Epigenetic Modification of Histone 3 Lysine 27

MEDIATOR SUBUNIT MED25 IS REQUIRED FOR THE DISSOCIATION OF POLYCOMB REPRESSIVE COMPLEX 2 FROM THE PROMOTER OF CYTOCHROME P450 2C9*

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Background: Mediator subunit MED25 associates with hepatocyte nuclear factor 4α to initiate activation of select genes.

Results: CYP2C9 activation by MED25 involves preinitiation complex formation, H3K27 acetylation, and chromatin conformation changes, and the absence of MED25 results in H3K27 trimethylation and Polycomb group recruitment.

Conclusion: MED25 is a key regulator of epigenetic mechanisms.

Significance: MED25 regulates human drug metabolism gene CYP2C9 by epigenetic modification.

The Mediator complex is vital for the transcriptional regulation of eukaryotic genes. Mediator binds to nuclear receptors at target response elements and recruits chromatin-modifying enzymes and RNA polymerase II. Here, we examine the involvement of Mediator subunit MED25 in the epigenetic regulation of human cytochrome P450 2C9 (CYP2C9). MED25 is recruited to the CYP2C9 promoter through association with liver-enriched HNF4α, and we show that MED25 influences the H3K27 status of the HNF4α binding region. This region was enriched for the activating marker H3K27ac and histone acetyltransferase CREBBP after MED25 overexpression but was trimethylated when MED25 expression was silenced. The epigenetic regulator Polycomb repressive complex (PRC2), which represses expression by methylating H3K27, plays an important role in target gene regulation. Silencing MED25 correlated with increased association of PRC2 not only with the promoter region chromatin but with HNF4α itself. We confirmed the involvement of MED25 for fully functional preinitiation complex recruitment and transcriptional output in vitro. Formaldehyde-assisted isolation of regulatory elements (FAIRE) revealed chromatin conformation changes that were reliant on MED25, indicating that MED25 induced a permissive chromatin state that reflected increases in CYP2C9 mRNA. For the first time, we showed evidence that a functionally relevant human gene is transcriptionally regulated by HNF4α via MED25 and PRC2. CYP2C9 is important for the metabolism of many exogenous chemicals including pharmaceutical drugs as well as endogenous substrates. Thus, MED25 is important for regulating the epigenetic landscape resulting in transcriptional activation of a highly inducible gene, CYP2C9.

Epigenetic modifications that impact gene activation coordinate to functionally remodel the chromatin architecture, allowing coactivators to populate the exposed DNA strand and facilitate polymerase recruitment (1, 2). Acetylation of nucleosomal core histone N-terminal lysines commonly leads to gene expression by neutralizing the electrostatic bond between the negatively charged DNA and positively charged protein, whereas lysine methylation has been shown to either inhibit or activate expression depending on the amino acid substrate as well as degree of methylation (3, 4). For example, numerous cytochrome P450 (CYP) genes, including CYP2C9 and CYP2E1, have been shown to be up-regulated with trichostatin A, a histone deacetylase inhibitor (5, 6), and expression of CYP7A1 can be induced by glucose through increased histone acetylation in the promoter region (7). Conversely, epigenetic inhibition of gene expression via histone modifications involves regulation by cofactors of the Polycomb repressive complex (PRC2), which methylate histone 3 lysine 27 residues (8), leading to chromatin compaction and a heterochromatin-like conformation. Directing PRC2 to the target genes likely occurs through multiple mechanisms that may be context-specific, and Polycomb complexes can have variable subunits that exhibit a multitude of enzymatic and non-enzymatic functions at their target genes including the blocking of transcriptional machinery (9).

Studies in HepG2 liver carcinoma cells and primary human hepatocytes have shown functional activation of CYP2C9 expression through the binding of the liver-enriched receptor hepatocyte nuclear factor 4α (HNF4α) (10). The mechanism of nuclear receptor-mediated activation involves recruitment of the Mediator complex by HNF4α, which we found to occur specifically through direct interaction with the variable Mediator subunit MED25 (11). The metazoan Mediator complex is composed of 30 distinct subunits, which are variably associated with transcriptional cofactors such as the glucocorticoid receptor (12), CREB–binding protein (CREBBP) (13), and the preini-
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In the present study, we tested the hypothesis that MED25 is important for the permissive open conformation state of CYP2C9 chromatin and is essential for the recruitment of the pol II preinitiation complex. We found a MED25-dependent shift in the H3K27 marker status at the HNF4α binding region that correlated with an open chromatin conformation, and silencing MED25 expression resulted in reduced PIC cofactor recruitment, accumulation of H3K27me3, and a condensed chromatin conformation. The mechanism of activation involved MED25-mediated dissociation of the Polycomb proteins responsible for H3K27 trimethylation and concurrent inactivation of HNF4α-mediated CYP2C9 expression.

