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Med25 is Required for Estrogen Receptor alpha (ER α)-mediated Regulation of Human CYP2C9 Expression

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Abstract

The CYP2C subfamily of cytochrome P450 enzymes is an important class of drug metabolizing enzymes in human liver. CYP2C9 is the most abundant member of the human CYP2C subfamily in liver and metabolizes ~15% of the therapeutic drugs as well as other xenobiotics and endogenous compounds. A number of nuclear receptors including xenobiotic-sensing receptors such as the constitutive androstane receptor (CAR), pregnane X receptor (PXR), and glucocorticoid receptor (GR) as well as liver enriched receptors hepatic nuclear factor 4 α (HNF4 α) and the estrogen receptor α (ER α) regulate CYP2C9 expression. Here, we show that Med25, a variable component of Mediator complex, enhanced ligand dependent ER α -mediated transcriptional activation of *CYP2C9* promoter and interacts with activated ER α by 17 β -estradiol through its C-terminal LXXLL motif. In conclusion, Med25 is identified as a new coactivator of ER α that is required for ER α -mediated regulation of CYP2C9 expression.

Keywords

CYP2C9; Med25; ER α ; Transcription Regulation

1. Introduction

Cytochrome P450 (CYP) enzymes play vital roles in the metabolism of xenobiotics and environmental toxins. The CYP2C subfamily (consisting of CYP2C8, CYP2C9, CYP2C18, and CYP2C19) is an important subfamily of cytochrome P450 enzymes. CYP2Cs enzymes are responsible for the metabolism of ~20-25% of all clinically prescribed therapeutics, and they also metabolize a number of environmental chemicals as well as many endogenous

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compounds [1, 2]. The CYP2C enzymes are expressed highest levels in human liver [2], although the proteins or mRNAs have also been reported at lower levels in extrahepatic tissues such as kidney, lung, heart, endothelial tissue, adrenal gland, mammary gland, and brain [3-6]. CYP2C9 is the principal CYP2C enzyme in human liver, metabolizing approximately 15% of all clinically prescribed drugs including the anticonvulsant phenytoin, the *S*-enantiomer of the anticoagulant warfarin, most nonsteroidal anti-inflammatory drugs such as ibuprofen, antidiabetic drugs such as tolbutamide and glipizide, and it also metabolizes endogenous compounds such as arachidonic acid to biologically active intermediates [1, 2].

Recent studies have identified that several nuclear receptors including the xenobiotic-sensing receptors such as the constitutive androstane receptor (CAR), pregnane X receptor (PXR), and glucocorticoid receptor (GR), as well as liver enriched receptors such as hepatic nuclear factor 4 α (HNF4 α) which bind to specific responsive elements (REs) within the *CYP2C* promoter cause the transcriptional up-regulation of *CYP2C* genes by drugs [7-11]. We also found that microRNAs regulate the *CYP2C* family post-transcriptionally in human liver [12]. Recently, a functionally relevant estrogen responsive element (ERE) half-site at position -149/-145 of the *CYP2C9* promoter has been identified, and through this site the estrogen receptor α (ER α) regulates *CYP2C9* expression in a ligand dependent manner [13]. However, which cofactors are involved in ER α -mediated activation of *CYP2C9* is unknown. Recently Mediator 25 (Med25), which is a member of the Mediator complex, was found to be involved in HNF4 α -dependent transcriptional activation of a number of gene promoters including the *CYP2C9* promoter by interacting with HNF4 α and thus binding to HNF4 α binding sites within the *CYP2C9* promoter region and through protein-protein interaction, bring RNA pol II as well as other members of the preinitiation complex to these sites initiating the transcription of *CYP2C9* and a number of other HNF4 α target genes [14].

Here, we identify Med25 as a new coactivator of ER α , and show it is required for ER α -mediated transcriptional activation of the *CYP2C9* promoter by ligand 17 β -estradiol (E2) stimulation. We also show that the C-terminal LXXLL motif of Med25 is the important binding site for interacting with ER α for this activation.

