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Molecular and Biochemical Studies of Several Novel Estrogen Receptor Alpha-Interacting Proteins in Breast Cancer Cells

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Abstract

Breast cancer is the second leading cause of cancer-related death in women, and approximately 70% of incidences are estrogen receptor (ER)-positive breast cancer. ERα and its interacting proteins play a key role in the development and progression of breast cancer. However, how ERα regulates its target gene expression and hence cell proliferation is not fully understood. To enhance our understanding of the molecular mechanism by which ERα regulates gene expression, we used a quantitative proteomic method to identify cellular proteins that interact with ERα. The first group of proteins that were identified to associate with ERα are heat shock proteins (Hsps). We identified 21 Hsps and 3 Hsp cochaperones that were associated with ERα. Co-immunoprecipitation assay demonstrated that Hsp70-1 and Hsc70, the two most abundant ERα-associated proteins, interacted with ERα in both transcriptionally active and inactive chromatin of MCF7 cells.

A novel of protein that was identified to interact with ERα is histone acetyltransferase 1 (HAT1). We showed that HAT1 physically binds ERα through the E domain of ERα, and silencing HAT1 by shRNA significantly increased the ERα-mediated transcription in MCF7 cells. Importantly, our data suggest that HAT1 regulates ERα transcriptional activity through affecting the interactions of ERα with histone proteins around the promoter region of ERα target genes in breast cancer cells**.**

We also identified and confirmed that protein arginine methyltransferase 5 (PRMT5) is a new ERα interacting partner, and PRMT5 interacts with ERα preferentially in the cytoplasm of MCF7 cells. Functionally, we found that overexpression of PRMT5 in MCF7 cells significantly decreased ERα transcriptional activity.

Finally, we demonstrated that chromatin target of PRMT1 (CHTOP) directly binds to ERα through the E domain of ERα. We found that knockout of CHTOP by CRISPR-Cas9

significantly decreased ERα transcriptional activity, and the effect is potentially through decreasing protein levels of MEP50, an ERα coactivator.

In summary, we identified and characterized several novel ERα-interacting proteins that play significant roles in regulating ERα transcriptional activities. Our results provide new insight into the molecular mechanisms by which ERα controls its target gene expression and regulates cell proliferation in ERα-positive cells.

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Abbreviations

AF1	Activation function 1
AF2	Activation function 2
AKT	Protein Kinase B
AMPK	AMP-activated protein kinase
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
BSA	Bovine serum albumin
cAMP	cyclin AMP
CHTOP	Chromatin target of PRMT1
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats-CRISPR associated protein 9
DBD	DNA binding domain
DMEM	Dulbecco modified eagle medium
DTT	Dithiothreitol
E2	17β- estradiol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMSA	Electrophoretic mobility shift assay
ERE	Estrogen response element
ERs	Estrogen receptors
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FBS	Fetal bovine serum
H3K14	Histone 3 lysine 14
H4K12	Histone 4 lysine 12
H4K5	Histone 4 lysine 5
HAT1	Histone acetyltransferase 1
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
Hsp70	70 kDa heat shock protein
Hsp90	90 kDa heat shock protein
IFNAR	IFN-α/β receptor
IGFR1	Insulin-like growth factor 1 receptor
IgG	Immunoglobulin G
IP	Immunoprecipitation
kDa	Kilo Dalton
LBD	Ligand binding domain
LXXLL	Leucine rich motif
MAPK	Mitogen-activated protein kinase
MEP50	Methylosome protein 50
MNAR	Modulator of nongenomic activity of estrogen receptor

MS	Mass spectrometry
NCoR	Nuclear receptor corepressor
NLS	Nuclear localization signal
PBS	Phosphate buffered saline
PELP1	Proline, glutamate and leucine rich protein 1
PI3K	Phosphoinositide 3-kinase
pICln	Chloride nucleotide-sensitive channel 1A
PRMT1	Protein arginine methyltransferase 1
PRMT5	Protein arginine methyltransferase 5
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real time- polymerase chain reaction
RbAp46	Nuclear receptor-associating protein of approximately 46 kDa
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERD	Selective estrogen receptor down-regulator
SERM	Selective estrogen-receptor modulator
shRNA	Small hairpin RNA
SILAC	Stable isotope labeling with amino acids in cell culture
siRNA	Small inhibitory RNA
SRC1	Steroid receptor coactivator 1
TAM	Tamoxifen
Tris	Tris-hydroxymethyl-aminomethane

List of published papers

Chapter2. Dhamad AE, 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. https://doi.org/10.1371/journal.pone.0160312

Introduction

1.1 Breast cancer

Breast cancer is the most common cancer in American women, after lung and bronchial cancers [1]. In 2017, it is estimated that 252,710 invasive and 63,410 noninvasive new cases of breast cancers will be diagnosed in women. Although recent incidence rates for breast cancer in women have decreased due to increased awareness, earlier detection, and better treatment, the survival rate is still low. One in 37 (about 2.7%) women who is diagnosed with breast cancer have high chance to die, putting breast cancer as the second leading cause of cancer death in women after lung cancer [2].

The risk to have breast cancer is related to many factors which can be mainly divided into non-controlled risk factors, including but not limited to the gender, age, and genetic inheritance [3-5]. Although male breast cancer is very rare, less than 1% of all breast carcinoma, recent studies have shown that the incidence of male breast cancer has constantly increased. In the United States, 900 males were estimated to have breast cancer in 1991, and this number was doubled to be 2240 males by 2014 [6]. Additional studies reported that younger females have less risk of having breast cancer than older females. Seven percent of breast cancer cases have been estimated to occur at an age under 40 years, and a female who has a first-degree relative with breast cancer has double the risk of having breast cancer [7]. Approximately 5 to 10% of breast cancer incidences were linked to bypass oncogene mutations, particularly in breast cancer 1 and breast cancer 2 genes (BRCA1 and BRCA2, respectively) from parents [8, 9]. Other risk factors for breast cancer that cannot be controlled are an unhealthy diet, being overweight, lack of exercise, excessive alcohol consumption, cigarette smoking, having a full-term pregnancy, and breastfeeding especially for longer than 12 months [7, 10-13].

In addition to histopathology, grade, stage, and molecular classification, breast cancer cells can be classified depending upon whether the cells can express one or more of these receptors: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HER2) [14]. This classification has high prognostic and therapeutic value in relation to breast cancer. It has been reported that ER-positive breast cancer cells, expressing ER, constitute approximately 70% of breast cancer, have a better prognosis, and can be treated with hormone therapy drug like tamoxifen and anastrozole [15-17]. On the other hand, triple-negative breast cancers (TNBC), expressing none of the above mentioned receptors (ER-/PR-/HER2-), constitute around 10-15% of breast cancer and are generally more aggressive than other types of breast cancers [14, 18, 19].

1.2 Estrogen receptors' structure and functions

1.2.1 ER structure

ERs contain two subtypes, ERα and ERβ [20], which are encoded by two different genes, ESR1 and ESR2 [21]. ERα and ERβ proteins are almost identical in structure, but different in ligand affinities and expression levels [22, 23]. ERα, like other nuclear receptors, consists five structural and functional domains: The N-terminal AB domain, which contains the transactivation domain 1 (AF-1), the DNA binding domain (DBD; C), and the hinge domain (D), the ligand binding domain (LBD; E) and the C-terminal F domain [24]. The E and F domains constitute the transactivation domain 2 (AF-2) [25]. While the AF-1 domain is necessary for ligand-independent interaction of the ERs with coactivators, the AF-2 domain facilitates the ligand-dependent interaction of the ERs with regulators [26, 27]. Both receptors (ERα and ERβ) share a high degree of homology in their amino acid sequences especially in the most conservative domains, DBD (97%) and LBD (56%)[23]. This allows these receptors to

bind similar ligands and interact with identical response elements [28]. Thus, it was hypothesized that ERβ is an altered copy of ERα; however, extensive research reveals that it has distinct expressions and functions. ERα is predominantly expressed in the breast, bone, and uterus, while ERβ is mainly expressed in the prostate, ovary, testis, lung, spleen, and thymus [29].

1.2.2 ERs functions: genomic and non-genomic action

1.2.2.1 Genomic action

Under this category, ER pathways can be divided into two types: classical and non-classical. In the classical pathway, ER is an inactive and monomeric molecule with a short half-life [30], until it binds to the ligand (E2). At this point, ERα dissociates from the chaperone protein, such as the heat shock protein 90 and 70, Hsp90 and Hsp70, respectively [31, 32], dimerizes, and translocates to the nucleus. In the nucleus, ERs directly bind to the estrogen response element (ERE; GGTCAnnnTGACC) of the target genes [33] and recruits coregulators (coactivators or corepressors) [24]. In the non-classical pathway, ERs regulate gene transcription in the absence of ERE sequence through binding to other transcriptional factors, such as specific protein 1(Sp1) and activator protein 1(Ap1) or nuclear factor-kappa B (NFkB) which have low affinity to interact with ERE [34, 35]. This pathway explains how ERs can interact and regulate gene promoters missing the ERE sequence, which represent 30% of total E2-target genes [35]. In addition, there are some genes with promoters that have an ERE-like sequence, half-ERE, or many copies of ERE which are considered more complex than the standard ERE-promoter and might require both classical and non-classical actions during their transcriptional regulation [36, 37].

1.2.2.2 Non-genomic action

Accumulated evidence shows that cells respond to estrogen very rapidly within a timeframe of action considered too short to take place through classical genomic action. This evidence suggests that this type of action must occur through a different pathway classified as a non-genomic action. Many studies demonstrated that the estrogen and ER subpopulation that localize at the cytoplasm and plasma membrane mediate the non-genomic action [38, 39]. Upon binding to estrogen, ER-plasma membrane will shortly activate the internal signaling pathways, such as MAPK (mitogen-activated protein kinase) and PI3K (Phosphoinositide 3-kinase) [40, 41]. This activation occurs via interaction of ER with either adapter proteins like proline-, glutamic acid- and leucine-rich protein 1(PELP1) or growth factor receptors including EGFR (epidermal growth factor receptor), HER2 and IGFR1 (insulin-like growth factor 1 receptor) [42, 43]. It is known that non-genomic action modulates several transcriptional factors including ERα itself and its coactivators, involves in endocrine therapy resistance, and affects a function of many target cells and tissues [44].

1.3 Estrogen receptor alpha

1.3.1 ERα transcriptional regulation

Depending on the ERα expression, breast cancer cells are classified into a positive or negative. In the positive breast cancer cells, ERα is expressed and linked to cell growth, proliferation, and hormone resistance. Many studies have shown that ERα expression is regulated by various factors including ERα enhancer region. The ERα enhancer element at -3.7kb, located on chromosome 6q25.1, plays an important role in ERα expression [45]. Upon binding by the Ap1 transcription factor, the enhancer promotes ERα expression in the positive breast cancer cells [45]. Additional study showed that ERα expression was decreased when the

regulatory sequence of the ESR1 locus was directly bound by zinc finger protein SNAI1 (Snail) [46].

Crosstalk with growth factor receptor signaling is another mechanism by which ERα loses its expression. In the ERα-negative breast cancer, cells express elevated levels of growth factor receptors, such as HER1, HER2, and EGFR [47]. It is reported that overexpression of HER1 and HER2 in the ERα-positive breast cancer cells (MCF7) can cause a reduction in ERα expression and lead to estrogen resistance. The reduction in the ERα expression was not because of ligand-independent activation of ERα, but more likely related to the MAPK and PI3K/AKT signaling pathway activations. When ERα-negative breast cancer cells were treated with MAPK inhibitors, ERα expression and antiestrogen sensitivity were restored in the cells [48, 49]. Additionally, it has been shown that HER2 was overexpressed in about 20% of breast cancers which is positively correlated with activation of MAPK signaling pathway and negatively with ER expression [50]. These findings led to the use of ERα and growth factor signaling inhibitors as a therapeutic approach to treat estrogen-resistant breast cancer. Indeed, several clinical trials showed that some patients who were treated with trastuzumab, anti-HER2, restored ERα expression [51].

Mutation and deletion are other factors that regulate ERα expression. About 19 variant point mutations have been identified in ERα some of which significantly affect ERα like a stop mutation at AA437, and K303R and Y537N that are related to hormone resistance [52-54]. Moreover, it is expected that homologous deletion of ERα region might diminish ERα expression, but there is no convincing evidence supporting this hypothesis [55].

Epigenetic modulations such as methylation are well known as effective factors that can influence protein expression, and ER is no exception. It has been shown that the

hypermethylation of CpG islands on the ER promoter can lead to a reduction in ER expression which is one way to explain why ER-negative breast cancer cells do not express ER [56-58]. Interestingly, removing methyl groups from the CpG islands on the ER promoter by demethylating agent 5-aza-2'-deoxycytidine results in re-expression of ER [59]. In addition, some studies found that twist-related protein 1 (TWIST1) expression was increased during cancer progression and negatively correlated with ERα expression [60]. After binding ERα promoter, TWIST1 enhances *de novo* methylation by recruiting DNA methyltransferase 3B which causes ERα loss and hormone resistance [61].

Acetylation is another epigenetic modulation that affects the ER expression. It is reported that histone acetyltransferases (HATs) enhance ER expression through acetylating histones at ER promoter which cause chromatin relaxation and make ER promoter more accessible to the transcriptional machinery [62, 63]. On the other hand, removal of acetyl groups from histones by histone deacetylases (HDAC) leads to transcriptional repression. Other studies have shown that ER expression was highly increased after HDAC was inhibited [56].

1.3.2 ERα Posttranslational Modifications

Like other proteins, ERα is subjected to various posttranslational modifications that influence its activity and stability. Up to now, at least six residues of ERα are known to be phosphorylated which are eventually involved in ligand-independent receptor activation and endocrine-therapeutic resistance. It is reported that ERα-S118 and ERα-S167 residues are phosphorylated via MAPK and PI3K/Akt signaling pathways and related to low-grade tumor and a positive response to hormone therapy [64, 65]. Additional study showed that COUP transcription factor 1(COUP-TF1), orphan nuclear receptor, can interact with ERα and phosphorylate ERα-S118 residue [66]. Many clinical and in-vitro studies demonstrated that the

phosphorylation of ERα on S305 and Y537 residues are linked to a tamoxifen resistance and poor clinical outcomes [54, 67].

Methylation and acetylation are other ERα posttranslational modifications. A Recent study showed that arginine methyltransferase 1(PRMT1) interacts with ERα and methylates ERα-R260 residue [68]. This modification promotes ERα to interact with PI3k and proto-oncogene tyrosine-protein kinase (SRC) in the cytoplasm and activate Akt-signaling pathway which is linked to ERα non-genomic action and endocrine resistance. ERα-K303 residue was found to be acetylated by HATs family members, such as CBP/p300 and linked to TAM resistance [69, 70].

1.3.3 ERα coregulators and interacting partners

Increasing evidence suggest that the modulations in ERα-coregulators' expression, stability, and activity would affect tumor cells respond to estrogen and tumor progression [71]. A series of ERα coregulators have been characterized and classified into coactivators and corepressors, increasing and decreasing ERα transcriptional activity, respectively. The first ER coactivators were identified by Halachmi et al., 1994 [72] and termed as ER-associated proteins 140 and 160 (ERAP140 and ERAP160, respectively). These coactivators are hormone-dependent and require AF-2 domain for interaction. Since then at least twenty ER coactivators have been identified, such as transcriptional intermediary factor 1 (TIF-1), human receptor potentiating factor 1 (hRPF1), thyroid hormone receptor associated proteins (TRAPs/DRIPs) [73]. Like other ER coactivators, SRC-1 (steroid receptor coactivator-1) interacts with ERα through a highly conserved LXXLL motif, called the nuclear receptor (NR) box, where L and X are leucine and any amino acid, respectively [26, 74]. It is well known that CBP/p300, histone

acetyltransferases, act as ERα coactivators via recruiting other proteins that eventually promote ERα transcription [75, 76].

About six ERα corepressors have been identified and with ERβ corepressors termed repressor of estrogen receptor activity (REA) [77, 78]. The nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) probably are the most well-known corepressors [79]. They interact with LBD of ERs through two NR-interacting domains (CoRNR boxes) which are similar to the NR boxes (LXXLL motifs) in the coactivators [80]. Both NCoR and SMRT bind to other cofactors, such as mSin3, a protein that associates with HDACs, to facilitate their repression activity on ERs [81]. BRCA1 is another ERα corepressor that directly binds to ERα C-terminus and inhibits ERα hormonal-transcriptional activity [82].

ERα-interacting proteins also play important roles in ERα functions. It is well understood that heat shock proteins like Hsp90 associates with ERα and regulates ER-mediated cell proliferation. Upon interacting with Hsp90-based chaperone protein complex, ERα is in a ligand-binding competent conformation status (inactive form). When binding estrogen, ERα dissociates from Hsp90, dimerizes, binds to EREs, and triggers the transcription of its target genes through recruiting coactivators [83-85]. Additionally, the interaction between ER and Hsps members such as Hsp70 and Hsp90 facilitates the receptor-ligand transportation and modulates the receptor affinity. It has been shown that ER associated with Hsps complex has a high affinity for a ligand and low affinity toward EREs [86, 87].

1.4 ERs as a therapeutic target in breast cancer

Although ERα is involved in breast cancer development and progression, its expression is beneficial in terms of treating breast cancer. Breast cancer cells that express ERα are more

sensitive to endocrine therapies than ER-negative breast cancer cells. Thus, blocking ERα signaling pathways is the most common approach used to treat ERα-positive breast cancer through two main strategies. The first strategy is directly targeting ERα with a selective estrogen receptor modulator (SERM) like TAM and toremifene. After binding with TAM, ERα undergoes several cascade steps including conformational change and recruitment of corepressors instead of coactivators that lead to represses the ERα-mediated gene expression. On the other hand, TAM acts as an estrogen agonist in certain tissues, such as the uterus and bones [88]. A selective estrogen receptor down-regulator (SERD) such as fulvestrant is another way to block ERα transcriptional activity. Fulvestrant is generally used after tamoxifen treatment to treat metastatic breast cancer[89].

Stopping estrogen production by aromatase inhibitors (AI) or ovarian ablation is the second strategy to hinder ERα signaling pathways. AI inhibits aromatase, an enzyme that is required for biosynthesis of estrogens from fat tissues and commonly used to treat premenopausal women because they produce a small amount of estrogen from fat tissues [90].

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Chapter1. Systematic proteomic identification of the heat shock proteins (Hsp) that interact with estrogen receptor alpha (ERα) and biochemical characterization of the ERα-Hsp70 interaction

Abstract

Heat shock proteins (Hsps) are known to associate with estrogen receptors (ER) and regulate ER-mediated cell proliferation. Historically, the studies in this area have focused on Hsp90. However, some critical aspects of the Hsp-ERα interactions remain unclear. For example, we do not know which Hsps are the major or minor ERα interactants and whether or not different Hsp isoforms associate equally with ERα. In the present study, through a quantitative proteomic method, we found that 21 Hsps and 3 Hsp cochaperones were associated with ERα in human 293T cells that were cultured in a medium containing necessary elements for cell proliferation. Four Hsp70s (Hsp70-1, Hsc70, Grp75, and Grp78) were the most abundant Hsps identified to associate with ERα, followed by two Hsp90s (Hsp90α and Hsp90β) and three Hsp110s (Hsp105, HspA4, and HspA4L). Hsp90α was found to be 2-3 times more abundant than Hsp90β in the ERα-containing complexes. Among the reported Hsp cochaperones, we detected prostaglandin E synthase 3 (p23), peptidyl-prolyl cis-trans isomerase FKBP5 (FKBP51), and E3 ubiquitin-protein ligase CHIP (CHIP). Studies with the two most abundant ERα-associated Hsps, Hsp70-1 and Hsc70, using human breast cancer MCF7 cells demonstrate that the two Hsps interacted with ERα in both the cytoplasm and nucleus when the cells were cultured in a medium supplemented with fetal bovine serum and phenol red. Interestingly, the ERα-Hsp70-1/Hsc70 interactions were detected only in the cytoplasm but not in the nucleus under hormone starvation conditions, and stimulation of the starved cells with 17β-estradiol (E2) did not change this. In addition, E2-treatment weakened the ERα-Hsc70 interaction but had no effect on the ERα-Hsp70-1 interaction. Further studies showed that significant portions of Hsp70-1 and Hsc70 were associated with transcriptionally active chromatin and inactive chromatin, and the two Hsps interacted with ERα in both forms of the chromatins in MCF7 cells.

Introduction

Estrogen receptor alpha (ERα) is a nuclear transcription factor that controls the expression of estrogen responsive genes. Like other members of the steroid receptor (SR) superfamily including androgen receptor, progesterone receptor, glucocorticoid receptor and mineralocorticoid receptor, the responsiveness of ERα to its ligands such as 17β-estradiol (E2) is regulated by heat shock proteins (Hsps) and their cochaperones [1-3]. In the absence of estrogenic ligands, ERα is assembled into an Hsp90-based chaperone protein complex, which keeps ERα in a ligand-binding competent but inactive state and prevents it from binding to estrogen-response elements [4-7]. Unliganded ERα is a short-lived protein with a half-life of 4-5 h and is constantly degraded [8]. The degradation is mediated by E3 ubiquitin-protein ligase CHIP (CHIP) and through the ubiquitin-proteasome pathway [9-11]. Upon binding of its ligands, ERα dissociates from Hsp90, dimerizes, binds to the estrogen-response elements, and induces transcription of its target genes through recruiting coactivators [12, 13]. Hsp90 is essential for ERα hormone binding [6], dimer formation [12], and binding to the estrogen-response elements [14].

The Hsps are highly conserved chaperones and play important roles in protein folding, assembly, trafficking and disposition, and stress responses [15, 16]. Human Hsps are classified into six families, Hsp110 (HspH), Hsp90 (HspC), Hsp70 (HspA), Hsp40 (DNAJ), small Hsps (HspB), and chaperonin (HspD/E and CCT) [17, 18]. Hsps vary substantially from one to another with regards to function, expression, and subcellular localization. Some Hsps are constitutively expressed such as Hsc70 and Hsp90β, whereas others are induced by stresses such as Hsp70-1 and Hsp90α [19, 20]. While some Hsps are localized in specific cellular compartments, such as Grp75 in mitochondria and Grp78 in endoplasmic reticulum, most Hsps

are localized in the cytoplasm and the nucleus [21, 22]. Hsp70 and Hsp90 are among the most abundant cellular proteins, with each family accounting for 1-2% of total cellular protein under normal conditions and 2-4% under stress conditions [23-26]. Despite the fact that Hsp70 and Hsp90 are among the main conserved protective systems in cells [27], they are substantially overexpressed in cancer cells, and the upregulations correlate with poor prognosis [28, 29]. Because of the important roles of Hsp70 and Hsp90 in regulating SRs, and the "addiction" of cancer cells to higher levels of Hsps, inhibitors of Hsp70 and Hsp90 are actively being pursued for treating cancers [23, 24, 28, 30-32].

The extensive studies on the interactions of Hsps with SRs including ERα over the past five decades have established the fundamental roles of Hsps, Hsp90 in particular, in regulating SRs [33]. However, some details are missing and in some cases results are controversial. For examples, because Hsp90α and Hsp90β share 86% sequence [34], it is expected that the two isoforms have similar functions in cells. Probably because of this reason, many publications on studying the roles of Hsp90 in regulating SRs even did not mention which isoforms they used. However, while Hsp90α-knockout mice are viable, Hsp90β-knockout mice are lethal [35, 36]. As myoblasts differentiate into myotubes, Hsp90α disappears and only Hsp90β remains, and the isoform switch is essential for the differentiation [37]. These results suggest that there are critical differences between the two isoforms. Through a quantitative proteomic approach, we have comprehensively identified cellular proteins that are associated with ERα in human embryonic kidney cells 293T cells that were grown in a "complete" culture medium [a medium that was supplemented with growth stimulating factors including phenol red and fetal bovine serum (FBS)]. Here we present the results revealing the interactions between ERα and Hsps/cochaperones at the proteome level. Our proteomic data demonstrate that four Hsp70

family members, Hsp70-1, Hsc70, Grp75 and Grp78, were the predominant Hsps that were associated with ERα in 293T cells, followed by two Hsp90 family members, Hsp90α and Hsp90β, and three Hsp110 family members, Hsp105, HspA4 and HspA4L. In addition, three Hsp cochaperones, prostaglandin E synthase 3 (p23), peptidyl-prolyl cis-trans isomerase FKBP5 (FKBP51) and CHIP, were also identified to associate with ERα. Studies with the two most abundant ERα-associated Hsps, Hsp70-1 and Hsc70, suggest that these two Hsps interact with ERα in the cytoplasm and the nucleus when human breast cancer MCF7 cells were cultured in the conventional laboratory conditions. However, under hormone starvation, the ERα-Hsp70-1/Hsc70 interactions were observed only in the cytosol, and E2 stimulation did not change the pattern. The E2-treatment weakened the ERα-Hsc70 interaction but had no effect on the ERα-Hsp70-1 interaction in the cytosol. Different from Hsp90α, significant portions of Hsp70-1 and Hsc70 were found to be associated with transcriptionally active chromatin and inactive chromatin, and the two Hsps interacted with ERα in both forms of the chromatins in MCF7 cells.

Materials and Methods

Cell culture, proteome labeling, and affinity purification. We used the SILAC/AACT (stable isotope labeling with amino acids in cell culture/amino acid-coded tagging) approach to label the proteome of cells [38, 39]. A population of human embryonic kidney 293T cells (American Type Culture Collection, Manassas, VA) were cultured in labeled (Arg-$^{13}C_6$ and Lys-$^{13}C_6{}^{15}N_2$) Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific, Waltham, MA) with 10% dialyzed FBS and 1% penicillin and streptomycin for two weeks and then transiently transfected with a plasmid expressing Flag tag alone. A second population of 293T cells were cultured in unlabeled DMEM with 10% FBS and 1% penicillin and streptomycin, and transiently transfected with a plasmid expressing Flag-ERα. The two population of cells were harvested 48 h after transfection, washed with cold PBS, and incubated in 5 packed cell pellet volumes of lysis buffer I [20 mM Tris-HCl, pH 7.5, 0.5% NP-40, 1 mM EDTA, 10 nM E2, protease inhibitors (Roche, Indianapolis, IN), and phosphatase inhibitors (1 mM Na3VO4, 10 mM NaF, and 10 mM β-glycerophosphate)] on ice for 30 min. The cells were then lysed by douncing with a 15-mL glass dounce homogenizer with a tight-fitting type B pestle (Kontes Glass Co., Vineland, NJ). After adding NaCl and glycerol to final concentrations of 125 mM and 10%, respectively, the extracts were centrifuged at 20,000 g for 15 min at 4°C. The resulting pellets were resuspended in lysis buffer I supplemented with 125 mM NaCl and 10% glycerol and extracted again with sonication (Branson Digital Sonifier 450, Branson Ultrasonics Co., CT) [40]. Protein concentration of the combined and cleared supernatant was determined, and equal amounts of the labeled and unlabeled cell extracts were separately incubated with pre-washed Flag M2 resin (Sigma-Aldrich, St. Louis, MO) for 5 h at 4°C with end-to-end rotation. The beads were then washed extensively with lysis buffer I supplemented with 125 mM NaCl and 10% glycerol. The

bound proteins were eluted with elution buffer (10 mM Tris-HCl, pH 7.5, 350 mM NaCl, 1 mM EDTA, 250 mM 3X Flag peptides, and protease inhibitors). The eluates of the two affinity purifications were mixed and fractionated with a 12% SDS-PAGE gel for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Human breast cancer MCF7 cells (American Type Culture Collection, Manassas, VA) were maintained in Minimum Essential Medium α (MEM α; Thermo Fisher Scientific, Waltham, MA) with 5% FBS and 1% penicillin and streptomycin.

LC-MS/MS, database search, and data analysis. In-gel digestion, LC-MS/MS analysis, and protein identification/quantification with the Maxquant (version 1.0.13.13) and Mascot (version 2.2; Matrix Science, Boston, MA) by searching against a composite target-decoy International Protein Index (IPI) human protein database (version 3.52) were performed as described previously [41]. In this SILAC/AACT approach, because the Flag-ERα expressing cells and the Flag expressing cells were cultured in the unlabeled medium and stable-isotope-labeled medium, respectively, and the eluates from the two affinity purifications of equal amounts of the unlabeled cell extract and labeled cell extract were mixed and analyzed by LC-MS/MS, the relative intensities of the paired isotopic peaks of peptides (i.e., light/heavy ratios: L/H ratios) reflect the binding profile of the protein to ERα. Whereas the L/H ratios for the nonspecific binding proteins were around 1, the ratios for the proteins that specifically bind to ERα were significantly larger than 1 due to affinity enrichment of the proteins [42, 43]. Search results were further processed by Scaffold software (version 4.4.7; Proteome Software Inc., Portland, OR) for viewing protein and peptide identification information. In the Scaffold analysis, protein identification probability with at least two peptides was set to 99% and the peptide identification probability was set to 95%. The normalized spectral abundance factors (NcSAFs) were

calculated as described [44, 45]. The normalization was applied only to the identified Hsps and cochaperones to estimate the relative level of each protein within the identified Hsps and cochaperones that were associated with ERα [44, 45]. Spectral counts for peptides shared among the identified Hsps were counted only once, and distributed based on the number of unique spectral counts to each isoform [46].

The E2 treatment and subcellular fractionation. The MCF7 cells were cultured in the phenol-red free MEM α supplemented with 5% charcoal-treated FBS (Hyclone, Logan, UT) for 3-4 days and then treated with either 100 nM E2 or ethanol (control) for 24 h. The cells were then harvested, washed twice with cold PBS, resuspended in 5 packed cell pellet volumes of hypotonic buffer (10 mM Tris-HCl, pH 8.0, 5 mM KCl, and 1.5 mM $MgCl_2$, and protease inhibitors) supplemented with 100 nM E2 for the E2-treated cells or ethanol for the control cells. The cells were incubated on ice for 20 min. After adding phosphatase inhibitors (1 mM Na3VO4, 10 mM NaF, and 10 mM glycerophosphate) to the cell suspension, the cells were lysed by douncing 12 times with a 15-mL glass dounce homogenizer with a tight-fitting type B pestle. After centrifugation at 500 g for 10 min at 4°C, the pellet was saved and the supernatant was cleared by centrifugation at 10,000 g for 15 min at 4ºC. The cleared supernatant was supplemented with 15 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1 % SDS and 3 mM EDTA, and saved as cytosolic fraction. The pellet from the 500xg centrifugation was resuspended in hypotonic buffer and dounced 5 times. After centrifugation at 500 g for 10 min at 4°C, the pellet was washed twice with hypotonic buffer and saved as nuclei. The isolated nuclei were resuspended in lysis buffer II (25 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 3 mM EDTA, 0.1 % SDS, protease inhibitors, and phosphate inhibitors) supplemented with 100 nM E2 for the E2-treated samples or ethanol for the control samples. The nuclei were then

sonicated on ice, centrifuged at 10,000 g for 15 min at 4ºC, and the resulting supernatant was designated as nuclear fraction.

Cross-linking, immunoprecipitation (IP), and Western blotting. In-cell cross-linking was performed using the cell-permeable cross-linking reagent dithiobis (succinimidylpropionate) (DSP) (Thermo Fisher Scientific, Waltham, MA). The MCF7 cells in plates were washed twice with PBS at room temperature and incubated with 1 mM DSP in DMEM at 37°C for 15 min. After removal of the cross-linker solution, the cells were incubated with quenching solution (100 mM Tris-HCl, pH 8.0 in DMEM) at 37°C for 10 min. Quenching solution was removed, and the cells were washed twice with PBS and lysed for IPs. The IPs and Western blotting were performed as described previously [47, 48]. Antibodies used in this study were purchased from the following commercial sources: Anti-ERα, p300, and NCoR antibodies from Santa Cruz Biotech (Santa Cruz, Dallas, TX; catalog no.: Anti-ERα, sc-8002; anti-p300, sc-584; anti-NCoR, sc-1609), anti-Hsp70-1 and Hsc70 antibodies from Enzo life science (Farmingdale, NY; catalog no.: anti-Hsp70-1, ADI-SPA-810; anti-Hsc70, ADI-SPA-815), anti-Hsp90α from Epitomics (Burlingame, CA; catalog no., 3670-1)), anti-histone H3 from Cell signaling (Danvers, MA; catalog no., 9715), and anti-tubulin from Sigma-Aldrich (St. Louis, MO; catalog no., T9026). Quantification of protein bands in Western blotting was performed using ImageJ software.

Extraction of chromatin-binding protein, and transcriptionally active chromatin and inactive chromatin. Chromatin-binding protein was extracted with 0.3% SDS and 250 units/mL benzonase as described by Yang *et al*. (2014) [49]. Briefly, after MCF7 cells were resuspended in a radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, and 1 mM EDTA) supplemented with 200 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate and protease inhibitors, the

cells were homogenized by passing through a 22G needle 10 times, followed by an incubation on ice for 20 min. The chromatin was separated from the soluble protein (S) by a centrifugation at 1,000 g, and the isolated chromatin was extracted with 0.3% SDS and 250 units/mL benzonase (EMD Millipore, Billerica, MA) on ice for 10 min. The digested chromatin was centrifuged at 1,000 g, and the resulting supernatant was designated as chromatin-binding protein (CB). Transcriptionally active chromatin and inactive chromatin were extracted with different concentrations of salt according to Henikoff *et al*. (2009) and Yang *et al*. (2014) [49, 50]. Briefly, after MCF7 cells were lysed with a lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, 340 mM sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, and protease inhibitors) on ice for 8 min, cytoplasmic protein (C) was separated from the nuclei with a 1,300xg centrifugation. The washed nuclei were digested with 2,000 gel units/mL micrococcal nuclease (New England Biolabs, Ipswich, MA) in the lysis buffer described above plus 1 mM $CaCl_2$ at 37 °C for 10 min, and the reaction was stopped by 2 mM EGTA. After centrifugation at 1,300 g for 10 min at 4°C, the supernatant (nuclear soluble protein: NS) was removed and the digested nuclei were washed and first treated with 150 mM NaCl at 4°C for 2 h for extracting active chromatin (Ch1) and then with 600 mM NaCl at 4°C overnight for extracting inactive chromatin (Ch2).

Statistical Analysis. Statistical analysis was performed using one-way ANOVA (PSI-PLOT, Pearl River, NY). A p-value of <0.05 was considered significant.

Results

Identification of Hsps and their cochaperones that associate with ERα. We used a SILAC/AACT-based quantitative proteomic method to systematically identify cellular proteins that were associated with ERα [42, 43]. Through this approach, a subset of Hsps and their cochaperones were identified to associate with ERα (Table 1). Most of the Hsps and cochaperones were identified with high confidence with LC-MS/MS, which can be reflected by the very low PEP (posterior error probability) values for the identifications (Table 1).

To examine the abundance of the identified Hsps and cochaperones that were associated with ERα, we calculated NcSAF for each protein [51]. NcSAF is based on spectral counting for each protein in LC-MS/MS analysis, and a larger NcSAF value reflects the higher abundance of the protein in biological samples [44, 46, 51, 52]. The most abundant Hsps that were associated with ERα were four Hsp70 family members, Hsp70-1, Hsc70, Grp75 and Grp78, with the NcSAF values in the range of 0.08-0.286. The L/H ratios for all the identified Hsp70s varied in a narrow range of from 8 to 12, suggesting they were enriched by affinity purification similarly.

Two Hsp90 family members and three Hsp110 family members were also identified to be abundant in the ERα-containing complexes, though at significantly less levels than the four Hsp70 family members described above (Table 1). Among the 5 reported Hsp90 members [17], Hsp90α and Hsp90β, which share 86% sequence homology [34], were identified to associate with ERα. The NcSAF values for Hsp90α and Hsp90β were 0.057 and 0.022, respectively, and thus the former was 2.6-fold of that of the latter, suggesting that Hsp90α is 2-3 times more abundant than Hsp90β in the ERα-containing protein complexes. It is known that while the expression of Hsp90α is inducible, Hsp90β is constitutively expressed [53]. The L/H ratios were similar for Hsp90α and Hsp90β (6.7 and 5.5, respectively), suggesting the proportions of those

that were specifically associated with ERα to those of non-specific bindings for the two isoforms were similar. The Hsp110 members are known as nucleotide exchanger factors (NEFs) of Hsp70 and interact with Hsc70 [17, 54]. Three Hsp110 members, Hsp105, HspA4 and HspA4L, were identified to abundantly associate with ERα (Table 1). The abundances of the three Hsp110 members were comparable to those of Hsp90α and Hsp90β, with the NcSAF values in the range of 0.012-0.032. HspA4 and HspA4L were originally considered as members of Hsp70 [55], but now are classified as members of the Hsp110 family [56]. It is noteworthy that Hsp105 and HspA4L were identified with high L/H ratios, suggesting that they were highly enriched by anti-Flag antibody.

The Hsp40 (DNAJ proteins) constitutes the largest subgroup of the Hsp family, up to 50 members, in human cells. One of the major functions of Hsp40 is to couple with Hsp70 to facilitate folding of Hsp70 client proteins [27]. We identified eight Hsp40 members in this study, and all of them were identified with smaller NcSAF values compared with other identified Hsps except for DNAJC9, which was identified with an NcSAF value comparable to those for the Hsp110 members. These results suggest that the majority of Hsp40 members are not abundant in the ERα-containing complexes. Based on the fact that Hsp40 physically interacts with Hsp70 [27], it is likely that Hsp40 interacts with ERα indirectly and the interactions are mediated by Hsp70.

Multiple Hsp cochaperones, including p23, FKBP51, FKBP52, protein phosphatase 5 (PP5) and cyclophilin 40 (Cyp40), have been reported to couple with Hsp90 to facilitate the function of SRs [1, 33]. Most of these cochaperones contain tetratricopeptide repeat domains, which bind to the EEDV motif of Hsp90/Hsp70 [57], and are typically assembled into SR complexes at the final stages of assembly to form the mature, hormone-competent states of SRs

[58, 59]. Among the reported cochaperones, we identified p23, FKBP51, and CHIP but were not able to detect FKBP52, Cyp40, and PP5 (Table 1). Notably, CHIP was identified with a larger NcSAF value (0.03), which was comparable to those for the two Hsp90 family members and the three Hsp110 family members, suggesting that CHIP is also abundantly associated with ERα. CHIP has been shown to interact with ERα via its tetratricopeptide repeat domain and mediates ERα degradation through the ubiquitin-proteasome pathway in the nucleus [10, 11].

Hsp70-1 and Hsc70 interact with ERα in the cytoplasm and the nucleus. The role of Hsp90 in regulating the assembly, trafficking, and transcriptional activity of ERα has been studied extensively [1]. Compared with Hsp90, much less is known about the role of Hsp70 in regulating ERα and some results are controversial [5, 7, 60]. In this study, we found that Hsp70-1 and Hsc70 were the two most abundant Hsps that were associated with ERα (Table 1). As the first step to characterizing these important interactions, we proceeded to verify the interaction of ERα with Hsp70-1/Hsc70 using IP and Western blotting. Consisting with our proteomic data, the IP results obtained with the 293T cells ectopically expressing Flag-ERα demonstrate that Flag-ERα interacted with endogenous Hsp70-1 and Hsc70 (Fig. 1). To examine if endogenous ERα interacts with endogenous Hsp70-1/Hsc70 and determine the subcellular site where the ERα-Hsp70-1/Hsc70 interactions occur in ERα-positive breast cancer cells, we performed IPs using cytosolic and nuclear proteins of human breast cancer MCF7 cells as starting materials, respectively. The results demonstrate that anti-ERα antibody precipitated significantly more Hsp70-1 than the control IgG precipitated in both the cytosolic fractions and the nuclear fractions (Fig. 2A, top row; Fig. 2B, left panel). However, the amounts of Hsc70 precipitated by anti-ERα antibody and the control IgG were not statistically significantly. In addition, we observed large variations on Hsc70 in the IP results among different sample preparations (Fig. 2A, middle row;

Fig. 2B, left panel). The interactions between SRs and Hsps are typically transient and weak by nature [61]. To confirm the interaction of endogenous ERα with Hsc70 and to further validate the specific ERα-Hsp70-1 interaction, we used the cell-permeable cross-linking reagent DSP to treat MCF7 cells and then used whole cell lysate of the DSP-treated cells to perform IPs and Western blotting. The results demonstrate that anti-ERα antibody precipitated significantly more Hsp70-1 and Hsc70 proteins than the IgG precipitated after the cross-linking treatment (Fig. 2C). These results suggest that Hsp70-1 and Hsc70 indeed specifically interact with ERα in addition to the nonspecific interactions. We have confirmed the effectiveness of our subcellular fractionation by performing Western blot analysis using markers of the cytoplasm and the nucleus (Fig. 2B, right panel).