EXPERIMENTAL PROCEDURES

Cell Culture—HepG2 cells were maintained in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1 mM sodium pyruvate in a 37 °C incubator with humidified air containing 5% CO2. HEK293 cells were maintained in Dulbecco’s MEM supplemented the same as HepG2 cells.

Formaldehyde-assisted Isolation of Regulatory Elements (FAIRE)—HepG2 cells were co-infected with CAR plus HNF4α with FLAG-MED25 or CAR plus HNF4α with shMED25 (11). After 48 h, chromatin was isolated, reverse-transcribed with the SuperScript III First Strand system (Invitrogen), and 100 ng/reaction was used in qPCR in triplicate. TaqMan probes included CYP2C9 TATA-binding protein (TBP) as an endogenous control. RNA transcript levels were measured using the TaqMan method.

Quantification of RNA Levels—An aliquot of cells was taken from each treatment group during the FAIRE experiment to measure the levels of CYP2C9 mRNA. Briefly, cells were rinsed and pelleted in cold PBS. Pellets were resuspended in 600 µl of buffer RLT (Qiagen), and total RNA was isolated as directed using an automated QiaCube machine (Qiagen). RNA (1 µg) was reverse-transcribed with the SuperScript III First Strand system (Invitrogen), and 100 ng/reaction was used in qPCR in triplicate. TaqMan probes included CYP2C9 TATA-binding protein (TBP) as an endogenous control. RNA transcript levels were measured using the TaqMan method.

Nucleosome Occupancy Prediction—The predicted occupancy was derived using a probabilistic nucleosome-DNA sequence surrounding the translation start site (TSS) and displayed the proximal 2 kb upstream of the TSS.

Chromatin Immunoprecipitation (ChIP)—HepG2 cells were cultured, infected, and cross-linked as described above for FAIRE experiments. Chromatin was isolated and sonicated to measure the levels of CYP2C9 mRNA. Briefly, cells were rinsed from each treatment group during the FAIRE experiment to measure the levels of CYP2C9 mRNA. Briefly, cells were rinsed and pelleted in cold PBS. Pellets were resuspended in 600 µl of buffer RLT (Qiagen), and total RNA was isolated as directed using an automated QiaCube machine (Qiagen). RNA (1 µg) was reverse-transcribed with the SuperScript III First Strand system (Invitrogen), and 100 ng/reaction was used in qPCR in triplicate. TaqMan probes included CYP2C9 TATA-binding protein (TBP) as an endogenous control. RNA transcript levels were measured using the TaqMan method.
MED25 Orchestrates Epigenetic Regulation for CYP2C9 were normalized to TATA-binding protein using the 2^−ΔΔCt method, and treatments were normalized to LacZ controls. Data are the means ± S.E., n = 3.

**Co-immunoprecipitation Assays**—HEK293 cells were transfected with expression plasmids pCR3.1-HNF4α and either FLAG-MED25 or siRNA (Thermo Fisher, L-014689-01) against MED25 using Lipofectamine 2000 (Invitrogen); cells were also co-transfected with pCMVHA-EED, pCMVHA-hEZH2 (26), pCMVHA-SUZ12 (27), or pT3-EF1a-BM11 (28). Nuclear extracts were prepared as described previously (29) and immunoprecipitated with 2 μg of HNF4α (rabbit, sc-8987, Santa Cruz Biotechnology), hemagglutinin (HA) (rat, catalog No. 11867423001, Roche Applied Science), or IgG (rabbit, catalog No. 10005291, Invitrogen) antibody overnight at 4 °C with protein G-agarose beads (Sigma). The beads were washed in PBS plus 0.1% Nonidet P-40, resuspended in SDS loading buffer, and boiled for 5 min at 100 °C, and the proteins were separated on a 4–20% Tris-glycine gel. Immunoblots were probed with antibodies against HNF4α (mouse, PP-H1415-00, Perseus Proteomics, Inc.), HA (mouse, sc-7392, Santa Cruz Biotechnology), or GAPDH (mouse, MAB374, Millipore).

**Chromatin Assembly**—Biotin-labeled probes were generated by PCR using a biotin-labeled forward primer (−258, /biotin/ACCCATGGCCGCCGGC; −410, /biotin/TGGCCATTCTGTAAATTATCAT; and −817, /biotin/TTCTCTAAGCAGCGTCTGGTA) and reverse primer (TCTGGTACCGGTCGAGATAT) that amplified the proximal promoter and partial coding region of the pGL3-2C9 plasmid. The amplified DNA product was purified using a PCR purification kit (Qiagen), and 1 μg was included in a chromatin assembly reaction as described in a chromatin assembly kit (Active Motif). A portion of the in vitro assembled chromatin was analyzed for nucleosome formation through enzymatic digestion and visualized on an agarose gel.