2. Materials and Methods

2.1. Cell culture

HepG2 cells were maintained in Eagle's minimal essential medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), and penicillin-streptomycin (Gibco) at 37°C under 5% CO₂. In addition, human fresh normal liver tissues were obtained from the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). Human primary hepatocytes were isolated according to a previous protocol [15]. Primary hepatocytes cultured as previously described [12, 14]. Hepatocytes were maintained in William's E medium without Phenol Red (Sigma-Aldrich, St. Louis, MO) supplemented with ITS+1 liquid media supplement (Sigma-Aldrich), HEPES buffer (Sigma-Aldrich), L-glutamine (Sigma-Aldrich), and dexamethasone (Sigma-Aldrich). The study was under

agreement of the donors and the protocol approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

2.2. Plasmids construction

A 1.9 kb of a *CYP2C9* luciferase promoter construct was generated in a pGL3-basic luciferase promoter vector (Promega, Madison, WI). Full length CAR or HNF4 α expression constructs were generated in pcr3.1 vector (Invitrogen), and full length Med25 was cloned into p3xFLAG-CMV-7.1 (Sigma-Aldrich) as described previously [11, 14]. Full length of Med25 was inserted into pBind vector (Promega). Mutation constructs of Med25 (FlagMed25BM, pBindMed25BM, LXXLL mutated to LXXAA) and CYP2C9-ERE mut-Luc (ERE half site at -149/-145 GGTC A mutated to TTAGT) were generated using site-directed mutagenesis kit (Stratagene, La Jolla, CA). Human ER α /pcDNA3.1 and 3XERE-TATA-pGL3 luciferase reporter plasmids were provided kindly by Dr. Korach's lab and Dr. Negishi's lab respectively (NIEHS/NIH, Research Triangle Park, NC). pACTER α construct was a kind gift of Dr. McDonnell's lab (Duke University, Durham, NC).

2.3. Promoter assay

HepG2 cells were seeded in 24-well plates and cultured in MEM media without phenol red, containing 10% dextran charcoal stripped serum, transiently transfected with various plasmids (200 ng/well 1.9 kb 2C9-Luc or 2C9-mut-Luc or 100 ng/well 3XERE-Luc, 100 ng/well ER α /pcDNA3.1, 200 ng/well FlagMed25 or FlagMed25BM) along with the 10 ng/well internal control pRL-TK plasmid (Promega) using Lipofectamine 2000 (Invitrogen). The final amounts of transfected DNAs were adjusted by adding empty vector (pcDNA3.1). After 24 h, the cells were treated with 10 nM E2 (Sigma-Aldrich) or 0.1% EtOH. Twenty-four hours later, the cells were harvested. Dual-Glo luciferase assay system (Promega) was performed to measure luciferase activities and firefly luciferase activities were normalized with *Renilla* luciferase activities.

2.4. Mammalian two-hybrid assay

A series of constructs (200 ng/well pG5-Luc, 100 ng/well pActER α , 200 ng/well pBindMed25 or pBindMed25BM) were transiently transfected into HepG2 cells in 24-well plates. After 24 h, the cells were treated with 10 nM E2. Twenty-four hours later, the cells were harvested. The luciferase activity was measured by the Dual-Glo luciferase assay system.

2.5. Nuclear extracts preparation, immunoprecipitation and immunoblot analyses

HepG2 cells were transiently transfected with constructs of ER α /pcDNA3.1 plus FlagMed25 (2 μ g/well each) or ER α /pcDNA3.1 plus FlagMed25BM (2 μ g/well each) in 6-well plates, and after 24h, the cells were treated with 10 nM E2 or the vehicle control 0.1% EtOH. Twenty-four hours later, the cells were harvested. Nuclear extracts were prepared from HepG2 cells by NE-PERTM nuclear and cytoplasmic extraction Reagents from Thermo Fisher Scientific (Rockford, IL) according to the manufacturer's instructions. Nuclear extracts were subjected to immunoprecipitation (IP) with EZviewTM Red anti-FLAG M2 affinity gel (Sigma-Aldrich). Precipitated proteins were subjected to SDS-PAGE, followed

