Z., Zhou, J., & Du, Y. (2016). Systematic Proteomic Identification of the Heat Shock Proteins (Hsp) that Interact with Estrogen Receptor Alpha (ERα) and Biochemical Characterization of the ERα-Hsp70 Interaction. *PLOS ONE*, *11*(8), e0160312. doi: 10.1371/journal.pone.0160312
38. Yi, P., Wang, Z., Feng, Q., Pintilie, G., Foulds, C., & Lanz, R. et al. (2015). Structure of a Biologically Active Estrogen Receptor-Coactivator Complex on DNA. *Molecular Cell*, *57*(6), 1047-1058. doi: 10.1016/j.molcel.2015.01.025
39. Kang, Y. K. et al. "The TRAP/Mediator Coactivator Complex Interacts Directly With Estrogen Receptors And Through The TRAP220 Subunit And Directly Enhances Estrogen Receptor Function In Vitro". *Proceedings Of The National Academy Of Sciences*, vol 99, no. 5, 2002, pp. 2642-2647. *Proceedings Of The National Academy Of Sciences*, doi:10.1073/pnas.261715899
40. Zhang, Xiaoting et al. "MED1/TRAP220 Exists Predominantly In A TRAP/ Mediator Subpopulation Enriched In RNA Polymerase II And Is Required For ER-Mediated Transcription". *Molecular Cell*, vol 19, no. 1, 2005, pp. 89-100. *Elsevier BV*, doi:10.1016/j.molcel.2005.05.015.
41. Knuesel, M. T. et al. "The Human CDK8 Subcomplex Is A Molecular Switch That Controls Mediator Coactivator Function". *Genes & Development*, vol 23, no. 4, 2009, pp. 439-451. *Cold Spring Harbor Laboratory*, doi:10.1101/gad.1767009.
42. Elmlund, H. et al. "The Cyclin-Dependent Kinase 8 Module Sterically Blocks Mediator Interactions With RNA Polymerase II". *Proceedings Of The National Academy Of Sciences*, vol 103, no. 43, 2006, pp. 15788-15793. *Proceedings Of The National Academy Of Sciences*, doi:10.1073/pnas.0607483103.
43. Foulds, Charles E. et al. "Proteomic Analysis Of Coregulators Bound To Erα On DNA And Nucleosomes Reveals Coregulator Dynamics". *Molecular Cell*, vol 51, no. 2, 2013, pp. 185-199. *Elsevier BV*, doi:10.1016/j.molcel.2013.06.007.
44. McDermott, Martina S.J. et al. "Inhibition Of CDK8 Mediator Kinase Suppresses Estrogen Dependent Transcription And The Growth Of Estrogen Receptor

Positive Breast Cancer". *Oncotarget*, vol 8, no. 8, 2017. *Impact Journals, LLC*, doi:10.18632/oncotarget.14894.

45. Mohammed, Hisham et al. "Endogenous Purification Reveals GREB1 As A Key Estrogen Receptor Regulatory Factor". *Cell Reports*, vol 3, no. 2, 2013, pp. 342-349. *Elsevier BV*, doi:10.1016/j.celrep.2013.01.010.
46. Poss, Zachary C. et al. "The Mediator Complex And Transcription Regulation". *Critical Reviews In Biochemistry And Molecular Biology*, vol 48, no. 6, 2013, pp. 575-608. *Informa UK Limited*, doi:10.3109/10409238.2013.840259.
47. Takimoto GS, Graham JD, Jackson TA, et al. Tamoxifen resistant breast cancer: coregulators determine the direction of transcription by antagonist-occupied steroid receptors. J Steroid Biochem Mol Biol 1999;69:45–50.
48. Chang, Bo-Yoon et al. "The Effect Of Selective Estrogen Receptor Modulators (Serms) On The Tamoxifen Resistant Breast Cancer Cells". *Toxicological Research*, vol 27, no. 2, 2011, pp. 85-93. *The Korean Society Of Toxicology*, doi:10.5487/tr.2011.27.2.085.
49. EBCTCG.Tamoxifen for early breast cancer: an overviewof the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet 1998;351:1451–67.
50. Cui, J. et al. "Cross-Talk Between HER2 And MED1 Regulates Tamoxifen Resistance Of Human Breast Cancer Cells". *Cancer Research*, vol 72, no. 21, 2012, pp. 5625-5634. *American Association For Cancer Research (AACR)*, doi:10.1158/0008-5472.can-12-1305.
51. Nagalingam, Arumugam et al. "Med1 Plays A Critical Role In The Development Of Tamoxifen Resistance". *Carcinogenesis*, vol 33, no. 4, 2012, pp. 918-930. *Oxford University Press (OUP)*, doi:10.1093/carcin/bgs105.
52. Robinson CV, Sali A, Baumeister W (2007) The molecular sociology of the cell. Nature 450(7172):973–982. doi:10.1038/nature06523
53. Mesa P, Deniaud A, Montoya G, Schaffi tzel C (2013) Directly from the source: endogenous preparations of molecular machines. Curr Opin Struct Biol 23(3):319–325. doi:10.1016/j.sbi.2013.01.005