Hsp70-1 and Hsc70 interact with ERα in transcriptionally active and inactive chromatins. To characterize the interactions of ERα with Hsp70-1/Hsc70, we fractionated MCF7 cell extracts into soluble protein (S), chromatin-binding protein (CB) and the remaining pellet (P), and analyzed those fractions with Western blotting. The results demonstrate that significant portions of Hsp70-1 and Hsc70 were associated with chromatin and the remaining pellets (Fig. 3A). In contrast, the amount of Hsp90α associated with chromatin was neglectable and none was detected in the remaining pellet. As expected, a large portion of ERα, a transcriptional factor, was also associated with chromatin and the pellet. The analysis of a marker of chromatin-binding protein, histone H3, confirmed that the method we used for extracting chromatin-binding protein was effective (Fig. 3A). To examine how Hsp70-1 and Hsc70 are associated with chromatin, we fractionated MCF7 cell extracts into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1), and inactive chromatin (Ch2) [49]. The results demonstrate that significant portions of Hsp70-1 and Hsc70 were associated with active

chromatin and inactive chromatin (Fig. 3B). In contrast, only a tiny amount of Hsp90α was associated with active chromatin and none was detected to associate with inactive chromatin. The portions of Hsp70-1, Hsc70, and Hsp90α that existed as nuclear soluble protein were comparable among the three Hsps (Fig. 3B). These results suggest that different from Hsp90α, which is localized almost exclusively in the cytoplasm and in the nucleus as non-chromatin-binding protein, Hsp70-1 and Hsc70 are also associated with active chromatin and inactive chromatin in addition to being localized in the cytoplasm and in the nucleus as non-chromatin-binding protein. Strikingly, a large portion of ERα was associated with inactive chromatin when the MCF7 cells were cultured in the "complete" medium. We have verified our active/inactive chromatin extraction protocol with a well-established coactivator – p300 and a corepressor – NcoR, which are typically associated with transcriptionally active chromatin and inactive chromatin, respectively [62, 63] (Fig. 3B, left panel).

To examine in which subcellular fraction Hsp70-1 and Hsc70 interact with ERα, we performed IPs using fractionated (cytoplasmic, nuclear soluble, active chromatin, and inactive chromatin fractions) proteins from MCF7 as starting materials. The results demonstrate that anti-ERα antibody precipitated significantly more Hsp70-1 and Hsc70 than the control IgG precipitated in all four fractions tested except for Hsc70 in the cytosolic fraction due to large variations among different sample preparations (Fig. 4). We have confirmed the presence of ERα in the expected samples by probing the membrane with anti-ERα body (Fig. 4, middle panel; Fig. S1). It seemed that the precipitated amounts of Hsp70-1 and Hsc70 correlated with the amount of ERα that was precipitated, which in turn seemed to be correlated with the level of ERα in input samples (Fig. 4, top and middle panels). In addition, despite that the majority of Hsp70-1 and Hsc70 were localized in cytoplasm and in the nucleus as soluble protein (Fig. 3B; Fig. 4, top

panel), significant portions of the ERα-Hsp70-1 and ERα-Hsc70 interactions occurred in the active chromatin and inactive chromatin (Fig. 4, middle and low panels), suggesting that the levels of Hsp70-1 and Hsc70 do not affect the amounts of the ERα-Hsp70-1 and ERα-Hsc70 interactions. In short, the results in this section demonstrate that Hsp70-1 and Hsc70 interact with ERα in both active chromatin and inactive chromatin.

ERα interacts with Hsp70-1/Hsc70 in the cytoplasm under conditions of hormone starvation/stimulation. To examine the effect of estrogens on the interaction of ERα with Hsp70-1/Hsc70 in ERα-positive cells, we cultured MCF7 cells in the phenol-red free MEMα supplemented with 5% charcoal-treated FBS for 3-4 days, and then treated the cells with either 100 nM E2 or ethanol (control) for 24 h. We then harvested the cells, fractionated the cell extracts into cytosolic and nuclear fractions, and performed IPs using the cytosolic and nuclear fractions, respectively, as starting materials. The results demonstrate that anti-ERα antibody immunoprecipitated more Hsp70-1 and Hsc70 than the IgG precipitated in the cytosolic fractions (Fig. 5A, low panel; compare lane 2 with lane 1, and lane 4 with lane 3; Fig. 5B, left panel), suggesting that ERα interacts with Hsp70-1 and Hsc70 in the cytoplasm under conditions of hormone starvation and the subsequent hormone stimulation. The E2 treatment had no significant effect on the ERα-Hsp70-1 interaction, but significantly weakened the interaction between ERα and Hsc70 in the cytoplasm (Fig. 5A, low panel; compare lane 4 with lane 2; Fig. 5B, left panel). These results are consistent with the previous observations, which showed that Hsp70 was still associated with progesterone receptors in the presence of progesterone but the levels of the association decreased compared with in the absence of progesterone [64, 65]. Anti-ERα antibody did not precipitate any detectable amount of Hsp70-1 and Hsc70 from the nuclear fractions either in the absence or presence of E2 (Fig. 5A, low panel; lanes 5-8). Compared with

the results shown in Fig. 2, which were obtained with the MCF7 cells cultured under conventional laboratory conditions (i.e., a culture medium supplemented with 5% FBS and phenol red), the ERα-Hsp70-1/Hsc70 interactions observed under E2 starvation/stimulation conditions appeared to be different: under the former conditions the interactions were observed in both the cytoplasm and the nucleus (Fig. 2) and under the latter conditions in the cytoplasm only (Fig. 5). These results suggest that certain factors, potentially not just E2, in the culture media dictate whether ERα interacts with Hsp70-1/Hsc70 in the cytoplasm or the nucleus.

To examine how estrogens affect the association of Hsp70-1 and Hsc70 with chromatin, we cultured MCF7 cells under hormone-starvation conditions for 4 days and then treated the cells with either 100 nM E2 or ethanol (control) for 24 h, fractionated the treated cells into cytoplasmic (C), nuclear soluble (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2) fractions, and analyzed those fractions with Western blotting. The E2 treatment caused significant reduction of ERα as a cytoplasmic protein and as a nuclear soluble protein, suggesting that E2 treatment causes translocation of ERα from the cytoplasm to the nucleoplasm, and eventually the majority of the soluble nuclear ERα to chromatin (Fig. 6). In addition, E2 significantly increased the distribution of Hsp90α in the nucleus as nuclear soluble protein. Compared with the dynamic changes in ERα and Hsp90α, E2 had no significant effect on the distribution of Hsp70-1 and Hsc70 among the different fractions (Fig. 6).

Discussion

Through a quantitative proteomic approach, we identified 21 Hsps and 3 Hsp cochaperones that associate with ERα. The most abundant Hsps that were identified to associate with ERα were four Hsp70 members, followed by two Hsp90 members and three Hsp110 members when 293T cells were cultured in "complete" medium. Within the Hsp70 family, Hsp70-1 and Hsc70 stood out as the most abundant Hsps that associate with ERα, followed by Grp75 that is localized in the mitochondria, and Grp78 that is localized in the endoplasmic reticulum. The two most common Hsp90 family members, Hsp90α and Hsp90β [53], were also identified to abundantly associate with ERα, though at much less abundant levels than the four Hsp70 family members. It is generally believed that ERα interacts with Hsp90 only in the absence of ligands, and dissociates from Hsp90 in the presence of ligands [1, 33, 57]. In this study, although we did not add any exogenous estrogenic ligands (such as E2) to the media for culturing the 293T cells for proteomic identification, we cultured the cells in "complete" medium that contains phenol red, which is known to act as a weak estrogen to stimulate proliferation of ERα-positive cells [66] and FBS, which contains steroid hormones [67]. In addition, we included 10 nM E2 in the lysis buffer for preparing total cellular protein for LC-MS/MS analysis. The identification of Hsp90α and Hsp90β as ERα interacting proteins under the present cell culture and affinity purification conditions suggests that Hsp90 could also complex with ERα, at least partially, in the presence of estrogenic ligands. This conclusion is consistent with the notion that the dynamic and transient interaction of steroid-bound SRs with Hsp90 may be required for the cytoplasmic-nuclear trafficking of SRs in cells [61].

Historically, the attention in studying the role of Hsps in regulating the assembly, trafficking, and transcriptional activity of ERα has been focused on Hsp90 [1, 33]. Through

conventional liquid chromatography or affinity purification, it has been well established that Hsp90 interacts with ERα in a variety of tissue/cells in the absence of ligands [33]. Because of its role in controlling SRs including ERs, and a separate role in protecting oncoproteins, Hsp90 inhibitors are in clinical trials for treating cancer [23, 24]. Compared with Hsp90, much less is known about Hsp70 in regulating ERα. In this study, we found that Hsp70-1 and Hsc70 were the most abundant Hsps that associate with ERα (Table 1). Interestingly, despite that the majority of Hsp70-1 and Hsc70 were localized in the cytoplasm, comparable amounts of cytoplasmic and nuclear Hsp70-1 and Hsc70 were precipitated by anti-ERα antibody (Fig. 2). Furthermore, significant portions of Hsp70-1 and Hsc70 were associated with active chromatin and inactive chromatin (Fig. 3), and the two Hsps interacted with ERα in both forms of the chromatins (Fig. 4). These results are consistent with the observation that the association of Hsp70 with SRs does not affect DNA binding activity of SRs [68]. In contrast, Hsp90α was almost exclusively localized in the cytoplasm and in the nucleus as non-chromatin-binding protein (Fig. 3), which is consistent with the previous observations that SR-Hsp90 complexes are not associated with DNA and that dissociation of Hsp90 from SRs leads to DNA-binding of SRs [69, 70]. Unlike the ERα-Hsp90 association that is normally hormone-dependent [1, 33, 57], Hsp70 is still associated with SRs in the presence of steroid hormones [64, 65, 68, 71], which was also observed in this study (Fig. 5). These results suggest that Hsp70 may play a dramatically different role in regulating ER biological activities compared with Hsp90. Perhaps, cells have evolved two distinct Hsp chaperone systems as repressors to keep ERα in the inactive states in transcription – one is "off-site" (not associated with chromatin) and ligand responsive, which is mediated by Hsp90, and one is "on-site" (associated with chromatin) and not/partially ligand responsive, which is mediated by Hsp70. If this is the case, it would be interesting to examine how these two

chaperone systems interplay to regulate ERα transcriptional activities in a broad context such as tissue development and homeostasis.

In addition to functioning as nuclear receptors and transcription factors in the nucleus, ERs also act as signaling molecules in the plasma membrane and are localized to the mitochondria and endoplasmic reticulum [72-74]. In this study, three mitochondrial Hsp members, Grp75, HSPE1, DNAJA3, were identified to associate with ERα. In particular, Grp75 was identified as a major ERα interactant (Table 1). When nuclear-gene-encoded proteins, such as ERα, are transported into mitochondria via posttranslational import, the proteins are imported into mitochondria in the unfolded states and need to be properly folded after the import. It would be interesting to determine whether Grp75, HSPE1, and DNAJA3 are merely responsible for folding imported ERα in the mitochondria or play additional roles in regulating ERα biological activities in the mitochondria. Several lines of evidence suggest that ERs may play important roles in the mitochondria. For example, it is known that a portion of cellular ERs are localized to mitochondria and the relative distribution of ERs into the mitochondrial pool is regulated by estrogens [47, 48, 75-78]. In addition, it has been shown that mitochondrial DNA contains estrogen response elements [79], and that mitochondrial structure and some important functions are influenced by estrogenic ligands. ERs are also associated with the endoplasmic reticulum [72]. However, the function of ERs in the endoplasmic reticulum remains poorly understood. In this study, we found that Grp78, an Hsp that is localized in the endoplasmic reticulum, was abundantly associated with ERα (Table 1). At present, it is not clear whether the identification of Grp78 as a potential ERα interactant reflects a need of this Hsp in mediating the function of ERα in this organelle.

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Figures

Fig. 1. Verification of the interaction between Flag-ERα and endogenous Hsp70-1/Hsc70. The 293T cells were transfected with a plasmid that expresses Flag alone (control) or Flag-ERα. Forty-eight hours after transfection, the cells were harvested, lysed, and the resulting total protein was pulled down by immobilized anti-Flag antibody. The bound proteins were analyzed with Western blotting using anti-Hsp70-1 and anti-Hsc70 antibodies.

Fig. 2. Endogenous ERα interacts with endogenous Hsp70-1 and Hsc70. (A) The cytosolic and nuclear extracts of MCF7 cells were immunoprecipitated by anti-ERα antibody or an isotype-matched, unrelated control IgG, and the immunoprecipitated protein was analyzed by Western blotting with the indicated antibodies. (B) Left panel, quantification of the IP protein bands in Western blots. Signal intensity values were arbitrary numbers obtained by analyzing the protein bands with ImageJ software. Right panel, validation of the cytosolic and nuclear fractionations. Tubulin and histone H3 were used as markers for the cytosolic and nuclear fractions, respectively. (C) The MCF7 cells were treated with the cell-permeable cross-linking reagent DSP and whole cell lysate of the DSP-treated cells was immunoprecipitated by anti-ERα antibody or a control IgG, followed by Western blot analyses with the indicated antibodies. Values in the Western blot quantifications in (B) and (C) were the means ± S.D. of three separate sample preparations. Cyto, cytosolic; Nuc, nuclear. * and *** denote $p < 0.05$ and $p < 0.001$, respectively.

Fig. 3. Hsp70-1 and Hsc70 are associated with chromatin. (A) The MCF7 cell extract was fractionated into soluble protein (S), chromatin-binding protein (CB), and the pellet (P), and then analyzed by Western blotting with the indicated antibodies (left panel). Right panel, quantification of Western blots. (B) The MCF7 cell extract was fractionated into cytoplasmic

protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and analyzed by Western blotting with the indicated antibodies (left panel). Right panel, quantification of Western blots. Histone H3, p300, and NCoR were used as markers of chromatin-binding protein, active chromatin, and inactive chromatins, respectively. Signal intensity values in the Western blot quantifications were arbitrary numbers obtained by analyzing the protein bands with ImageJ software. Values in the Western blot quantifications were the means ± S.D. of three separate sample preparations.

Fig. 4. Hsp70-1 and Hsc70 interact with ERα in chromatin. Anti-ERα antibody (ERα) and an isotype-matched, unrelated control IgG were used to immunoprecipitate proteins from cytoplasmic (C), nuclear soluble (NS), transcriptionally active chromatin (Ch1), and inactive chromatin (Ch2) fractions prepared from MCF7 cells. The subcellular proteins were prepared as for Fig. 3 except that the inactive chromatin (Ch2) was obtained through sonication instead of elution with 600 mM NaCl. Signal intensity values in the Western blot quantifications were arbitrary numbers obtained by analyzing the protein bands with ImageJ software. Values in the Western blot quantifications were the means ± S.D. of three separate sample preparations. W, whole cell lysate. *, **, and *** denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

Fig. 5. ERα interacts with Hsp70-1 and Hsc70 in the cytoplasm under conditions of hormone starvation/stimulation. (A) The MCF7 cells were cultured under hormone starvation conditions for 3-4 days and then treated with either 100 nM E2 or ethanol (control) for 24 h. The cytosolic and nuclear extracts of the treated cells were then immunoprecipitated by anti-ERα antibody or a control IgG, and the immunoprecipitated protein was analyzed by Western blotting with the indicated antibodies. (B) Left panel, quantification of Western blots. Only the Hsp70-1 and Hsc70 protein bands in the cytosolic fractions were quantified. Signal intensity values in the

Western blot quantifications were arbitrary numbers obtained by analyzing the protein bands with ImageJ software. Values in the Western blot quantifications were the means ± S.D. of four separate sample preparations. Right panel, validation of the cytosolic and nuclear fractionations. Tubulin and histone H3 were used as markers for the cytosolic and nuclear fractions, respectively. W, whole cell lysate. Ctr, control. * and ** denote $p < 0.05$ and $p < 0.01$, respectively.

Fig. 6. Estradiol does not affect the association of Hsp70-1 and Hsc70 with chromatin. The MCF7 cell extract was fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and analyzed by Western blotting with the indicated antibodies (top panel). Signal intensity values in the Western blot quantifications were arbitrary numbers obtained by analyzing the protein bands with ImageJ software. Values in the Western blot quantifications were the means ± S.D. of three separate sample preparations. * denotes $p < 0.05$.

Table.1

Family	Gene names	Protein names (short names)	UniProt ID	Unique peptides	Unique spectra	Sequence coverage (%)†	NcSAF	L/H ratios*	PEP‡
Hsp70	HspA1A	Heat shock 70 kDa protein 1A/1B (Hsp70-1)	P08107	33	927	60.5	0.286	8.9	0
	HspA2	Heat shock-related 70 kDa protein 2	P54652	12	38	37.6	0.012	8.0	3.2E-198
	HspA5	78 kDa glucose-regulated protein (Grp78)	P11021	35	266	51.4	0.080	10.7	0
	HspA6	Heat shock 70 kDa protein 6	P17066	6	15	22.9	0.005	8.0	1.1E-121
	HspA8	Heat shock cognate 71 kDa protein (Hsc70)	P11142	40	810	60.5	0.248	11.7	0
	HspA9	Stress-70 protein, mitochondrial (Grp75)	P38646	33	425	49.9	0.124	12.1	0
Hsp90	Hsp90AA1	Heat shock protein Hsp 90-alpha (Hsp90α)	P07900	33	221	45.4	0.057	6.7	3.3E-195
	Hsp90AB1	Heat shock protein Hsp 90-beta (Hsp90β)	P08238	19	72	47.1	0.022	5.5	1.7E-144
Hsp110	HspH1	Heat shock protein 105 kDa (Hsp105)	Q92598	26	74	34.1	0.016	24.6	3.7E-241
	HspA4	Heat shock 70 kDa protein 4 (HspA4)	P34932	40	154	54.0	0.032	4.7	0
	HspA4L	Heat shock 70 kDa protein 4L (HspA4L)	O95757	22	60	35.9	0.012	26.2	9.2E-163
Hsp40	DNAJA2	DnaJ homolog subfamily A member 2	O60884	2	4	6.1	0.002	2.1	9.0E-22
	DNAJA3	DnaJ homolog subfamily A member 3, mitochondrial	Q96EY1	5	16	16.9	0.005	12.7	1.0E-55
	DNAJB1	DnaJ homolog subfamily B member 1	P25685	4	6	12.6	0.003	5.6	1.7E-15

Table.1 Cont.

Family	Gene names	Protein names (short names)	UniProt ID	Unique peptides	Unique spectra	Sequence coverage (%)†	NcSAF	L/H ratios*	PEP‡
	DNAJB4	DnaJ homolog subfamily B member 4	Q9UDY4	2	4	5.9	0.002	4.7	2.6E-07
	DNAJB6	DnaJ homolog subfamily B member 6	O75190	3	8	9.2	0.004	2.3	1.2E-12
	DNAJC7	DnaJ homolog subfamily C member 7	Q99615	4	8	9.9	0.003	2.0	4.9E-25
	DNAJC9	DnaJ homolog subfamily C member 9	Q8WXX5	10	21	35.0	0.013	14.7	0.068
	DNAJC10	DnaJ homolog subfamily C member 10	Q8IXB1	2	6	2.8	0.001	5.9	1.2E-07
Small Hsps	HspB8	Heat shock protein beta-8 (Hsp22)	Q9UJY1	2	4	9.7	0.003	12.0	0.0035
Chaperonin	HspE1	10 kDa heat shock protein, mitochondrial	P61604	3	10	31.4	0.016	15.6	8.3E-27
Cochaperones	STUB1	E3 ubiquitin-protein ligase CHIP (CHIP)	Q9UNE7	14	57	45.5	0.030	21.6	1.5E-111
	FKBP5	Peptidyl-prolyl cis-trans isomerase FKBP5 (FKBP51)	Q13451	18	39	38.5	0.014	16.5	8.0E-108
	PTGES3	Prostaglandin E synthase 3 (p23)	Q15185	4	12	15.6	0.012	171.1	2.0E-08

*Ratios of light peptides (derived from Flag-ERα-expressing cells) versus heavy peptides (derived from Flag alone-expressing cells).

†Coverage of all peptide sequences matched to the identified protein sequence (%).

‡PEP: posterior error probability.

Fig. 1

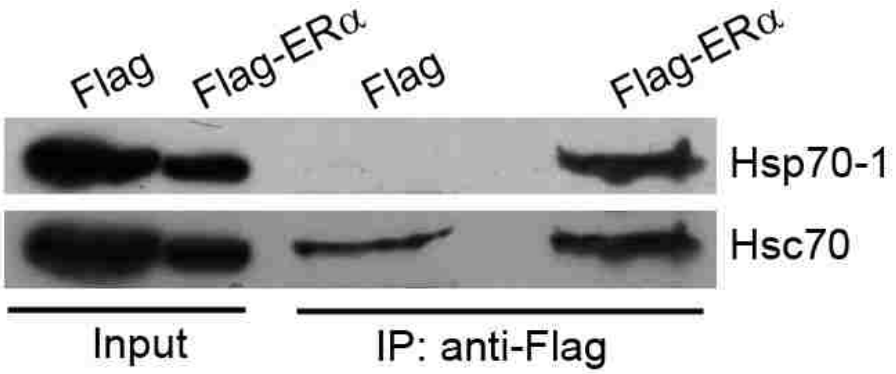
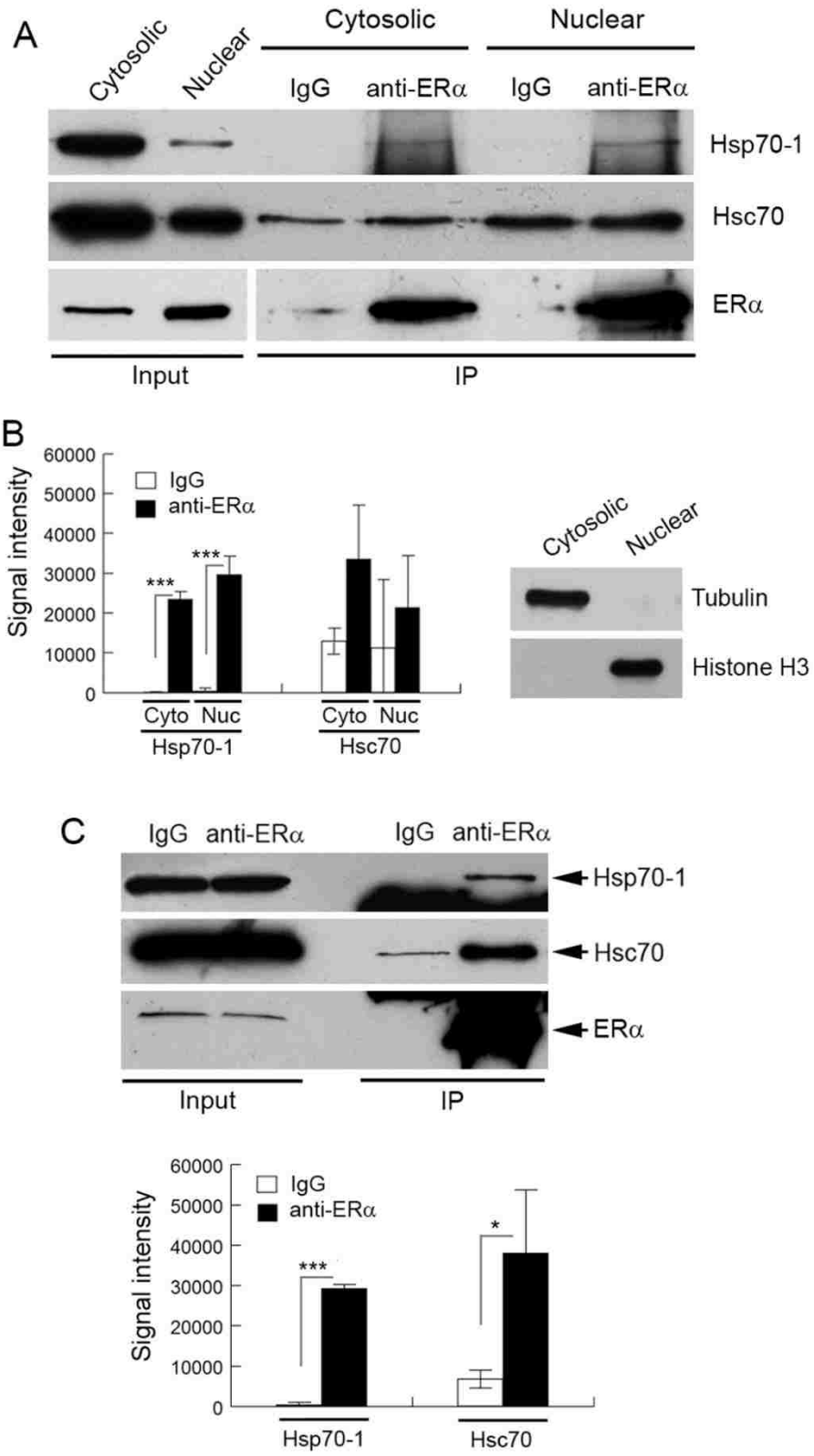


Fig. 2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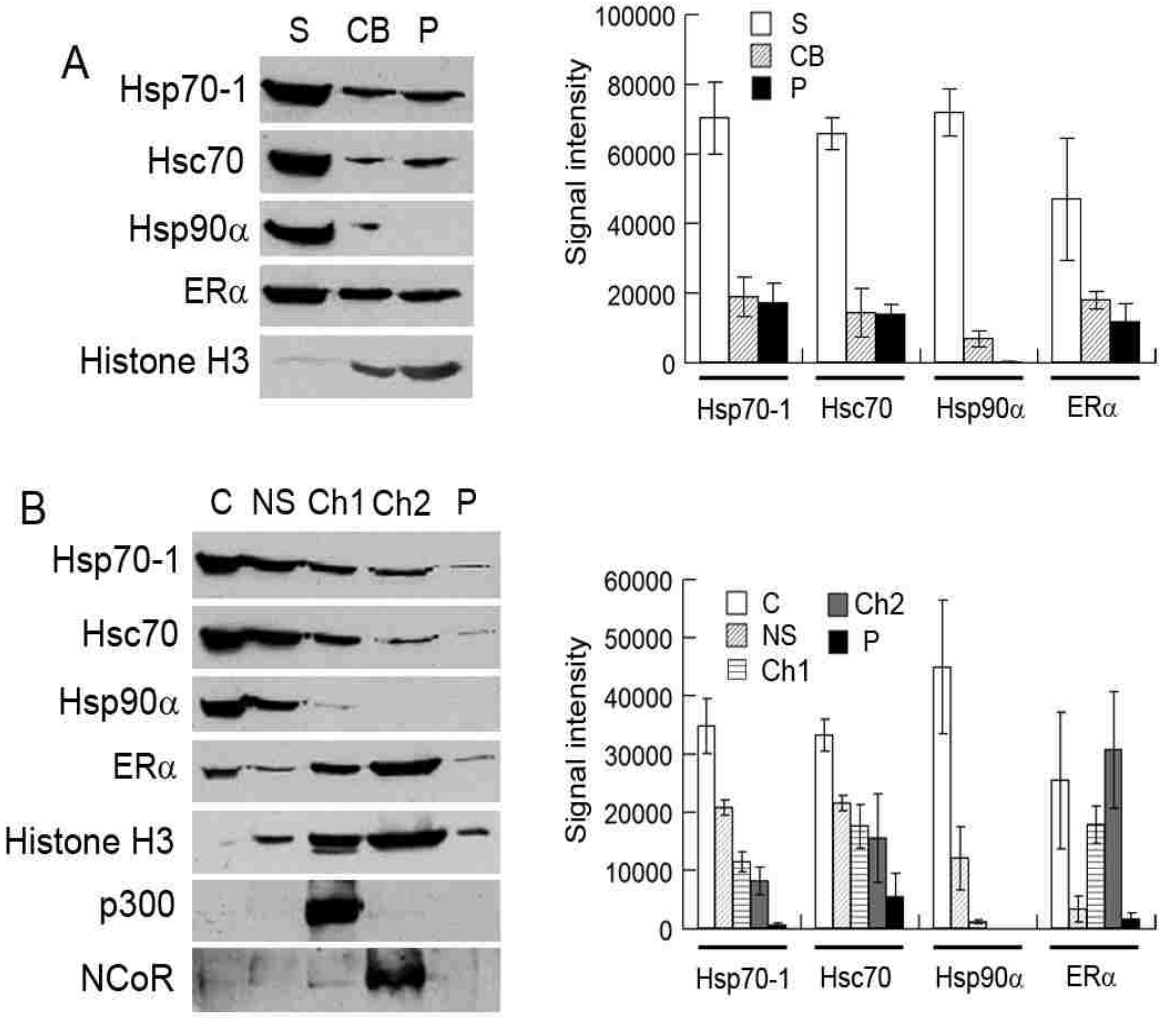
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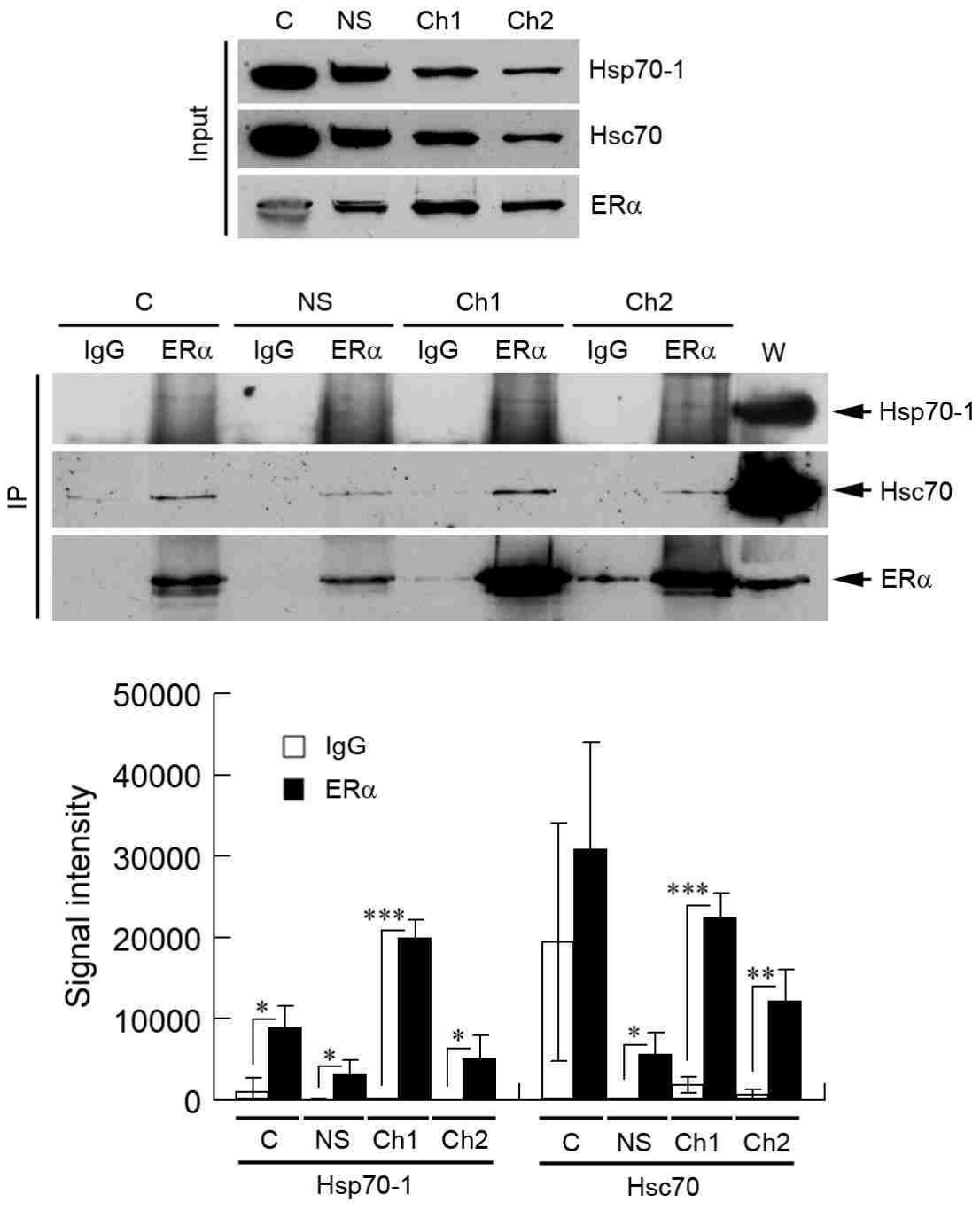
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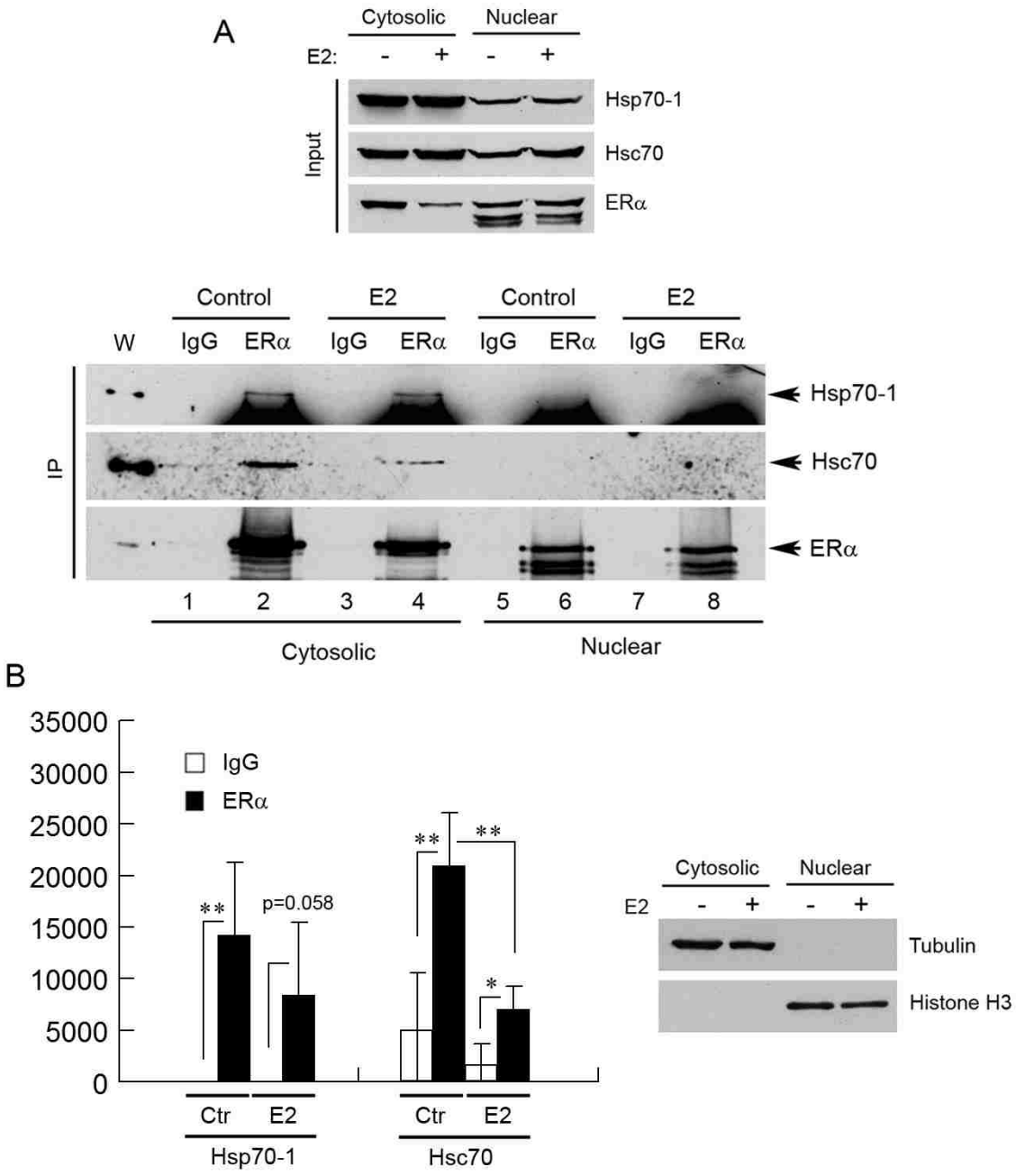
Fig. 5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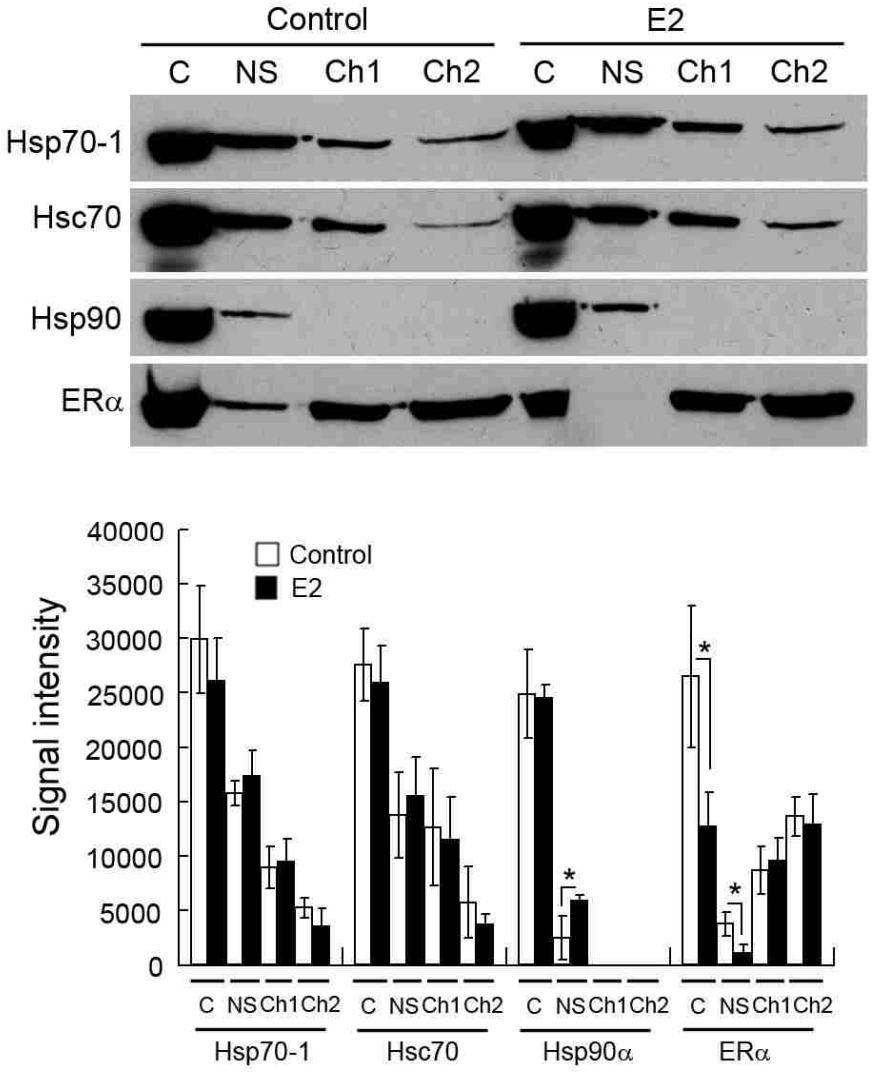
Histone H3

Fig. 6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Control
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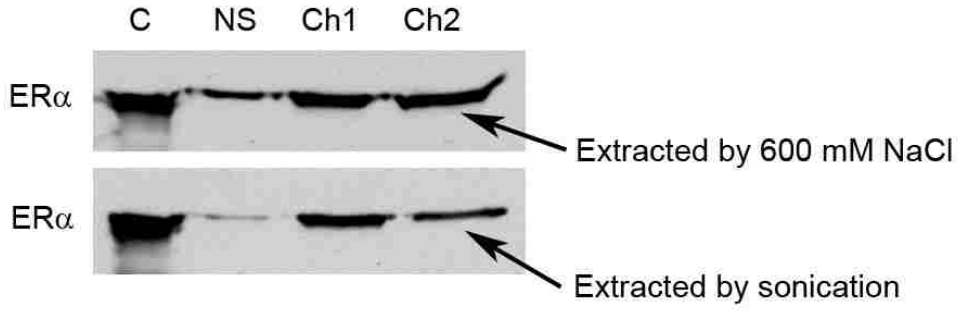
Hsc70
Hsp90α
ERα

Appendix A

S1. Comparison of extraction of inactive chromatin with 600 mM NaCl and sonication.