by Western blotting. Samples were separated by 4-12% NuPAGE Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane (Invitrogen). The membrane was blocked with 5% nonfat milk blocking buffer and blotted with rabbit-anti-FLAG (Sigma-Aldrich) or rabbit-anti-ER α (MC-20) (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody. Goat anti-rabbit-HRP (Pierce Biotechnology, Rockford, IL) was used as a secondary antibody. The protein bands on the membranes were developed using SuperSignal Western Femto kit (Pierce Biotechnology) followed by exposure on Kodak BioMax MR film (Kodak).

2.6. Confocal microscopy

Coverslips (Thermo Fisher Scientific) were treated with 1 mg/ml poly-lysine (Sigma-Aldrich) in H₂O. HepG2 cells were plated on the coverslips and transfected with ER α /pcDNA3.1 plus FlagMed25 (1 μ g each). After 24 hours, cells were treated with 10 nM E2. Twenty-four hours later, cells were washed thrice with PBS (pH 7.4) prior to fixation. Cells were fixed with 3% formaldehyde for 20 min at room temperature (RT). Fixed cells were blocked with 2% Goat serum (Gibco)/PBS for 30 min at RT. Then cells were stained with primary antibodies, rabbit-anti-ER α (MC-20) or anti-FLAG M2 (Sigma-Aldrich), followed by Alexa Fluor-conjugated secondary antibodies, Alexa Fluor 488 goat anti-rabbit IgG (A-11008), Alexa Fluor 568 goat anti-mouse IgG (A-11004), respectively (Molecular Probes/Invitrogen). Nucleic DNA was stained with DAPI (4'-6-diamidino-2-phenylindole) (Invitrogen) according to the manufacturer's protocol. Stained cells were washed with PBS, mounted on microscope slides with ProLong Gold (Invitrogen), and allowed to cure overnight. Cells were imaged using a Zeiss LSM-510 inverted confocal microscope.

2.7. Chromatin immunoprecipitation (ChIP) analysis

HepG2 cells were transfected with 12 μ g empty vector (pcDNA3.1), ER α /pcDNA3.1, FlagMed25, or ER α /pcDNA3.1 plus FlagMed25 (12 μ g each) in 10 cm dish. The final amounts of transfected DNAs were adjusted by adding empty vector. The cells were treated with 10 nM E2. Chromatin immunoprecipitation (ChIP) analysis was performed using MAGnify ChIP system (Invitrogen) according to the manufacturer's protocol. Antibody ER α (MC-20) (Santa Cruz Biotechnology) or FLAG M2 (Sigma-Aldrich) or control IgG was used in immunoprecipitation. The inputs and immunoprecipitated samples were subjected to PCR. The following ChIP primers were used: forward, 5'-ACAGAGTGGACAATGGAACGAAGG-3'; reverse, 5'-CACCTAGGTCCACTATATGC-3'. The PCR was carried out for 35 cycles. The bands on the gel were confirmed by sequencing. The ChIP primer set spanned ERE half site at position -149/-145 of *CYP2C9* promoter.

2.8. Quantitative Real time PCR

Human primary hepatocytes were transiently transfected with 2 μ g/well empty vector (pcDNA3.1), ER α /pcDNA3.1, FlagMed25, or ER α /pcDNA3.1 plus FlagMed25 (2 μ g/well each) in 6-well plates. The final amounts of transfected DNAs were adjusted by adding empty vector. After 24h, the cells were treated with 10 nM E2. Twenty-four hours later, the cells were harvested. For gene silencing experiments by small interfering RNA (siRNA), 50

pmol Med25 siRNA (h) (sc-97106), or control siRNA (sc-37007) (Santa cruz Biotechnology) were transfected into primary hepatocytes using Lipofectamine 2000. Total RNA was extracted by RNeasy Mini prep system (Qiagen, Valencia, CA). Then cDNA was synthesized from total RNA by MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA). Quantitative real time PCR was performed with Taqman Universal PCR Master Mix (Applied Biosystems), Taqman probes for CYP2C9 and Med25, and Applied Biosystems 7900HT sequence detection system. The TaqMan GAPDH probe was used as an internal control. Relative quantities were normalized to endogenous control values and were calculated by using the 2^{-CT} method.