54. Tolia, Niraj H, and Leemor Joshua-Tor. "Strategies For Protein Coexpression In Escherichia Coli". *Nature Methods*, vol 3, no. 1, 2006, pp. 55-64. *Springer Nature*, doi:10.1038/nmeth0106-55.
55. Rosano, GermÃ¡n L., and Eduardo A. Ceccarelli. "Recombinant Protein Expression In Escherichia Coli: Advances And Challenges". *Frontiers In Microbiology*, vol 5, 2014. *Frontiers Media SA*, doi:10.3389/fmicb.2014.00172.
56. Smith, G E et al. "Production Of Human Beta Interferon In Insect Cells Infected With A Baculovirus Expression Vector.". *Molecular And Cellular Biology*, vol 3, no. 12, 1983, pp. 2156-2165. *American Society For Microbiology*, doi:10.1128/mcb.3.12.2156.
57. Kitts, Paul A. et al. "Linearization Of Baculovirus DNA Enhances The Recovery Of Recombinant Virus Expression Vectors". *Nucleic Acids Research*, vol 18, no. 19, 1990, pp. 5667-5672. *Oxford University Press (OUP)*, doi:10.1093/nar/18.19.5667.
58. Berger, Imre et al. "Baculovirus Expression System For Heterologous Multiprotein Complexes". *Nature Biotechnology*, vol 22, no. 12, 2004, pp. 1583-1587. *Springer Nature*, doi:10.1038/nbt1036.
59. Winuthayanon, W. et al. "Uterine Epithelial Estrogen Receptor Is Dispensable For Proliferation But Essential For Complete Biological And Biochemical Responses". *Proceedings Of The National Academy Of Sciences*, vol 107, no. 45, 2010, pp. 19272-19277. *Proceedings Of The National Academy Of Sciences*, doi:10.1073/pnas.1013226107.
60. McKenna, Neil J., and Bert W. O'Malley. "Combinatorial Control Of Gene Expression By Nuclear Receptors And Coregulators". *Cell*, vol 108, no. 4, 2002, pp. 465-474. *Elsevier BV*, doi:10.1016/s0092-8674(02)00641-4.
61. Acevedo, M. L., and W. L. Kraus. "Mediator And P300/CBP-Steroid Receptor Coactivator Complexes Have Distinct Roles, But Function Synergistically, During Estrogen Receptor -Dependent Transcription With Chromatin Templates". *Molecular And Cellular Biology*, vol 23, no. 1, 2003, pp. 335-348. *American Society For Microbiology*, doi:10.1128/mcb.23.1.335-348.2003.

62. Allen, Benjamin L., and Dylan J. Taatjes. "The Mediator Complex: A Central Integrator Of Transcription". *Nature Reviews Molecular Cell Biology*, vol 16, no. 3, 2015, pp. 155-166. *Springer Nature*, doi:10.1038/nrm3951.
63. Malik, Sohail, and Robert G. Roeder. "The Metazoan Mediator Co-Activator Complex As An Integrative Hub For Transcriptional Regulation". *Nature Reviews Genetics*, vol 11, no. 11, 2010, pp. 761-772. *Springer Nature*, doi:10.1038/nrg2901.
64. Tecalco-Cruz, Angeles C. et al. "Nucleo-Cytoplasmic Transport Of Estrogen Receptor Alpha In Breast Cancer Cells". *Cellular Signalling*, vol 34, 2017, pp. 121-132. *Elsevier BV*, doi:10.1016/j.cellsig.2017.03.011.
65. Yuan CX, Ito M, Fondell JD, Fu ZY, Roeder RG. The TRAP220 component of a thyroid hormone receptor- associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion. Proc Natl Acad Sci U S A. 1998;95:7939–7944.
66. Ito M, Yuan CX, Malik S, Gu W, Fondell JD, Yamamura S, Fu ZY, Zhang X, Qin J, Roeder RG. Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. Mol Cell. 1999;3:361–370.
67. Kagey, Michael H. et al. "Mediator And Cohesin Connect Gene Expression And Chromatin Architecture". *Nature*, vol 467, no. 7314, 2010, pp. 430-435. *Springer Nature*, doi:10.1038/nature09380.
68. Schiff R1, Massarweh S, Shou J, Osborne CK. “Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response”. Clinical Cancer Research, 2003 Jan;9(1 Pt 2):447S-54S.
69. Jiang, P. et al. "Key Roles For MED1 Lxxll Motifs In Pubertal Mammary Gland Development And Luminal-Cell Differentiation". *Proceedings Of The National Academy Of Sciences*, vol 107, no. 15, 2010, pp. 6765-6770. *Proceedings Of The National Academy Of Sciences*, doi:10.1073/pnas.1001814107.
70. Nishikawa, Joy L. et al. "Inhibiting Fungal Multidrug Resistance By Disrupting An Activator–Mediator Interaction". *Nature*, vol 530, no. 7591, 2016, pp. 485-489. *Springer Nature*, doi:10.1038/nature16963.