**In Vitro Chromatin Recruitment Assay**—In vitro chromatin (up to 100 ng of DNA) was incubated with 100 μg of HepG2 nuclear extract prepared as described (29) expressing HNF4α with either FLAG-MED25 or shMED25 (11) overnight at 4 °C for cofactor analysis or at 30 °C for 1 h and 1 h with HeLa nuclear extract for histone marker analysis. Biotin DNA-protein complexes were captured on 15 μl of streptavidin-coupled Dynabeads (Invitrogen) as suggested by the manufacturer. The chromatin-coupled beads were washed with PBS plus 0.1% Nonidet P-40, eluted with SDS loading buffer, and subjected to Western blotting. Aliquots of nuclear extracts were used as input controls. Briefly, equal amounts of in vitro chromatin were loaded on a 4–20% Tris-HCl Criterion gel (Bio-Rad), and protein complexes were analyzed using antibodies targeting HNF4α, polymerase II, phospho-serine 5 polymerase II, CREBBP, MED25 (Santa Cruz Biotechnology), histone 3, and H3K27ac (Abcam).

**In Vitro Transcription**—Chromatin was assembled as described above, and transcription was carried out essentially as described previously (30). In the order-of-addition dissociation experiment, 5 ng (DNA) in 1× transcription buffer was incubated with 1 μl of in vitro transcribed HNF4α with or without 1 μl of in vitro transcribed MED25 for 20 min at room temperature. Samples were incubated with 10 μg of HEK293 whole cell lysate expressing EZH2-HA, SUZ12-HA, EED-HA, or BMI1-HA individually, or in vitro transcription buffer for control reactions for 15 min at 30 °C. In the order-of-addition blocking experiment, 5 ng (DNA) was preincubated with PRC-expressing HEK293 lysates for 15 min at 30 °C and then incubated with translated HNF4α with or without MED25, and transcription was initiated as described above. Isolated RNA was loaded on a 5% TBE (Tris borate-EDTA)-urea acrylamide gel, and the bands were visualized by autoradiography.

**Confocal Microscopy**—Colocalization studies were performed as described previously (11). pEGFPC2-MED25 and pCR3.1-HNF4α plasmids were co-transfected, and cells were prepared for immunofluorescence. Antibodies targeting histone modifications included H3K27ac (Cell Signaling Technology), H3K27me3 and H3K27me1 (Millipore), H3K27me2 and H3K4me2 (Abcam), and HNF4α (Santa Cruz Biotechnology). Cells were visualized with a Zeiss LSM-510 UV confocal microscope. Fluorescence intensity profiling data were obtained from intensity values along the midline of the nucleus to generate correlation coefficient values for at least three nuclei, which were then averaged and graphed (ZEN 2009 software).

**Statistical Analysis**—Where reported, n values represent technical replicates from a representative result from at least two independent experiments. Expression data for FAIRE, ChIP, and RNA isolation were analyzed by analysis of variance followed by Bonferroni post hoc testing for multiple comparisons. Data are displayed as the means ± S.E., and significance between the indicated data is displayed as *, p < 0.05; **, p < 0.01; and (**), p < 0.001.

**RESULTS**

**MED25 Regulates the Epigenetic Landscape of CYP2C9**

**Chromatin**—Epigenetic regulation of genes involved in homeostasis and pathophysiological conditions has been well documented. However, many genes that are involved in the metabolism of a large number of therapeutically prescribed drugs and are activated by xenobiotics have not been shown to be regulated by epigenetic modification. In this study, we focused on the changes in histone modifications and chromatin structure occurring in the promoter region of human CYP2C9 during its activation and inactivation.

Using ChIP assays, we assessed the levels of numerous histone code modifications known to be associated with gene activation or repression. We investigated H3K9ac, which is associated with enhancers (31), activating markers such as H3K4ac, -me2, and -me3, and repressive markers such as H3K79me3, H4K20me3, and H3K9me3. We did not observe appreciable differences for any of these modifications at the HNF4α binding region of CYP2C9 after overexpression of HNF4α, CAR, and MED25 compared with expression of CAR and HNF4α while silencing MED25. However, we observed a MED25-dependent change in H3K27 epigenetic marker status that is consistent with the activating function of MED25. The repressive marker H3K27me3 was detected at the HNF4α binding region strictly when MED25 expression was silenced (Fig. 1A), indicating that the chromatin maintains a heterochromatin-like histone code when MED25 is absent. After overexpression of CAR, HNF4α, and MED25, we observed a trend toward reduction in H3K27 methylation and an increase in H3K27 acetylation in the prox-
imal HNF4α binding region compared with overexpression of CAR and HNF4α with silencing MED25. H3K27me2, which typically correlates with gene repression (32), was most highly enriched after silencing MED25 compared with the other treatments (Fig. 1B), but it also showed a lower yet significant enrichment upon CAR, HNF4α/H9251, and MED25 overexpression. H3K27me1, which was shown to be deposited intragenically by PRC2 in actively expressed genes (33), showed no significant differences in enrichment of the HNF4α binding region that were due to MED25 expression (Fig. 1C). Finally, antibody to acetylated H3K27 showed 2-fold higher enrichment in the HNF4α binding region when MED25, HNF4α/H9251, and CAR were overexpressed compared with silencing MED25 in the presence of overexpression of CAR and HNF4α (Fig. 1D). When CAR and HNF4α were overexpressed individually, there was a minimal increase in the enrichment of H3K27ac. The observation that silencing MED25 in the presence of overexpression of CAR and HNF4α did not significantly lower H3K27ac enrichment compared with overexpression of CAR and HNF4α alone (in the presence of endogenous MED25) may reflect the fact that we assayed the average acetylation level within the chromatin region targeted by the primers and not the effects of a single nucleosome. Moreover, we do not know the actual level of MED25 in the groups containing endogenous MED25 versus those in which MED25 was silenced. Certainly with overexpression very high levels are reached (11). The cumulative differ-