2.9. P450 Glo CYP2C9 Assay

Human primary hepatocytes were transiently transfected 2 μ g/well empty vector (pcDNA3.1), ER α /pcDNA3.1, FlagMed25, or ER α /pcDNA3.1 plus FlagMed25 (2 μ g/well each) constructs or 50 pmol Med25 siRNA (h) (sc-97106), or control siRNA (sc-37007) (Santa cruz Biotechnology) using Lipofectamine 2000 in 6-well plates. The final amounts of transfected DNAs were adjusted by adding empty vector. After 24h, the cells were treated with 10 nM E2. Twenty-four hours later, the cells were harvested. Hepatocytes homogenates were prepared in a homogenate buffer containing 50 mM Tris-HCL (pH7.0), 150 mM KCL, and 2 mM EDTA. The homogenates were centrifuged at 1,000 $\times$ g for 1 min, and the supernatants used for P450 Glo CYP2C9 Assays and luminescence was measured according to the protocol of the manufacturer (P450 Glo CYP2C9 Assay, Promega).

2.10. Statistical Analyses

Data indicate the mean $\pm$ S.D. of three independent experiments or triplicate determinations. Multiple comparisons were determined using Dunnett's test following the ANOVA by using JMP 8.0 software. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Med25 mediated ER α -dependent transactivation of the CYP2C9 promoter

The Mediator complex plays crucial roles in transcriptional activation by interacting with transcription factors and receptors and recruiting RNA polymerase II to initiate transcription [16-18]. ER α , a ligand-activated transcription factor, is activated by the sex hormone estrogen, then directly binds to ERE's located in the promoter regions of target genes. A recent study showed that the CYP2C9 promoter region contains a functionally relevant ERE half site at position -149/-145 [13]. To investigate whether Med25, a variable member of the Mediator complex is required for ER α -mediated transcriptional activation of CYP2C9, we performed luciferase gene reporter assays in HepG2 cells. We transiently transfected a 1.9 kb of a CYP2C9 luciferase promoter construct along with Med25 and ER α full length expression plasmids into HepG2 cells. The cells were treated with E2 or vehicle EtOH. As shown in Fig. 1A, Med25 and ER α had no significant effect on the CYP2C9 promoter activity in the absence of ligand. However, promoter activity was 14-fold increased when Med25 and ER α were both present followed by treatment with the ligand E2. Furthermore, we generated a mutated ERE half site at position -149/-145 of 1.9 kb of CYP2C9 luciferase promoter construct (2C9mut). As shown in Fig. 1B, Med25 and ER α failed to activate the

CYP2C9 ERE mutant luciferase promoter activity after ligand E2 treatment. The luciferase activity of the mutated promoter significantly reduced compared with the wild-type *CYP2C9* promoter. Additionally, we also performed a 3XERE-luciferase reporter assay. The ERE luciferase reporter construct was transiently transfected into HepG2 cells along with Med25 and ER α full length expression plasmids. The cells were treated with the ligand of ER α , E2 or vehicle EtOH. As shown in Fig. 1C, after E2 treatment, we found 3XERE-luciferase reporter activity was much higher when both Med25 and ER α were transfected than in the ER α alone group ($p<0.05$).

3.2. Identification of Med25 interacting with ER α

Med25 contains a single C-terminal motif LXXLL which has been identified as a binding site interacting with some nuclear receptors such as HNF4 α [14]. To know whether the binding site motif LXXLL in Med25 was responsible for this activation, a construct (Med25 BM) with mutations LXXAA in the LXXLL motif of full length Med25 was generated. The enhanced effect of Med25 on ER α -mediated transcriptional activation of *CYP2C9* promoter was essentially abolished when the motif LXXLL was mutated (Fig. 1B).