There was an inconsistency between Figs 3 and 4 in the main text with regard to the relative content of ERα in inactive chromatin (Ch2). When inactive chromatin was extracted with 600 mM NaCl, which was the case for Fig. 3, ERα content in inactive chromatin was the highest among the five fractions examined (Fig. 3). However, when inactive chromatin was extracted with sonication, which was the case for Fig. 4, ERα content was lower in inactive chromatin than in active chromatin (Fig. 4, top panel). To examine whether the inconsistency was caused by different extraction methods, we extracted cytoplasmic (C), nuclear soluble (NS), and active chromatin (Ch1) from two populations of MCF7 cells as described in the main text, followed by extraction of inactive chromatin from the first population of cells with 600 mM NaCl and from the second population of cells with sonication. The results demonstrate that sonication extracted less ERα in inactive chromatin fraction compared to 600 mM NaCl extraction (S1 Fig.), suggesting that the lower input ERα content in inactive chromatin fraction shown in the Fig. 4 resulted from less efficient extraction of inactive chromatin by sonication compared to 600 mM NaCl extraction.



Appendix B

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Chapter2. Histone acetyltransferase 1 interacts with estrogen receptor alpha (ERα) and affects ERα-mediated transcription

Abstract

Transcriptional regulation of estrogen receptor alpha (ERα) is a complex and multistep process. To identify novel proteins that are involved in ERα-mediated transcription, we used a quantitative proteomic method to identify cellular proteins that interact with ERα. Histone acetyltransferase 1 (HAT1) is one of the identified proteins. We have verified ERα-HAT1 interaction by performing coimmunoprecipitation and in-vitro binding assay. In addition, we found that the interaction occurred in the nucleus more than in the cytoplasm. Domain mapping assay showed that ERα bound HAT1 primarily through the ligand binding E domain. In a luciferase assay, we found that knockdown of HAT1 by shRNA resulted in a significant increase in ERα-mediated transcription in breast cancer MCF7 cells, suggesting that HAT1 is functionally linked to ERα. Moreover, electrophoretic mobility shift assay (EMSA) results showed that HAT1 inhibitory role on ERα transcriptional activity was not by blocking ERα from binding estrogen response elements (EREs). An enzyme-dead mutant HAT1 showed similar effect on ERα transcriptional activity as the wild-type HAT1, suggesting that the enzyme activity of HAT1 is not involved in its effect on ERα transcriptional activity. Interestingly, knockout of HAT1 abolished acetylation of histone H4 at lysine 5 and 12 in the cytoplasmic portion of MCF7 cells. Lastly, we demonstrate that the effect of HAT1 on ERα transcriptional activity is gene specific. Our data suggest that HAT1 regulates ERα-mediated transcription through affecting the interactions of ERα with histone proteins around the promoter region of ERα target genes in breast cancer cells. We also examined the function of RbAp46, a regulatory subunit of the HAT1, in the HAT1-ERα complex. Co-immunoprecipitation (CO-IP) results demonstrated that RbAp46 interacted with HAT1 in the cytoplasm and nucleus while interacted with ERα preferentially in transcriptionally active chromatin (Ch1). Our results conclude that HAT1and

RbAp46 play an important role in regulating ERα-mediated gene expression in breast cancer cells.

Introduction

Estrogen receptors (ERs), are key regulators of cell survival, growth, and differentiation in the mammary gland [1, 2] and important factors in breast cancer development. ERs contains two subtypes, ERα and ERβ [3], which are encoded by two different genes, ESR1 and ESR2 [4]. ERα and ERβ proteins are almost identical in structure, but different in ligand affinities and expression levels [5, 6]. ERα, like other nuclear receptors, consists six structural and functional domains: The N-terminal AB domain, which contains the transactivation domain 1 (AF1), the DNA binding domain (DBD; C), and the hinge domain (D), the ligand binding domain (LBD; E) and the C-terminal F domain [7]. The E and F domains constitute the transactivation domain 2 (AF2) [8]. While the AF-1 domain is necessary for ligand-independent interaction of the ERs with coactivators, the AF-2 domain facilitates the ligand-dependent interaction of the ERs with regulators [8, 9]. Functionally, binding of the ERα to estrogens, of which 17β-estradiol (E2) is a major component in cells, triggers ERα conformational changes, translocation into the nucleus, dimerization, and association with estrogen receptor elements (EREs) [10, 11] that reside in the promoters of ERα target genes [12]. Once binding to EREs, ERα promotes the expression of its target genes via recruiting transcriptional co-regulators (coactivator and corepressor) [13] [14, 15], such as CBP/p300, SRC, NCOA1 through AF-1 and/or AF-2 transactivation domains [16-18].

Histone acetylation is one of the most important mechanisms by which ER transcriptional activity is regulated [19]. In the eukaryotic cells, different histone acetyltransferase (HATs) have been discovered, including but not limit to MYST family, CBP/p300, and TFIIIC [20] [21]. Based on subcellular localization, HATs are divided into type A and type B [22]. Type A HATs are exclusively located in the nucleus and act as coactivators by acetylating histones around

promoter regions to make the promoters more accessible to transcriptional machinery. On the other hand, type B HATs are mainly located in the cytosol. HAT1 is the first type B HAT that was identified from cytosolic extract [23]. Later studies showed that HAT1 subcellular location varies depending on cell type and physiological conditions [24]. It has been reported that HAT1 translocates between the cytoplasm and nucleus during cell development [25, 26]. In Oocytes, HAT1 was largely in the nucleus and then redistributed to the cytoplasm during embryogenesis [24]. In fully differentiated *Xenopus* cells, HAT1 was identified in both the cytoplasm and nucleus [24, 27]. In DT40 cells and yeast cells, HAT1 were found to be predominantly located in nucleus [22, 28, 29]. Functionally, HAT1, is believed to be responsible for acetylating soluble histone H4 at lysine 5 and 12 (H4K5,12) sites, but not the histone H4 in nucleosomes [26, 30]. HAT1 may also be involved in histone deposition, chromatin assembly, and DNA double-strand break (DSB) repair [31]. Like its yeast counterpart, human HAT1 holoenzyme contains of two subunits, Hat1 and RbAp46 (retinoblastoma-associated protein 46). RbAp46 binds to core histones and significantly stimulates the acetyltransferase activity of HAT1 [26, 32]. RbAp46 and RbAp48, a homology of RbAp46, involve in chromatin remodeling and transcription repression. Both proteins (RbAp46 and RbAp48) were reported as ERα-interacting proteins and have ability to influence ERα transcriptional activity [33].

To further elucidate the molecular mechanisms underlying ER transcriptional regulations, we used a SILAC (stable isotope labeling by amino acids in cell culture)-based quantitative proteomic approach to identify potential proteins that interact with ERα. We found that HAT1 interacts with ERα in in-vivo and in-vitro and the interaction was mainly mediated by the E domain. Functional studies demonstrate that HAT1 regulates ERα-mediated transcription

through affecting the interactions of ERα with histone proteins around the promoter region of ERα target genes in breast cancer cells.

Material and Methods

Cell culture and transfection. Human embryonic kidney 293T cells (HEK293T) were routinely maintained in D-MEM supplemented with 10% FBS and 1% penicillin and streptomycin. ER-positive breast cancer cells (MCF7) were maintained in α-MEM with 5% FBS and 1% penicillin and streptomycin. For transient transfection experiments, cells were transfected with pcDNA3.1 plasmids that express the indicated gene using the calcium-phosphate method or ViaFect reagent (Bio-Rad).

Cell labeling and affinity Purification. Human 293T cells were cultured in labeled DMEM ($R^{13}C_6$, $K^{13}C_6{}^{15}N_2$) with 10% dialyzed FBS and 1% penicillin and streptomycin for two weeks and then transiently transfected with plasmids expressing Flag tag alone. A second population of 293T cells was cultured in unlabeled DMEM with 10% FBS and 1% penicillin and streptomycin and transiently transfected with plasmids that express Flag tagged ERα. The two population of cells were harvested 48 h after transfection, washed with cold PBS, and then lysed in 5 packed cell pellet volumes of lysis buffer 1 [20 mM Tris- HCl, pH 7.5, 125 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM EDTA and 10 nM E2 supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitors (1 mM Na3VO4, 10 mM NaF and 10 mM β-glycerophosphate)] by incubating the cells on ice for 30 min followed by douncing 50 times. After centrifugation at 20,000 g for 15 min at 4°C, the pellets were further extracted twice with 2 ml of the lysis buffer 1 and sonication. The combined and cleared supernatant was incubated with 200 μl pre-washed Flag M2 resin (Sigma) for 5 h at 4°C with end-to-end rotation. The beads were then washed 7 times (1 ml each time) with lysis buffer 1. The bound proteins were eluted with an elution buffer (10 mM Tris-HCl, pH 7.5, 350 mM NaCl, 1 mM EDTA, 250 mM 3 × Flag peptides and protease inhibitors), and fractionated by a 12% SDS-PAGE gel.

MS Analysis and Database Search. In-gel digestion was performed as described previously [34] [35] and LC-MS/MS analysis was carried out using a LTQ-Orbitrap XL mass spectrometer (Thermo, San Jose, CA) at the Proteomic Facility at the University of Arkansas for Medical Sciences (Little Rock, AR). Briefly, the entire protein lane was cut into 9 slices, and proteins in gel slices were digested with trypsin (Promega, Madison, WI) overnight at 37°C and the resulting peptides were dissolved in 20 µl 0.1% formic acid for LC-MS/MS analysis. Protein identification and quantification were performed with Maxquant (version 1.0.13.13) and Mascot (version 2.2; Matrix Science, Boston, MA) by searching against a composite target-decoy international Protein Index (IPI) human protein database (version 3.52) as described previously [36].

Knockdown and knockout HAT1 in MCF7 cells. The Retroviral Gene Suppressor System (San Diego, CA) with shRNA were used to knock down HAT1 in MCF7 cell. Positive cells were selected with 1 mg/ml G418 (Invitrogen, Carlsbad, CA). To knock out HAT1 in MCF7 cells, the CRISPR-Cas9 genome editing system was used as described [37]. Briefly, two specific guide RNAs (sgRNA) that target HAT1 coding region (S1) were designed and inserted in pSpCas9(BB)-2A-Puro using BbsI restriction enzyme. MCF7 cells were transfected with sgRNAs and selected with 0.8 mg/ml puromycin. Western blot was used to check HAT1 protein expression in the knockdown (ShHAT1) and knockout (KO-HAT1) MCF7 cells.

Protein expression, in-vitro binding assay, and ERα-protein domain mapping. pET-21a plasmid was used to express Flag-tagged full-length human ERα or Flag-tagged ERα domains (AB, C, CD, CDE, CDEF, DE, DEF, E, and EF) in Rosetta cells (Invitrogen) while pGEX-6P-2 plasmid (gift of Dr. Ralph Henry) was used to produce GST-tagged human HAT1, GST-tagged

human RbAp46, and GST-tag alone (as control) in BL21 StarTM (DE3) One Shot cells (Invitrogen). The expressed proteins were purified as described previously [38] . The purity of purified proteins was checked with SDS-PAGE. For in-vitro binding assay, two-fold molar amounts of Flag-ERα or its domains were mixed with the one fold molar amount of GST tag or GST-HAT1 in binding buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 0.1% Triton X-100 and protease inhibitors), incubated for 2.5 h at 4°C. Fifteen microliter of glutathione Agarose Resin (Gold Biotechnology, St. Louis, MO) were added to the mixtures and incubated for an additional 1.5 h at 4°C with end-to-end rotation. Beads were collected by1000 g centrifugation for 2 min at 4°C and washed 3 times with wash buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 0.1% Triton X-100). Bound proteins were eluted with elution buffer (50 mM Tris-HCl pH 8.0, 10 mM reduced glutathione) and examined by Western blotting.

The 17β-Estradiol (E2), Tamoxifen (TAM), and DNase treatment. Cells were cultured under starvation conditions (phenol-red-free α-MEM (Invitrogen) with 5% charcoal-treated FBS and 1% penicillin and streptomycin (Hyclone, Logan, UT)) for at least 3 days and indicated concentration of E2 (Sigma, St. Louis, MO) and TAM (MP Biochemicals, Irvine, CA), or ethanol as control were added to the cells for 24 h. The cells were harvested and washed twice with PBS. Cell pellets were either used directly or saved in -80 for later analysis. For DNase treatment, cells were cultured in α-MEM with 5% FBS and 1% penicillin and streptomycin. After cells were harvested, washed twice with 1xPBS, and lysed in lysis buffer, cell lysate was divided into 2 parts (control and treatment). One unit/10 µl of DNase I recombinant (cat# 04716728001) was mixed with treated proportion. Both the control and the treatment were incubated for 20 min at 37^{o}C and then held on ice for further experiments.

Cell fractionation, co-immunoprecipitation, and sucrose gradient fractionation. Cells were cultured and fractionated as described [39]. For co-immunoprecipitation, cytosolic and nuclear fractions were clarified by mixing them with empty beads for 1 h at 4^{o}C with end-to end rotation, and then incubated with antibody-conjugated beads overnight at 4°C with end-to end rotation. Bound proteins were eluted by elusion buffer after the beads were washed at least 3 times with wash buffer. For sucrose gradient fractionation, cells pellet was re-suspended in sucrose gradient buffer (10 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1 mM EDTA, 0.1% NP-40) supplemented with protease and phosphatase inhibitors, sonicated, and the resulting lysate was centrifuged at 21,000xg for 15 min at 4ºC. The supernatant was mock-treated or treated with DNaseI (1unite/10 μl) and resulting solution was fractionated with onto a 10-30% sucrose gradient by centrifugation with a SW40 rotor at 37,000 rpm for 17.5 h at 4ºC. The fractionated proteins were collected and analyzed by Western blotting.

Extraction of chromatin-binding protein and separation of transcriptionally active chromatin and inactive chromatin. Chromatin-binding protein and transcriptionally active chromatin and inactive chromatin were extracted as described previously [39].

Dual-luciferase reporter assay. MCF7 cells were seeded in 24-well plates overnight in α-MEM (Invitrogen) with 5% charcoal-treated FBS (Hyclone, Logan, UT) and 1% penicillin and streptomycin before transfection. Cells were co-transfected with 500 ng of 3XERE-TATA-luc plasmid [16] and 10 ng pRL-TK Renilla luciferase normalization vector (Promega, Madison, WI) using ViaFect reagent (Bio-Rad). Twenty-four hours after transfection, the medium was replaced with fresh medium, and the cells were incubated for another 24 h. The dual-luciferase assays were conducted according to the manufacturer's protocols (Promega). At least three

independent repeats were performed for each sample. The results are showed as relative light unit (RLU) = Firefly luciferase reading/ Renilla luciferase reading.

Electrophoretic Mobility Shift Assay (EMSA). Two hundred fmol of 5' biotin-labeled estrogen response elements (ERE) (Forward: 5'-GGATCTAGGTCACTGTGACCC CGGATC-3'; Reverse:5'-GATCCGGGGTCACAGTGACCTAGATC-3') were mixed with indicated amounts of purified recombinant ERα, ERα-domains, HAT1, or bovine serum albumin (BSA) (control) in binding buffer (10 mM Tris- HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 M DTT and 5% glycerol). The mixtures were incubated at room temperature for 20 min, fractionated by a 5 % non-denaturing polyacrylamide gel at 4°C, and transferred by semi-dry transferring into a positively charged Nylon membrane (Nytran SPC, Whatman) at 4.5 mA/cm^2 for 1 h. The membrane was blocked with Odyssey blocking buffer (Lincoln, Nebraska) plus 0.1% SDS for 30 min at room temperature and probed with Streptavidin-IRDye 800W for 30 min at room temperature. The membrane was then rinsed three times with PBS and washed three times with PBS containing 0.1 Tween-20 before scanned by Odyssey infrared imaging system (Lincoln, Nebraska). (In case of combinations of ERα-domains and HAT1, appropriate amounts of recombinant proteins were mixed in above binding buffer and incubated for 2.5 h at 4°C with rotation before they were mixed with 5' biotin-labeled estrogen response elements (ERE).

HAT1 enzyme-dead mutations. To diminish HAT1 enzyme activity, two mutations E187Q and E276Q (glutamate (E) was replaced by glutamine (Q) at 187 and 276 sites, respectively) were introduced into the encoded DNA sequence of HAT1 using site-specific mutagenesis by overlap extension protocol as described [40]. After confirmation of the mutations by DNA sequencing, the wild-type and mutated HAT1-DNA sequences were in-frame cloned into pcDNA3.1 plasmid for cell transfections.

DNA affinity precipitation assay. Cells were harvested, washed with 1xPBS, and fractionated into the cytosolic and nuclear fractions (nuclear soluble protein, active chromatin and inactive chromatin proteins) as described [39] without washing steps after nuclear pellets were produced. Nuclear proteins were desalting by a Zeba spin desalting column (Thermo) with binding buffer (20 mM HEPES-NaOH, pH 7.9, 0.1 M KCl, 20 mM EGTA, 0.5 mM DTT, 0.01% NP-40, 10% glycerol) and mixed (1mg) with 0.1μg/μl poly (dI-dC) Amersham Pharmacia (Piscataway, NJ) (Sigma, P4929) final concentration for 15 min on ice. Fifteen microliters of streptavidin-coupled beads and 40 pmoles 5'- biotinylated 3x ERE were added to the mixture, and the mixture was incubated for 1 h at 21°C with end-to end rotation. The beads were then washed four times with PBS50 (10 mM PO4 and 50mM NaCl, pH 7.4) + 0.1%Tween-20 and once with 50 mM NH4HCO3. The bound proteins were eluted by 50 μl elution buffer (5 mM biotin in 50 mM NH4HCO3) for 2 h at 21°C two times. The eluted proteins were examined with Western blotting with indicted antibodies.

Statistical Analysis-The p values were calculated using One-way ANOVA (PSI-PLOT, Pearl River, NY). Data were presented as the mean ± S.D.

Results

HAT1 interacts with ERα. A SILAC-based quantitative proteomic approach was used to identify proteins that are associated with ERα. Two populations of 293T cells, unlabeled and isotope labeled, were transiently transfected with Flag-ERα plasmid and Flag tag alone plasmid, respectively. Affinity purification using Flag M2 beads was conducted and eluted proteins from both groups were mixed with 1:1 ratio, fractionated by a SDS-PAGE gel, digested, and analyzed by MS. One of the identified proteins was histone acetyltransferase1 (HAT1), a member of the type B Histone acetyltransferases family whose biological function is not fully understood. Results from immunoprecipitation (IP) experiments demonstrated that endogenous HAT1 interacts with endogenous ERα (Fig. 1).

HAT1 interacts with ERα in the cytoplasm, nuclear matrix, and on transcriptionally active chromatin. To characterize the interactions of HAT1 with ERα, we first performed sucrose gradient ultracentrifugation analysis. Whole cell lysate of MCF7 cells were fractionated by 10-30% sucrose gradient ultracentrifugation, followed by Western blotting. The results showed that cellular HAT1 fractions overlapped with most of the ERα fractions that had smaller molecular weight (Fig. 2A). To further elucidate in which subcellular compartments HAT1 and ERα interact, MCF7 cells were fractioned into soluble protein (S), chromatin binding protein (CB) and remaining pellet (P), and the resulting fractions were analyzed by Western blotting. The result showed that the great majority of HAT1 was soluble protein and a small amount of it was associated with chromatin, whereas for ERα, a large amount of it was associated with chromatin and the remaining pellet (Fig. 2B). To investigate whether HAT1 and ERα would overlap in soluble and chromatin fractions, MCF7 cells were fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin

(Ch2), and analyzed by Western blotting. The result demonstrated that while the majority of HAT1 were cytoplasmic soluble protein and nuclear soluble protein, a small part appeared as protein associated with transcriptionally active chromatin. In contrast, ERα was mainly associated with transcriptionally active and inactive chromatin (Fig. 2C). Our active/inactive chromatin extraction protocol was validated with a well-known coactivator–p300 and a corepressor–NcoR, which are typically associated with transcriptionally active chromatin and inactive chromatin, respectively [41, 42] (Fig. 2C). To examine in which subcellular fraction HAT1 interacts with ERα, 293T cells were transiently transfected with either Flag tagged ERα or Flag tagged EGFP as control and fractionated into C, NS, Ch1, and Ch2 fractions, followed by co-immunoprecipitation with anti-Flag antibody. The result demonstrated that ERα-HAT1 interaction occurred mainly in the cytoplasm (C) and nuclear matrix (NS), and a small portion of the interaction occurred in the transcriptionally active chromatin fractions (Ch1) (Fig. 2D).

HAT1 directly binds to ERα through ERα-E domain. A preliminary data from our laboratory group showed that HAT1 physically interacts with ERα. So, we were interested to know which part of ERα interacts with HAT1. A domain mapping assay was conducted by expressing GST-tagged HAT1 (GST-HAT1), GST tag alone (GST) as control, full-length Flag-ERα, and nine Flag tagged ERα-domains (AB, C, CD, CDE, CDEF, DE, DEF, E, and EF) in *E. coli* and purified by affinity chromatography (Fig. 3A). Purified recombinant GST-HAT1 or GST were mixed and incubated with the purified recombinant domains along with the full-length Flag-ERα. The mixtures were affinity-pulled down by glutathione agarose resin, and eluted complexes were analyzed by Western blotting. The results demonstrated that the E domain, the ligand binding domain (LBD), was the domain that binds HAT1 (Fig. 3B, upper panel). Interestingly, it appeared that while the C domain, the DNA-binding domain, obstructed the binding of the E

domain to HAT1, and the D domain, the short hinge domain, promoted the binds of the E domain to HAT1 (Fig. 3B, upper and lower panels).

HAT1-ERα interaction was regulated by TAM but not by 17β-Estradiol (E2). To understand how E2 affects the interaction between HAT1 and ERα, we first examined whether E2 affects HAT1 and ERα proteins levels and distributions. MCF7 cells were cultured under starvation conditions (phenol-red-free α-MEM with 5% charcoal-treated FBS and 1% penicillin and streptomycin) for 4 days and then treated with indicated concentrations of E2 for 24 h, followed by Western blotting. The result demonstrated that while ERα protein level was decreased by increasing E2 concentrations, which is in agreement with published results [43, 44] [45], HAT1 protein levels were not affected by E2 (Fig. 4A). Meanwhile, increasing TAM concentrations have no influences on HAT1 protein level, but have on ERα protein levels (Fig. 4B), which were increased by increasing TAM concentrations, consistent with previous results that showed that ERα protein levels were raised above than basal level after adding TAM in MCF7 cells [45].

To determine effect of E2 on HAT1 and ERα subcellular distributions, MCF7 cells were cultured under starvation conditions for 4 days, treated with 100 nM E2 or ethanol (vehicle) for 24 h, and then fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2). The Western blotting results showed HAT1 protein was present as soluble cytoplasmic protein in the cytoplasm and soluble protein in the nucleus, and there was no detectable HAT1 that was associated with the transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2) under starvation conditions (Fig. 4C, upper panel). This result was in contrast with the results observed when the cells were cultured in the normal conditions (completed medium), which shows that a small amount of HAT1 was associated with transcriptionally active chromatin (Ch1) (Fig. 2C).

Interestingly, E2 treatment resulted in increased distribution of HAT1 to the nucleus as nuclear soluble protein (Fig. 4C). Finally, we examined whether E2 impacts HAT1-ERα binding, MCF7 cells were cultured under starvation conditions for 4 days, treated with 100 nM E2 or ethanol for 24 h, and then the cell lysate was immunoprecipitated with anti-HAT1 antibody. The result demonstrated that HAT1- ERα binding was not affected by E2 (Fig. 5A). The result is consistent with the results on in-vitro binding assay, which showed that E2 had no effects on HAT1-ERα binding (Fig. 5B). Unlike E2, TAM enhances the binding between HAT1and ERα (Fig. 5B).

HAT1 was not associated with DNA, and the HAT1-ERα interaction is DNA independent. Because the majority of ERα was associated with transcriptionally active chromatin and inactive chromatin (Fig. 2C), it would be interesting to examine whether HAT1-ERα interaction was mediated by DNA. For this purpose, we treated MCF7 cell lysate with DNase I, fractionated the treated lysate with sucrose gradient, and analyzed the fractionated proteins with Western blotting. The results showed that, as expected, the amounts of ERα appeared in the low fractions were decreased after the lysate was digested with DNase I compared to mock-treated samples (e.g., fractions 5-8) (Fig. 6A, upper panels), suggesting that ERα in those fractions was associated with DNA. In contrast, the distribution of HAT1 was not obviously affected by the DNase I treatment (Fig. 6A, lower panels), suggesting the HAT1 was not associated with DNA. To test whether ERα-HAT1 interaction was mediated by DNA, MCF7 cell lysate was mock-treated or treated with DNase I, and treated lysate was immunoprecipitated with anti-HAT1 antibody. The results demonstrated that HAT1 pulled down a similar amount of ERα from the mock-treated lysate and DNase I treated lysate (Fig. 6B), suggesting that HAT1-ERα interaction was DNA independent.

HAT1 inhibits ERα transcriptional activity. Having established that HAT1 directly binds ERα, we sought to determine whether HAT1 affects ERα transcriptional activity. For this purpose, we first generated a stable cell line in which the *HAT1* gene in MCF7 cells was knocked out using CRISP-Cas9 gene editing system (KO-HAT1). We also generated a control cell line, for which the guide-RNA against parts of GFP gene sequence were used in the CRISP-Cas9 knockout process (KO-EGFP) (Fig. 7A). We then performed dual-luciferase report assays with the control and HAT1-knockout cells. The result demonstrated that ERα transcriptional activity in KO-HAT1 cells significantly higher (about 1.5 times) than that in KO-EGFP cells (Fig.7B), suggesting HAT1 inhibits ERα transcriptional activity in normal MCF7 cells.

HAT1 has no influence on ERα protein expression and distribution in MCF7 cells. Since previous results showed that HAT1 inhibits ERα transcriptional activity, it is possible that it was through affecting ERα protein expression and/or distribution. To investigate this possibility, ERα protein levels were checked in KO-HAT1 and KO-EGFP cells with Western blotting. The results demonstrated that HAT1 had no effect on ERα protein expression (Fig. 8A). In addition, HAT1 also has no effect on the distribution of ERα protein among different subcellular compartments (Fig.7B). The data presented here clearly indicate that the HAT1 inhibitory role on ERα-mediated gene expression was not through affecting ERα protein expression and subcellular distribution in MCF7 cells.

HAT1 doesn't block ERα-ERE interaction. To understand the mechanism by which HAT1 inhibits ERα transcriptional activity, we performed electrophoretic mobility shift assay (EMSA) using purified recombinant HAT1 and ERα to examine whether HAT1 blocks ERα from binding to (ERE). The result showed that HAT1 did not block ERα from binding to ERE (Fig. 9A, lane3) even when 4-fold molar excess of HAT1 used in the EMSA assay (Fig. 9A, lane 4). To confirm

these results, fixed amount of the purified C domain of ERα, the DNA binding domain (DBD), were incubated with increasing concentrations of purified recombinant HAT1 in the EMSA assay. The result demonstrated that as expected while the C domain bound to ERE and caused a shift, HAT1 did not cause a shift of ERE because HAT1 does not bind to DNA (Fig. 9B, lanes 2 and 3). Increasing concentrations of HAT1 caused progressive further shifts of C domain-ERE complexes (Fig. 9B, lanes 4-8), presumably resulted from the binding of HAT1 protein to the C domain to support this interpretation, we included an anti-GST antibody, which would bind to GST tagged HAT1, in the EMSA assay. Indeed, the anti-GST antibody caused a further shift of the ERE complex (Fig. 9B, line 9), suggesting that HAT1 interacts with the C domain of ERα and the interaction does not interfere the binding of the C domain to the ERE. We were interested to check other ERα-domains that might interact with ERE and might be blocked by HAT1. First, we incubated eight ERα domains (AB, E, CD, DE, EF, CDE, DEF, and CDEF) with ERE separately (Fig. 9C) in the EMSA assays. The result showed that, as expected, only domains that contain the C domain (CD, CDE, and CDEF) can bind ERE (Fig. 9C, lanes 4, 7, and 9, respectively). We then examined whether HAT affects the bind of CD, CDE, and CDEF domains to the ERE using EMSA. The results showed that none of these domains were blocked by HAT1 from binding ERE (Fig. 9D, lane 4, 6, and 8). In summary, these results suggest that HAT1 inhibits ERα transcriptional activity without blocking ERα-ERE interaction.

HAT1 enzyme activity is not required for the effect of HAT1 on ERα-mediated transcription. It has been reported that HATs can acetylate histone and non-histone proteins and change their activity [46], and since HAT1 is a member of acetyltransferase family, it is possible that HAT1 inhibits ERα transcriptional activity through its acetylation-enzyme activity. To test this possibility, we generated enzyme-dead mutant HAT1(mHAT1) by using site-specific

mutagenesis by overlap extension protocol [40]. A wild type HAT1(WT-HAT1) and mHAT1 proteins were produced, purified, and tested regard acetylation-enzyme activity. The result showed that the acetylation states of histone H4 at lysine 5 (H4K5) and histone H4 at lysine 12 (H4K12), HAT1 substrates, were significantly decreased in the presence of mHAT1 compared to the WT-HAT1 (Fig. 10A), suggesting that the mutations were significantly effective and mHAT1 has very weak enzyme activity compared to the wild type. Then, we examined whether HAT1 enzyme activity is involved in the inhibiting ERα transcriptional activity. HAT1 was knocked down in MCF7 (ShHAT1) with the technique of small hairpin RNA (Fig. 10B), and then HAT1-silenced MCF7 cells (ShHAT1) were transiently transfected with either WT-HAT1 or mHAT1 followed by a dual-luciferase reporter assay. The result demonstrated that knockdown of HAT1 significantly enhanced the ERα transcription activity (Fig. 10C), consistent with our previous results (Fig. 7B). Introducing wild-type and the enzyme-dead mutant HAT1 into HAT1-silenced cells both repressed ERα transcriptional activity, and no difference was observed between introduction of WT and the mutant HAT1 (Fig. 10C). These results suggest that that HAT1 enzyme activity is not required for the effect of HAT1 on ERα transcriptional activity.

HAT1 does not affect ERα coregulator interactions. It is well known that nuclear transcription factors recruit coregulators (coactivator and corepressor) in promoter region of their downstream genes to control the gene expression [47]. To test whether HAT1 affects ERα transcriptional activity by recruiting ERα or coregulators of ERα to ERE, we performed DNA affinity purifications using the nuclear protein that was extracted from HAT1-silenced (ShHAT1) and wild type MCF7 cells. The results showed that similar amounts of ERα bound to ERE in the wild-type and HAT1-silenced HAT1 MCF7 cells (Fig. 11, upper panel). These results are

consistent with our previous results from EMSA (Fig. 9A, B, C, and D), which show that HAT1 did not impact ERα-ERE interaction. Importantly, we observed that there was no significant difference in the amounts of a well-known coactivator (p300) [41, 42] that were associated with ERE between wild type and HAT1-silenced cells (Fig. 11, middle panel). We could not detect NCoR, a well-known corepressor, in the IP:ERE lanes (Fig. 11, lower panel) which may be because NCoR amount that bound ERα-ERE was not detectable or NCoR did not bind ERα-ERE under this experimental conditions. The results suggest that HAT1 has no effect on recruiting ERα or its coactivator (p300) to at ERE.

HAT1 affects ERα transcriptional activity by decreasing ERα-histone proteins associations. HAT1 has been shown to affect binding of histone proteins to chromatin [25, 48, 49]. To test whether HAT1 affects ERα transcriptional activity through influencing the association of histones to ERα, wild-type MCF7 cells or ShHAT1 cells were fractionated into cytosolic and nuclear proteins followed by immunoprecipitation with anti-ERα antibody. The result showed that ERα interacted with histones H2A, H3, and H4 in the nucleus fractions and the interactions were moderately increased after HAT1 was silenced (Fig.12A). To confirm these results, we performed DNA affinity precipitation assay. Nuclear proteins extracted from a wild-type MCF7 and ShHAT1 cells were incubated separately with ERE followed by ERE-immunoprecipitation. The result showed that, as expected, ERα and histone proteins (H2A, H3, H4K12, and H3K14) were associated with the ERE (Fig. 12B). Interestingly, the interaction between histone H2A, H3, and H4K12 and ERE were noticeably increased when HAT1 was knocked down (Fig. 12B).

Endogenous RbAp46 interacts with endogenous HAT1 and the interaction was E2 and DNA-independent. It is known that RbAp46, the regulatory subunit of HAT1 holoenzyme (14), interacts with HAT1, but whether the RbAp46-HAT1 interaction was in the cytoplasm and/or

nucleus of ERα-positive breast cancer cells was not studied. To test this, MCF7 cells were fractionated into cytoplasmic and nuclear fractions followed by immunoprecipitation by anti-HAT1 antibody. The result demonstrated that endogenous RbAp46 was mainly localized in nucleus which is in agreement with published results [26], and it associated with endogenous HAT1 in both the cytoplasm and the nucleus of MCF7 cells (Fig. 13A). To investigate whether E2 mediates RbAp46-HAT1 interaction, MCF7 cells were cultured under starvation conditions for 4 days and then treated with 100 nM E2 or ethanol for 24 h. The cell lysate from the treated cells was then immunoprecipitated with anti-HAT1 antibody. The result showed that E2 has no effect on RbAp46-HAT1 interaction (Fig. 13B). Since the majority of RbAp46 is located in the nucleus, and is associated with chromatin [23], we wanted to know whether DNA is involved in the RbAp46-HAT1 interaction. To test this, MCF7 cell lysate was treated with DNase I followed by immunoprecipitation by anti-HAT1 antibody. The results demonstrated that the interaction between endogenous RbAp46 and endogenous HAT1 was not mediated by DNA (Fig. 13C).

RbAp46 expression is HAT1-independent. To know whether HAT1 affects RbAp46 expression in ER-positive breast cancer cells, we compared RbAp46 protein expression in KO-HAT1 cells and KO-EGFP control cells with Western blotting. The results showed that RbAp46 protein expression was not affected by the knockout of HAT1 in MCF7 cells (Fig. 14).

RbAp46 binds to ERα preferentially in transcriptionally active chromatin. It has been shown that RbAp46 is associated with ERα [33]. To determine in which subcellular compartment RbAp46 interacts with ERα, 293T cells were transiently transfected with Flag tagged ERα or Flag tagged EGFP, 48 h after the transfection, the cells were harvested and cell lysate extracted from the transfected cells was fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and

fractionated proteins were immunoprecipitated with followed anti-Flag antibody. The result showed that RbAp46 interacts with ERα mainly in the nucleus (NS, Ch1, and Ch2) and preferentially in the transcriptionally active chromatin (Ch1) (Fig. 15).

Knockout of HAT1 doesn't affect acetylation status of H4K5, H4K12 in the nucleus of MCF7 cells. HAT1 is known to play a key role in acetylating histones H4 and H3 [50, 51]. In consistent with this, we found that knockout of HAT1 dramatically reduced the levels of acetylated histones H4K5 and H4K12 but had no effect on the levels of acetylated histones H4K8, H4K16, and H3K14 in the whole cell lysate of MCF7 cells (Fig. 16A). To test whether HAT1 affects the subcellular localization of these acetylated histones, we fractionated MCF7 cells into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2) and analyzed each fraction with Western blotting. The results demonstrated that acetylated histones H4K5 and H4K12 in cytoplasmic protein (C) in HAT1 knockout cell were diminished, whereas those in other fractions were not changed (Fig. 16B). These results suggest that HAT1 is responsible for acetylation of histone H4K5 and H4K12 only in the cytosol but not in the nucleus, and another histone acetylase(s) can acetylate H4K5 and H4K12 and is responsible for maintain the acetylation state of H4K5 and H4K12 in the nucleus. The H4K8, H4K16 and H3K14 were not acetylated in the cytoplasm and the acetylation states of them were not affected by knockout of HAT1 (Fig. 16 A-C).

Discussion

Through SILAC-based quantitative proteomic method and immunoprecipitation we identified and confirmed that HAT1 is a novel ERα-interacting protein. We have demonstrated that HAT1 interacts with endogenous ERα (Fig. 1) and the interaction occurs more in the nucleus particularly in nuclear matrix and transcriptionally active chromatin (Ch1) (Fig. 2D). In addition, domain mapping assay showed that HAT1 directly binds ERα through E domain of ERα (Fig. 3B). Interestingly, we observed that HAT1 protein subcellular distribution were dramatically different under starvation conditions compared to normal conditions (completed medium) (Fig. 4C and 2C), and E2 treatment of the hormone-starved cells resulted in translocation of HAT1 from cytoplasm into nucleus (Fig. 4C). These results suggest that HAT1 may be involved in estrogen responses in mammalian cells. It has been reported that HAT1 translocates from nucleus to cytoplasm during the maturation of *Xenopus* oocyte into an egg [24].

Since HAT1 has no effects on the ERα-ERE interaction (Fig. 9A-D) and ERα coregulator recruitment (Fig. 11), and HAT1 enzyme activity did not mediate ERα-mediated transcription (Fig. 10A-C), it is possible that HAT1 affects ERα transcriptional activity through affecting the interaction between ERα and histone proteins. Indeed, the co-immunoprecipitation result showed that the interactions between ERα and core histones (H2A, H3, and H4) were significantly increased after silencing HAT1 in MCF7 cells (Fig. 12A). In addition, DNA affinity precipitation assay (DAPA) results suggest that HAT1 reduced the interaction between ERE and histones H2A, H3, and H4K12 (Fig. 12B). RbAp46 is the regulatory subunit of the HAT1 holoenzyme [26] [52]. Our co-immunoprecipitation results demonstrated that RbAp46 interacted with HAT1 in the cytoplasm and nucleus (Fig. 13A), while preferentially interacted with ERα in transcriptional active chromatin (Ch1) (Fig.15). It is also noteworthy that both HAT-ERα

binding (Fig. 2D) and RbAp46-ERα binding (Fig. 15) were significantly overlapped in transcriptional active chromatin (Ch1). These results support the notion that HAT1 inhibits ERα transcriptional activity by decreasing ERα-histone proteins interactions, and RbAp46 further inhibits ERα transcriptional activity by promoting HAT1-ERα interaction via decreasing ERα-histone interactions (Fig. 17).