71. Kornberg, Roger D. "The Eukaryotic Gene Transcription Machinery". *Biological Chemistry*, vol 382, no. 8, 2001. *Walter De Gruyter Gmbh*, doi:10.1515/bc.2001.140.
72. Robinson, Philip J. et al. "Structure Of A Complete Mediator-RNA Polymerase II Pre-Initiation Complex". *Cell*, vol 166, no. 6, 2016, pp. 1411-1422.e16. *Elsevier BV*, doi:10.1016/j.cell.2016.08.050.
73. Malik, S. et al. "Structural And Functional Characterization Of PC2 And RNA Polymerase II-Associated Subpopulations Of Metazoan Mediator". *Molecular And Cellular Biology*, vol 25, no. 6, 2005, pp. 2117-2129. *American Society For Microbiology*, doi:10.1128/mcb.25.6.2117-2129.2005.
74. Sato, Shigeo et al. "A Set Of Consensus Mammalian Mediator Subunits Identified By Multidimensional Protein Identification Technology". *Molecular Cell*, vol 14, no. 5, 2004, pp. 685-691. *Elsevier BV*, doi:10.1016/j.molcel.2004.05.006.
75. Meka, H. (2005). Crystal structure and RNA binding of the Rpb4/Rpb7 subunits of human RNA polymerase II. *Nucleic Acids Research*, 33(19), pp.6435-6444.
76. Wang, W., Yao, X., Huang, Y., Hu, X., Liu, R., Hou, D., Chen, R. and Wang, G. (2013). Mediator MED23 regulates basal transcription in vivo via an interaction with P-TEFb. *Transcription*, 4(1), pp.39-51.
77. Yang, F., Vought, B., Satterlee, J., Walker, A., Jim Sun, Z., Watts, J., DeBeaumont, R., Mako Saito, R., Hyberts, S., Yang, S., Macol, C., Iyer, L., Tjian, R., van den Heuvel, S., Hart, A., Wagner, G. and Näär, A. (2006). An ARC/Mediator subunit required for SREBP control of cholesterol and lipid homeostasis. *Nature*, 442(7103), pp.700-704.
78. R. Kumar and E. B. Thompson, "The structure of the nuclear hormone receptors," Steroids, vol. 64, no. 5, pp. 310–319, 1999.
79. Beato, M. (1989). Gene regulation by steroid hormones. *Cell*, 56(3), pp.335-344.
80. Y. L. Wu, X. Yang, Z. Ren et al., "Structural basis for an unexpected mode of SERM-mediated ER antagonism," Molecular Cell, vol. 18, no. 4, pp. 413–424, 2005.
81. M. M. Montano, V. Muller, A. Trobaugh, and B. S. Katzenellenbogen, "The carboxy-terminal F domain of the human estrogen receptor: role in the

transcriptional activity of the receptor and the effectiveness of antiestrogens as estrogen antagonists," Molecular Endocrinology, vol. 9, no. 7, pp. 814–825, 1995.

82. A. Koide, C. Zhao, M. Naganuma et al., "Identification of regions within the F domain of the human estrogen receptor alpha that are important for modulating transactivation and protein-protein interactions," Molecular Endocrinology, vol. 21, no. 4, pp. 829–842, 2007.

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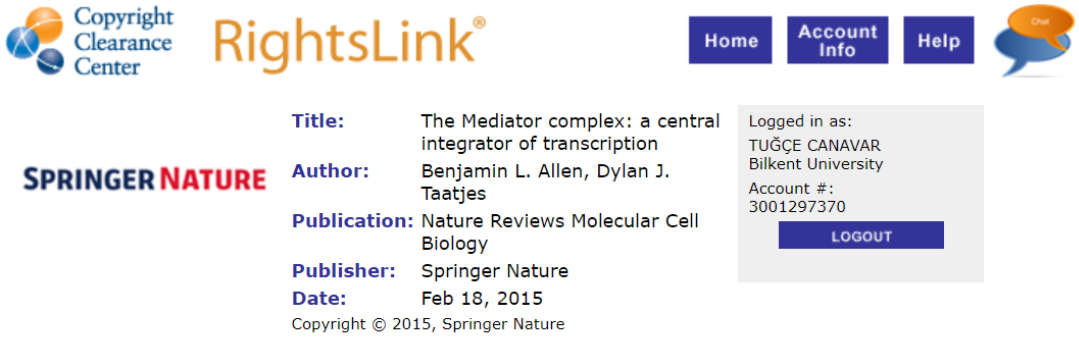

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