To further confirm the importance of the C-terminal LXXLL motif in the activation of *CYP2C9* by ER α , we performed a mammalian two-hybrid assay and immunoprecipitation analysis. A series of constructs for this system were transiently transfected into HepG2 cells. The cells were treated with E2. As shown in Fig. 2A, Med25 interacted with ER α . However, the interaction was abolished when a mutated Med25 construct containing LXXAA instead of LXXLL. Furthermore, we performed immunoprecipitation analysis to assess the interaction between Med25 and ER α . The full length expressing constructs of ER α , and FLAG-tagged Med25 or Med25 mutant (Med25BM) were transiently transfected into HepG2 cells. The cells were treated with E2 or vehicle EtOH. As shown in Fig. 2B, when cells were immunoprecipitated with FLAG antibody to bring down Med25 in the presence of E2, ER α was detected in the immunoprecipitates expected followed by treated with E2, whereas the mutation of the LXXLL motif in Med25 abolished the immunoprecipitation of ER α .

Additionally, we did confocal microscopy analysis. HepG2 cells were transiently transfected with FlagMed25 and ER α constructs. The cells were treated with E2. As shown in Fig.2C, Med25 colocalized with ER α in the nucleus.

3.3. Med25 is recruited to ER α -mediated *CYP2C9* promoter

Chromatin immunoprecipitation assay was performed in HepG2 cells to investigate whether Med25 is recruited to ER α -mediated *CYP2C9* promoter. As shown in Fig.3, when the sheared chromatin extracts were pulled down with Flag antibody, FlagMed25 was observed to be recruited to the ERE half site (position -149/-145) of the *CYP2C9* promoter when FlagMed25 was coexpressed with ER α after E2 treatment.

3.4. Med25 is necessary for ER α regulated *CYP2C9* gene expression

Our results above suggest that Med25 is required for ER α -mediated transcriptional activation of the *CYP2C9* promoter. To further investigate the effect of this activation on

CYP2C9 gene expression, human primary hepatocytes were transiently transfected with constructs overexpressing full length of Med25, ER α , or empty vector as control, and cells were treated with E2. We then measured the mRNA level of *CYP2C9*. Consistent with *CYP2C9* luciferase promoter assay results, Med25 and ER α significantly induced *CYP2C9* mRNA after stimulation with E2, while Med25 or ER α alone did not have significant effect on *CYP2C9* gene expression (Fig. 4A).

As shown in Fig. 4B and 4C, we transiently transfected with control siRNA and Med25 siRNA to silence Med25 in human primary hepatocytes. Cells were treated with E2. Med25 mRNA levels significantly reduced when Med25 was silenced. *CYP2C9* mRNA levels significantly decreased both in SiMed25 and ER α +SiMed25 groups.

3.5. The effect of Med25 on *CYP2C9* enzyme activity in P450 Glo Assay

Human primary hepatocytes were transiently transfected with constructs expressing full length of Med25, ER α , or empty vector as control, and cells were treated with E2. As shown in Fig.5A, *CYP2C9* enzyme activity significantly increased when Med25 and ER α both overexpressed. To investigate the effect of Med25 on *CYP2C9* enzyme activity, Med25 was silenced. However, silencing Med25 significantly diminished *CYP2C9* enzyme activity (Fig.5B).