It has been known that HAT1 is responsible for acetylating soluble histone H4 at lysine 5 and 12 (H4K5 and H4K12, respectively) sites and can't acetylate histones H4K5 and H4K12 that were already incorporated in chromatin [25]. Additionally, HAT1 can acetylate histone H2A not histone H2B [26]. On the other hand, it was unclear whether HAT1 is the only acetyltransferase that can acetylate cytosolic H4K5 and H4K12. For example, it has been reported that deletion of *HAT1* in chicken cells (DT40) or yeast cells resulted in a significant decrease, but not completed, in acetylation states of the cytosolic histone H4 (H4K5 and H4K12) [53, 54]. Another study showed that there was subtle change in the acetylation state of the cytosolic H4K12 after HAT1 was knocked out by siRNA in mammalian cells [32]. Interestingly, we found that knockout of HAT1 resulted in depletion of only acetylate histones H4K5 and H4K12 in the cytosol but has no effect on the acetylation states of nuclear H4K5 and H4K12 (Fig. 16B). These results strongly suggest that 1) HAT1 is the sole acetyltransferase that acetylates H4K5 and H4K12 in the cytosol, and 2) HAT1 is not involved in acetylating H4K5 and H4K12 in the nucleus. Thus, there must be another acetyltransferase that is responsible for acetylating histone H4K5 and H4K12 in the nucleus. Indeed, it has been shown that p300, an acetyltransferase, can acetylate H4K5 and H4K8 [55]. If the nuclear acetyl H4K5 and H4K12 are not from the cytosolic acetyl H4K5 and H4K12, it implies that acetyl H4K5 and H4K12 need to be de-acetylated before they go into the nucleus. Alternatively, it is also possible that acetyl H4K5 and H4K12 in the nucleus are

essential for cells. In the presence of HAT1, the cytosolic acetyl H4K5 and H4K12 translocate into the nucleus for fulfill their essential function in the nucleus. While, in the absence of HAT1, cells initiate an alternative pathway to acetylated H4K5 and H4K12 in the nucleus. However, the first mechanism is more likely to be occurred because deacetylation of histones H3 and H4 is required for chromatin maturation [25, 56].

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Figures

Fig. 1. Verification of the interaction between ERα and HAT1. Endogenous HAT1 interacts with endogenous ERα. Whole cell lysate of MCF7 cells were incubated with either anti-ERα or an anti-isotype-matched, unrelated control IgG, separately. The immunoprecipitated proteins were probed with anti-HAT1 antibody by Western blotting. As shown, more HAT1 was immunoprecipitated by anti-ERα antibody compared to the control antibody.

Fig. 2. HAT1 partially overlap with ERα in cells and interacts with ERα in both the cytosol and the nucleus. A, MCF7 whole cell lysate was fractionated by a10-30% sucrose gradient, and the proteins in each fraction were analyzed by Western blotting. B, MCF7 cells (2×10^6) were fractionated into soluble protein (S), chromatin binding protein (CB), and the remaining pellet (P), and proteins in each fraction were analyzed by Western blotting with indicated antibodies (upper panel). Lower panel, quantification of Western blots. C, the whole cell lysate of MCF7 cells (2×10^6) were fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and analyzed by Western blotting with the indicated antibodies (left panel). Right panel, quantification of Western blots. Histone H3, p300, and NCoR were used as markers of chromatin-binding protein, active chromatin, and inactive chromatins, respectively. Signal intensity values in the Western blot quantifications were arbitrary numbers obtained by analyzing the protein bands with ImageJ software. Values in the Western blot quantifications were the means ± S.D. of three separate sample preparations. D, 293T cells transiently transfected with either Flag-ERα or Flag- EGFP were lysed, fractionated as in the part (C) except that Ch2 was obtained through sonication instead of elution with 600 mM NaCl, immunoprecipitated with Flag M2 beads. The immunoprecipitated proteins were probed with anti-HAT1 antibody in Western blotting.

Fig. 3. **HAT1 directly binds ERα through ERα-E domain**. A, a schematic diagram of recombinant ERα domains expressed and purified. Each domain was tagged with a Flag tag, expressed in E coli, and purified by affinity purification using M2 resins. B, a purified GST tag or GST-HAT1 were incubated with purified Full length Flag-ERα or each of the purified Flag tagged ERα domains, the mixture was pulled down by glutathione beads, and the eluted proteins were analyzed by Western blotting.

Fig. 4. Effect of E2 and TAM on HAT1 and ERα proteins levels and subcellular distribution. A and B, MCF7 cells were cultured under hormone starvation conditions for 4 days and then treated with indicated E2, TAM concentrations or ethanol (control) for 24 h. Whole cell lysates were analyzed by Western blotting with the indicated antibodies, actin serves as loading control. C, MCF7 cells (2×10^6) were cultured as in above and treated with 100 nM E2 for 24 h. Cells extract was fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and analyzed by Western blotting with the indicated antibodies (upper panel). Low panel, quantification of Western blots. Signal intensity values in the Western blot quantifications were arbitrary numbers obtained by analyzing the protein bands with ImageJ software (see [39]). Values in the Western blot quantifications were the means ± S.D. of three separate sample preparations. * denotes $p < 0.05$.

Fig. 5. HAT1-ERα interaction was not mediated by E2. A, Whole cell lysate of MCF7 cells that were cultured under hormone starvation conditions for 3-4 days and then treated with either 100 nM E2 or ethanol (control) for 24 h were immunoprecipitated by anti-HAT1 antibody. The immunoprecipitated proteins were analyzed by Western blotting with ERα antibody. B, in-vitro binding assay, purified recombinant Flag-ERα was incubated with purified recombinant GST-HAT1 in a buffer contain either 100 nM E2, 100 nM tamoxifen (TAM), or ethanol, and GST-

HAT1 was then pulled down with glutathione agarose resin. The Eluted proteins were analyzed by Western blotting with anti-Flag antibody.

Fig. 6. HAT1-ERα interaction is DNA-independent. A, sucrose gradient fractionation. MCF7 whole cell lysate, either mock-treated or treated with DNase I (1unite/10 μl), was fractionated with a 10-30% sucrose gradient. Fractionated proteins were analyzed by Western blotting. B, MCF7 cell lysate, mock-treated or treated with DNase I (1 unite/10 μl), were immune-precipitated by anti-HAT1 antibody. The immunoprecipitated proteins were analyzed by Western blotting with anti-ERα antibody.

Fig. 7. HAT1 negatively regulates ERα transcriptional activity. A, Western blot analysis of HAT1 expression in the HAT1 knockout MCF7 cells (KO-HAT1) and knockout MCF7 cells (KO-EGFP, control). B, dual luciferase reporter assay. RULs values were the means ± S.D. of three separate sample preparations. * denotes $p < 0.05$.

Fig. 8. knockout of HAT1 doesn't affect ERα expression and subcellular distribution in MCF7 cells. A, equal amounts (60 μg) of whole cell lysate from KO-HAT1or KO-EGFP were fractionated by 10% SDS-PAGE and probed with indicated antibodies in Western blotting. Tubulin serves as loading control. B, KO-HAT1or KO-EGFP cells (2×10^6 cells/ each) were fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and analyzed by Western blotting with indicated antibodies.

Fig. 9. HAT1 doesn't block ERα-EREs interaction. A, electrophoretic mobility shift assay (EMSA). Purified recombinant ERα and HAT1, or BSA (control) were incubated with Biotin labeled 3xERE oligos. The resulting mixtures were fractionated by a 5% nondenaturing acrylamide gel, probed with streptavidin labeled IRDye-800CW and visualized by Odyssey

infrared imaging system. B, increasing amounts of purified recombinant HAT1 was mixed with a fixed amount of purified recombinant ERα-C domain, and the mixtures were incubated with biotin labeled 3xERE oligos. Regards to super shift (lane 9), anti-GST antibody was added to the incubation mixture. C and D, purified recombinant ERα domains or BSA were mixed with biotin labeled 3xERE oligos and EMSAs were conducted as in A.

Fig. 10. The effect of HAT1 on ERα transcriptional activity is enzyme activity-independent. A, HAT1 enzyme activity, the purified recombinant mHAT1 and WT-HAT1 were mixed and incubated with histone H4 peptide, separately. The mixtures were resolved by Western blotting with indicated antibodies**.** B, Western blot analysis of the WT-MCF cells and stable MCF7 cells in which the expression of HAT is silenced by shRNA (ShHAT1). C, HAT1 enzyme activity is not involved in ERα transcriptional activity. ShHAT1 cells were transfected with plasmid which either expresses wild type HAT1 or enzyme-dead mutant HAT1 (HAT1M), the transfected cells were used to measure ERα transcriptional activity using luciferase assays. MCF7-ShGFP cells used as control. * denotes $p < 0.05$.

Fig. 11. HAT1 doesn't mediate ERα coactivator interactions. Nuclear protein extractions from wild type MCF7 and ShHAT1 cells were mixed with biotinylated 3xERE and then were pulled down by streptavidin beads. Eluted proteins were analyzed by Western blotting with indicated antibodies.

Fig. 12. Knockdown of HAT1 enhances the interaction of ERα with histones. A, the cytosolic and nuclear extracts of wild type or shHAT1 MCF7 cells were immunoprecipitated by anti-ERα antibody and the immunoprecipitated proteins were analyzed by Western blotting with the indicated antibodies. Tubulin serves as cytosolic marker. B, DNA affinity precipitation assay was conducted as in figure 11 with indicated antibodies.

Fig. 13. RbAp46 binds HAT1 in cytoplasm and nucleus of MCF7 cells and the interaction is E2 and DNA independent. A, the cytosolic (C) and nuclear (N) extracts of MCF7 cells were immunoprecipitated by anti-HAT1 antibody and the immunoprecipitated protein was analyzed by Western blotting with the indicated antibodies. Actin and histone H3 were used as markers for the cytosolic and nuclear fractions, respectively. B, MCF7 cells were cultured under hormone starvation conditions for 4 days and then treated with either 100 nM E2 or ethanol (control) for 24 h. The treated cells were lysed and immunoprecipitated by anti-HAT1 antibody. The immunoprecipitated proteins were analyzed by Western blotting with anti-RbAp46 antibody. C, MCF7 cell extracts were treated as described in Fig. 2A, immunoprecipitated with anti-HAT1 antibody, and the immunoprecipitated proteins were analyzed by Western blotting with anti-RbAp46 antibody.

Fig. 14. RbAp46 expression is HAT1-independent. A, Western blot analysis of RbAp46 expression. Equal amount of whole cell lysates of KO-HAT1 and KO-EGFP cell analyzed by Western blotting with indicated antibodies. Tubulin serves as loading marker.

Fig. 15. RbAp46-ERα binding was preferentially in transcriptionally active chromatin. 293T cells were transfected with plasmids that express Flag-ERα or Flag-EGFP (control). Forty-eight hours after the transfection, the cells were lysed, fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and the fractionated proteins were immunoprecipitated with Flag M2 beads. The eluted proteins were probed with anti-RbAp46 antibody in Western blotting.

Fig. 16. HAT1 is responsible solely for acetylating histone 4 at lysine 5 and 12 in cytosol of MCF7 cells. A, Western blot analysis of equal amounts of whole cell lysate HAT1 knockout (KO-HAT1) MCF7 cells and control knockout MCF7 cells (KO-GFP). B, the cell extracts of

KO-HAT1 and KO-EGFP cells were fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and analyzed by Western blotting with the indicated antibodies. C, quantification of Western blots , signal intensity values in the Western blot quantifications were arbitrary numbers obtained by analyzing the protein bands with ImageJ software (see [39]). Values in the Western blot quantifications were the means ± S.D. of three separate sample preparations. *** denotes $p < 0.001$.

Fig. 17. A model for HAT1 and RbAp46 function in ERα-regulated gene expression. A, in the absence of E2, ERα is weakly bound to the promoter region (e.g. ERE) and has no transcriptional activity. B, upon ligand binding, ERα tightly binds to the promoter region and histone proteins (e.g. H2A, H3, and H4) leads to a high ERα-target gene expression. C, Binding the E domain of ERα, HAT1 decreases the binding between ERα and the histone proteins and ERα transcriptional activity. D, RbAp46 (p46) can bind both HAT1 and ERα and recruit more HAT1 into the RbAp46-HAT1-ERα complex which led to increase the inhibitory role of HAT1 on ERα transcriptional activity.

Fig. 1

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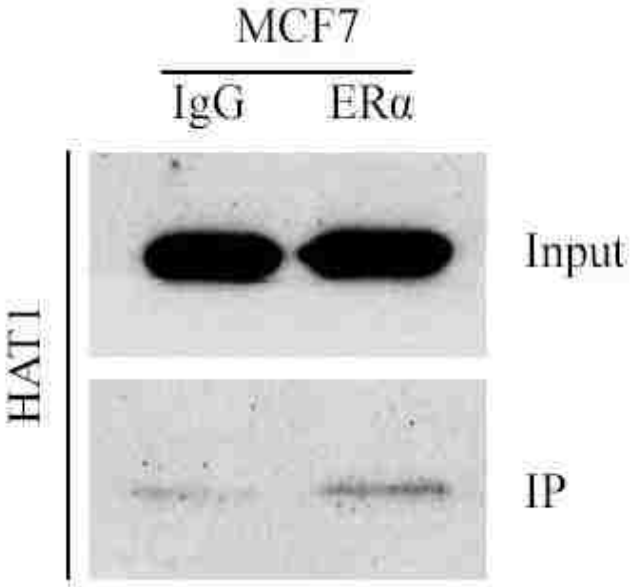
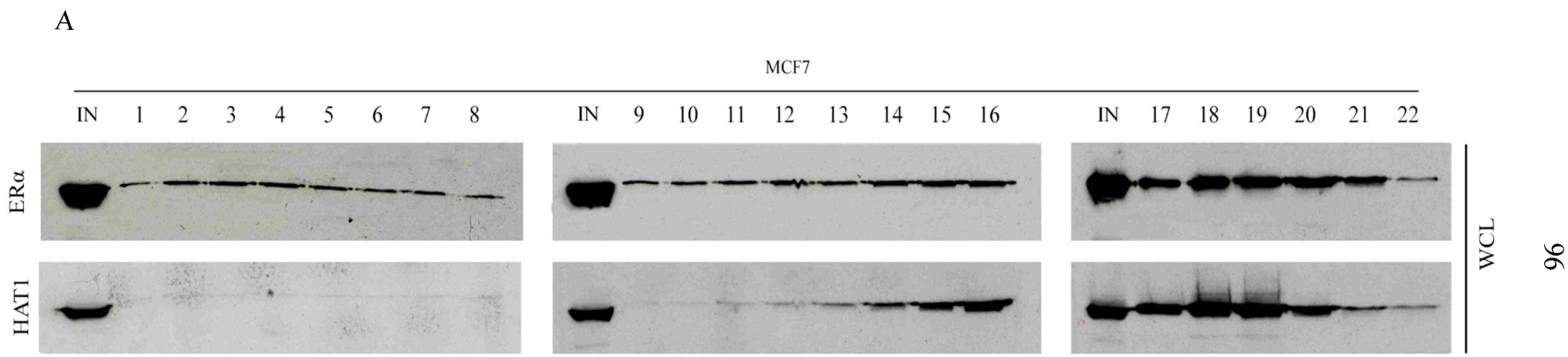


Fig.2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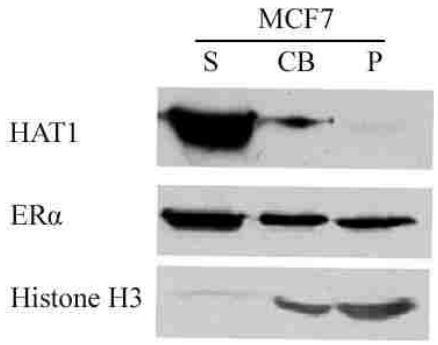
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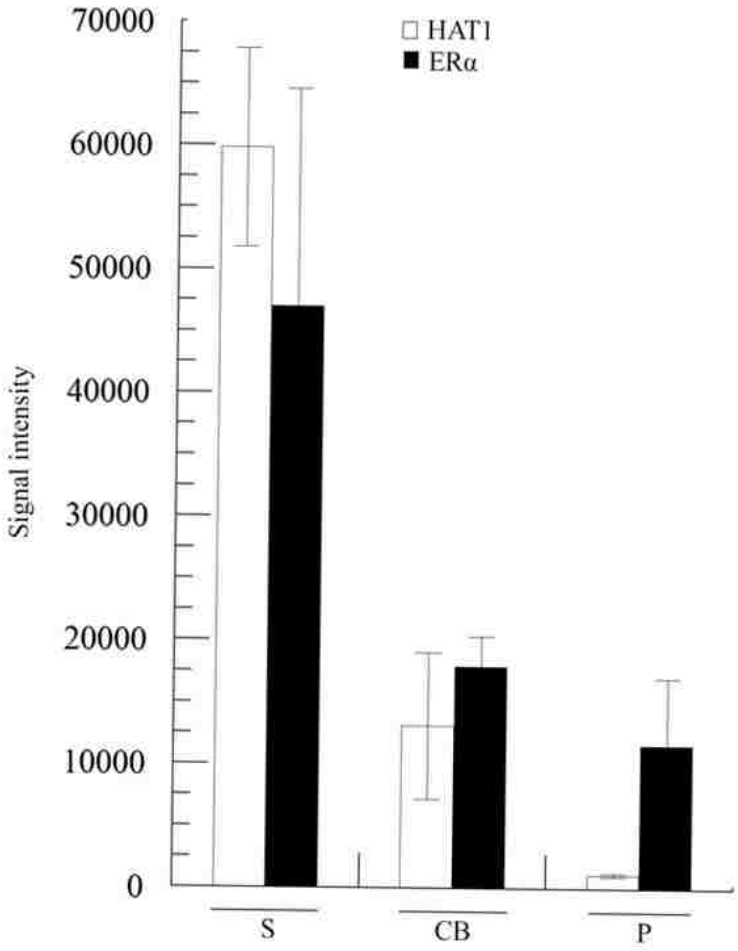
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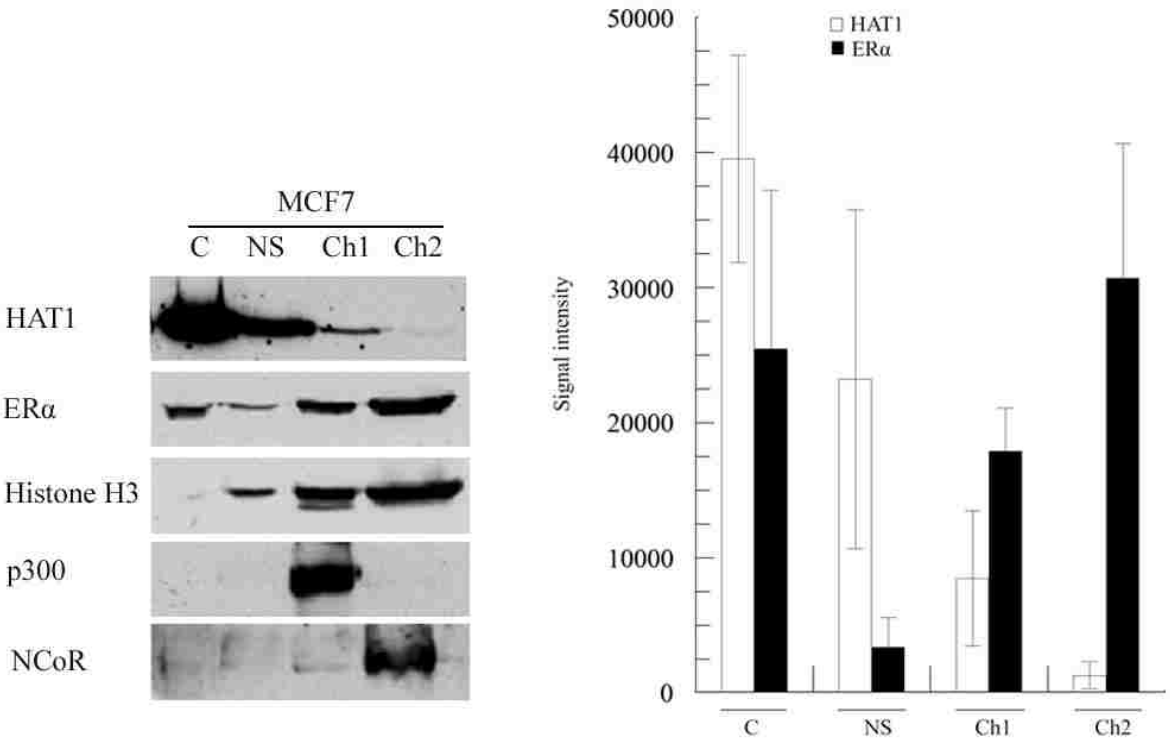
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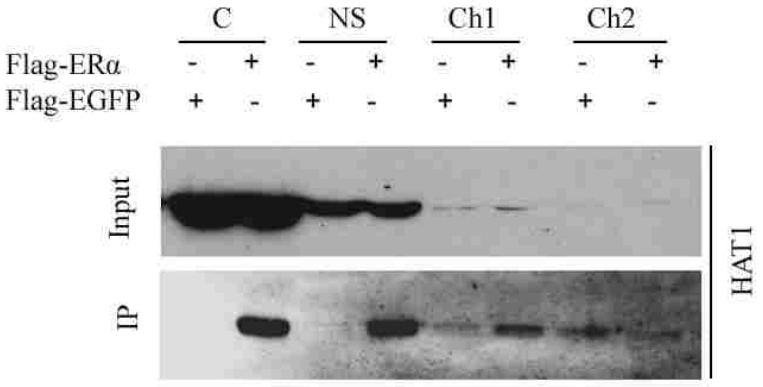
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HAT1

Fig. 3

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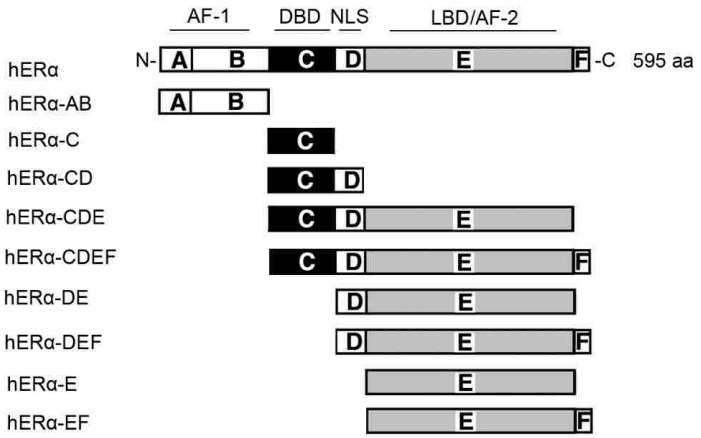
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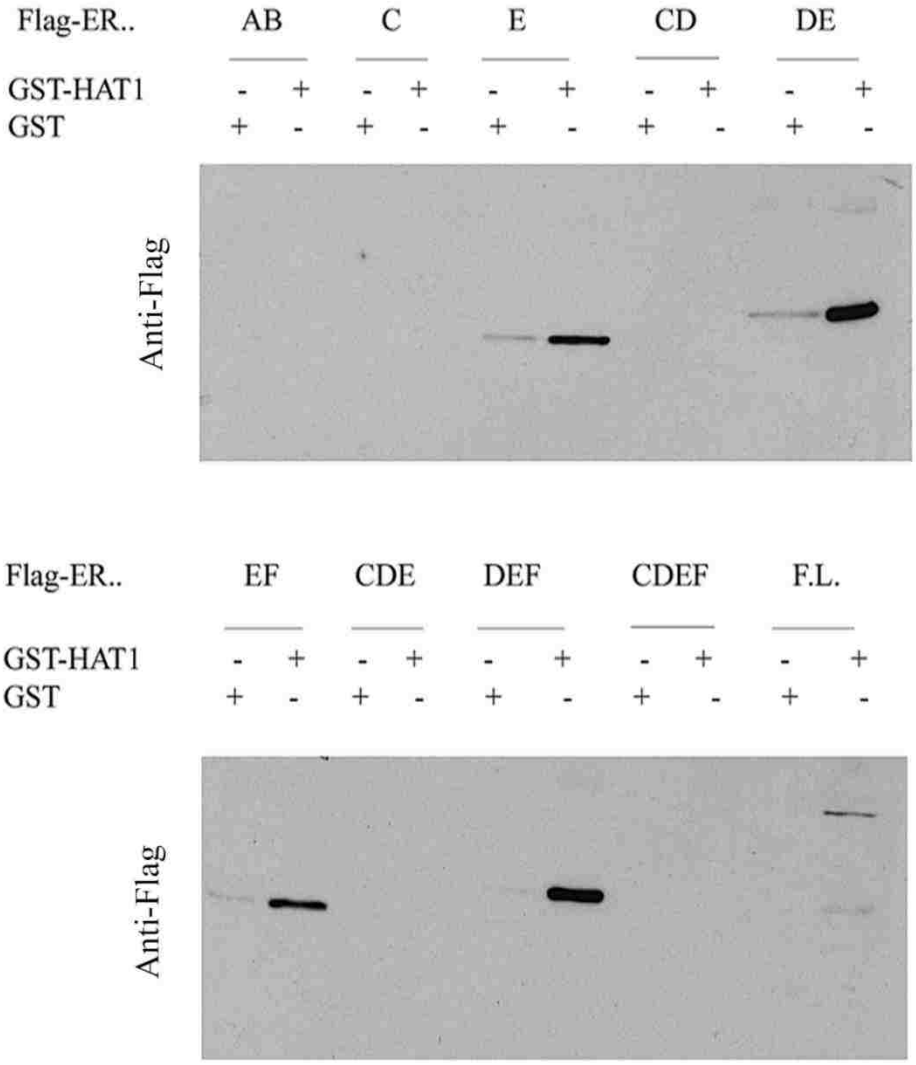
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Fig. 4

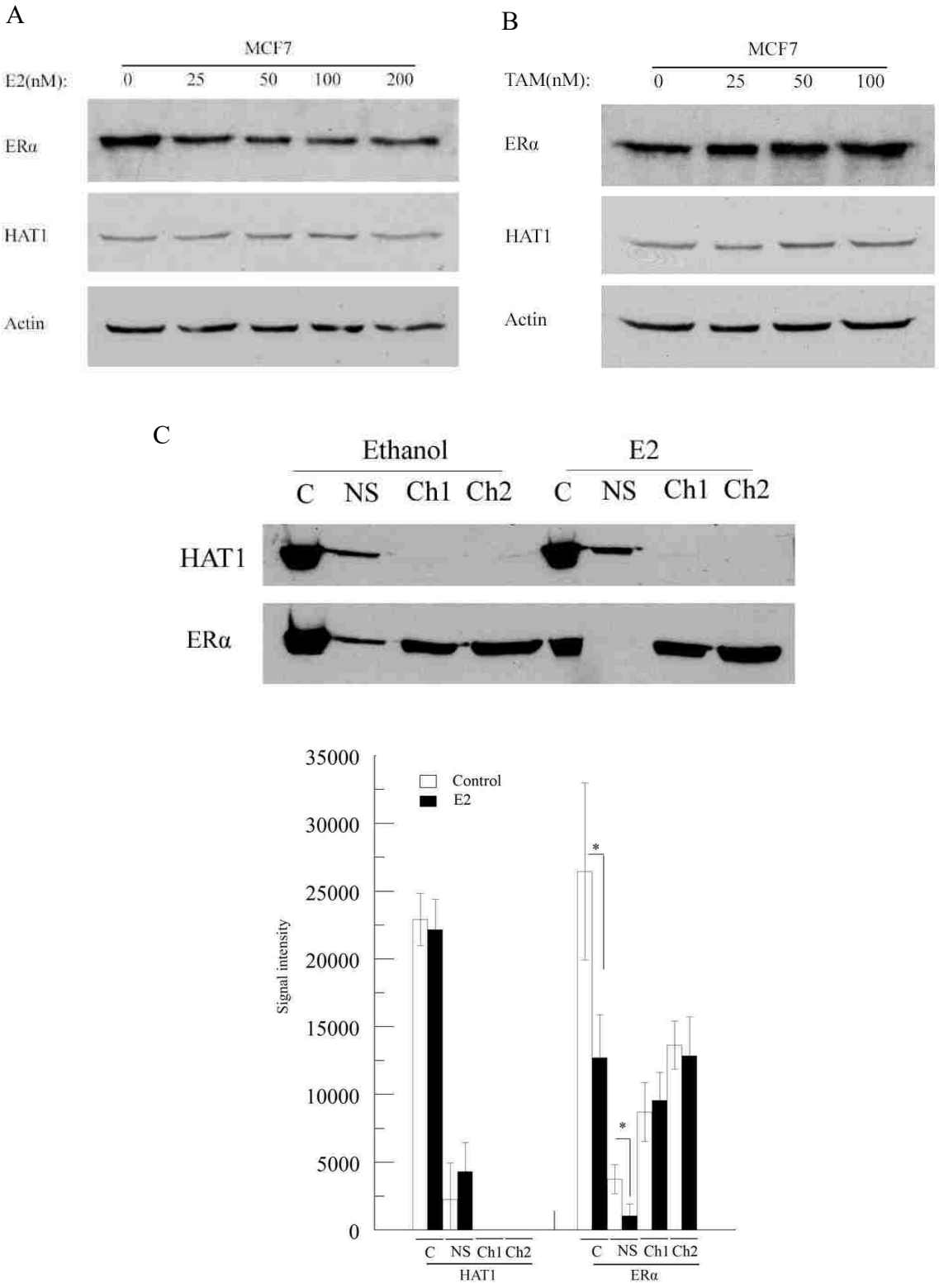
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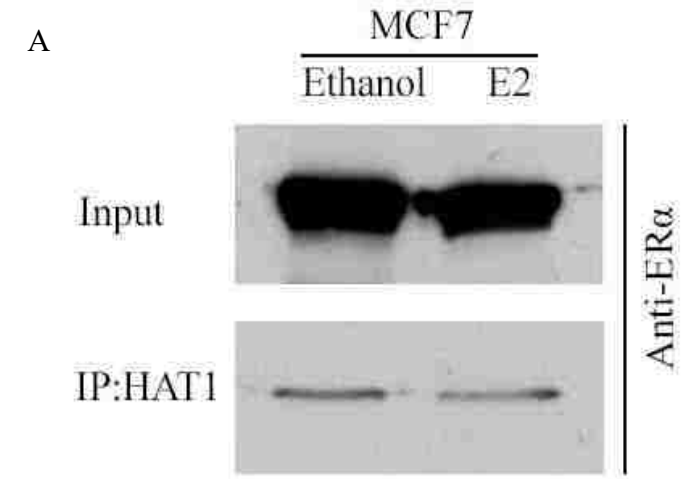
Fig.5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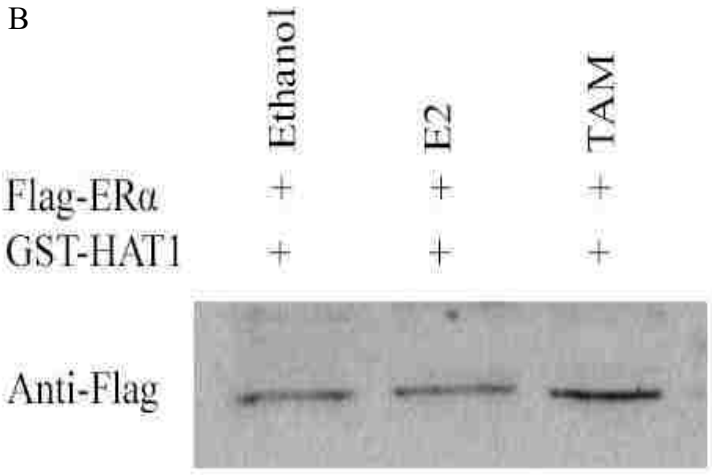
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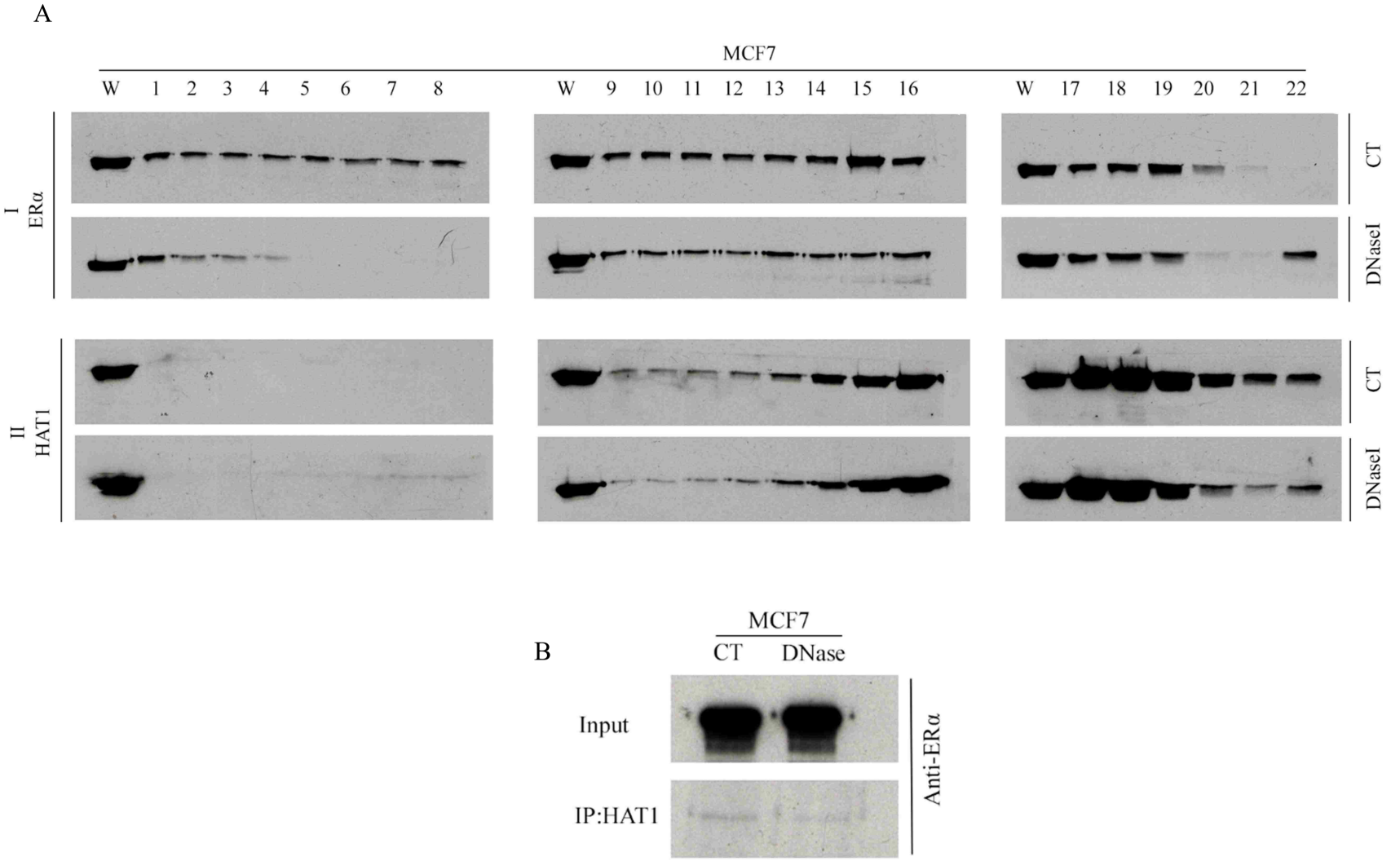
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Fig.7

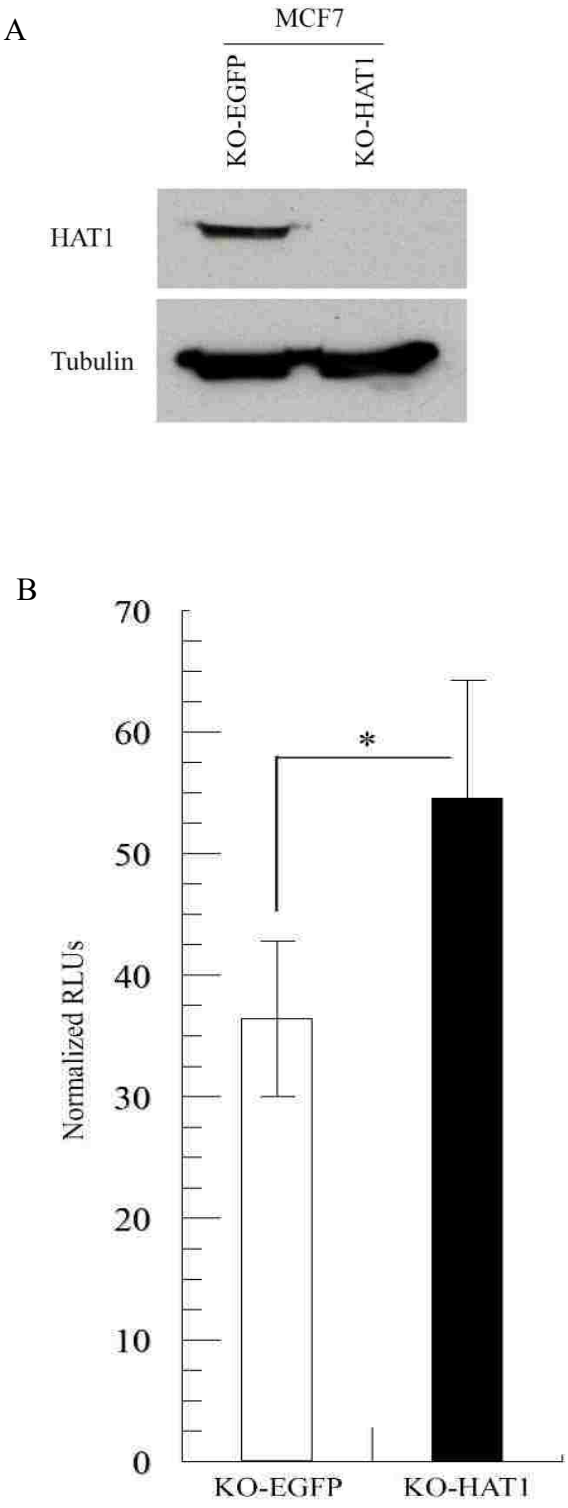
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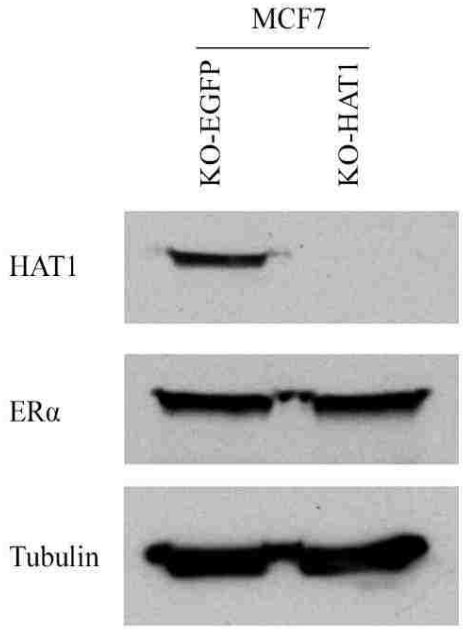
Fig. 8

.