FIGURE 1. The HNF4α binding region is enriched for activating H3K27 markers in the presence of MED25. ChIP assays in HepG2 cells infected with adenovirus expressing the indicated factors targeted the HNF4α binding region of the CYP2C9 proximal promoter. A, ChIP assay using anti-H3K27me3 antibody with expression of indicated proteins. B–D, H3K27me2 (B), H3K27me1 (C), and H3K27ac (D) antibodies were used to immunoprecipitate chromatin. Data are the means ± S.E. (n = 3). Significance from LacZ control: *, p < 0.05; **, p < 0.001. Significantly different from CAR + HNF4α###, p < 0.001. The arrow (below A–D) signifies the general trend of histone modification function during regulation of gene expression. E, representative confocal images of HEK293 cells expressing GFP-MED25 and incubated with antibodies against HNF4α, H3K27ac, H3K27me1, H3K27me2, or H3K27me3. F, fluorescence intensity data from the green and red channels were taken from the entire nuclear space, and the mean correlation (R) value was graphed. Data are the means ± S.E. (n ≥ 3).
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ence of enrichment, dependent on MED25 expression from a repressive marker (H3K27me3) to an activating marker (H3K27ac), was 35-fold. These data suggest that the presence of MED25 induces changes in chromatin toward a state of activation by mediating histone modification. An examination of histone N-tail modifications suggested a dynamic chromatin landscape in the HNF4α binding region in response to gene activation/inactivation.

Colocalization of MED25 with Epigenetic Histone Markers—The human Mediator complex is variable, and its composition is postulated to be dependent on target promoter context. Using confocal microscopy, we investigated whether the activation of HNF4α binding regions by MED25 could be the result of a more global mechanism. In HepG2 cells, which express low levels of HNF4α, HNF4α and GFP-MED25 were transiently co-transfected, and we quantified the correlation coefficient between the GFP-MED25 protein and histone markers (Fig. 2). As expected, two other members of the Mediator complex, MED1 and MED14, were enriched in the HNF4α binding region upon CAR, HNF4α, and MED25 coexpression but not when MED25 was silenced (Fig. 2, C and D). These Mediator subunits represent the middle and tail segments, respectively, consistent with the hypothesis that MED25 recruits a functionally active Mediator complex to the HNF4α binding region. These Mediator subunits were not enriched in the HNF4α binding region in the absence of ectopic MED25. It could be noted that the assay may be at the limits of detection because values for MED1 and MED14 are lower than the IgG controls. Analysis of CREBBP, a chromatin remodeling factor that displays histone acetyltransferase (HAT) activity, revealed that it bound to the chromatin region in conditions of CYP2C9 activation by overexpression of CAR and HNF4α with MED25 but not when MED25 was silenced, despite overexpression of CAR and HNF4α (Fig. 2E). CREBBP is a crucial member of HNF4α-containing complexes at active genes (34, 35). MED25 has been shown to recruit CREBBP to active retinoic acid receptor complexes (13), and we have shown previously that CREBBP immunoprecipitates with MED25 (11). These data are consistent with a role for the involvement of MED25 in the recruitment of PIC components at the CYP2C9 HNF4α binding region.