3.6. Comparison of the effect of Med25 on nuclear receptors-mediated induction of *CYP2C9* promoter activation

A number of nuclear receptors have been shown to bind to the *CYP2C9* promoter region and regulate *CYP2C9* transcription [7-11]. To further assess the role of Med25 in regulation of *CYP2C9* by some of these receptors, HepG2 cells were transfected with a 1.9 kb *CYP2C9* promoter reporter construct along with Med25 and the nuclear receptors ER α , HNF4 α , and CAR individually or in combination as shown in Fig.6. Med25 and ER α activated *CYP2C9* only in the presence of ligand (E2) as shown previously. Med25 dramatically increases HNF4 α -mediated transactivation of *CYP2C9* as reported previously [14]. Exogenous ligand is not necessary for activity of recombinant HNF4 α presumably because of the presence of bacterially expressed lipids in the ligand binding pocket. But Med25 did not have a significant effect on CAR-mediated activation of *CYP2C9* even in the presence of ligand, although it has been found to increase the synergistic effects of HNF4 α and CAR on the *CYP2C9* promoter in previous studies in one of our laboratories [14]. CAR is a receptor which is constitutively active even in the absence of ligand. Although the induction of *CYP2C9* promoter activation in ER α plus Med25 group is not as strong as that in HNF4 α plus Med25 group, Med25 is critical for ER α -mediated induction of *CYP2C9* promoter activation in E2 dependent manner.

4. Discussion

The CYP2C subfamily of cytochrome P450 enzymes is an important class of drug metabolizing enzymes as well as other xenobiotics and endogenous compounds in human liver. *CYP2C9* is an important member of CYP2C subfamily. To date, a number of nuclear receptor binding sites have been identified within the *CYP2C9* promoter region. Among

these nuclear receptors CAR, PXR, GR, HNF4 α , and ER α bind to *CYP2C9* promoter specific recognition sites and transcriptionally regulate *CYP2C9* expression [7, 8, 19]. In the present study, we first demonstrate that Med25 is an important coactivator of ER α , and functionally involved in ER α -mediated transcriptional regulation of *CYP2C9* in a ligand-dependent manner.

Nuclear hormone receptors are ligand-activated transcription factors that play essential roles in nearly every aspect of vertebrate development and adult physiology [20, 21]. In the presence of ligand, nuclear hormone receptors activate the transcription of target genes by recruiting specific coactivators as complexes to the target genes promoter regions. ER α is a classical hormone nuclear receptor and can be activated by lipophilic compounds such as E2. Activated ER α is a transcription factor, generally binding to specific recognition sites termed ERE in the regulatory region of target genes and thus regulate the transcription of target genes [22]. In the presence of ligand, ER α regulated *CYP2C9* expression through an ERE-half site within *CYP2C9* promoter region [13]. However, the involvement of coactivators in this regulation has not been previously studied. The Mediator complex is important in the initiation of transcription of nuclear receptors and recruitment of transcription factors. By ERE luciferase assay, we found Med25, a variable subunit of the Mediator complex, significantly enhanced ERE activation when Med25 and ER α were both transfected into HepG2 cells after E2 stimulation (Fig. 1C), indicating Med25 plays a vital role in ER α -mediated transcriptional regulation of the target gene which has an ERE in the promoter region. Preliminary studies by Han et al found Med25 has an impact on a selective set of nuclear receptors such as ER α and HNF4 α -mediated transactivation apparently with an ERE-luciferase response element and a HNF4 α -luciferase responsive element when transfected in Hela cells [23]. We identified coexpression of Med25 and ER α significantly enhanced *CYP2C9* promoter activity through ERE half site in the *CYP2C9* promoter region (Fig.1A), whereas this activation failed in the mutated promoter (Fig.1C). Our data shows that Med25, a pivotal cofactor for ER α , is essential for ER α -mediated transcriptional regulation of hepatic *CYP2C9* in a ligand-stimulated fashion. However, Mwinyi et al. reported E2 marginally suppressed *CYP2C9* promoter activity [13]. The difference results may due to the absence of Med25 in their studies.

The Mediator is a multi-subunit protein complex that is a key regulator of transcription by RNA polymerase II [16, 24-27]. In humans, Mediator was first identified as a transcriptional coactivator bound to the thyroid hormone receptor. Then later, Mediator has been shown to be an essential ligand-dependent coactivator for a broad range of nuclear hormone receptors as well as other signal-activated transcription factors [28]. The subunit Med 1 is responsible for binding to many but not all receptors and bringing the mediator complex to the receptor site. Med25 is a variable of the subunit of the Mediator complex acting as a molecular bridge between certain DNA-bound transcriptional activators and the general transcription machinery [17]. The C-terminal LXXLL motif of Med25 can directly bind to retinoic acid receptor α [29] and HNF4 α [14]. Our immunoprecipitation and mammalian two-hybrid analyses demonstrated that Med25 directly interacted with ER α in a ligand-dependent manner. We further identified the functional interaction of Med25 with ER α occurred through the C-terminal LXXLL motif of Med25. Mutation of LXXLL in Med25 resulted in