A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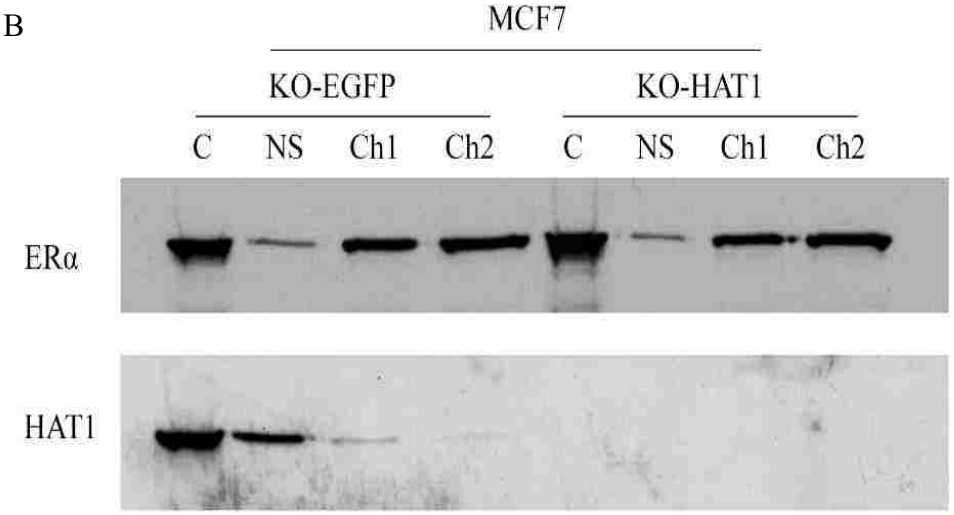
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ERα
HAT1

Fig. 9

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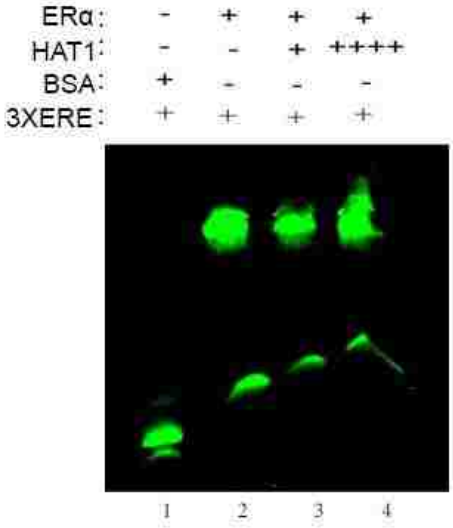
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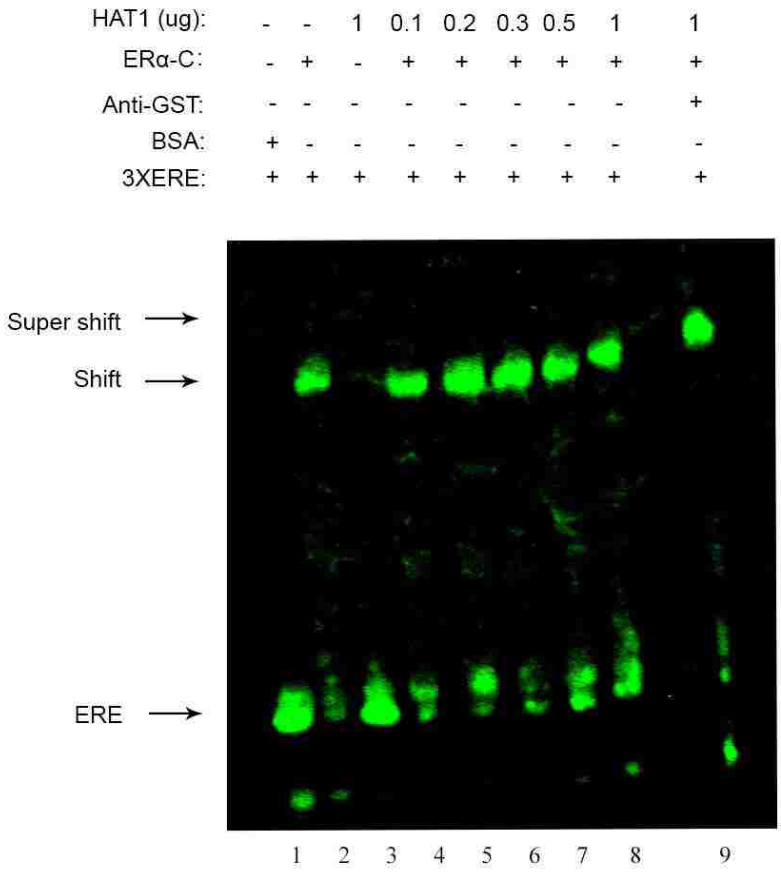
B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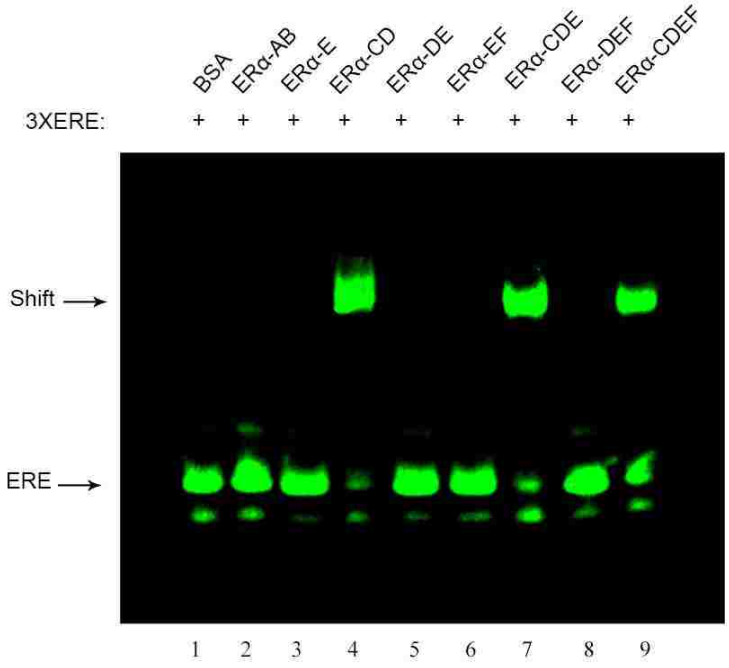
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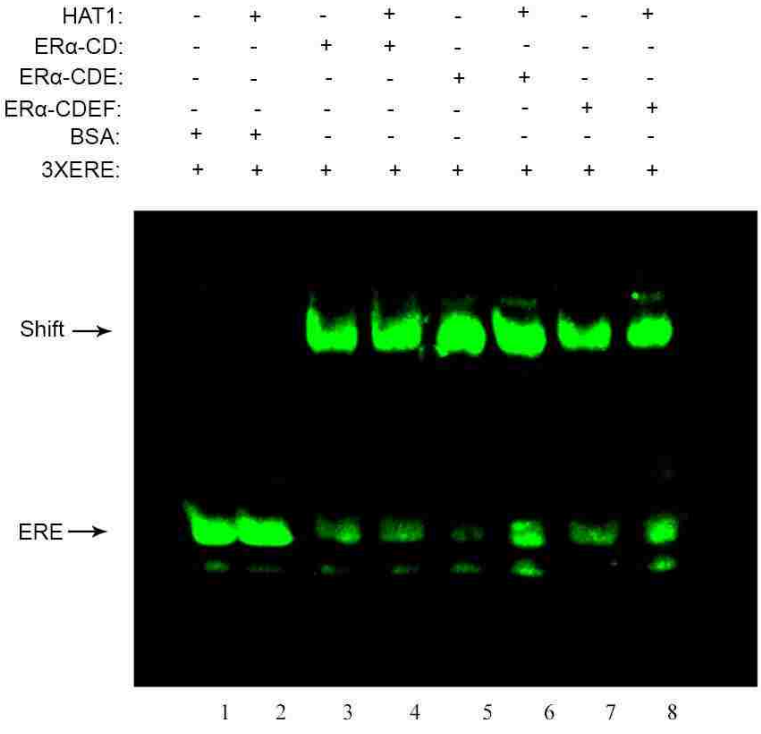
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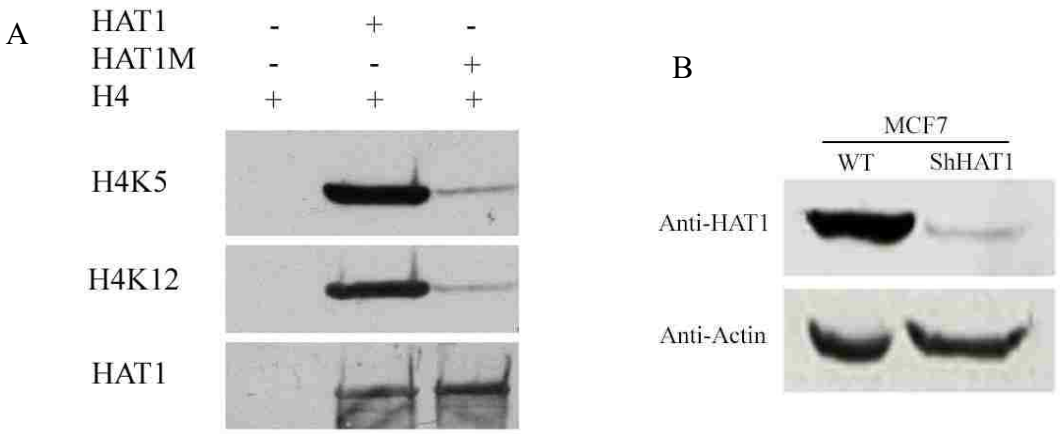
ERα-CD: - - + + - - - -
ERα-CDE: - - - - + + - -
ERα-CDEF: - - - - - - + +
BSA: + + - - - - - -
3XERE: + + + + + + + +
Shift
ERE
1 2 3 4 5 6 7 8

Fig. 10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HAT1M - - +
H4 + + +
H4K5
H4K12
HAT1
B
MCF7
WT
ShHAT1
Anti-HAT1
Anti-Actin

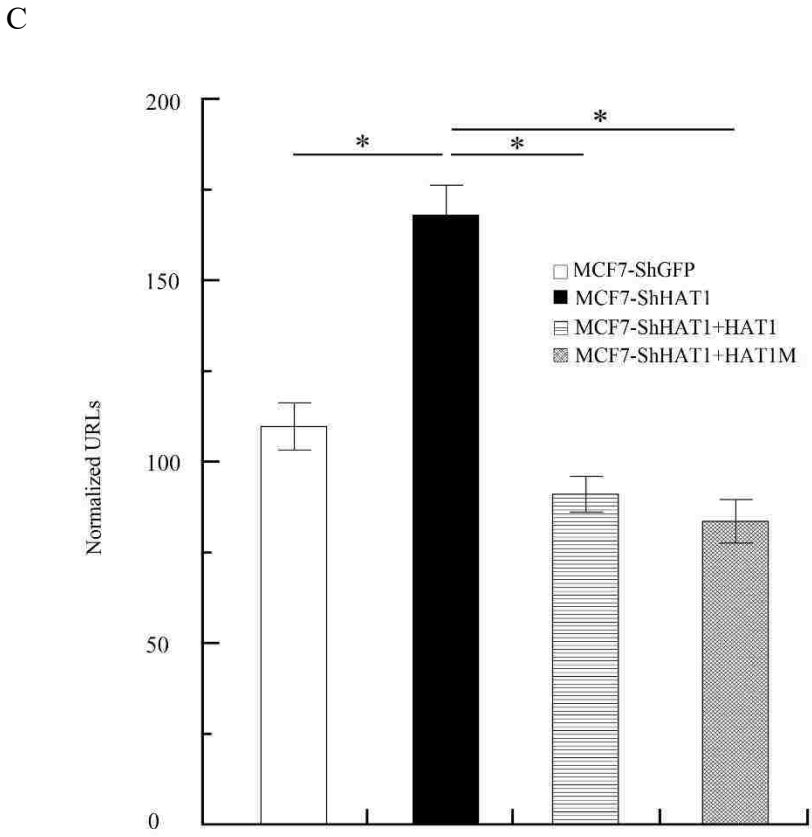

C
200
150
100
50
0
Normalized URLs
*
*
*
MCF7-ShGFP
MCF7-ShHAT1
MCF7-ShHAT1+HAT1
MCF7-ShHAT1+HAT1M

Fig. 11

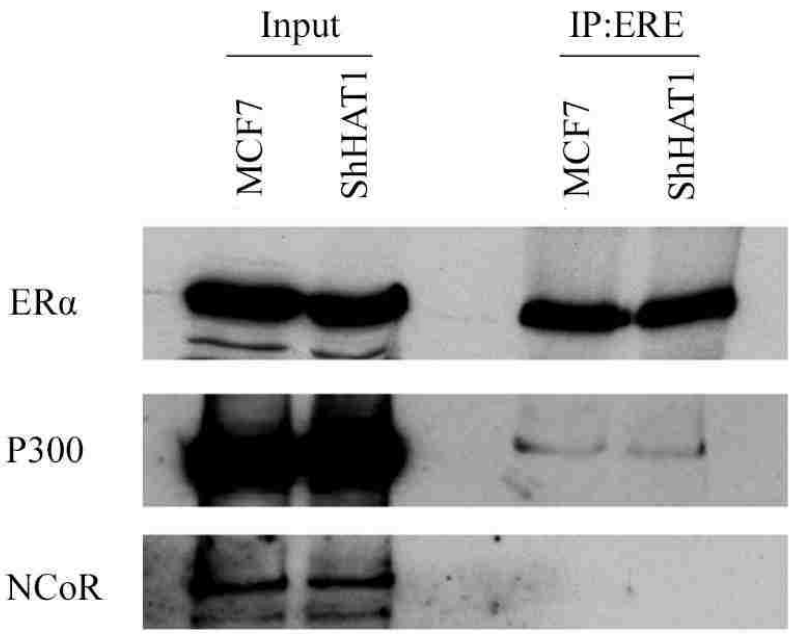
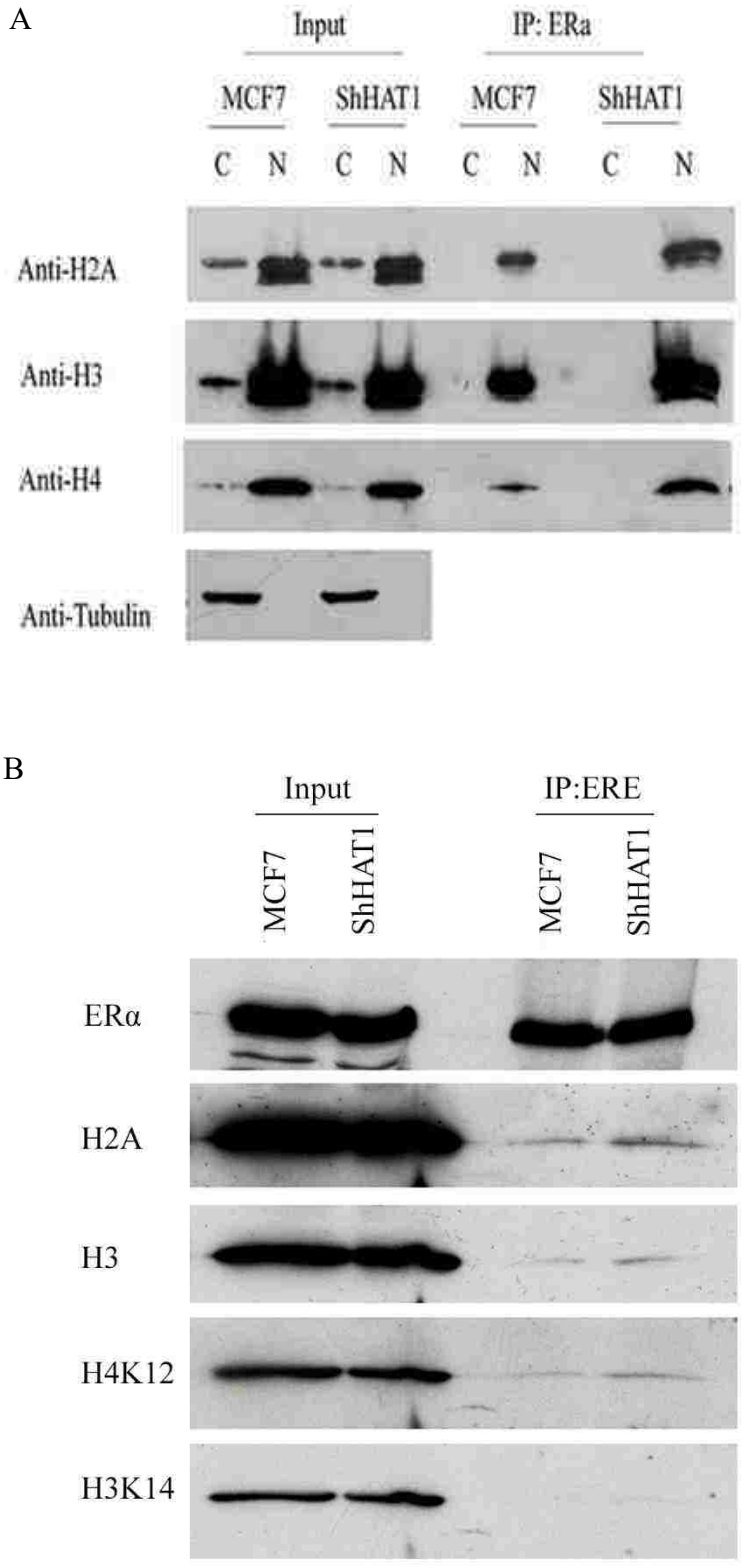


Fig.12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MCF7
ShHAT1
MCF7
ShHAT1
C N C N C N C N
Anti-H2A
Anti-H3
Anti-H4
Anti-Tubulin
B
Input
IP:ERE
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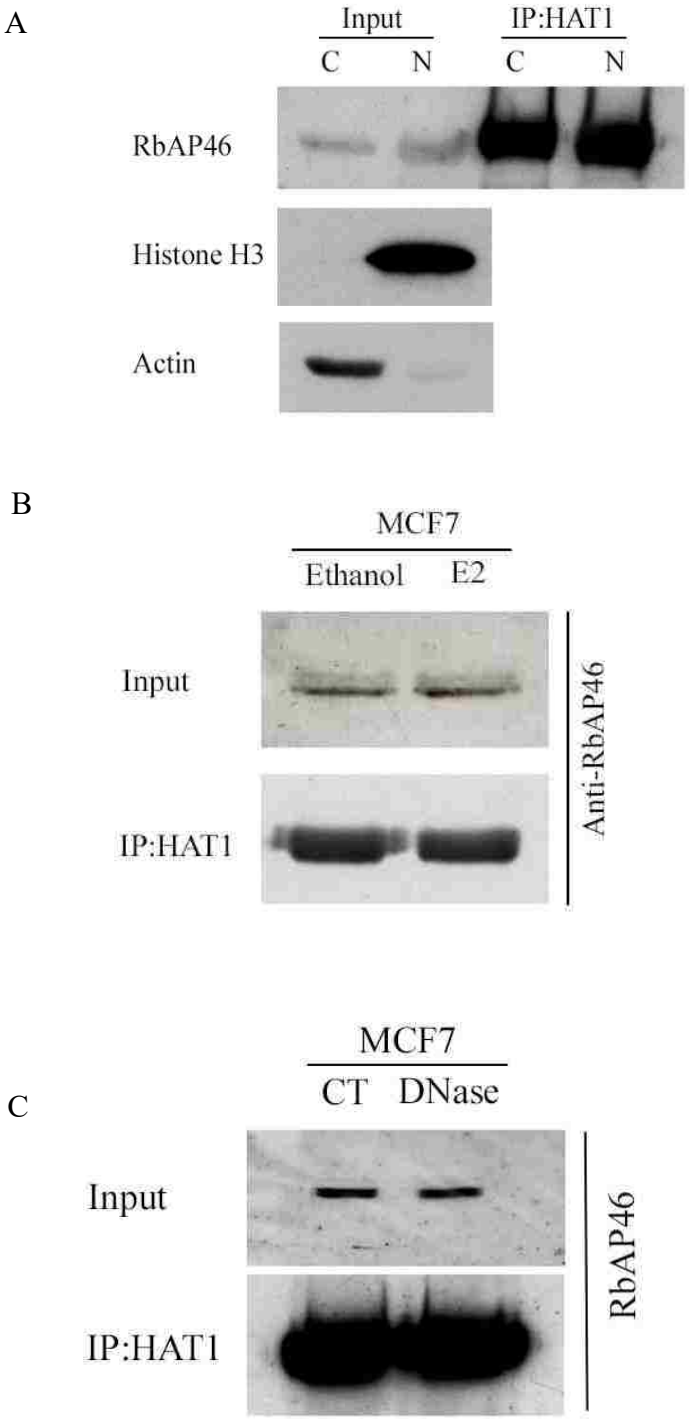
ShHAT1
MCF7
ShHAT1
ERα
H2A
H3
H4K12
H3K14

Fig. 13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Input
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C
N
C
N
RbAP46
Histone H3
Actin
B
MCF7
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E2
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Anti-RbAP46
C
MCF7
CT
DNase
Input
IP:HAT1
RbAP46

Fig.14

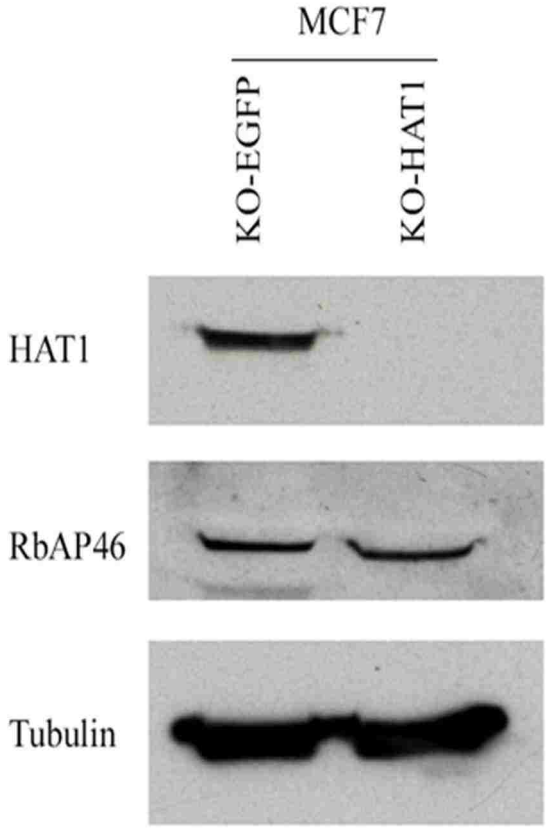


Fig. 15

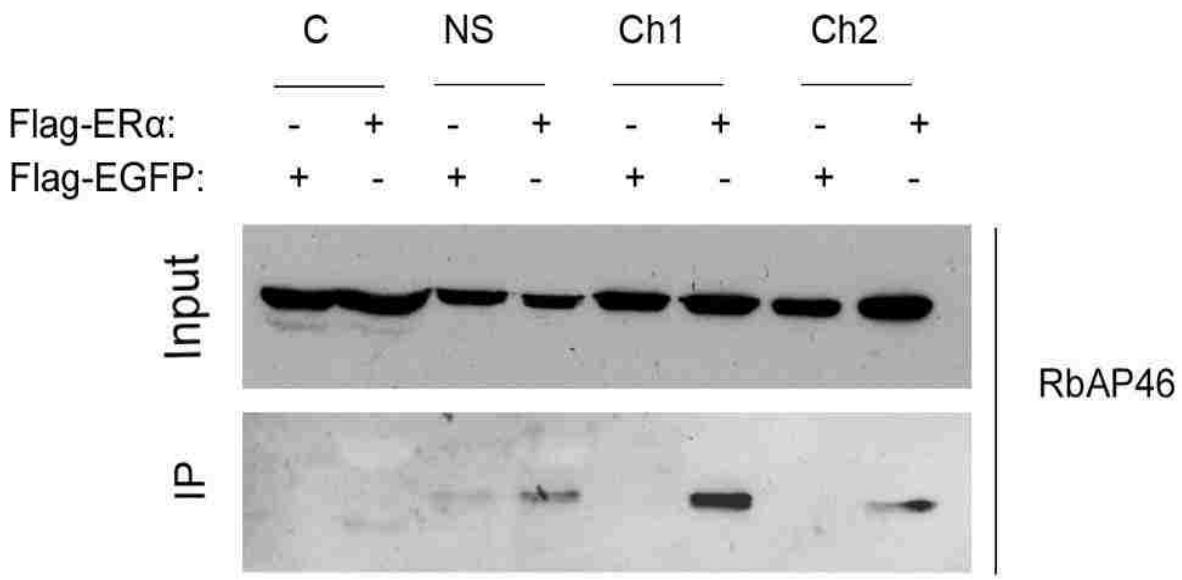
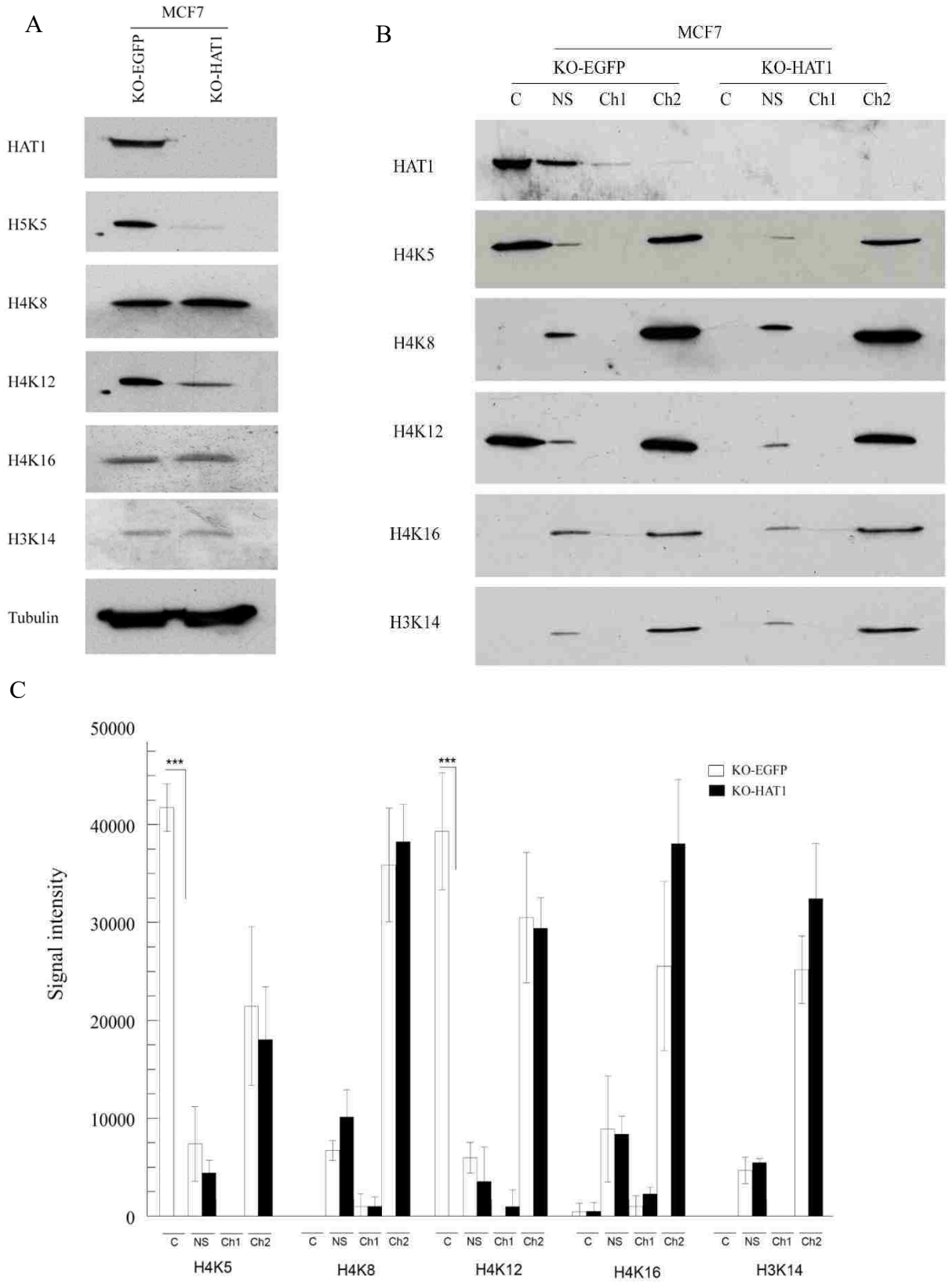


Fig.16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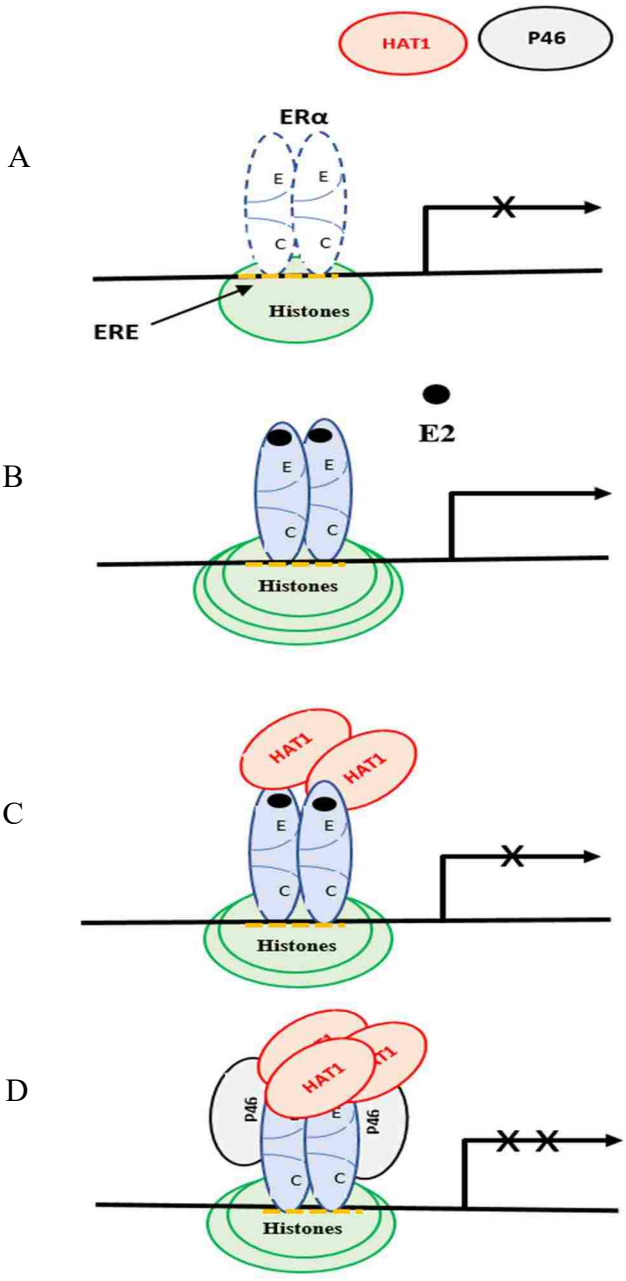
H5K5
H4K8
H4K12
H4K16
H3K14
Tubulin
B
MCF7
KO-EGFP
KO-HAT1
C
NS
Ch1
Ch2
C
NS
Ch1
Ch2
HAT1
H4K5
H4K8
H4K12
H4K16
H3K14
C
Signal intensity
50000
40000
30000
20000
10000
0
***
***
KO-EGFP
KO-HAT1
C
NS
Ch1
Ch2
H4K5
H4K8
H4K12
H4K16
H3K14

Fig. 17


HAT1
P46
A
ERα
E
E
C
C
Histones
ERE
E2
B
E
E
C
C
Histones
HAT1
HAT1
C
Histones
D
HAT1
P46
P46
Histones

Supporting information

S1. CRISPR Cas9-sgRNAs for knocking out HAT1 in MCF7 cells

HAT1-F1 (5'-CACCGCTACGCTCTTTGCGACCGT-3')

HAT1-R1 (5'-AAACACGGTCGCAAAGAGCGTAGC-3')

HAT1-F2 (5'-CACCGACACGTGGCCGGGTTTTGTC-3')

HAT1-R2 (5'-AAACGACAAAACCCGGCCACGTGTC-3')

S2. PCR primers to generate enzyme-dead mutant HAT1

HAT1-mutant1(187)-F (5'-GATGTGGTTTATTCAAACTGCTAGC-3')

HAT1-mutant1-R (5'-GCTAGCAGTTTGAATAAACCACATC-3')

HAT1-mutant2(276)-F (5'-GATATTACAGCGCAAGATCCATCC-3')

HAT1-mutant2-R (5'-GGATGGATCTTGCGCTGTAATATC-3')

Chapter3. Protein arginine methyltransferase 5 (PRMT5) is a novel ERα-interacting protein that negatively regulates ERα transcriptional activity

Abstract

Protein arginine methyltransferase 5 (PRMT5) is a member of type II PRMTs and responsible for methylation of mono- and symmetric arginine of histone and non-histone proteins. Previous studies have shown that PRMT5 methylates histone H3 at arginine 8 (H3R8) and histone H4 at arginine 3 (H4R3) and acts as a growth inhibitor in prostate cancer cells. Through a quantitative proteomic approach, we identified PRMT5 as a novel ERα-associated protein and the PRMT5-ERα interaction has been verified by co-immunoprecipitations. Cell fractionation and immunoprecipitation assays show that PRMT5 interacted with ERα preferentially in the cytoplasm of ERα-positive breast cancer cells (MCF7). We found that PMRT5 interacted with chloride nucleotide-sensitive channel 1A (pICln), and that both proteins were predominantly localized in the cytoplasm and much less associated with the transcriptionally active chromatin. Functionally, we found that overexpression of PRMT5 in MCF7 cells significantly decreased ERα transcriptional activity, suggesting that PRMT5 has a key role in regulating ERα function in breast cancer cells. A dual-luciferase reporter assay with a PRMT5 inhibitor showed that the methylation enzyme activity of PRMT5 is not required for the inhibitory effect of PRMT5 on ERα transcriptional activity. Importantly, we found that overexpression of PRMT5 in MCF7 cells significantly decreased ERα protein levels, which may explain the mechanism by which PRMT5 inhibited ERα-mediated gene expression.

Introduction

Estrogen receptor alpha (ERα), a member of the nuclear receptor (NR) superfamily of ligand-inducible transcription factors[1], plays a significant role in ERα-positive breast cancer cell growth, proliferation, and resistance of first-line endocrine therapies [2, 3]. There are two main mechanisms by which ERα is activated and regulates cell proliferation. First, the classical pathway (genomic action), in which estrogen binds ERα and triggers its conformational change, which in turn leads to ERα-chaperone protein disassociation [4]. ERα molecules then dimerize with either themselves (homodimer) [5] or ERβ (heterodimer), both of which bind with estrogen receptor elements (EREs) [6] and recruit coactivator or corepressor to regulate downstream genes [7, 8]. In addition to the classical (ligand- and ERE-mediated) pathway, ERα can exerts its influence on the physiology of cells through non-genomic action [9, 10], where ERα interacts with growth factor receptors, such as epidermal growth factor receptor (EGFR), insulin-like growth factor 1 (IGFR), the intracellular effector cAMP, and human epidermal growth factor receptor 2 (HER2) or adaptor proteins such as the modulator of nongenomic activity of estrogen receptor (MNAR) [11], and activates intracellular signaling pathways, including MAPK [12, 13] and PI3K [14, 15].

Posttranslational modification is an important process and participates in gene expression regulation, RNA assembly, and protein function [16, 17]. Protein arginine methyltransferases (PRMTs), the enzymes that transfer methyl groups from methyl donor (S-adeniosylmethionine) to arginine residues of variant proteins, are classified into four types depend in on the number and position of methylated arginine residues. Type I PRMTs catalyze ω-N^G-monomethylarginine (MMA) and asymmetric ω-N^G-dimethylarginine (aDMA) [18] and are linked to transcriptional activation. Type II PRMTs catalyze ω-N^G-monomethylarginine (MMA) and symmetric ω-N^G-

dimethylarginine (aDMA) and are linked to transcriptional repression [19, 20]. Type III and IV PRMTs catalyze monomethylarginine and δ-N^G-monomethylarginine that limited to yeast Rmt2, respectively [21]. PRMT5, also known Hs17, Jbp1, Capsuleen, Skb1, or Dart5, is the main type II PRMT. PRMT5 methylates non-histones and histones protein (histone H3 at arginine 8 (H3R8), histone H4 at arginine 3 (H4R3), and Histone H2A) [22] and is involved in gene transcription, protein biosynthesis [23], cell cycle checkpoints [24], cell reprogramming [25] and migration [26], primordial germ cells [27], and signaling modulation [28, 29]. PRMT5 also plays a key role in mRNA metabolism by methylating spliceosomal proteins [30, 31]. PRMT5 complex symmetrically dimethylates Sm proteins D1, D3 and B/B', which are in turn bound by pICln that acts as assembly chaperone. pICln among other PRMT5 binding partners [32], such as Menin/Men1, RioK1, and CoPR5, serves as PRMT5 adapter protein and regulates PRMT5 in substrate selection [27].

It has been reported that PRMT5 overexpression was linked to poor prognosis of breast cancer [33]. However, the information on the role of PRMT5 in breast cancer is very limited. In this study, we found that PRMT5 is a new ERα-interacting protein that preferentially associates with ERα in cytoplasm of MCF7 cells. In addition, we found that PRMT5 acts as a ERα suppresser potentially through decreasing ERα protein levels. Furthermore, we observed that PRMT5 methylation enzyme activity is not involved in the PRMT5 inhibitory effect on ERα.

Materials and Methods

Materials. Alpha minimal essential medium (α-MEM), and Opti-MEM I reduced serum medium were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was from Atlanta biologicals (Norcross, GA, USA). Protein A beads, were obtained from Sigma-Aldrich (Sigma, St. Louis, MO, USA). Protease inhibitor cocktails were from Roche (Indianapolis, IN). Benzonase nuclease and Micrococcal nuclease were from EMD Millipore (Billerica, MA, USA) and New England Biolabs (Ipswich, MA, USA), respectively. Mouse monoclonal anti-ERα (F-10) and anti-pICln (C-5); goat polyclonal anti-PRMT5 (C-20), anti-NCoR (C-20), and anti-Actin (I-19); Rabbit polyclonal anti-p300 (N-15); non-immune IgG, and secondary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-Histone H3 was from Cell signaling (Danvers, MA, USA). RNase A was from affymetrix (Santa Clara, CA, USA) and RNase T1 was from Therom (Walkersviller, MD, USA). Dual-luciferase reporter assay system was from Promega (Madison, WI, USA). PRMT5 inhibitor (EPZ015666) was from Cayman chemical (Ann Arbor, MI, USA).

Cell culture, plasmid construction, and cell transfection. ER-positive breast cancer cells (MCF7) and human embryonic kidney 293T cells (HEK293T) were routinely cultured in DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 5% fetal bovine serum, 1% penicillin and streptomycin. Coding sequences of PRMT5 or EGFP were cloned into the BamHI and XhoI sites of pcDNA 3.1 (Invitrogen). The plasmids were transiently transfected into MCF7 cells using ViaFec reagent (Bio-Rad) or into 293T cells using calcium-phosphate method.

Proteome labeling, and affinity purification. The SILAC-labeling was performed as previously described [4]. In brief, A population of human embryonic kidney 293T cells were cultured in labeled ($R^{13}C_6$, $K^{13}C_6{}^{15}N_2$) Dulbecco's Modified Eagle's Medium (Thermo Fisher

Scientific, Waltham, MA) with 10% dialyzed FBS and 1% penicillin and streptomycin for two weeks and then transiently transfected with plasmids expressing Flag tag alone. A second population of 293T cells were cultured in unlabeled ($R^{12}C_6$, $K^{12}C_6{}^{14}N_2$) Dulbecco's Modified Eagle's Medium with 10% FBS and 1% penicillin and streptomycin, and transiently transfected with plasmids that express Flag-ERα. The two population of cells were harvested 48 h after transfection, washed, and incubated in 5 packed cell pellet volumes of lysis buffer I [20 mM Tris-HCl, pH 7.5, 0.5% NP-40, 1 mM EDTA, 10 nM 17β-estradiol, protease inhibitors (Roche, Indianapolis, IN), and phosphatase inhibitors (1 mM Na3VO4, 10 mM NaF, and 10 mM β-glycerophosphate)] on ice for 30 min. The cells were then lysed and supplied with NaCl and glycerol to final concentrations of 125 mM and 10%, respectively. The extracts were centrifuged and the resulting pellets were resuspended in lysis buffer I supplemented with 125 mM NaCl and 10% glycerol and extracted again with sonication (Branson Digital Sonifier 450, Branson Ultrasonics Co., CT) [34].The combined and cleared supernatant was incubated with pre-washed Flag M2 resin (Sigma-Aldrich, St. Louis, MO) for 5 h at 4°C with end-to-end rotation. The beads were then washed extensively with lysis buffer I supplemented with 125 mM NaCl and 10% glycerol. The bound proteins were eluted with elution buffer and fractionated with a 12% SDS-PAGE gel for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS, database search, and data analysis. In-gel digestion and LC-MS/MS analysis were performed as previously described [35, 36]. Shortly, Protein identification and quantification were performed with Maxquant (version 1.0.13.13) and Mascot (version 2.2; Matrix Science, Boston, MA) by searching against a composite target-decoy International Protein Index (IPI) human protein database (version 3.52) as described previously [35]. The LC-MS/MS spectral data were also analyzed with the Scaffold (version 3.4.5; Proteome Software

Inc., Portland, OR). For the Scaffold analysis, the following values were used: 95% peptide probability, 99.0% protein probability, and a minimum of 2 peptides/protein. The normalized spectral abundance factors (NcSAFs) were calculated as described [37, 38].