MED25 Promotes Reorganization of the CYP2C9 Promoter Chromatin—The recruitment of a HAT complex to the promoter region and the acetylation of H3K27 observed with CAR, HNF4α, and MED25 overexpression prompted us to investigate the organization of the CYP2C9 promoter chromatin. To this end, we performed FAIRE analysis by targeting the CYP2C9 locus with qPCR. We tested whether the dynamic histone alterations observed with ChIP translated into remodeling of the CYP2C9 promoter chromatin. Regions of “open” chromatin, which are considered to be nucleosome-free and thus transcriptionally active (36), were isolated using the FAIRE technique. Under conditions of low CYP2C9 expression in cells expressing CAR or HNF4α alone, there was no significant enrichment of FAIRE-accessible DNA compared with the LacZ control (Fig. 3A). However, activation by CAR and HNF4α with endogenous MED25 expression or with ectopic MED25 expression resulted in a significant increase of FAIRE-accessible DNA, suggesting that the nucleosome occupancy in this region was reduced compared with constitutive expression conditions; FAIRE DNA enrichment patterns correlated with those of CYP2C9 mRNA expression (Fig. 3B). A previous report from our laboratory indicates that expression of CAR and HNF4α with silencing of MED25 represses CYP2C9 mRNA expression (11). Here, overexpressing CAR and HNF4α while concomitantly silencing MED25 resulted in the abrogation of FAIRE enrichment levels supporting a compaction of the HNF4α binding region in the chromatin.

Further analysis of the CYP2C9 promoter and coding region revealed varying levels of constitutive and “openness” of the chromatin conformation. An upstream region (starting at −1235 from the TSS) of the CYP2C9 promoter neither containing HNF4α response elements nor thought to be relevant to regulation by MED25 showed a slight increase in FAIRE accessibility with CAR, HNF4α, and MED25 overexpression but not under other treatment conditions (Fig. 3C). Similarly, a region of exon 2 was not dramatically altered in response to overexpression of CAR and HNF4α with MED25 overexpression or silencing (Fig. 3D). In contrast, the FAIRE signal was significantly increased at the CAR response element (−1839 bp from the translation start site) when CAR and HNF4α were coexpressed in the presence of endogenous or ectopic MED25 expression; FAIRE enrichment was reduced to control levels after silencing MED25 in the presence of CAR and HNF4α.
coexpression (Fig. 3E). These data support the Mediator-dependent recruitment of transcriptional cofactors for CYP2C9 activation through a mechanism that involves looping of the DNA between the CAR and HNF4α binding regions, which are separated by 1.7 kb (37).

Finally, we asked whether the CYP2C9 promoter region displayed intrinsic nucleosome binding affinity based on the DNA sequence. First, we noted that there were no CpG islands in the CYP2C9 promoter based on methylation-specific PCR. Therefore, the conventional GC content and hypomethylated DNA of this region should not impart the structural characteristics normally associated with CpG islands (38). Second, using a nucleosome occupancy model first developed in yeast (25), we observed that the TSS and the HNF4α binding region between −211 and −150 bp upstream of the TSS were predicted to be moderately nucleosomal (0.62 and 0.53, respectively), whereas the CAR binding site at −1839 bp had a lower predicted nucleosomal affinity (0.42) (Fig. 3F). The intervening region between the CAR and HNF4α binding regions showed a periodicity of four strong nucleosome probabilities; the region at −1235 bp

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**FIGURE 2.** PIC cofactors are recruited to the HNF4α response element of the CYP2C9 promoter. ChIP assays targeted the HNF4α binding region of the CYP2C9 proximal promoter in HepG2 cells infected with adenovirus expressing the indicated factors, and resultant DNA enrichment was quantified by qPCR. ChIP assays were conducted with anti-HNF4α (A), -FLAG (for FLAG-MED25) (B), -MED1 (C), -MED14 (D), and -CREBBP (E) antibodies. Data were normalized to IgG controls and are the means ± S.E. (n = 3). Significantly different from LacZ control: ***, p < 0.001; **, p < 0.01. Significantly different from CAR + HNF4α: ###, p < 0.001; ##, p < 0.01. Significantly different from CAR + HNF4α + FLAG-MED25: ++, p < 0.001. The positions of qPCR primers are indicated at the top of the figure.
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In Vitro Association of MED25 with PIC Components—The association of coactivators with the CYP2C9 HNF4α binding region is dependent on the function of MED25 in vivo. The loss or absence of MED25 resulting in a diminished association with HNF4α results in a closing of the chromatin region and a shift in H3K27 modification. This observation led us to examine whether the recruitment function of MED25 to HNF4α could be initiated using chromatin encompassing the proximal CYP2C9 promoter in vitro.

Biotin-labeled DNA fragments containing up to 817 bp of the upstream regulatory region were PCR-amplified and used to assemble chromatin with recombinant HeLa histones. All fragments were able to form at least two nucleosomes based on a micrococcal nuclease digestion analysis (Fig. 4A). The 817-bp assembled chromatin strand was chosen as a recruitment template based on its ability to form a multinucleosomal array. As depicted in Fig. 4B, endogenous HNF4α expression was detected in HepG2 nuclear extracts; ectopic expression of HNF4α increased the expression levels regardless of MED25 status. MED25 protein expression was evident in control nuclear extracts, which increased with ectopic expression (HNF4α + FLAG-MED25) but was strongly reduced by shRNAs targeting MED25. Thus, incubation of the 817-bp chromatin template and analysis of the isolated protein complexes revealed low levels of endogenous HNF4α and MED25 recruitment from untreated HepG2 nuclear extracts. Increasing the expression of HNF4α and MED25 allowed for increased recruitment to the chromatin template, whereas complete abrogation of MED25 recruitment was seen with silencing MED25 expression.