the loss of interaction with ER α and the abolishment of the ER α -mediated transactivation of CYP2C9, indicating the C-terminal LXXLL motif in Med25 is important for Med25-ER α interaction. Additionally, by confocal analysis, we further found Med25 colocalized with ER α in the nucleus. These findings indicate that Med25 is a pivotal coactivator of ER α , interacting with ER α through its C-terminal LXXLL motif, and plays vital role in the transcriptional activation of CYP2C9 by ER α . Our ChIP analysis shows that when coexpression of Med25 and ER α , Med25 is recruited to ERE half site at position -149/-145 of CYP2C9 promoter in an estrogen-dependent manner.

Med25 is necessary for ER α regulated CYP2C9 gene expression. CYP2C9 mRNA levels induced significantly when Med25 and ER α both present while when Med25 was silenced, mRNA levels of CYP2C9 were markedly reduced. Med25 has vital effect on ER α -mediated increases in CYP2C9 gene expression.

Coexpression of Med25 and ER α in human primary hepatocytes significantly increased CYP2C9 enzyme activity after E2 treatment, while the enzyme activity was highly reduced when Med25 was silenced. This indicates that Med25 has an important effect on ER α -mediated the induction of CYP2C9 enzyme activity.

Various cofactors can be involved in nuclear hormone receptors mediated activation of target gene. The TRAP/Mediator coactivator complex has been shown to interact with ER α and ER β ligand binding domains through TRAP220/Med1 in an E2-dependent manner in vitro [30]. Our future studies will investigate the role of other cofactors and the recruitment of the preinitiation complex in the Med25-ER α regulation of CYP2C9. In the present study Med25 is identified as an important coactivator of ER α and is required for ER α -mediated regulation of CYP2C9 expression.

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Abbreviations

CYP	cytochrome P450
Med	Mediator
CAR	constitutive androstane receptor
PXR	pregnane X receptor
GR	glucocorticoid receptor
HNF4α	hepatic nuclear factor 4 α
ERα/β	estrogen receptor α / β
E2	17 β -estradiol

RE	responsive element
ERE	estrogen responsive element
h	human
Ab	antibody
IP	immunoprecipitation
CITCO	6-(4-chlorophenyl)imidazo[2,1- <i>b</i>]-[1,3]thiazole-5-carbaldehyde <i>O</i> -(3,4-dichlorobenzyl)oxime
DMSO	dimethyl sulfoxide
siRNA	small interfering RNA
DAPI	4',6-diamidino-2-phenylindole

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A

B

C

Fig.1.

Med25 regulated ERα-mediated activation of *CYP2C9* promoter in ligand-dependent manner in HepG2 cells. (A) Cells were transiently transfected with a 1.9 kb *CYP2C9* luciferase promoter construct along with ERα, Med25 and Med25BM (LXXLL mutant) full length expression plasmids. (B) A 1.9 kb *CYP2C9* ERE mutated luciferase promoter construct (2C9mut) along with ERα and Med25 full length expression plasmids were transiently transfected into cells. (C) Cells were transiently transfected with 3×ERE-TK pGL3 luciferase construct along with ERα and Med25. The cells were treated with the ligand of 10 nM E2 or the vehicle 0.1% EtOH. Data are shown as fold changes of luciferase activity relative to the activity of *CYP2C9* or its mutant luciferase promoter construct treated with EtOH. Data represent mean ± S.D. of three independent experiments. *, significantly different between groups indicated by bars ($P < 0.05$). #, the transactivation of the *CYP2C9* promoter in the mutant group (ERα plus Med25BM) was significantly decreased after E2 treatment compared with that of ERα plus unmutated Med25 ($P < 0.05$).

A

B

ER α +FlagMed25

Fig.2.