Subcellular fraction assays. To fractionate MCF7 cells into cytoplasmic and nuclear fractions, we followed our established protocol in the PLoS ONE paper [4]. Briefly, cells were cultured in the α-MEM supplemented with 5% FBS (Hyclone, Logan, UT) and 1% penicillin and streptomycin, harvested, washed twice with cold 1x PBS, resuspended in 5 packed cell pellet volumes of hypotonic buffer (10 mM Tris-HCl, pH 8.0, 5 mM KCl, and 1.5 mM MgCl2) supplemented with protease inhibitors, and incubated on ice for 20 min. The cells were lysed by douncing after adding phosphatase inhibitors (1 mM Na3VO4, 10 mM NaF, and 10 mM glycerophosphate) to the cell suspension. After centrifugation, the supernatant was cleared by centrifugation, supplemented with 15 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1 % SDS and 3 mM EDTA, and saved as cytosolic fraction. The pellet was resuspended in hypotonic buffer, dounced, and centrifuged. The pellet was washed twice with hypotonic buffer and saved as nuclei. The isolated nuclei were resuspended in lysis buffer (25 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 3 mM EDTA, 0.1 % SDS, protease inhibitors, and phosphate inhibitors), sonicated on ice, centrifuged, and the resulting supernatant was designated as nuclear fraction. MCF7 cells were fractionated into soluble protein (S), chromatin-binding protein (CB), and pellet (P) and into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2) as described before in chapter two and three.

Immunoprecipitation. MCF7 or transfected 293T cells lysate was centrifuged at 16,000 g for 15 min at 4°C, and pre-cleared with protein A beads (Sigma) at 4°C for 1 h with end-to-end

rotation. The pre-cleared supernatant was incubated with ERα, pICln, or IgG-conjugated protein A beads overnight at 4°C with end-to-end rotation. After washing at least 3 times with the washing buffer, the bound proteins were eluted by boiling in 1x SDS sample buffer.

Sucrose gradient separation assay and RNases treatment. Extracted proteins from whole cell lysate or fractionated MCF7 cells were cleared by centrifugation 21,000xg for 15 min at 4ºC. For RNases treatment, RNase A (0.2 μg/μl) and RNase T1 (0.5 U/μl) were mixed with a cleared supernatant and the treated and mock-treated samples were incubated for 20 min at 37ºC, centrifuged 10,000xg for 5 min at 4ºC. Supernatants were fractionated by sucrose gradient separation assay as described in chapter three. The fractions were analyzed by Western blotting with indicated antibody.

PRMT5 overexpression in MCF7 cells. MCF7 cells were seeded in 12 wells plates overnight in α-MEM (Invitrogen) with 5% charcoal-treated FBS (Hyclone, Logan, UT) and 1% penicillin and streptomycin and then transiently transfected with increasing concentrations of a plasmid that expresses PRMT5 as indicated. The difference in total amount of DNA that added into the cells was compensated by the empty vector. After 24 h, the medium was replaced with the fresh medium and cells were incubated for 2 days. The cells were harvested, washed with cold 1x PBS, and lysed in the lysis buffer (10 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1 mM EDTA, 0.1% NP-40) supplied with protease inhibitors and phosphate inhibitors (1mM Na3VO4, 10 mM NaF, and 10 mM glycerophosphate). Equal amounts of extracted proteins from each concentration were fractionated by 10% SDA-PAGE and analyzed by Western blot with indicated antibodies.

PRMT5 enzyme inhibitory assay. MCF7 cells were cultured overnight in 12 wells plates in α-MEM (Invitrogen) supplement with 5% FBS (Hyclone, Logan, UT) and 1% penicillin and streptomycin and then treated with different concentrations of PRMT5 inhibitor (EPZ014666) or

ethanol as indicated for 4 days. The cells were harvested, washed twice with cold 1x PBS, and lysed in the lysis buffer (50 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1% SDS, protease inhibitor mixture). 60 µg extracted proteins from each concentration were resolved by Western blot with indicated antibodies.

Dual-luciferase reporter assay. Cells were seeded in 24-well plates overnight in α-MEM (Invitrogen) with 5% charcoal-treated FBS (Hyclone, Logan, UT) and 1% penicillin and streptomycin and co-transfected with either PRMT5 or empty plasmids and 3 × ERE-TATA-luc [39] and pRL-TK (Promega) Renilla luciferase normalization vector (Promega, Madison, WI) with 50:1 ratio, respectively, by ViaFect reagent (BioRad). After 24 h, the medium was replaced with the fresh above medium, and cells were incubated for another day. For PRMT5 inhibitor (EPZ015666) effects on ERα transcriptional activity cells were treated with 1uM of PRMT5 inhibitor or ethanol as control for 24 h. The dual-luciferase reporter assay was performed for at least three independent repeats as described in chapter 3.

Statistical Analysis. The p values were calculated using a One-way ANOVA (PSI-PLOT, Pearl River, NY). Data were presented as the mean ± S.D.

Results

Identification of the proteins that potentially associate with ERα. A SILAC-based quantitative proteomic approach was performed to identify proteins that are probably associated with ERα. One protein identified that is potentially associates with ERα is PRMT5. To determine how cellular PRMT5 protein is distributed in cells in relation to cellular ERα protein, we fractionated whole cell lysate of MCF7 cells with sucrose gradient separation ultracentrifugation and analyzed the fractionated proteins with WB. The results demonstrated that cellular PRMT5 protein appeared in two major peaks in sucrose gradient, one in fractions 10-15 and one in fractions 18-20 (Fig. 1, II row). The second peak (fractions 18-20) appears to overlap with a major peak of cellular ERα protein (Fig.1, compare II row with I row). pICln is the adaptor protein of PRMT5 and is one component of the methylosome that consists of PRMT5, methylosome protein 50 (MEP50) and pICln. Interestingly, the majority of cellular did not perfectly overlap with the second peak of PRMT5 (fractions 18-20), and a small portion of pICln perfectly overlaps with the first peak of PRMT5 (fractions 10-15) (Fig. 1, compare III row with II row). Importantly, MEP50 primarily appeared in one peak which highly overlaps with the first peak of PRMT5 and pICln peak (Fig. 1, compare IV row with II and III rows, respectively) confirming published results that showed that PRMT5, pICln, and MEP50 were found in one complex [40].

PRMT5 interacts with ERα in the cytoplasm of MCF7 cells. To verify the interaction between PRMT5 and ERα, we carried out co-immunoprecipitation using 293T cells that were transfected with plasmid that expresses ERα. The extracted proteins were immunoprecipitated by anti-ERα or an isotype-matched, unrelated control IgG, and the eluted proteins were analyzed by Western blotting. The result shows that anti-ERα precipitated more PRMT5 than did the control

IgG (Fig. 2A). To examine whether endogenous PRMT5 interacts with endogenous ERα and where the interaction might take place in cells, MCF7 cells were fractionated into cytoplasmic protein and nuclear protein, and the fractionated proteins were immunoprecipitated by antibodies against ERα. The bound proteins were analyzed by Western blotting. The result demonstrated that endogenous PRMT5 was mainly localized in the cytoplasm, which is consistent with the results from other research groups [41, 42]. Results from IPs showed that endogenous PRMT5 interacts with endogenous ERα in the cytoplasm (Fig. 2B). We have confirmed the effectiveness of our subcellular fractionation by performing Western blot analysis using markers of the cytoplasm and the nucleus, tubulin and histone H4, respectively (Fig. 2B). As expected, pICln interacts with PRMT5 (Fig. 2C).

PRMT5 and ERα subcellular colocalization in MCF7 cells. It is well known that PRMT5 subcellular localization is important for its function in the cells [43]. Studies have shown that PRMT5 in the cytoplasm forms methylosome, a 20S protein arginine methyltransferase complex consisting of PRMT5, pICln and MEP50, whereas in a nucleus it associates with different proteins (e.g. SWI/SNF chromatin remodelers) [20]. Thus, we examine the subcellular colocalization of PRMT5, its adaptor protein (pICln), MEP50, and ERα in MCF7 cells. First, MCF7 cells were fractionated into soluble protein (S), chromatin-binding protein (CB), and remaining pellet (P), and the extracted proteins from the fractions were analyzed by Western blotting with indicated antibodies. The result demonstrated that the majority of PRMT5, pICln, and MEP50 were localized in the cytosol and a minor portion of the two proteins were associated with chromatin. (Fig. 3A). Second, we fractionated MCF7 cells into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and the fractionated proteins were analyzed by Western blotting. The results showed that

the majority of PRMT5, pICln, and MEP50 were colocalized in the cytosol, and a significant portion of them also exist as soluble nuclear protein in the nucleus (C) (Fig. 3B). Compared with PRMT5 and MEP50 the portion of pICln protein that is associated with chromatin is much less.

Most cellular PRMT5 protein is not associated with RNAs. It has been well established that PMRT5 complex is involved in RNA metabolism [29, 44], which predominantly occur in the cytoplasm of mammalian cells [45]. To determine whether PRMT5, PICln, MEP50 associate with RNAs in relation to ERα, MCF7 cells were fractionated into cytoplasm and nucleus, and the fractionated proteins were digested with RNase A and T1. The RNase-digested and mock-digested portions were separated by sucrose gradient (10-30%) and analyzed by Western blotting. The results demonstrated that there is slight difference in PRMT5 protein distribution in the sucrose gradient fractionation between RNase-digested and mock-digested protein in both cytoplasmic protein and nuclear protein (Fig. 4A and B, II panel, compare RNase row with C.T row), suggesting that most cellular PRMT5 protein is not associated with RNAs. In contrast, the distribution of a small portion of cytosolic PICln and most nuclear PICln was altered by RNase digestion (Fig. 4A and B, III panel, compare RNase row with C.T row), suggesting that a significant portion of PICln, nuclear PICln in particular, is associated with RNAs. Like PRMT5, MEP50 protein distribution in cytoplasmic fractions was slightly changed after RNases digestion (Fig. 4A, IV panel, compare RNase row with C.T row). But, a meaningful change was occurred in MEP50 protein distribution in the nuclear factions after RNases digestion (Fig. 4B, IV panel, compare RNase row with C.T row). Surprisingly, ERα protein is associated with large complexes with very high molecular weight (e.g., fractions 1 to 4), and all these complexes were sensitive to RNases digestion (Fig. 4A, I panel), suggesting that most cytosolic ERα protein is associated with RNAs. The distribution of nuclear ERα in large complexes with very large

molecular weight (e.g., fractions 1 to 9) were not altered RNases digestion (Fig. 4B, I panel), presumably in those complexes the ERα is associated with DNAs. However, the distribution of nuclear ERα in complexes with smaller molecular weight (e.g., fractions 13-19) was substantially altered by the RNases digestion, suggesting that these ERα complexes contain RNAs.

PRMT5 inhibits ERα-mediated gene expression, and the enzyme activity of PRMT5 is not involved in the inhibitory effect. PRMT5 has been shown to be involved in transcriptional silencing of regulatory and tumor suppressor genes [20, 46] and in STAT3-mediated transcriptional repression [47]. To examine the effect of PRMT5 on ERα transcriptional activity, we performed dual-luciferase reporter assay. The result shows that ERα transcriptional activity was significantly decreased by exogenous expression of PRMT5 (Fig. 5A), suggesting that PRMT5 inhibits ERα-mediated gene expression in MCF7 cells. To test whether PRMT5 enzyme activity is involved in inhibitory effect of PRMT5 on ERα-mediated gene expression, we performed the dual-luciferase reporter assay with PRMT5 inhibitor (EPZ015666). The result shows that ERα transcriptional activity was significantly decreased after PRMT5 was overexpressed and addition of PRMT5 enzyme inhibitor EPZ015666 showed no significant change in the PRMT5 inhibitory effect on ERα transcriptional activity (Fig. 5B). These results suggest that PRMT5 enzyme activity is not required for its inhibitory effect on ERα transcriptional activity.

PRMT5 inhibits ERα-mediated gene expression through decreasing ERα-protein level in MCF7 cells. To understand how PRMT5 inhibits ERα transcriptional activity, we examined whether PRMT5 affects ERα-protein level. PRMT5 was overexpressed in MCF7 cells and the extracted proteins were analyzed by Western blotting. The results demonstrated that ERα protein

level was significantly decreased by moderate overexpression of PRMT5 (Fig. 6A). To further this result, we repeated the Western blotting with increasing amounts of the plasmid express PRMT5. The Western blotting result demonstrated that increased expression of PRMT5 protein levels resulted in reduced levels of ERα protein (Fig. 6B). These results suggest that PRMT5 may inhibit ERα-mediated gene expression by suppressing the expression of ERα in the cells. Additionally, neither pICln nor MEP50 protein levels were affected when PRMT5 was overexpressed in MCF7 cells (Fig. 6B), suggesting that PRMT5 selectively affected ERα protein levels. To determine whether PRMT5 methyltransferase activity is involved in inhibiting ERα-protein levels in the cells, MCF7 cells were treated with increasing concentrations of PRMT5 inhibitor (EPZ015666) or ethanol (vehicle) as indicated and whole cell lysate from the treated cells were analyzed by Western blotting with indicated antibodies. The result shows that the protein levels of PRMT5, pICln, MEP50, and ERα protein levels were not changed by PRMT5 inhibitor (Fig. 6C). These results support the view that the PRMT5 catalytic activity has no influence on ERα expression or stability in MCF7 cells.

Discussion

PRMT5 has been linked to many fundamental cellular processes, including but not limited to RNA metabolism, gene expression, and cell signaling [20, 48, 49]. In this study, through a SILAC -based quantitative proteomic method and co-immunoprecipitation, we identified and confirmed that PRMT5 is a novel ERα-interacting protein. We have demonstrated that PRMT5 interacts with ERα (Fig. 1A) and the interaction was preferentially in the cytoplasm of ERα-positive breast cancer cells (Fig. 1B).

It has been reported that PRMT5 subcellular localization affects its function and how it complexes with other proteins. Recent studies showed that the iRioK1 (Rio domain-containing protein) interacts with PRMT5 in the cytoplasm and potentially influences PRMT5 temporal and spatial activity [40]. We found in this study that PRMT5, pICln, and MEP50 were mainly colocalized in the cytosol of MCF7 cells (Fig. 3A and B).

It has been shown that PMRT5 influences the stability and function of some transcription factors, such as E2F-1. Silencing PRMT5 leads to increasing E2F1 protein levels and expression of its downstream genes in U2OS cells [50]. Our results from dual-luciferase reporter assays showed that ERα transcriptional activity was significantly decreased by overexpression of PRMT5 (Fig. 5A). It has been well established that PRMT regulates gene expression through posttranslational modifications. For example, recent studies showed that PRMT1, type I PRMTs [44], can methylate ERα at arginine 260 (R260) that modulates ERα function and leads to activate Akt pathway [51]. The methylation enzyme activity of PRMT5 was also shown to be important in regulating the transcriptional function of several transcription factors, such as E2F-1[52], p53 [53], and NF-KB [54]. Our results suggest that PRMT5 affect ERα transcriptional activity through the enzyme-independent mechanism (Fig. 5B). Further studies suggest that

PRMT5 inhibits ERα transcriptional activity through suppressing cellular protein levels of ERα, and the suppression is also PRMT5 enzyme activity-independent (Fig. 6 A-C). In summary, we identified through quantitative proteomic base technique that PRMT5 is a novel ERα-interacting protein and PRMT5 associates with ERα preferentially in the cytoplasm. We found that PRMT5 inhibits ERα-mediated gene expression through decreasing cellular ERα protein levels and this action was PRMT5 enzyme activity-independent.

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Figures

Fig. 1. Fractionation of cellular protein with sucrose gradient. MCF7 cells were harvested, washed with 1x PBS, and lysed with a lysis buffer. The extracted proteins were separated by10-30% sucrose gradient. The fractionated proteins were separated and analyzed by Western blotting. IN, input; WCL, whole cell lysate.

Fig. 2. PRMT5 interacts with ERα in the cytoplasm. A, 293T cells were transiently transfected with plasmids expressing ERα. Cell lysate of the transfected cells were immunoprecipitated with anti-ERα or an isotype-matched, unrelated control IgG. The immunoprecipitated proteins were analyzed by Western blotting. B, co-immunoprecipitation of endogenous PRMT5 and endogenous ERα in MCF7 cells. Cells were harvested, washed, and fractionated into cytoplasmic protein and nuclear protein, which were immunoprecipitated by anti-ERα. The immune-precipitated proteins were analyzed by Western blotting. Tubulin and Histone H4 were used as markers for the cytoplasmic and nuclear fraction, respectively. Cyto, cytosolic; Nuc, nuclear. C, MCF7 whole cell lysate were immunoprecipitated by anti-pICln antibody and the eluted proteins were analyzed by Western blotting.

Fig. 3. Subcellular distribution of PRMT5, pICln and MEP50. Whole cell lysate of MCF7 cells (2×10^6) were fractionated into either A, soluble protein (S), chromatin binding protein (CB), and pellet (P) or B, cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2). The fractionated proteins were analyzed by Western blotting with the indicated antibodies. Histone H3, p300, and NCoR were used as markers of chromatin-binding protein, active chromatin, and inactive chromatins, respectively.

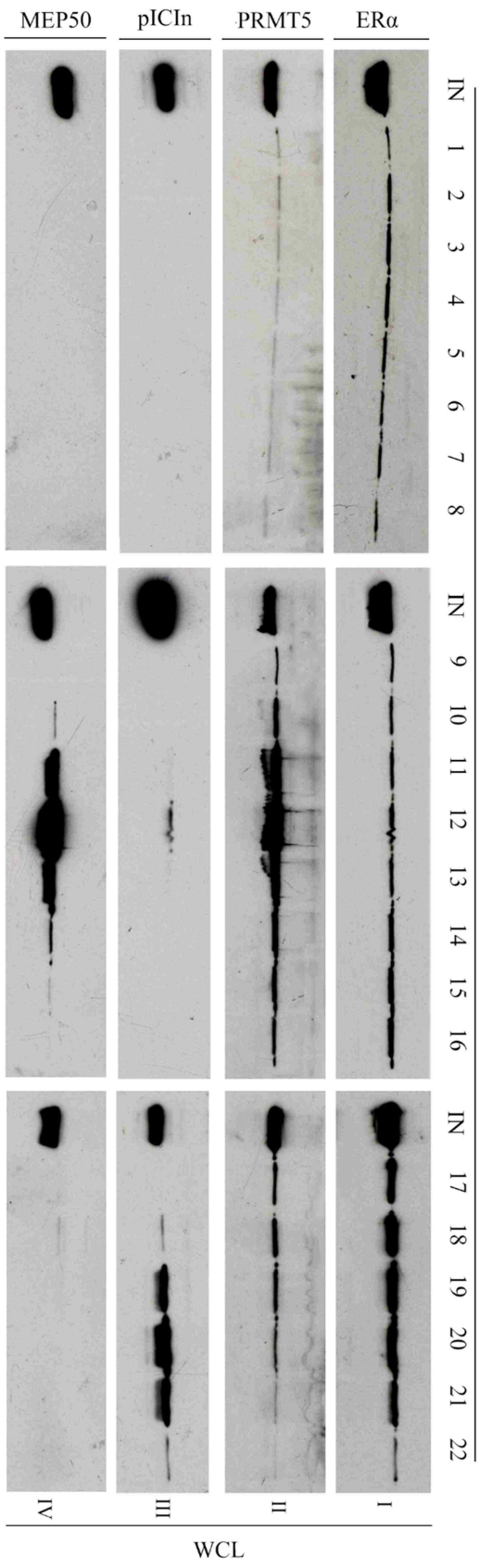
Fig. 4. Majority of cellular PRMT5 protein is not associated with RNAs. MCF7 cells were fractionated into cytoplasmic protein (A) and nuclear protein (B), and fractionated proteins were

mock-digested or digested with RNase A and RNase T1. The mock-digested and RNase digested proteins were separated by10-30% sucrose gradient, and the fractionated proteins were analyzed by Western blotting with indicated antibodies. IN, whole cell lysate; RNases and C.T, RNase-treated and mock-treated (control) with RNases, respectively.

Fig. 5. PRMT5 inhibits ERα transcriptional activity. A, MCF7 cells were co-transfected with reporter plasmid 3×ERE-TATA-luc, Renilla luciferase normalization vector pRL-TK, and plasmid expressing PRMT5 or empty plasmids, and the transfected cells were monitored for expression of reporter gene. B, MCF7 cells were treated with 1μM PRMT5 inhibitor (EPZ015666) or ethanol for 24 h, and the expression of the reporter gene was measured as described in A. The results are showed as relative light unit (RLU) = Firefly luciferase reading/ Renilla luciferase reading. ** denote $p < 0.01$.

Fig. 6. PRMT5 suppresses ERα protein level in MCF7 cells. A, Cells were transfected with either PRMT5 or empty vector and whole cell lysate was analyzed by Western blotting with indicated antibodies. B, MCF7 cells were transfected with fixed amount of plasmid expressing ERα and increasing amount of plasmid expressing PRMT5. The transfected cells were harvested, washed, and lysed and whole cell lysate was analyzed by Western blotting with indicated antibodies. C, MCF7 cells were treated with either different concentrations of PRMT5 inhibitor (EPZ015666) or equivalent amounts of ethanol as control. Extracted proteins were analyzed by Western blotting with indicated antibodies. Actin servers as loading control. NT, none-treated cells.

Fig. 1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pICIn
PRMT5
ERα
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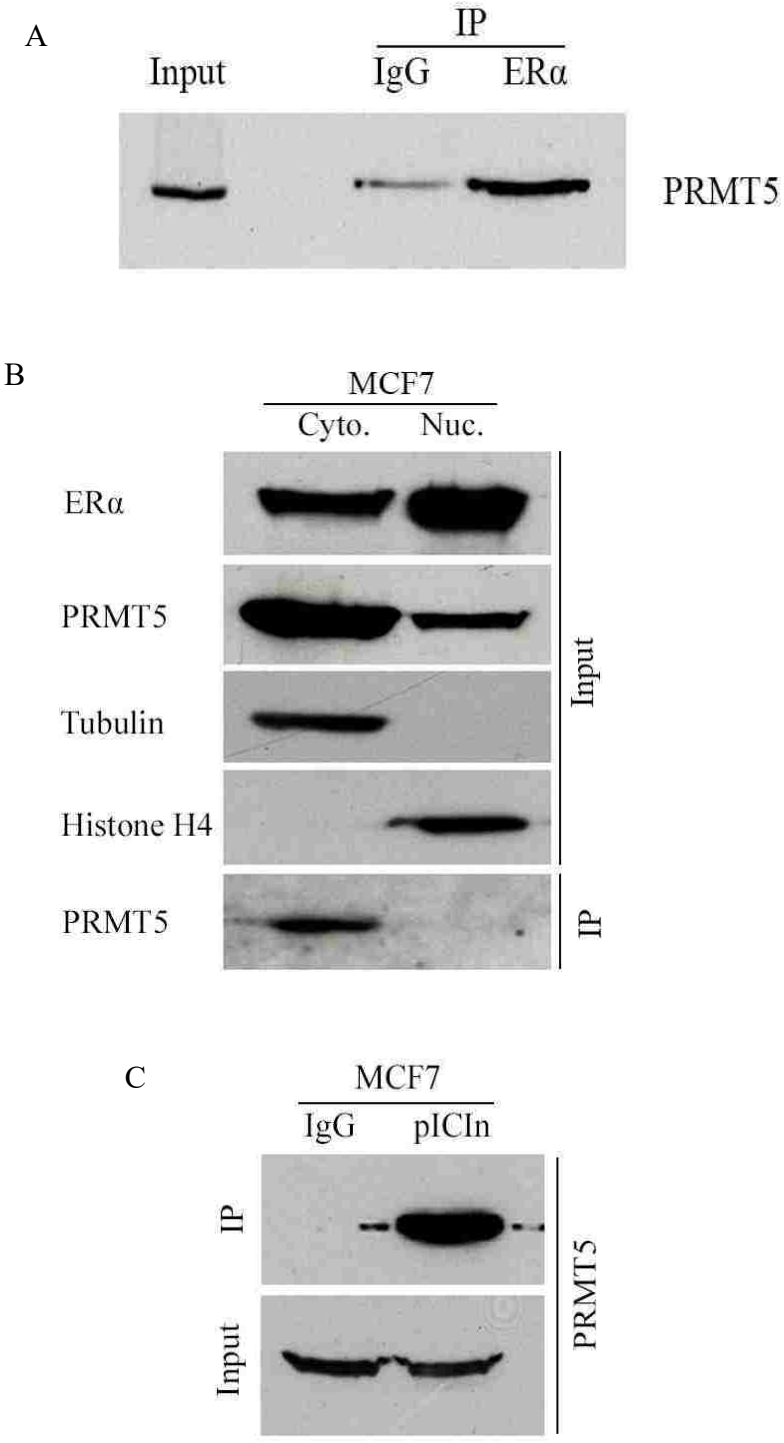
MCF7
IV
III
II
I
WCL

Fig. 2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IP
Input
IgG
ERα
PRMT5
B
MCF7
Cyto.
Nuc.
ERα
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Tubulin
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PRMT5
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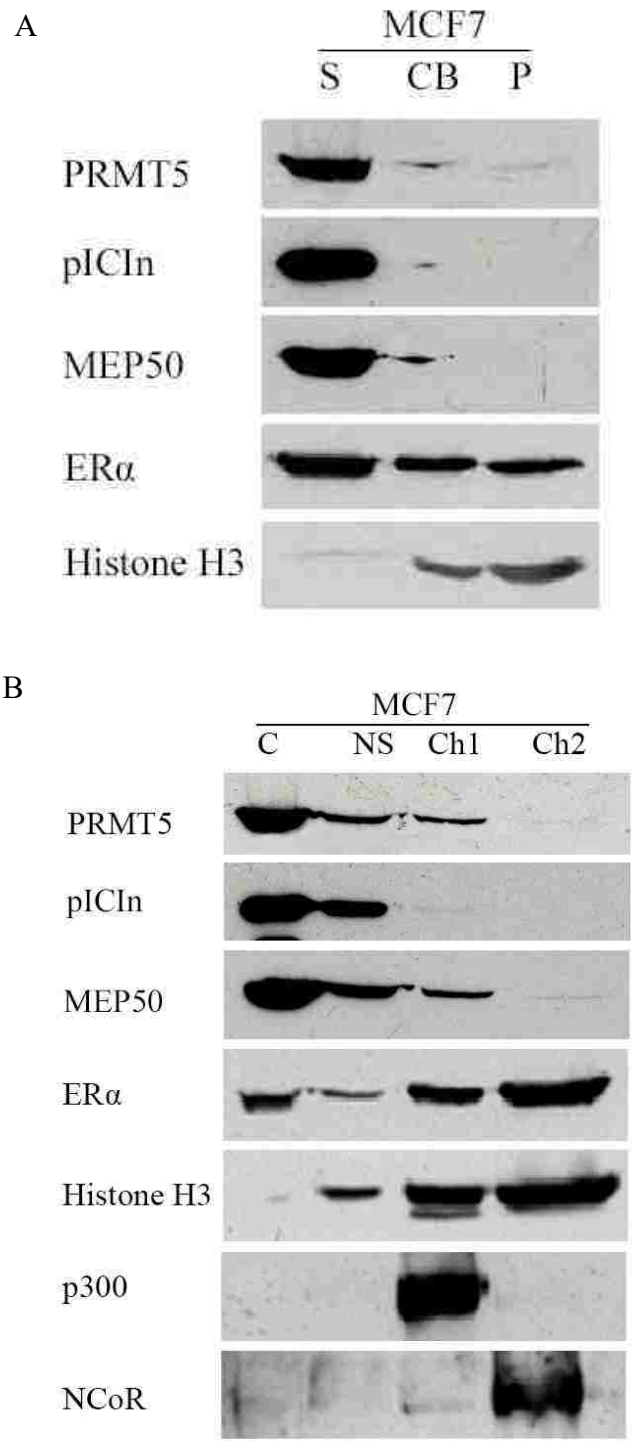
C
MCF7
IgG
pICIn
IP
Input
PRMT5

Fig. 3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MCF7
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Histone H3
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ERα
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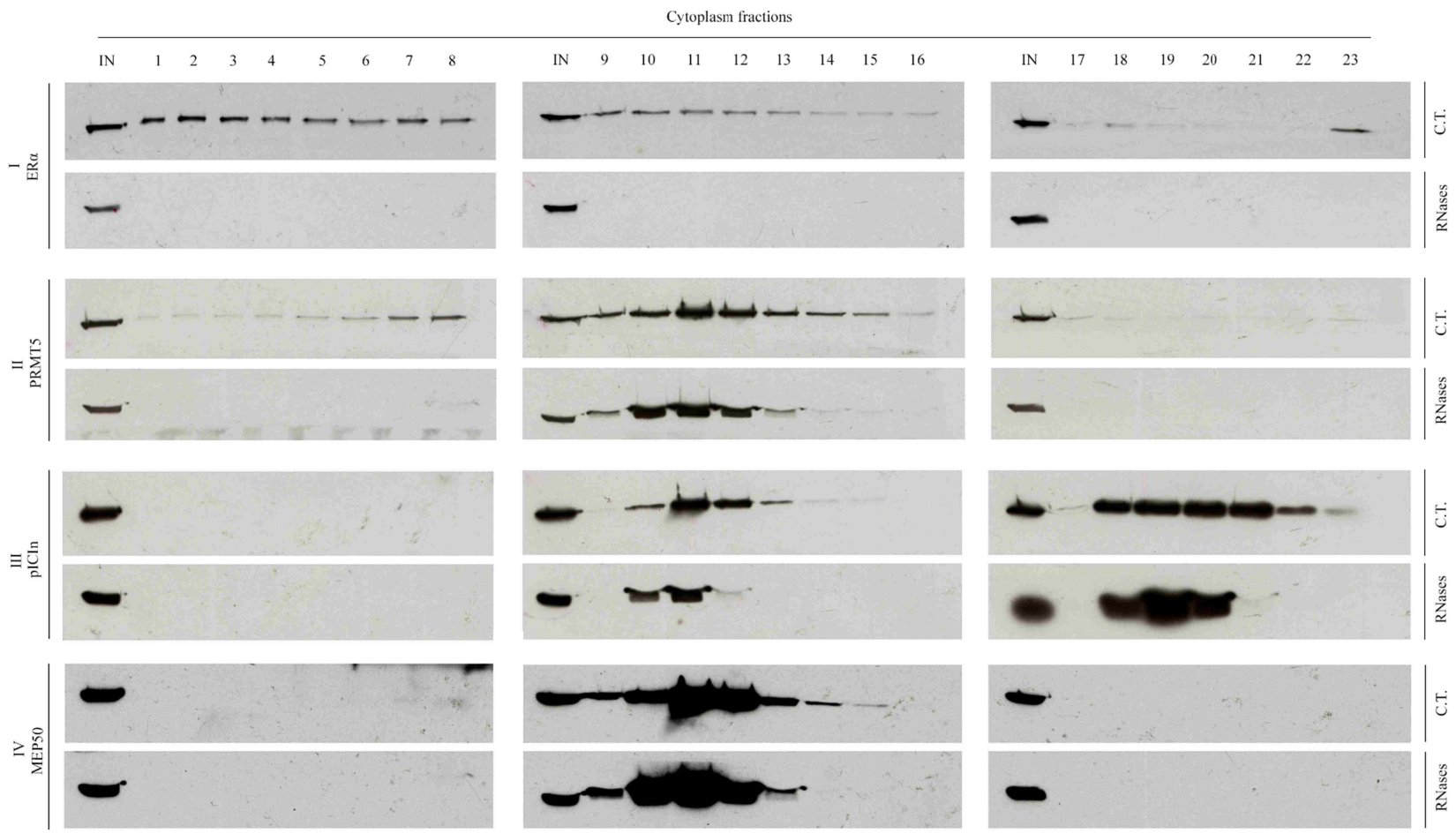
NCoR

Fig. 4


A
Cytoplasm fractions
IN 1 2 3 4 5 6 7 8
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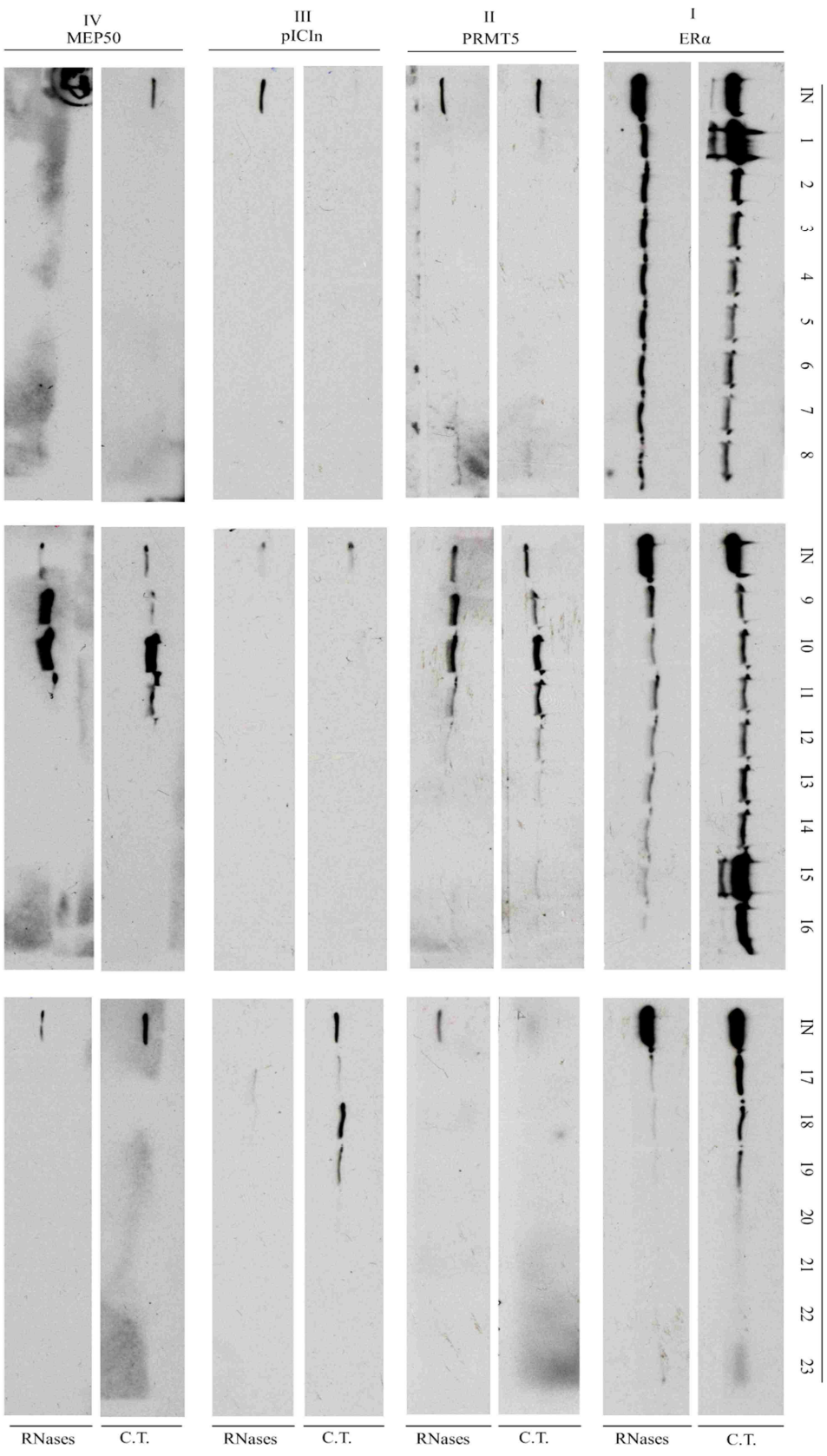
I ERα
II PRMT5
III pICln
IV MEP50
C.T.
RNases


B
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ERα
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PRMT5
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IN 9 10 11 12 13 14 15 16
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Nucleus fractions
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RNases
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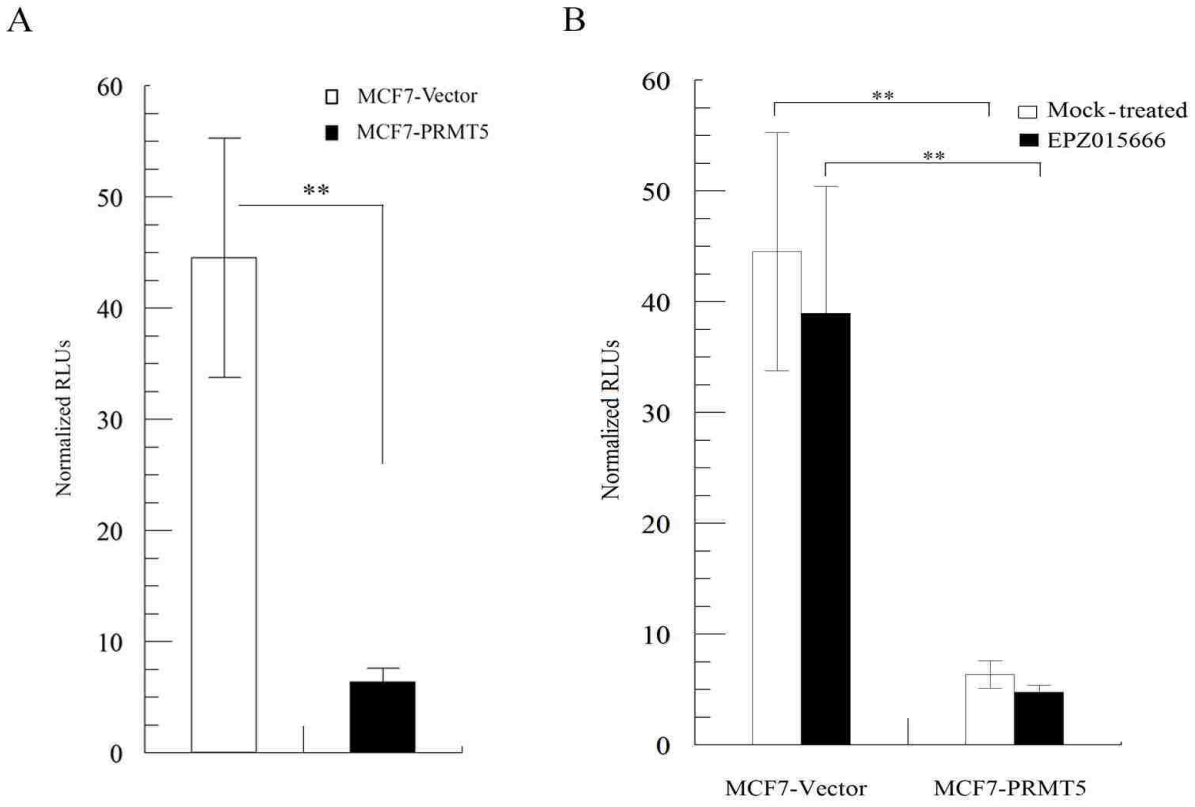
RNases
C.T.