The Mediator complex is an essential component of the pre-initiation complex with pol II and the general transcription factors. We tested the ability of our chromatin template to recruit pol II to the HNF4α binding region in vitro. We confirmed that HNF4α was recruited to the chromatin template regardless of MED25 expression and that MED25 was recruited upon overexpression, indicating HNF4α binds to its response element first in the order of association and recruitment (Fig. 4C). Interestingly, recruitment of total pol II to the chromatin template was similar between overexpression and silencing of MED25, but the phosphorylation of pol II at serine 5 was diminished when MED25 expression was silenced. Importantly, pSer-5 pol II has been shown to be present at gene promoters (39). Similar results were obtained for the histone acetyltransferase CREBBP, which showed a reduced recruitment pattern upon MED25 silencing. CREBBP may be an important histone modifier of this chromatin template because we also observed a similar reduction in the H3K27ac marker after silencing MED25 expression. Total H3 histone levels were unchanged throughout the experiment. Densitometric analysis of pSer-5 pol II, CREBBP, and H3K27ac in vitro recruitment revealed a 65, 50, and 54% reduction, respectively, after MED25 silencing compared with overexpression (Fig. 4C, right). This suggests that although MED25 is important for PIC recruitment to the HNF4α binding region, there may be secondary interactions between the PIC and HNF4α. H3K27me3 (not shown) was not altered in response to MED25 expression in this in vitro system, which may not provide suitable conditions for the enzymatic
conversion of HeLa core histone H3K27 to a trimethylated state.

The Association of PRC Proteins with HNF4α in the Presence and Absence of MED25—PRC proteins are associated with a large number of genes that determine various functions in development and differentiation. Sequence specific transcription factors such as MYCN (40), CpG islands (41), and noncoding RNAs (42) have been identified as putative mechanisms of PRC recruitment to target genes (43), and yet we are not aware of published data that identify typical nuclear receptors as PRC interaction partners. In our study, co-immunoprecipitation analyses of transiently transfected HA-tagged PRC proteins indicated that EED-HA, SUZ12-HA, EZH2-HA, and BMI1-HA associated with HNF4α (Fig. 5). The reciprocal IP reaction and subsequent detection of HA-tagged proteins generated multiple bands, which we identified as the predicted molecular weight PRC bands (*) and alternate bands (**). In the presence of MED25, the HA antibody detected a predominant band migrating with the predicted mobility of EZH2-HA as well as two alternate lower molecular weight bands in the input. When MED25 expression was silenced, the pattern was similar except that the alternate bands were somewhat more predominant in the IPs. The stability and activity of EZH2 relies on O-GlcNAcylation by the enzyme OGT, which results in increased H3K27me3 deposition (44). The relative intensity of the alternate bands is consistent with a 2.8-fold reduction in OGT expression when MED25 is silenced (11). In addition, frameshift mutations in EZH2 in myeloid malignancies were predicted to lead to premature chain termination (45). When MED25 was overexpressed, EED-HA associated with HNF4α with two bands, a major band with the predicted molecular weight and a minor, alternate band with a higher molecular weight. When MED25 was silenced, only the band with the predicted molecular weight was detected in the IPs. Multiple isoforms for EED have been reported that are derived from internal translation start sites from a single mRNA species (46), indicating the presence of complex protein processing mechanisms for PRC proteins. These variant proteins could differ in their affinities for HNF4α.

FIGURE 4. Silencing MED25 reduces PIC formation and alters H3K27 marker status in an in vitro system using biotin-labeled CYP2C9 chromatin probes assembled with histones. A, successively larger fragments of the CYP2C9 proximal promoter and coding region surrounding the translation start site were amplified by PCR and used to assemble chromatin in vitro. The chromatin was digested for verification of assembled nucleosomes, and 300 ng of DNA was separated on an agarose gel. Positive and negative controls for assembly are included, and the start positions upstream of the TSS for each chromatin fragment are indicated. B, Western blots of in vitro chromatin recruitment assays using nuclear extracts and HNF4α + FLAG-MED25 expression (HFM) or HNF4α + silencing MED25 (HsiM) studies. C, Western blots of in vitro recruitment of associated PIC cofactors and histone markers. Input protein levels and recruitment of MED25-associated cofactors are shown. A densitometric analysis of in vitro recruitment bands from the gel image in C is shown on the right. The data are means ± S.E.
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FIGURE 5. PRC2 proteins associate with HNF4α in the absence of MED25 expression. HepG2 cells were transfected with plasmids coding for individual HA-tagged PRC2 proteins along with either HNF4α + FLAG-MED25 (HFM) or HNF4α + siRNAs targeting MED25 (HsiM). Nuclear extracts were incubated with control anti-IgG, -HNF4α, or -HA antibodies (2 μg) at 4 °C overnight, and antibody-protein complexes were collected on protein G-agarose beads. Complexes were separated on a polyacrylamide gel and probed as indicated. The predicted molecular weight (*) and alternate isoforms (**) of the HA-tagged proteins are indicated. WB, Western blot.