In vivo interaction of Med25 with ER α . (A) Mammalian two-hybrid analysis. HepG2 cells were transiently transfected with luciferase construct pG5, pActER α , and pBindMed25, and its mutant pBindMed25BM. The cells were treated with 10 nM E2. Data are shown as fold changes of luciferase activity relative to the activity of pG5 luciferase promoter construct (control). Data represent mean $\pm$ S.D. of three independent experiments. *, significantly different from control (P 0.05). #, significantly lower than that of ER α plus Med25 (P 0.05). (B) Immunoprecipitation analysis. HepG2 cells were transiently transfected with full length of ER α and FLAG-tagged Med25 and its mutant Med25BM. The cells were treated with 10 nM E2 or 0.1% EtOH. The nuclear extracts were immunoprecipitated with IgG as negative control, with FLAG antibody (lower panels) for Med25, and then probed with ER α antibody (upper panels). The inputs were from ER α plus FlagMed25 or ER α plus

FlagMed25BM expressed nuclear extracts. Ab, antibody. IP, immunoprecipitation. (C) Confocal microscopy. HepG2 cells were transiently transfected with full length of FLAG-tagged Med25 and ER α . The cells were treated with 10nM E2. Med25 colocalized with ER α in the nucleus.

Fig.3.

Med25 is recruited to the *CYP2C9* promoter at ERE half site -149/-145 in HepG2 cells. The recruitment of Med25 to *CYP2C9* promoter was analyzed by ChIP assay. HepG2 cells were transiently transfected with full length of ERα, FLAG-tagged Med25 or ERα plus FlagMed25, or empty vector (pcDNA3.1) as control. The cells were treated with 10 nM E2 or 0.1% EtOH. Sheared chromatin extracts were immunoprecipitated by ERα, Flag antibodies and IgG as a control. The immunoprecipitates and inputs were subjected to PCR with ChIP primers spanning ERE half site at position -149/-145 of *CYP2C9* promoter.

A

B

C

Fig.4.

Med25 is important for ER α -mediated regulation of CYP2C9 gene expression after ligand stimulation. (A) Human primary hepatocytes were transiently transfected with empty vector (pcDNA3.1) as control and full length of Med25 and ER α constructs. The cells were treated with 10 nM E2. Data represent mean $\pm$ S.D. of triplicate determinations. *, significantly different from control (empty vector) (P 0.05). (B and C) Human primary hepatocytes were transiently transfected with full length of Med25 and ER α constructs, Med25 siRNA or control siRNA. The cells were treated with 10 nM E2. Data represent mean $\pm$ S.D. of triplicate determinations. *, significantly different from control siRNA group (P 0.05).

A

B

Fig.5.

The effect of Med25 on CYP2C9 enzyme activity in P450 Glo assay. Human primary hepatocytes were transiently transfected with (A) empty vector (pcDNA3.1) as control and full length of Med25 and ERα constructs (B) Med25 siRNA, or control siRNA, control siRNA plus Med25 and ERα constructs. The cells were treated with 10 nM E2. The supernatants of cell homogenates used for P450 Glo CYP2C9 Assay. Data represent mean $\pm$ S.D. of triplicate determinations. *, significantly different from control (empty vector) (P 0.05). #, significantly different from control (Con-SiRNA) (P 0.05).

Fig.6.

The effect of Med25 on induction of *CYP2C9* promoter activation by various nuclear receptors. HepG2 cells were transiently transfected with a 1.9 kb *CYP2C9* luciferase promoter construct (200 ng/well) along with ERα (100 ng/well), Med25 (200 ng/well), CAR (100 ng/well), or HNF4α (100 ng/well) full length expression plasmids. The cells were treated with the ligand of 10 nM E2 for ERα or 100 nM CITCO for CAR and HNF4α or vehicle 0.1% (EtOH or DMSO). Data are shown as fold change of luciferase activity relative to the activity of *CYP2C9* luciferase promoter construct treated with vehicle (control). Data represent mean ± S.D. of three independent experiments.