Fig. 5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MCF7-PRMT5
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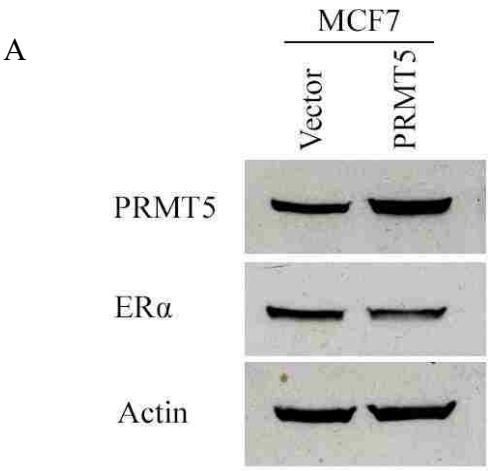
Mock-treated
EPZ015666
MCF7-Vector
MCF7-PRMT5

Fig. 6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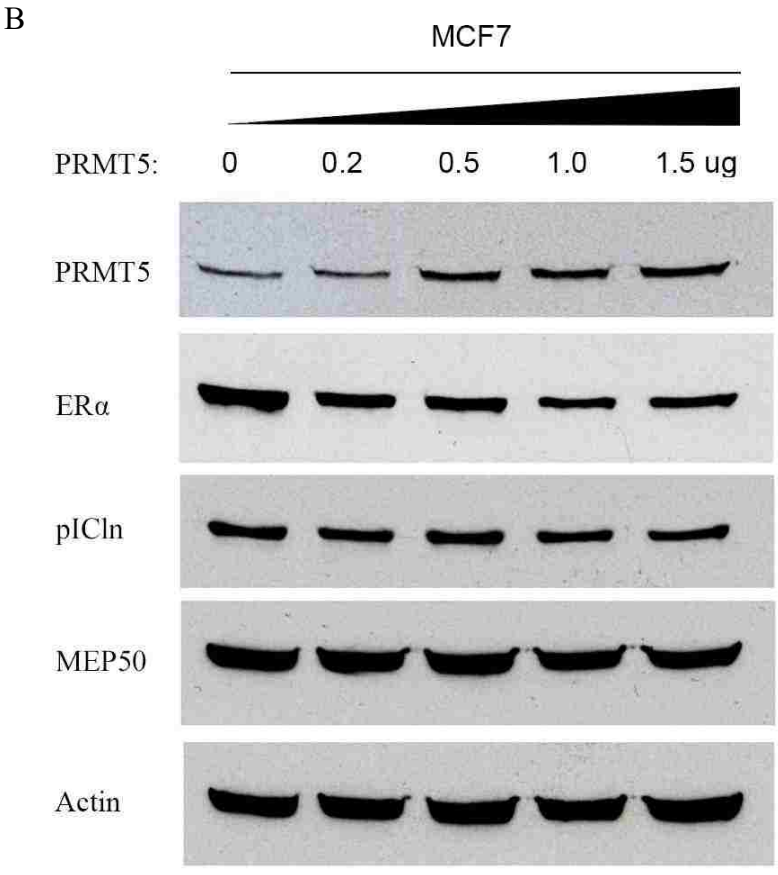
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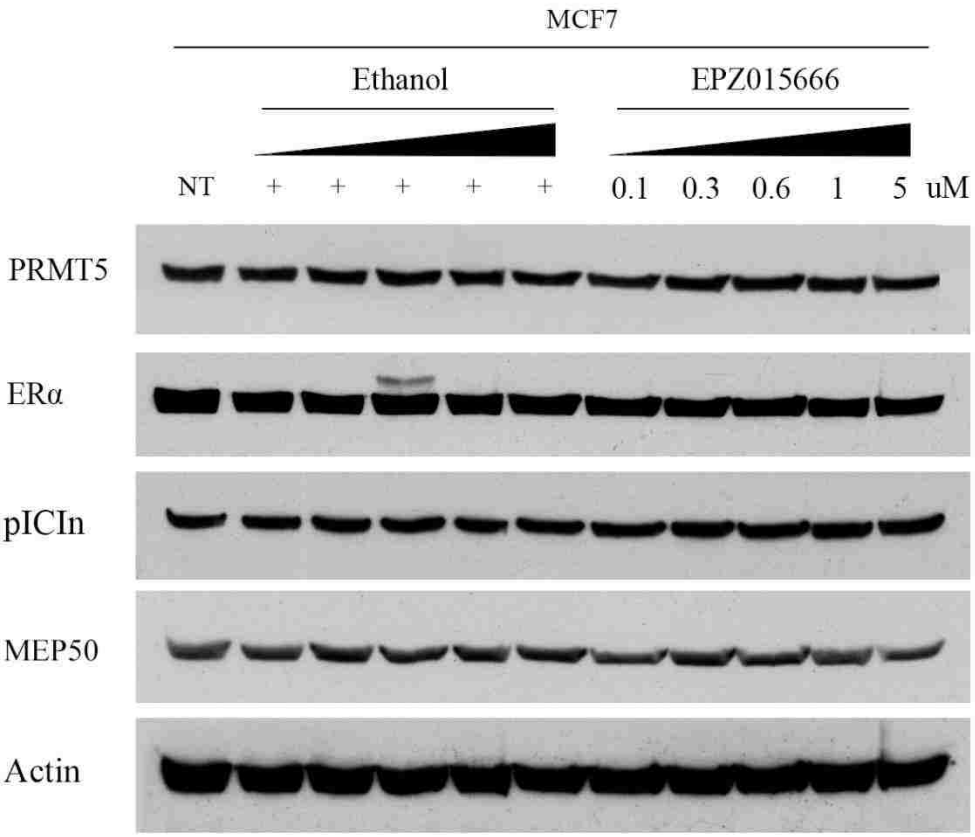
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Ethanol
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NT + + + + + 0.1 0.3 0.6 1 5 uM
PRMT5
ERα
pICIn
MEP50
Actin

Chapter4. Chromatin target of PRMT1 (CHTOP) binds ERα and regulates its transcriptional activity through MEP50 in breast cancer cells

Abstract

Chromatin target of PRMT1 (CHTOP) is a nuclear protein with a very conservative arginine-glycine-rich region (GAR). CHTOP is involved in cell proliferation, gene expression, and hormone-dependent activation of estrogen receptors (ERs). Recent studies have suggested that CHTOP may be involved in tumor development. However, the data on the function of CHTOP and the molecular mechanism of action are still very limited. Through a SILAC (stable isotope labeling by amino acids in cell culture)-based quantitative proteomic method and co-immunoprecipitation, we have identified and confirmed that CHTOP is a novel ERα-interacting protein. The in-vitro binding and protein domain mapping assays further established that CHTOP directly binds ERα and the binding is mediated the E domain, the ligand binding domain (LBD) of ERα. The results from electrophoretic mobility shift assay (EMSA) demonstrated that CHTOP directly bound to ERα, but not to estrogen receptor elements (EREs) and the CHTOP-ERα binding had no effect on ERα-ERE interaction. Importantly, we found that 17β-estradiol (E2) significantly enhances CHTOP-ERα binding under in-vitro conditions. Also, we have shown that the subcellular distribution, but not the expression of CHTOP was impacted by E2. Luciferase reporter assay reveals that knockout of CHTOP with the CRISPR-Cas9 gene editing system significantly decreased ERα transcriptional activity, suggesting that CHTOP is functionally linked to ERα-mediated gene expression. Interestingly, we found that knockout of CHTOP significantly decreased levels of MEP50 protein, a member of protein complex involves in the ER and AR transcriptional activity and methylosome pathway. Furthermore, we found that the decreased levels of MEP50 resulted from degradation of MEP50 through proteasome degradation pathway. In addition, we demonstrated that the nuclear MEP50 was dramatically shifted into the cytoplasm under hormone starvation conditions, and stimulation of starved

MCF7 cells with E2 slightly moved MEP50 back into the nucleus. Our results suggest that CHTOP may regulate ERα transcriptional activity through MEP50 in ERα positive mammalian cells.

Introduction

More than 70% of breast tumor cells express estrogen receptors (ERs), members of the nuclear receptor (NR) superfamily [1], that include estrogen receptors alpha and beta, ERα and ERβ [2]. These two receptors are encoded by different genes, but they have similar structures [3]. ERs, like other nuclear receptors, consists of six domains: The A/B domain, called activation function 1 (AF1), participates in the ligand-independent transactivation of ER [4]. The C and D domains are the DNA-binding domain (DBD) and hinge domain, respectively. The C domain is responsible for ERs binding to the ERE for target genes [5] while the D domain has a nuclear localization signal (NLS). The E domain, ligand-binding domain (LBD) [6], consists of 12 helices and primarily mediates the interaction between the receptor and its ligands, such as 17β-estradiol (E2) [7, 8]. Lastly, the E and F domains comprise the activation function 2 (AF2) region, which is involved in the ligand-dependent transactivation. ERα is inactive and monomeric molecule with a short half-life about 4-5 hours [9] until it binds to the ligand (e.g., E2), which triggers the ERα classical hormone activation pathway. Upon binding to estrogen, ERα dissociates from the chaperon protein [10, 11], dimerizes, translocates to the nucleus, and binds to the ERE [12]. Upon binding to ERE, ERα recruits coregulators (coactivators or corepressors) by which it controls the transcription of target genes [13]. It is well established that ERα plays an important role in breast cancer development [14], growth, and proliferation [15].

CHTOP, chromatin target of PRMT1, also known as FOP, SRAG, pp7704, C1orf77, FL SRAG, and C10orf77 [16], is relatively a small nuclear protein, about 27 kDa. CHTOP consists of the arginine-glycine-rich Region (GAR), which facilitates binding to DNA and RNA either directly or through nucleotide-binding proteins [17, 18]. Human CHTOP is encoded by previously unknown function gene called *C1orf77* located on chromosome 1 at 1q21.3. A shorter

CHTOP isoform, lacking the first 25 amino acids at the amino terminus, was identified in human and mouse [19]. Recently, CHTOP was found to be related to the cell proliferation and fetal globin gene expression regulation [17, 20]. It was reported that CHTOP interacted with protein arginine methyltransferase1 (PRMT1) [19] and was associated with the methylosome, an arginine methyltransferase complex consisting of arginine methyltransferase1(PRMT5), methylosome protein50 (MEP50), and enhancer of rudimentary homolog (ERH) [21, 22]. It was also reported that CHTOP was involved in glioblastomagenesis and required for the tumorigenicity of glioblastoma cells [22].

MEP50 was previously designated as a WD40 repeat protein because it has seven putative WD40 repeats [23]. The WD40 repeat proteins are known to play significant roles in protein-protein and protein-DNA interactions and posttranslational modifications [24, 25]. Previous studies showed that MEP50 acts as a coactivator of ER and androgen receptor (AR) and involves in the effects of hormone during ovarian tumorigenesis [26, 27]. The subcellular localization of MEP50 crucially impacts its function in cells. For example, while MEP50 is primarily located in benign prostate in the cytoplasm, in prostate cancer cells it is mainly located in the nucleus [28, 29]. A similar finding was reported for benign and malignant testicular tumors [30]. It has been reported that nuclear MEP50 in the breast and ovarian cancer cells enhances cell proliferation and invasiveness, whereas, the cytoplasm MEP50 inhibits both cell proliferation and invasiveness [26, 31].

In this study, we found that CHTOP interacts with ERα. In vitro binding assays demonstrated that CHTOP directly bound ERα, and the binding is mediated by E domain of ERα. The CHTOP-ERα binding was significantly increased by E2 under in-vitro conditions, and the binding had no effects on ERα-ERE interaction. Importantly, we found that knockout of

CHTOP significantly decreased ERα transcriptional activity. Concomitantly, we found that CHTOP mediated proteasome-degradation of MEP50 in MCF7 cells. Our results suggest that CHTOP, a novel ERα-interacting protein, acts as an ERα coactivator in the ER+ breast cancer cells.

Materials and methods

Cell culture, transfection, and knockout of CHTOP. Human ERα-positive breast cancer MCF7 cells, and human embryonic kidney 293T cells were routinely cultured in α-MEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 5% fetal bovine serum, 1% penicillin and streptomycin. Coding sequences of CHTOP or EGFP were cloned into the BamHI and XhoI sites of pcDNA 3.1 (Invitrogen). The plasmids were transiently transfected into MCF7 or 293T cells using ViaFec reagent (Bio-Rad) and calcium-phosphate method, respectively. To knock out CHTOP in MCF7 cells, CRISPR-Cas9 genome editing system was used as described [32]. Briefly, three specific guide RNAs (sgRNAs) that target CHTOP coding region were designed and inserted in pSpCas9(BB)-2A-GFP using BbsI restriction enzyme (Appendix A). MCF7 cells were transfected with the plasmids expressing the sgRNAs, and the transfected cells were analyzed by flow cytometry for GFP expression. CHTOP expression was analyzed by Western blotting and knockout of CHTOP was confirmed by DNA sequencing.

SILAC (Stable Isotope labeling by amino acid in cell culture). Two populations of human embryonic kidney 293T cells were used. The first group was grown in the labeled DMEM containing arginine-$^{13}C_6$ and lysine-$^{13}C_6{}^{15}N_2$, while the second group was grown in unlabeled DMEM for two weeks. The two populations of cells were transiently transfected with plasmids that express Flag alone and Flag-ERα, respectively. Cells were harvested, washed and lysed in 5 packed cell pellet volumes of lysis buffer I [20 mM Tris-HCl, pH 7.5, 0.5% NP-40, 1 mM EDTA, 10 nM 17β-estradiol, protease inhibitors (Roche, Indianapolis, IN), and phosphatase inhibitors (1 mM Na3VO4, 10 mM NaF, and 10 mM β-glycerophosphate)]. After adding NaCl and glycerol to final concentrations of 125 mM and 10%, respectively, the extracts were centrifuged and incubated with pre-washed Flag M2 resin (Sigma-Aldrich, St. Louis, MO) for 5

h at 4°C with end-to-end rotation. The beads were washed extensively and the bound proteins were eluted with elution buffer (10 mM Tris-HCl, pH 7.5, 350 mM NaCl, 1 mM EDTA, 250 mM 3X Flag peptides, and protease inhibitors), and fractionated with a 12% SDS-PAGE gel for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS and data analysis. In-gel digestion and LC-MS/MS analysis were performed as described previously [33, 34].

Co-immunoprecipitation and Western Blotting. The cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 0.1% Triton X-100 and protease inhibitors), and the Co-IP was carried out as previously described [10, 35]. In brief, the cells lysate was pre-cleaned with empty beads for 1 h at 4°C, and the cleared proteins were immunoprecipitated with antibody-conjugated beads overnight at 4°C with end-to-end rotation. After washing the beads three times with washing buffer, the bound proteins were eluted by either elution buffer or boiling in 1x SDS sample buffer for 4 min. The eluted proteins were separated by 10% SDS-PAGE and analyzed with Western blotting using enhanced chemiluminescence (ECL) system.

In-vitro binding assay and ERα-CHTOP protein domain mapping. pGEX-6P-2 plasmid was used to produce GST-tagged human CHTOP and GST-tag alone (as control) in BL21 StarTM (DE3) One Shot cells (Invitrogen). and the Flag-tagged full-length human ERα or Flag-tagged ERα domains (AB, C, CD, CDE, CDEF, DE, DEF, E, and EF) were expressed as described in the third chapter. The expressed proteins were purified as previously described [35] and checked by SDS-PAGE gel. Two-fold molar excess of Flag-ERα or its domains were mixed with GST tag or GST-CHTOP in the binding buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl2, 0.1% Triton X-100 and protease inhibitors), and incubated for 2.5 h at

4°C. In the case of examining the effect of E2 on binding between CHTOP and ERα , 100 nM E2 was added into the mixtures. 15ul of pre-washed glutathione agarose resin (Gold Biotechnology, St. Louis, MO) were added to mixtures after the overnight incubation and incubated for an additional 1.5 h at 4°C with end-to-end rotation. The beads were collected and washed 3 times with wash buffer. Bound proteins were eluted with elution buffer (50 mM Tris-HCl pH 8.0, 10 mM reduced glutathione) and analyzed by Western blotting.

Electrophoretic Mobility Shift Assay (EMSA). Purified recombinant Tag-ERα and Tag-CHTOP or bovine serum albumin (BSA) as control were mixed with 200 fmol of 5' biotin-labeled estrogen response elements (EREs; Appendix B) in the binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $MgCl_2$, 0.5 M DTT and 5% glycerol). The mixtures were incubated for 20 min at room temperature, fractionated by a 5 % non-denaturing polyacrylamide gel at 4 °C, and transferred to a positively charged Nylon membrane (Nytran SPC, Whatman). The membrane was blocked in the Odyssey blocking buffer (Lincoln, Nebraska) plus 0.1% SDS and probed with the Streptavidin-IRDye 800W for 30 min at room temperature. The Odyssey infrared imaging system (Lincoln, Nebraska) was used to scan the membrane after it was washed 3 times with the wash buffer.

Subcellular fractionations and sucrose gradient separation assay. Extraction of chromatin-binding protein and separation of transcriptionally active chromatin and inactive chromatin were carried out as described previously [10, 36]. For sucrose gradient separation, MCF7 cells (15×10^6 cells) were harvested, washed twice with cold PBS. The cells pellets were re-suspended in lysis buffer (40 mM Tris-HCl, pH 7.5, 100 mM NaCl, 6 mM MgCl2, 1mM $CaCl_2$, and 0.1% NP-40) supplied with the protease and phosphatase inhibitors, sonicated, and centrifuged. The supernatant was divided into two: one was treated with 1 unite/10 µl of DNase I 20 min at 37°C

and the second was mock-treated. The treated lysate was centrifuged at 21,000xg for 15 min at 4ºC, fractionated with a 10-30% sucrose gradient, and centrifuged again by ultrahigh-speed centrifuge 37,000 rpm for 17.5 h at 4ºC. The sucrose fractions were collected and analyzed by Western blotting with indicated antibodies.

E2 and tamoxifen (TAM) treatments. MCF7 cells were cultured under starvation conditions (phenol-red-free α-MEM with 5% charcoal-treated FBS and 1% penicillin and streptomycin (Hyclone, Logan, UT)) for 3 days. The cells were treated with indicated concentration of E2 (Sigma, St. Louis, MO) and TAM (MP Biochemicals, Irvine, CA), or ethanol as a control. After 24 h, the cells were harvested and washed twice with PBS. The Cell pellets were either lysed in the lysis buffer or fractionated by following the fractionation protocol.

Dual-luciferase reporter assay. To check whether CHTOP mediates ERα transcriptional activity, knocking out CHTOP or EGFP (control) in MCF7 cells (KO-CHTOP and KO-EGFP, respectively) were seeded in 24-well plates overnight and then transfected with 500 ng of 3 × ERE-TATA-luc [37] and 10 ng pRL-TK (Promega) Renilla luciferase normalization vector (Promega, Madison, WI) using ViaFect reagent (BioRad). After 48 h, dual-luciferase reporter assays were performed as described in chapter three for at least three independent repeats.

Proteasome inhibitor (MG132) treatment. KO-CHTOP and KO-EGFP cells were cultured in α-MEM supplied with 5% FBS and 1% penicillin and streptomycin for 5 days and then treated with 10 μM MG132 (N-[(Phenylmethoxy)carbonyl]-L-leucyl-N-[(1S)-1-formyl-3-methylbutyl]-L-leucinamide), a Potent cell-permeable inhibitor of proteasome, or ethanol for 10.5 h. The cells were harvested, washed twice with PBS, and lysed in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM KCl, 1.5 mM $MgCl_2$, 340 mM sucrose, 10% glycerol, 1 mM DTT, 0.1%

Triton X-100, and protease inhibitors). Protein extracts were fractionated by 10% SDS-PAGE and analyzed by Western blotting with indicated antibodies.

Statistical Analysis. The p values were calculated using a One-way ANOVA (PSI-PLOT, Pearl River, NY). Data were presented as the mean ± S.D.

Results

CHTOP was identidied to be a potential ERα-associated protein in a quantitative proteomic method. We used a SILAC-based quantitative proteomic method to identify proteins that are associated with ERα. 293T cells were cultured in unlabeled and isotope labeled media and then transiently transfected with plasmids that express Flag-ERα and Flag tag alone, respectively. Affinity purified with Flag M2 resin was performed and eluted proteins from both groups were mixed in 1:1 ratio, fractionated by a SDS-PAGE gel, digested and analyzed by MS. After the MS result analysis, CHTOP was found to be enriched by ERα and it is a potential ERα-interacting protein.

CHTOP is confirmed to interact with ERα. To confirm the interaction between CHTOP and ERα, 293T cells were co-transfected with HA-CHTOP and ERα. Cell lysates were coimmunoprecipitated by either ERα or an isotype-matched, unrelated control IgG. The result shows that anti-ERα antibody immunoprecipitated HA-CHTOP while anti-IgG antibody failed to do so (Fig. 1A), which suggests that CHTOP specifically interacts with ERα. Furthermore, we examined whether CHTOP can pull down ERα. 293T cells were transfected with plasmids expressing either HA-EGFP or HA-CHTOP and ERα, and the lysate was immunoprecipitated with HA antibody. The results demonstrate that HA-CHTOP precipitated more ERα than did HA-EGFP (Fig. 1B), confirming that CHTOP indeed interacts with ERα.

CHTOP directly binds ERα through the E domain of ERα. To determine whether CHTOP physically interacts with ERα, purified recombinant Flag tagged ERα (Flag-ERα) and GST-CHTOP (GST-CHTOP) were used to perform the in-vitro binding assay. The result showed that CHTOP directly bound to ERα (Fig. 2A, lanes 1 and 4). Interestingly, E2 moderately enhanced the interaction between CHTOP and ERα (Fig. 2A, compare lane 4 with lane 2). To determine

which part of ERα is responsible for binding to CHTOP, 9 Flag-tagged ERα-domains (AB, C, CD, CDE, CDEF, DE, DEF, E, and EF) plus Flag-tagged ERα full length (ERα F.L.) (Fig. 2B) were expressed in *E coli* and purified by affinity chromatography. Then, the in-vitro binding assays were conducted as described above. The result showed that CHTOP pulled down more full-length ERα than did the control (Fig. 2C, lanes 19 and 20), confirming our previous finding that showed that CHTOP directly bound to ERα. Interestingly, we observed that CHTOP binds all ERα-domains tested except for the C and DE domains (Fig. 2C, upper and down panels). Importantly, we observed that CHTOP bound more to ERα-E domain or ERα-domains that contain the E domain (= CDE, DEF, and CDEF) except for DE domain, which binds similarly to GST-CHOTP and GST. These results suggest that the E domain may play an important role in mediating the ERα-CHTOP interaction. Interestingly, both the A/B and EF domains showed low affinity to bind CHTOP. Taken together, these results demonstrate that CHTOP physically binds to ERα and the interaction may be mediated by the E domain of ERα.

Majority of cellular CHTOP is associated with transcriptionally inactive chromatin.

CHTOP protein is known to be associated with chromatin [19] .To examine how CHTOP protein is associated with chromatin, we first treated whole cell lysate of MCF7 cells with DNase I (mock-treated for control), fractionated the treated lysate with 10-30% sucrose gradient, and analyzed fractionated proteins with Western blotting. The result showed CHTOP protein in control samples (CT) appeared in three main peaks in sucrose gradient: peak 1 (1-3 fractions), peak 2 (12-15 fractions), and peak 3 (17-19 fractions) (Fig. 3, CT rows). CHTOP in high-molecular weight complexes in peak 1 were mostly resistant to DNase I digestion, (Fig. 3, II panel, compare DNase I row with CT row), suggesting CHTOP in these fractions is likely to be densely packed in chromatin. CHTOP complexes in peak 2 were sensitive to DNase I digestion

(Fig. 3, II panel, compare DNase I row with CT row), and after digestion, the CHTOP shifted to peak 3 (Fig. 3, II panel, compare fraction 15 with fraction 17). CHTOP in peak 3 did not shift upward after DNase I digestion (Fig. 3, II panel, compare peak 3 with peaks 1 and 2 in DNase I row). These results suggest CHTOP in peak 2 is not so densely packed into chromatin, and CHTOP in peak 3 was not associated with DNA. By comparison, ERα complexes in a wide range of fractions (fractions 2-16) showed sensitivity to DNase I digestion (Fig. 3, I panel, compare DNase I row with CT row). To further examine how CHTOP is localized in cells, we fractionated MCF7 cells extraction into soluble protein (S), chromatin binding protein (CB) and remaining pellet (P) and analyzed the fractionated protein with Western blotting. The result showed that the majority of CHTOP was associated with chromatin (Fig. 4A). (Meanwhile, large amounts of ERα, a transcriptional factor, were associated with chromatin binding protein (CB) and remaining pellet (P) (Fig. 4A). Histone H3, chromatin binding protein, was used to validate the fractionation. To further understand how CHTOP distributed in cells and how it is associated with chromatin, we fractionated MCF7 cells extraction into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2) and analyzed the fractionated protein with Western blotting. The result indicated that the majority of CHTOP was associated with transcriptional inactive chromatin (Ch2) and a small portion of it was associated with transcriptionally active chromatin (Ch1) and existed as soluble cytoplasmic protein (C) (Fig. 4B). Our active/inactive chromatin extraction protocol was validated with a well-known coactivator–p300 and a corepressor–NcoR, which are typically associated with transcriptionally active chromatin and inactive chromatin, respectively [37, 38] (Fig. 4B).

E2 affects CHTOP subcellular distribution but not its expression. As an effort to examine if CHTOP is functionally linked to ERα, we first examined whether E2 affects CHTOP expression

in MCF7 cells. MCF7 cells were cultured under starvation conditions (phenol-red-free α-MEM with 5% charcoal-treated FBS and 1% penicillin and streptomycin) for 4 days and then treated with different concentrations of E2 or TAM for 24 h, followed by Western blot analysis. The result showed that E2 and TAM have no effect on cellular levels of CHTOP (Fig. 5A and B). Consisting with published results [38], we observed that while E2 decreased ERα levels (Fig. 5A, middle row), TAM had the opposite effect (Fig. 5B, middle row). To understand whether E2 regulates subcellular distribution of CHTOP protein, we cultured MCF7 cells under starvation conditions for 4 days and then treated with 100 nM E2 for 24 h. The cells extractions were then fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2) and analyzed by Western blotting. The results demonstrated that E2 treatment resulted in translocation of CHTOP from the cytosol as cytoplasmic protein (C) into the nucleus as transcriptionally active chromatin (Ch1) (Fig. 5C, compare lanes 1 and 3 with lines 5 and 7). Concomitantly, we observed similar translocation pattern for ERα (Fig. 5C).

CHTOP positively regulates ERα transcriptional activity. To determine whether CHTOP affects ERα transcriptional activity, we knocked out *CHTOP* gene (KO-CHTOP) in MCF7 cells by CRISP-Cas9 gene editing system (Fig. 6A). We then performed the dual-luciferase reporter assay using the above cell lines. The results demonstrated that knockout of CHTOP decreased ERα transcriptional activity (Fig. 6B), consistent with published results [19].

CHTOP does not affect binding of ERα to the ERE. To test how CHTOP may affect ER binding of ERα to ERE, we first test if CHTOP affects the binding of ERα to the ERE using electrophoretic mobility shift assay (EMSA) with purified recombinant GST-CHTOP and Flag-ERα proteins. The result showed that, unlike ERα (Fig. 7, lane 2), CHTOP cannot bind ERE

(Fig. 7, lane 3). Also, we observed that CHTOP directly bound ERα and the binding did not affect binding of ERα to the ERE even at 3-fold molar excess of CHTOP (Fig. 7, lane 4 and 5).

Knockout of CHTOP decreases MEP50 protein level through proteasome pathway. It is known that MEP50, a component of the methylosome [23, 39], can act as a coactivator of ERs and androgen receptors (AR) [26]. To investigate whether the effect of CHTOP on ERα transcriptional activity is mediated by MEP50, we checked MEP50 expression in the KO-CHTOP and the control KO-EGFP cells by Western blotting. The result showed that, unlike ERα, MEP50 protein level was significantly decreased when CHTOP was knocked out (Fig. 8A and B). To examine how knockout of CHTOP decrease MEP50 protein levels, KO-CHTOP and KO-EGFP cell lines were cultured and then treated with 10 μM MG132 (potent cell-permeable inhibitor of proteasome) or ethanol for 10.5 h. Proteins extracted from the treated cells were analyzed by Western blotting. The result demonstrated that while in control KO-EGFP cells, MG132 did not affect CHTOP levels (Fig. 8B, compare lane 2 with lane 1), in KO-CHTOP cells MG132 treatment results elevated levels of MEP50 compared to ethanol treated cells (Fig. 8B, compare lane 3 with lane 4). These results suggest that in CHTOP-non-silenced cells, MEP50 protein is stable and not subject to proteasome-mediated degradation. However, in the absence of CHTOP in the cells, MEP50 protein becomes unstable and subject to proteasome-mediated degradation. Because MEP50 is a coactivator of ERα, it is likely that CHTOP enhances ERα transcriptional activity through maintaining necessary cellular MEP50 protein levels.

CHTOP binds with MEP50. In our previous results, we noticed that MEP50 protein level was directly related to CHTOP. So, we were interested to see whether CHTOP binds to MEP50. 293T cells were transiently transfected with either HA-CHTOP or HA-EGFP, extracted proteins were the lysate of the transfected cells was immunoprecipitated by the anti-AH antibody, and the

bound proteins were analyzed by Western blotting. The result demonstrated that HA-CHTOP immunoprecipitated more MEP50 than did HA-EGFP (Fig. 9), suggesting that CHTOP interacts with MEP50 in the cells.

CHTOP decreases MEP50 in ERα+ breast cancer cell. It has been reported that MEP50 subcellular localization affects the physiological function of MEP50 in cells [28-30]. For instance, it has been shown that MEP50 was translocated from the nucleus into the cytoplasm when benign epithelia become cancer cells [31]. To determine how CHTOP may affect subcellular localization of MEP50, we fractionated cell lysate of KO-CHTOP and KO-EGFP cells into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2) and analyzed the fractionated proteins with Western blotting. The result demonstrated that while the majority of MEP50 was in the cytosol, CHTOP was predominantly associated with transcriptionally inactive chromatin (Fig. 10). Knockout of CHTOP proportionally decreases the levels of MEP50 in all cell portions (C, NS, Ch1, and Ch2) (Fig. 10, compare lanes 5-8 with lanes 1-4) suggesting that CHTOP systematically regulates MEP50 protein levels in MCF7 cells.

E2 affects MEP50 subcellular distribution but not its expression and degradation. It is reported that MEP50 plays a vital role in tumorigenesis of breast cancer through mediating the hormone-dependent action of ERα [31], we examined how E2 may affect MEP50 subcellular distribution in cells. MCF7 cells were cultured under either hormone starvation conditions or a normal condition (completed medium) for 5 days. Starved cells were then treated with 100 nM E2 or ethanol for 24 h. The whole cell lysate of the treated cells was fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2) and the fractionated protein were analyzed by Western blotting.

The result showed that, as shown in Fig. 10, MEP50 was predominately localized in the cytosol when cells were cultured in complete medium (Fig. 11A, upper row). Surprisingly, the nuclear soluble MEP50 (NS), and the MEP50 associated with transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2) dramatically shifted into cytosol (C) under starvation conditions. The addition of E2 slightly moved MEP50 back into the nucleus and did not change the distribution pattern (Fig. 11A, lower row). These results suggest that hormone conditions affect MEP50 subcellular distribution and MEP50 may be profoundly involved in hormone-mediated physiological effect in cells. The data presented here strongly indicate that MEP50 plays an important role in breast cancer, which is in agreement with recent studies that showed that moving MEP50 between cytoplasm and nucleus significantly influences the proliferation and invasiveness of breast [31], prostate [40], ovarian [26], and lung cancer [41] cells. Taken together, these results propose a new mechanism by which breast cancer cell decreases its proliferation rate under hormone starvation conditions, and known this mechanism may shed light on a novel approach to treating breast cancer.

To check whether E2 influences MEP50 degradation after knocking out CHTOP, cells were starved for 4 days and then treated with 100 nM E2 or ethanol for 24 h. Equal amounts of extracted proteins were analyzed by Western blotting. The results demonstrated that E2 had no effect on MEP50 expression (Fig. 11B, upper row).

Discussion

Through SILAC-based quantitative proteomic method we found that CHTOP is a novel ERα-interacting protein. We confirmed the CHTOP-ERα interaction by co-immunoprecipitation. In-vitro binding and protein domain mapping assays showed that CHTOP directly bound to ERα (Fig. 2A and 2C), and the binding is mediated the E domain of ERα (Fig. 2C, lower panel). Interestingly, the C domain does not bind to CHTOP and both the A/B and E/F domains have low affinity to CHTOP (Fig. 2C). In the case of the E/F domain, the results imply that the F domain interferes the binding between CHTOP and the E domain. E2 binds ERα and triggers the conformational changes of the receptor, which eventually leads to recruitment of transcriptional coregulators (CBP/p300, SRC, and NCOA1) [42, 43]. We examined if E2 affect CHTOP-ERα binding by performing the in-vitro binding assay. Interestingly, E2 enhanced the binding between CHTOP and ERα (Fig. 2A, compare lane 4 with lane 2). In addition, we examined whether E2 influences ERα and CHTOP distributions and found that like ERα, CHTOP shifted into transcriptionally active chromatin after MCF7 cells were stimulated by E2 (Fig. 5C) without a significant effect on the CHTOP expression (Fig. 5A). These results support the notion that CHTOP is involved in estrogen-mediated cell physiology.

Luciferase reporter assay demonstrated that the ERα transcriptional activity was decreased after knocking out CHTOP (Fig. 6B). This finding is in line with published result, which indicated that ERα-pS2 promoter binding was significantly decreased after silencing CHTOP [19]. To understand how CHTOP regulates ERα transcriptional activity, we targeted MEP50, an ER and AR coactivator. Interestingly, we found that knocking out CHTOP significantly decreases MEP50 protein level in MCF7 cells (Fig. 8A), and the reduction was

through proteasome machinery (Fig. 8B). These finding suggests that CHTOP effects on ERα transcriptional activity may be mediated by MEP50.

It is well known that MEP50 function is tightly related to its subcellular localization. In breast and ovarian cancer cells, the nuclear MEP50 enhances cell proliferation and metastasis, whereas the cytoplasmic MEP50 decreases both the cell proliferation and metastasis [26, 31]. we observed that knockout of CHTOP proportionally decreased MEP50 amounts in all MCF7 cells portions (C, NS, Ch1, and Ch1) (Fig. 10), which may explain how the MEP50 reduction affects ERα transcriptional activity.

It has also been reported that MEP50 acts as a mediator of the ERα hormone-dependent action [31]. Our subcellular fractionation assay showed that MEP50 subcellular distribution was dramatically affected by cell culture condition regarding with or without hormone. We observed that nuclear MEP50 dramatically shifted into the cytosol when MCF7 cells were cultured under hormone-starvation condition compared to the non-starved condition (Fig. 11A, compare lower row with upper row). Moreover, we observed that stimulating starved cells with E2 slightly shifted MEP50 back into the nucleus (Fig. 11A, lower row). This observation is in agreement with the recent study by Ligr, *et. al* (2011) [26], who showed that estrogen promoted nuclear localization of MEP50 and cell proliferation in ovarian cancer (OVCAR-3) cells. These results demonstrate that MEP50 plays a crucial role in breast cancer cell proliferation and may be targeted for treating breast cancer.

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Figures

Fig. 1. CHTOP interacts with ERα. A, 293T cells were transiently co-transfected with plasmids that express HA-CHTOP and ERα. After 2 days of transfection, cells were harvested, washed, and lysed in a lysis buffer. Extracted proteins were immunoprecipitated with either an antibody against an isotype-matched, unrelated control IgG or anti-ERα. The immuno-precipitated proteins were analyzed by Western blotting with the anti-HA antibody. B, 293T cells were transfected with a plasmid that expresses either HA-CHTOP or HA-EGFP. Cells lysates were incubated with anti-HA-beads, and the bound proteins were eluted, fractionated and analyzed by Western blotting with anti-ERα antibody.

Fig. 2. CHTOP directly binds ERα via the E domain of ERα. A, Equal amounts of a purified recombinant Flag-ERα and purified recombinant GST-CHTOP or GST tag alone were mixed in the presence of E2 or ethanol. The mixtures pulled down with glutathione agarose resin. The bound proteins were eluted, fractionated by a 10% SDS-PAGE gel, and analyzed by Western blotting with indicated antibodies. F.T., flow through. B, schematic diagram of recombinant human ERα domains expressed and purified. Full-length Flag tagged ERα (ERα F. L) plus nine Flag tagged ERα domains (AB, C, CD, CDE, CDEF, DE, DEF, E, and EF) were expressed in *E. coli* and purified by affinity purification using M2 resins. C, protein domain mapping assays. Purified recombinant GST tag or GST-CHTOP was incubated with purified recombinant full-length Flag-ERα or each of the Flag tagged ERα domains, and the mixtures were immunoprecipitated by glutathione beads. The eluted proteins were analyzed by Western blotting.

Fig. 3. Sensitivity of cellular CHTOP- and ERα-complexes to DNase I digestion. Whole cell lysate of MCF7 was either mock-treated or treated with DNase I (1unite/10 μl), the treated lysate

was fractionated by 10-30% sucrose gradient, and fractionated proteins were analyzed by Western blotting with indicated antibodies.

Fig. 4. CHTOP is predominantly associated with transcriptionally inactive chromatin. A, MCF7 cells (2.5×10^6) were fractionated into soluble protein (S), chromatin binding protein (CB), and remaining pellet (P). The fractionated proteins were analysed by Western blotting with indicated antibodies. B, MCF7 cells (2.5×10^6) extract was fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and analyzed by Western blotting with the indicated antibodies. Histone H3, p300, and NCoR were used as markers of chromatin-binding protein, active chromatin, and inactive chromatins, respectively. * denote indicates the small isoform of CHTOP.

Fig. 5. Effect of E2 on CHTOP expression and subcellular distribution. A and B, MCF7 cells were cultured in 12-well plate under hormone starvation conditions for 4 days and then treated with indicated E2, TAM concentrations or ethanol (control) for 24 h. Equal amounts of extracted protein from each treatment were analyzed by Western blotting with the indicated antibodies. Actin serves as loading control. C, MCF7 cells (2.5×10^6) were cultured as above and treated with 100 nM E2 for 24 h. Cells extract was fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and analyzed by Western blotting with the indicated antibodies

Fig. 6. Knockout of CHTOP decreases ERα transcriptional activity. A, CHTOP expression analysis of knocking out CHTOP (KO-CHTOP) or EGFP (KO-EGFP) in MCF7 cells. Tubulin serves as a loading control. B, dual luciferase reporter assay. RULs values were the means ± S.D. of three independent sample preparations.

Fig. 7. CHTOP-ERα binding has no effect on ERα-EREs interaction. Electrophoretic mobility shift assay (EMSA) was performed by incubating equal amounts (1 μg) of purified recombinant ERα and CHTOP, or BSA (control) along with biotin-labeled 3xEREs oligos. The resulting mixtures were fractionated on a 5% nondenaturing acrylamide gel, probed with streptavidin labeled IRDye-800CW, and visualized by Odyssey infrared imaging system.