predominant in the input and IP, whereas a minor alternate molecular weight band was observed. When MED25 was silenced, the alternate molecular band was dominant. Only a small amount of the input protein with the predicted molecular weight was seen when MED25 was silenced, although it was concentrated by HNF4α in the IP. We performed the experiments with SUZ12-HA and EED-HA three times with qualitatively similar results. For BMI1-HA, when MED25 was overexpressed, we observed a minor band with the predicted molecular weight of BMI1-HA and a major alternative band in the input, but the IP showed predominantly the predicted molecular weight band. After silencing MED25, a similar pattern was observed. MED25 expression similarly affected processing of the proteins in the cases of BMI1-HA, EED-HA and SUZ12-HA. These data, for the first time, represent a snapshot of inhibitory proteins belonging to the Polycomb group interacting with a nuclear receptor, which is important for the maintenance of proper drug metabolism.

Silencing of MED25 Expression in the Presence of CAR and HNF4α Overexpression Results in Association of PRC Proteins with the HNF4α Binding Region—The increased methylation of H3K27 upon CAR and HNF4α overexpression with silencing of MED25 led us to investigate whether the histone methyltransferase EZH2 and its PRC2 partners SUZ12 and EED were recruited to the HNF4α binding region under these conditions. As shown in Fig. 6, A–C, ChIP experiments revealed that EZH2, SUZ12, and EED were most highly enriched in the HNF4α binding region upon CAR and HNF4α overexpression when MED25 was silenced; the other treatment groups expressing either endogenous or ectopic MED25 showed variable PRC enrichment over LacZ controls. However, when MED25 was overexpressed with CAR and HNF4α, recruitment of SUZ12 and BMI1 was significantly increased over recruitment in samples treated with CAR and HNF4α with endogenous MED25, whereas there was a small but not statistically significant increase in EZH2 and EED recruitment. A region of the GAPDH promoter that does not contain HNF4α response elements was included to contrast the enrichment of the CYP2C9 HNF4α binding region by PRC proteins in the absence of MED25 expression. The PRC2 complex specifically catalyzes the H3K27me3 modification and thus represents a putative mechanism for epigenetic inactivation of CYP2C9 expression. The presence of a complete PRC2 is required for an active enzymatic complex, as loss of any of the individual components results in complex dissociation (47). Additionally, the binding of PRC proteins to this site could not be explained by the proposed mechanism of recruitment by CpG islands (41), as there were none noted in the CYP2C9 promoter.

Human PRC1 complex components BMI1 and RING1B were also detected at the HNF4α binding region in a pattern similar to the PRC2 proteins but were not detected at the GAPDH promoter (Fig. 6, D and E). The canonical PRC1 complex containing the ubiquitin E3 ligase RING1B is known to be responsible for chromatin compaction at target sites (48). The prevalent model of PRC1 targeting to promoters is thought to involve the recognition of preexisting H3K27me3, and yet there is evidence that DNA-binding factors such as REST and E2F6 may target certain PRC1 complexes to genomic loci (47). We have included HNF4α ChIP data as a control (Fig. 6F). The recruitment of HNF4α to its site was greatest in the presence of CAR, HNF4α, and MED25. This may reflect changes in the chromatin in this area after various treatments.

MED25 Induces Functional Transcription from in Vitro Chromatin—Chromatin generated in vitro contained the CYP2C9 HNF4α binding region plus ~150 bp downstream of the transcription start site and had a poly nucleosome conformation based on digestion analysis. To validate the functional output of MED25 binding to the chromatin, we performed in vitro transcription assays. The chromatin template was incubated with in vitro translated (TNT®, Promega) HNF4α with or without TNT MED25. Core transcriptional machinery was provided by HeLa nuclear extracts. The results demonstrated that transcription was initiated by HNF4α alone, which was further enhanced by the addition of MED25 (Fig. 7). We further tested this cell-free system for the ability of PRC proteins to regulate transcription by blocking or repressing the activity of MED25, as our co-immunoprecipitation experiments showed that individual PRC members showed an association with HNF4α. In the first case, PRC-HA proteins were incubated after formation of the MED25-containing PIC complex on the chromatin to demonstrate repression of transcription. In the second case, HA-tagged PRCs were incubated with the chromatin prior to HNF4α or MED25 to demonstrate the ability to block transcription.