Fig. 8. knockout of CHTOP resulting in proteasome-mediated degradation of MEP50. A, expression of MEP50 and ERα in KO-CHTOP and KO-EGFP cells revealed by Western blotting. Right panel, quantification of MEP50 band density in Western blots. Signal intensity values were arbitrary numbers obtained by analyzing the protein bands with ImageJ software for at least three independent experiments. B, KO-CHTOP and KO-EGFP cells were cultured in the hormone-depleted medium for 5 days and then treated with 10 μM MG132, or ethanol for control for 10.5 h. Cells were lysed in a lysis buffer, and the extracted proteins were analyzed by Western blotting. Tubulin was used as a loading control. * denote $p < 0.05$

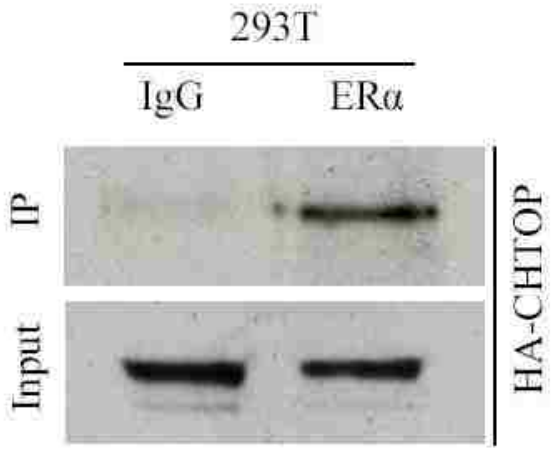
Fig. 9. CHTOP interacts with MEP50. 293T cells were transiently transfected with a plasmid that expresses either HA-CHTOP or HA-EGFP. Forty-eight h after transfection, the cells were harvested, washed and lysed in a lysis buffer. The extracted proteins were immunoprecipitated by anti-HA antibody, and the bound proteins were eluted and analyzed by Western blotting with the anti-MEP50 antibody.

Fig. 10. CHTOP affects MEP50 abundance in different MCF7 cell portions. KO-CHTOP and KO-EGFP cells were fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2), and the extracted proteins were analyzed by Western blotting with the indicated antibodies.

Fig. 11. E2 affects MEP50 subcellular distribution but not its expression and degradation. A, MCF7 cells were cultured under either normal condition (completed medium) or hormone-starvation conditions. After the cells were harvested and washed, the cell extraction was fractionated into cytoplasmic protein (C), nuclear soluble protein (NS), transcriptionally active chromatin (Ch1) and inactive chromatin (Ch2). The fractionated proeins were analyzed by Western blotting. B, KO-CHTOP and KO-EGFP cells were cultured under starvation conditions for 4 days and then treated with either 100 nM E2 or ethanol (vehicle) for 24 h. Total protein extrated from the treated cells were analyzed by Western blotting. Tubulin serves as a loading control.

Fig. 1

A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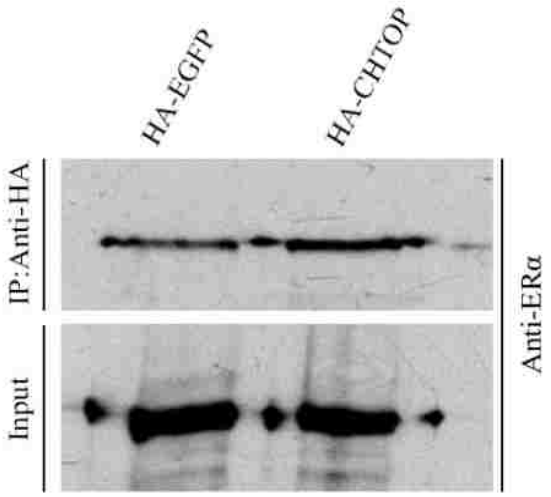
IgG
ERα
IP
Input
HA-CHTOP

B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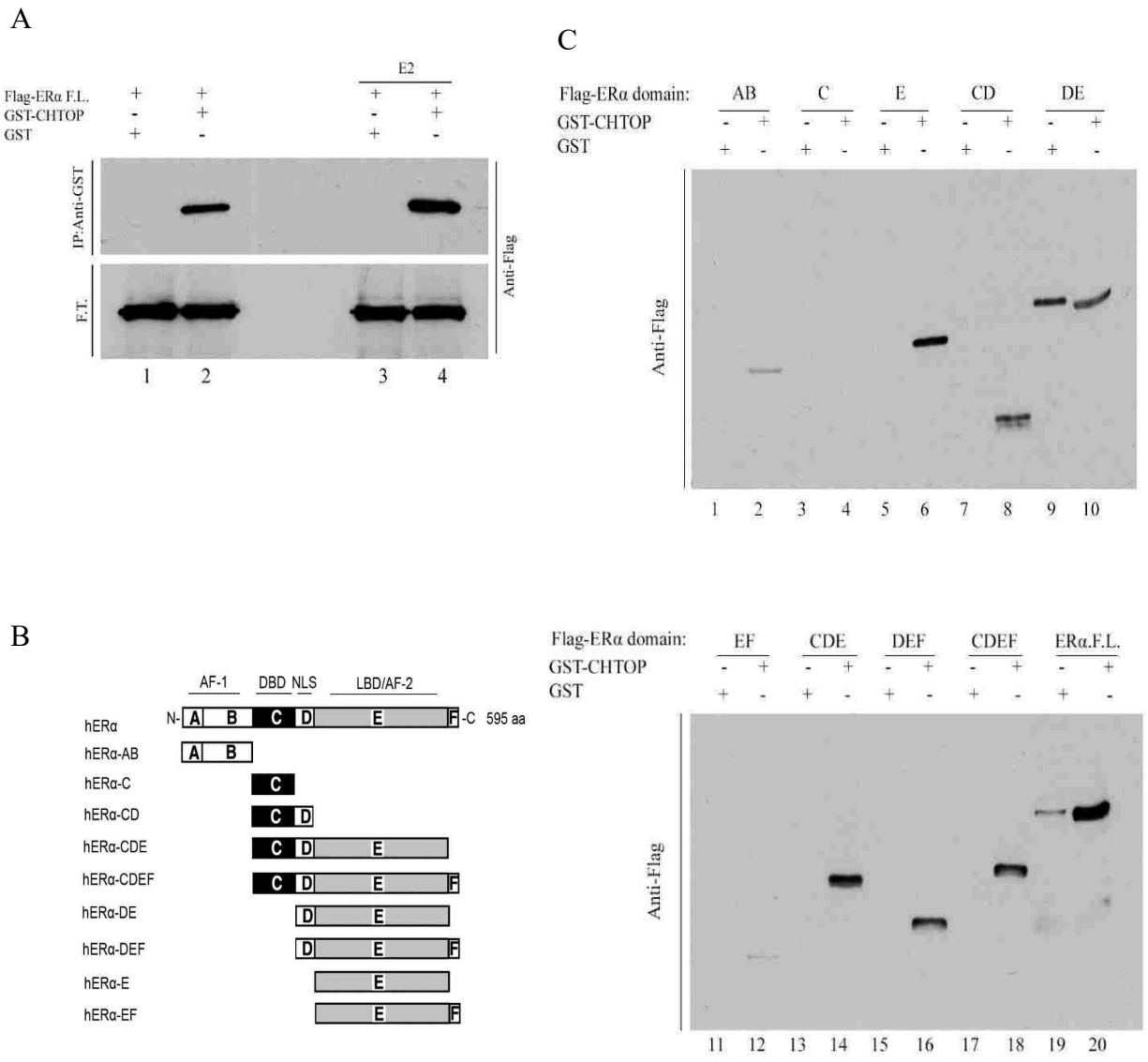
HA-CHTOP
IP:Anti-HA
Input
Anti-ERα

Fig. 2


A
E2
Flag-ERα F.L. + + + +
GST-CHTOP - + - +
GST + - + -
IP:Anti-GST
F.T.
Anti-Flag
1 2 3 4
C
Flag-ERα domain: AB C E CD DE
GST-CHTOP - + - + - + - + - +
GST + - + - + - + - + -
Anti-Flag
1 2 3 4 5 6 7 8 9 10
B
AF-1 DBD NLS LBD/AF-2
hERα N- A B C D E F -C 595 aa
hERα-AB A B
hERα-C C
hERα-CD C D
hERα-CDE C D E
hERα-CDEF C D E F
hERα-DE D E
hERα-DEF D E F
hERα-E E
hERα-EF E F
Flag-ERα domain: EF CDE DEF CDEF ERα.F.L.
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GST + - + - + - + - + -
Anti-Flag
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Fig. 3

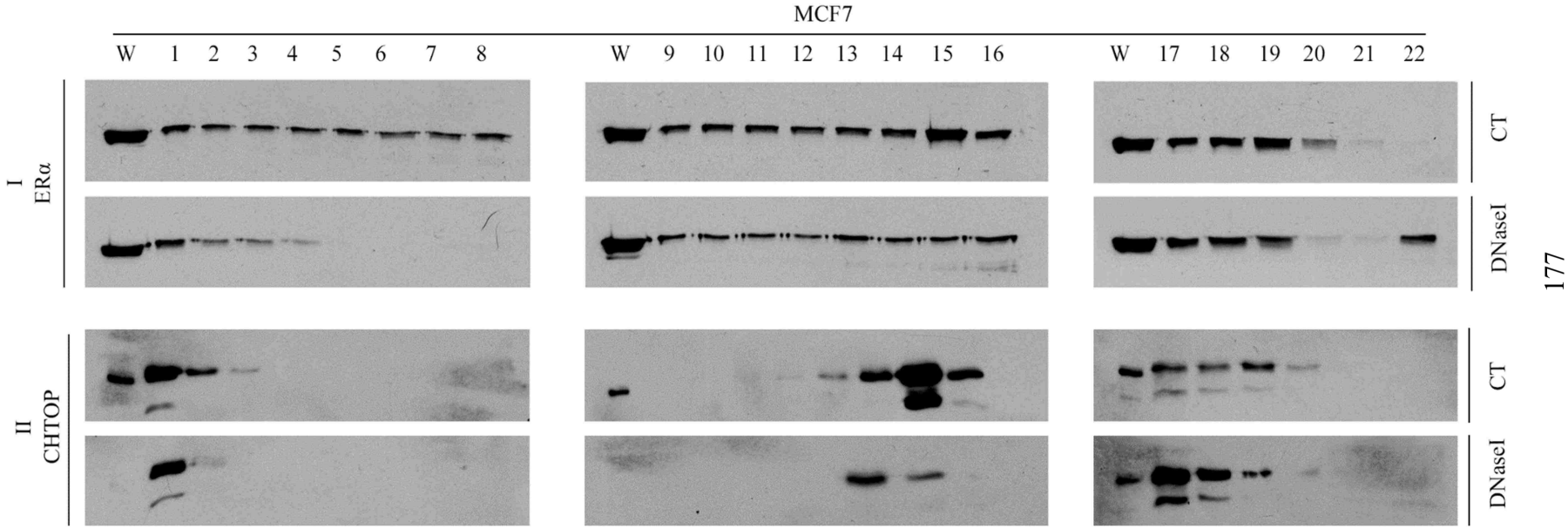

MCF7
W 1 2 3 4 5 6 7 8
W 9 10 11 12 13 14 15 16
W 17 18 19 20 21 22
I
ERα
II
CHTOP
CT
DNaseI
CT
DNaseI

Fig. 4

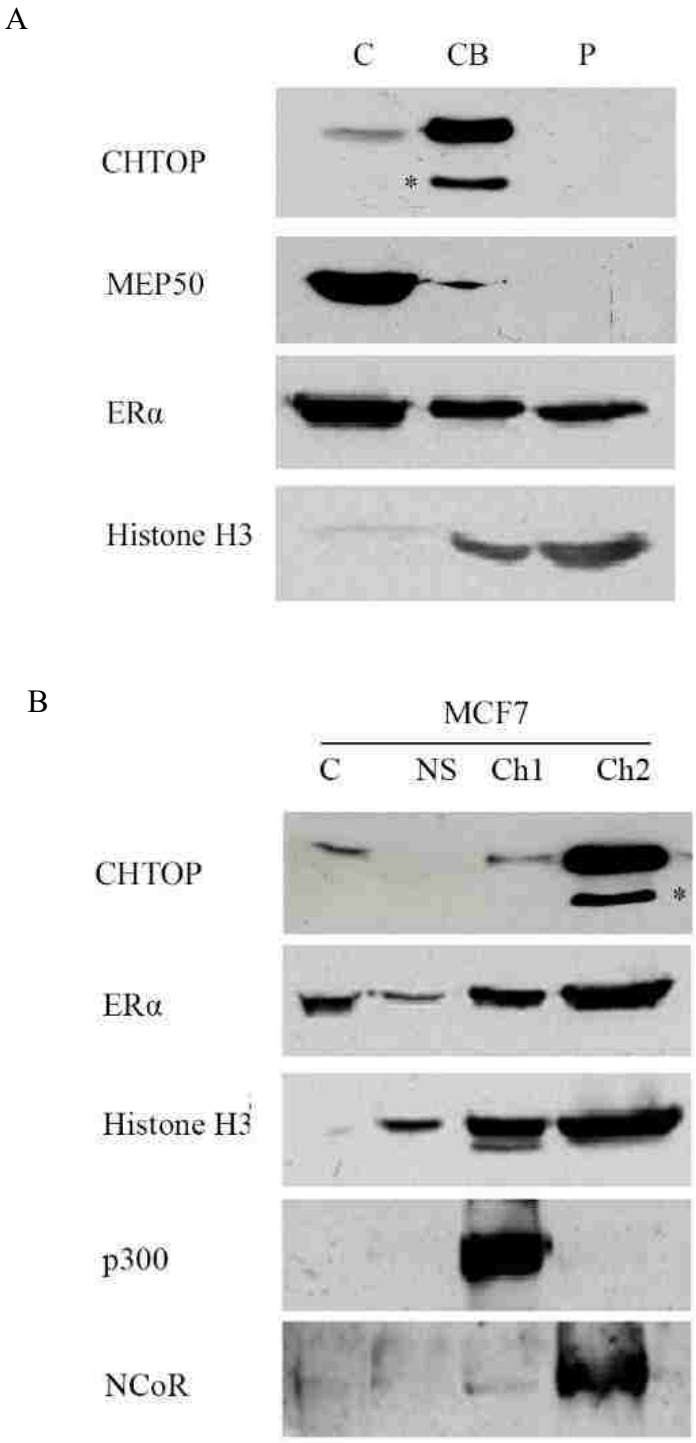
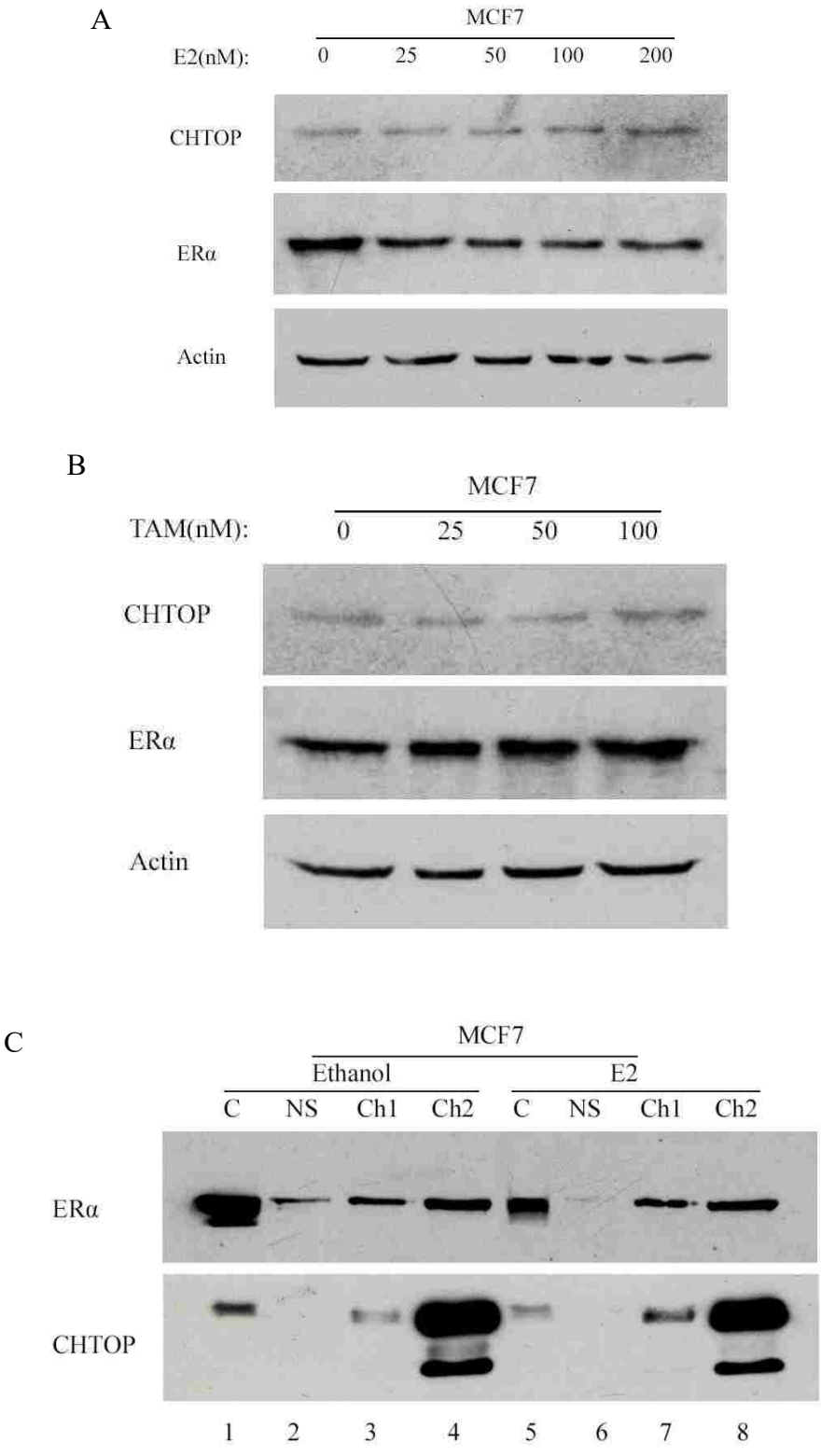

A
C
CB
P
CHTOP
*
MEP50
ERα
Histone H3
B
MCF7
C
NS
Ch1
Ch2
CHTOP
*
ERα
Histone H3
p300
NCoR

Fig. 5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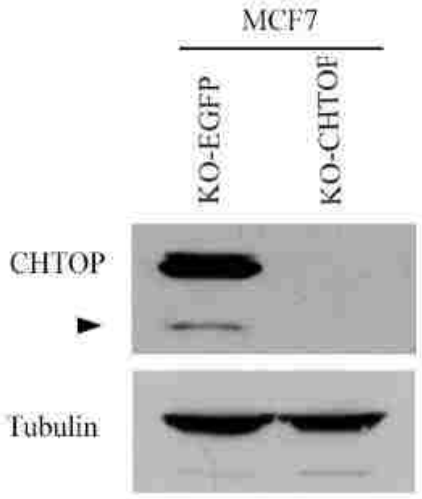
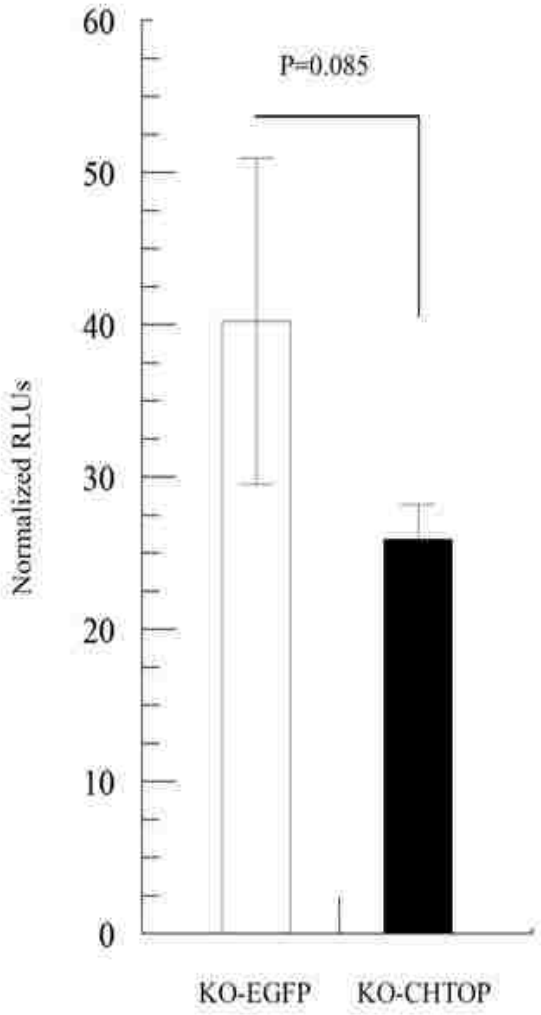
MCF7
E2(nM):
0
25
50
100
200
CHTOP
ERα
Actin
B
MCF7
TAM(nM):
0
25
50
100
CHTOP
ERα
Actin
C
MCF7
Ethanol
E2
C
NS
Ch1
Ch2
C
NS
Ch1
Ch2
ERα
CHTOP
1
2
3
4
5
6
7
8

Fig. 6

A


MCF7
KO-EGFP
KO-CHTOF
CHTOP
Tubulin

B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50
40
30
20
10
0
KO-EGFP
KO-CHTOP

Fig. 7

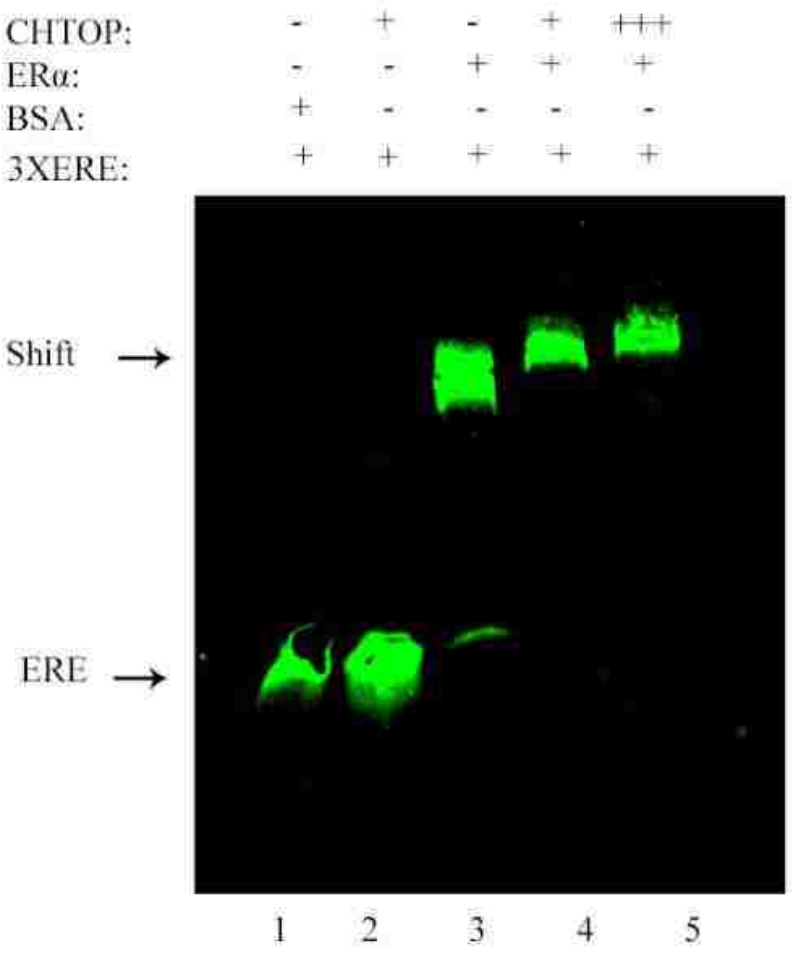
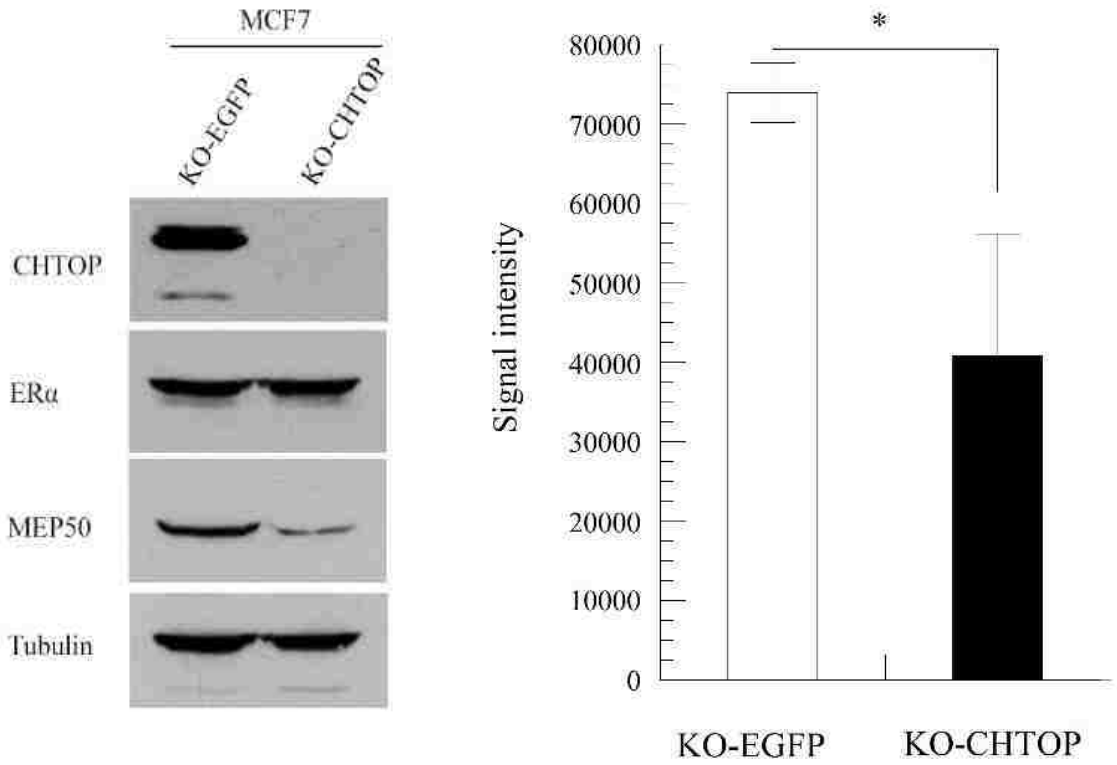


Fig. 8

A


MCF7
KO-EGFP
KO-CHTOP
CHTOP
ERα
MEP50
Tubulin
*
Signal intensity
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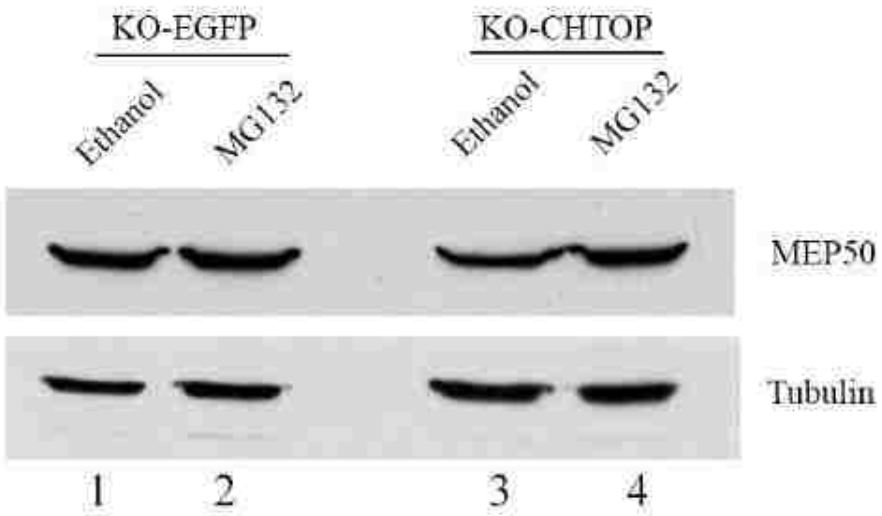
70000
60000
50000
40000
30000
20000
10000
0
KO-EGFP
KO-CHTOP

B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KO-CHTOP
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MG132
Ethanol
MG132
MEP50
Tubulin
1
2
3
4

Fig. 9

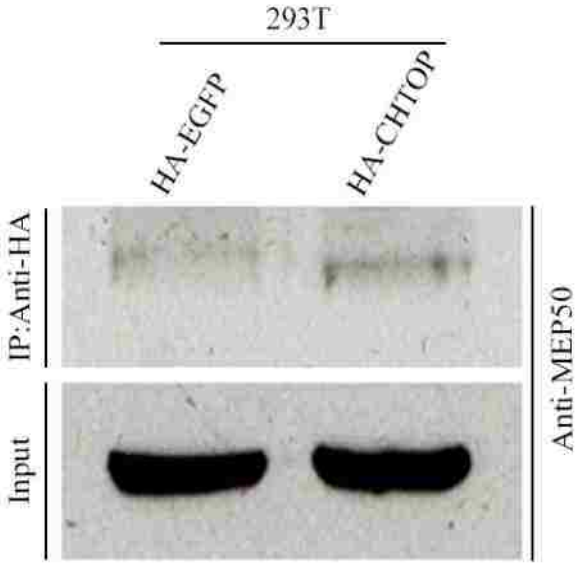


Fig. 10

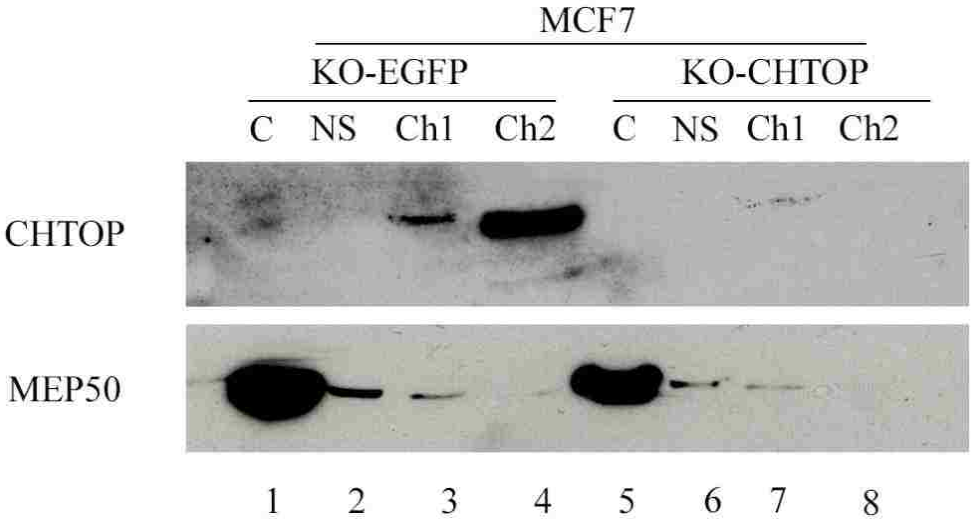
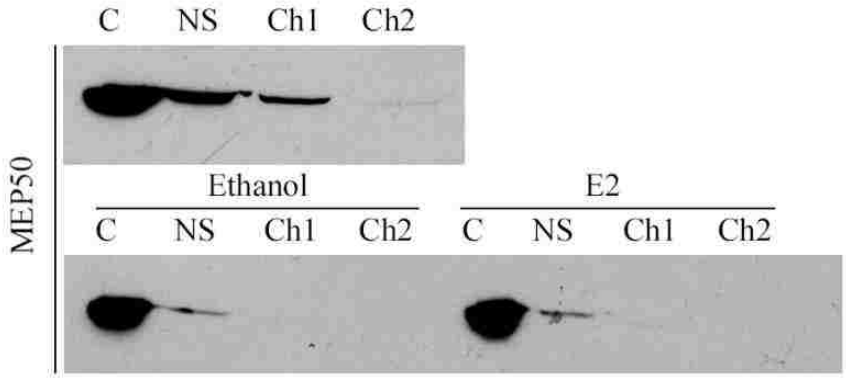

MCF7
KO-EGFP
KO-CHTOP
C
NS
Ch1
Ch2
C
NS
Ch1
Ch2
CHTOP
MEP50
1
2
3
4
5
6
7
8

Fig. 11

A


C
NS
Ch1
Ch2
MEP50
Ethanol
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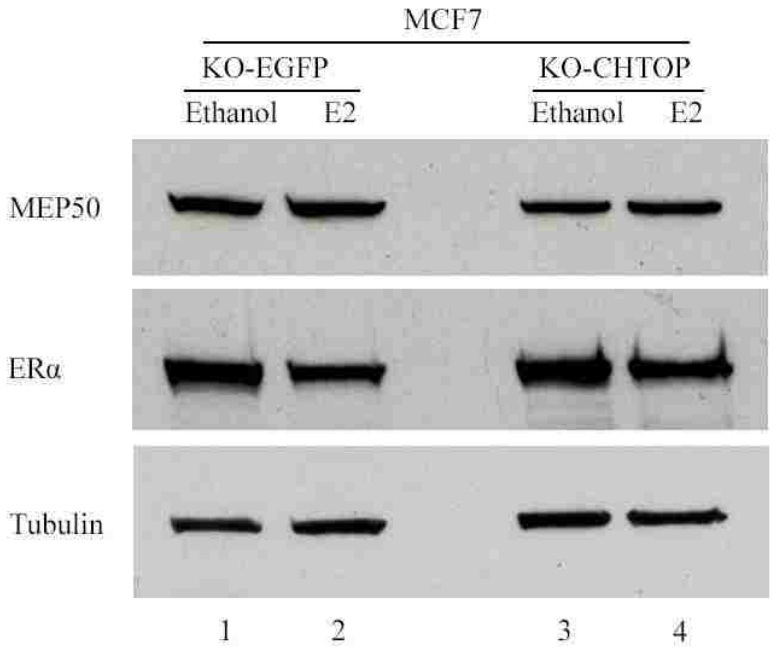
C
NS
Ch1
Ch2
C
NS
Ch1
Ch2

B


MCF7
KO-EGFP
KO-CHTOP
Ethanol
E2
Ethanol
E2
MEP50
ERα
Tubulin
1
2
3
4

Appendices

A, sgRNAs for knocking out CHTOP in MCF7 cells

CHTOP-F1 (5'-CACCGACGGTTAGGCCGACCCATA G-3')

CHTOP-R1 (5'- AAACCTATGGGTCGGCCTAACCGTC-3')

CHTOP-F2 (5'-CACCGGCCCGAATATTCACTGGCG T-3')

CHTOP-R2 (5'-AAACACGCCAGTGAATATTCGGGCC-3')

CHTOP-F3 (5'-CACCGCTCATTTAGAGACATCTTGG-3')

CHTOP-R3 (5'-AAACCCAAGATGTCTCTAAATGAGC-3')

B, 5' biotin-labeled estrogen response elements (EREs)

Forward (5'-GGATCTAGGTCACTGTGACCCCGGATC-3')

Reverse (5'-GATCCGGGGTCACAGTGACCTAGATC-3')

General discussion and conclusion

ERα is a key player in endocrine therapy resistance. However, the molecular mechanism of action of ERα in breast cancer is not fully understood. In this study, we characterized several novel ERα-interacting proteins in ERα-positive breast cancer cells. First, we systematically analyzed heat shock proteins (Hsps) that were identified to interact with ERα. We found that 21 Hsps and 3 Hsp cochaperones were associated with ERα in human 293T. Through various molecular and biochemical methods, we demonstrated that the two most abundant ERα-associated Hsps, Hsp70-1, and Hsc70, interacted with ERα in both the cytoplasm and nucleus when the cells were cultured in a medium supplemented with a fetal bovine serum and phenol red. Interestingly, the ERα-Hsp70-1/Hsc70 interactions were detected only in the cytoplasm but not in the nucleus under hormone starvation conditions, and the stimulation of the starved cells with 17β-estradiol (E2) did not change this. In addition, E2-treatment weakened the ERα-Hsc70 interaction but had no effect on the ERα-Hsp70-1 interaction. Further studies showed that significant portions of Hsp70-1 and Hsc70 were associated with transcriptionally active chromatin and inactive chromatin, and the two Hsps interacted with ERα in both forms of the chromatins in MCF7 cells. Whether Hsp70 interacts with ERα has been controversial for a long time. While some studies showed that Hsp70 was associated with ERα [1], others failed to detect the interaction [2]. Through detailed biochemical studies, we firmly established that Hsp70 and Hsc70 are genuine interacting partners of ERα. Furthermore, our data suggest that Hsp70 may play important roles in regulating ERα biological functions in ERα-positive cells.

ERα regulates the expression of its target genes through recruiting regulators, which normally control transcription via modifying chromatin near the promoter regions of the

target genes [3-5]. One of the major ways to modify chromatin structure is through posttranslational modifications of proteins involved in transcriptions, such as histone proteins [6]. Interestingly, we found that histone acetyltransferase 1(HAT1), a member of the histone acetyltransferases family, is a novel ERα-interacting protein. HAT1 is believed to be responsible for acetylating a newly synthesized histone H4 at lysine 5 and 12 (H4K5,12) sites, but not the histone H4 in nucleosomes [7]. HAT1 may also be involved in histone deposition, chromatin assembly, and DNA double-strand break (DSB) repair [8-10]. We found that knockdown of HAT1 by shRNA in MCF7 cells significantly increased ERα transcriptional activity, suggesting that HAT1 is functionally linked to ERα. Co-immunoprecipitation results demonstrated that ERα interacted with histones H2A, H3, and H4 in the nuclear fractions, and the interactions were moderately increased after HAT1 was silenced in MCF7 cells. We confirmed these results by performing DNA affinity precipitation assays, which showed that ERα and histone proteins (H2A, H3, and H4K12) interacted with the ERE, and the interactions were noticeably increased when HAT1 was knocked down. These results suggest that HAT1 regulates ERα-mediated transcription through affecting the interactions of ERα with histone proteins around the promoter region of ERα target genes in breast cancer cells.

In addition, we characterized two new ERα-interacting proteins: PRMT5 (protein arginine methltrasferase5) and CHTOP (chromatin target of PRMT1) in MCF7 cells. As shown by the luciferase reporter assay overexpression of PRMT5 led to a significant decrease in ERα transcriptional activity. We showed that the ERα protein level was significantly decreased by moderate overexpression of PRMT5 in MCF7 cells. The result was confirmed by increasing expression levels of the PRMT5 protein which resulted in

reduced levels of ERα protein. These results suggest that PRMT5 may inhibit ERα-mediated gene expression by suppressing the expression of ERα in the cells.

Knockout of CHTOP by CRISPR-Cas9 decreased the transcriptional activity of ERα. We showed that in the presence of CHTOP, MEP50 protein is stable and not subject to proteasome-mediated degradation in MCF7 cells. However, in the absence of CHTOP, MEP50 protein becomes unstable and subject to proteasome-mediated degradation. Since MEP50 is a coactivator of ERα [11, 12], it is likely that CHTOP enhances ERα transcriptional activity through maintaining necessary cellular MEP50 protein levels.

ERα is a key factor that affects breast cancer development and treatment options. Despite extensive studies, the molecular mechanisms by which ERα controls gene expression and cell proliferation are not fully understood. Lack of such knowledge is a major obstacle in preventing and treating breast cancer. For example, TAM has been used for decades to treat ERα-positive breast cancer. However, a significant portion of patient develops resistance to TAM [13, 14]. Due to the lack of knowledge in understanding the molecular mechanism of action of ERα, we still cannot rationally design effective therapeutics to overcome TAM resistance. Although the results obtained in this study cannot provide a direct answer to the questions mentioned above, they suggest that ERα regulates gene expression and cell proliferation through very complex processes, which are much more complex than the reported canonical genomic and non-genomic actions of ERα [15, 16]. Much more fundamental research concerning understanding the mechanism of action of ERα will be needed before we can effectively prevent and/or treat breast cancer.

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Appendix

UNIVERSITY OF ARKANSAS | Research & Economic Development
Research Compliance

May 17, 2016

MEMORANDUM

TO:	Dr. Yuchun Du
FROM:	Ines Pinto, Biosafety Committee Chair
RE:	Protocol Renewal
PROTOCOL #:	07028
PROTOCOL TITLE:	Proteomic/biochemical studies of protein complexes/expression in mammalian cells
APPROVED PROJECT PERIOD:	Start Date April 15, 2010 Expiration Date April 14, 2019

The Institutional Biosafety Committee (IBC) has approved your request, dated April 4, 2016, to renew IBC # 07028, "Proteomic/biochemical studies of protein complexes/expression in mammalian cells".

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.