We performed in vitro transcription assays to determine whether PRCs could dissociate the coactivators and repress transcription from chromatin containing the CYP2C9 HNF4α binding region. The results indeed confirmed that individual PRC members functionally repressed the HNF4α- and MED25-
mediated activation of transcription (Fig. 7A). In this experiment, EZH2 repressed the MED25 effect as well as some of the HNF4α/H9251-mediated activation, whereas EED did not inhibit the MED25 effect as strongly as activation by HNF4α/H9251 alone. There remained a basal level of transcription from the template that was presumably attributable to the activity of HNF4α. The addition of EED, SUZ12, and EZH2 together resulted in an almost complete repression of transcription from the template.

The blocking of transcriptional activation by PRC proteins recapitulates its role in the maintenance of gene silencing and chromatin compaction. Our results revealed that preincubation of the CYP2C9 chromatin fragment with PRC2 proteins blocked activation of transcription (Fig. 7B). Minor differences between incubation with HNF4α and the addition of MED25 were observed, with SUZ12 showing a stronger inhibition of the MED25 effect. These data suggest that each PRC member contributes to the blocking of transcription with different functional activities. Similar to the repression experiment, none of the individual PRC members completely abrogated transcription from the chromatin, as a basal level was observed consistently. However, preincubation with the three PRC2 proteins resulted in a more complete inhibition than observed for individual PRC members. Our data provide evidence that transcription from the CYP2C9 HNF4α binding region is functionally disrupted or inactivated by PRC1 and PRC2 in vitro.

**DISCUSSION**

The Mediator complex is recruited to gene promoters by nuclear receptors and serves as a scaffold for the cofactors responsible for remodeling the local chromatin architecture. In the case of many, but not all, HNF4α inducible genes, MED25 recruits the Mediator complex, pol II (11), and as the present study shows, enzymes involved in modifying the chromatin structure. An enrichment of the CYP2C9 HNF4α binding region by MED1 and MED14 suggests that a multimeric Mediator complex is recruited in coordination with MED25, with MED25 serving as one of the Mediator docking sites for specific transcriptional activation domains of transcription factors (49, 50). This recruitment constitutes the initial step of MED25-mediated activation of HNF4α target genes prior to full PIC formation. The role of Mediator as a component of the PIC has been substantiated in yeast and humans, and the Mediator complex associates with chromatin-modifying factors (51).
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![Diagram](image)

**FIGURE 7. In vitro transcription is inhibited by PRC2 proteins.** In vitro transcription was performed in two ways. **A**, in vitro chromatin was preincubated with in vitro transcribed (Tnt) HNF4α with or without Tnt MED25 for 20 min at 27 °C followed by lysates expressing individual PRC proteins for 15 min at 30 °C (inhibition assay). **B**, in vitro chromatin was preincubated with lysates followed by Tnt HNF4α with or without Tnt MED25 (blocking assay). HeLa nuclear extracts were added, and in vitro transcription assays produced 32P-UTP-labeled RNA, which was then separated on a 5% polyacrylamide Tris borate-EDTA-urea gel; the bands were visualized by autoradiography.

The functional output from the dynamic recruitment of cofactors includes modification of histone N-terminal tail amino acids. Indeed, MED25 exhibited a role in altering H3K27 status at the HNF4α binding region. The H3K27ac marker, initiated by the conserved CREBBP/p300, prevents H3K27 trimethylation of PRC target genes in Drosophila, but although PRC2-related H3K27me3 in chromatin does not prevent CREBBP binding it can block CREBBP HAT activity (52, 53).

We show that H3K27 is increasingly methylated upon CAR and HNF4α overexpression with silencing of MED25, suggesting that at certain HNF4α-responsive promoters, H3K27me3-rich heterochromatin-like conformation occurs and HAT activity is lost, presumably in concurrence with the loss of CREBBP binding, as seen with ChIP and in vitro recruitment. MED25 may represent the regulatory switch for certain HNF4α target genes between CREBBP-mediated H3K27ac activation and antagonizing PRC2-mediated H3K27me3. We present a schematic model for the activation of CYP2C9 by MED25 and its associated epigenetic modifiers, whereas epigenetic inactivation involves the association of PRCs at the HNF4α binding region and a compaction of the surrounding chromatin (Fig. 8).

MED25 and its associated Mediator complex not only impact histone modifications but also have a direct effect on chromatin conformation. The induction of CYP2C9 mRNA levels is mirrored in chromatin accessibility at the HNF4α binding region, but CAR and HNF4α overexpression combined with silencing MED25 expression results in chromatin condensation and a reduction in CYP2C9 mRNA expression. A similar FAIRE enrichment has been observed in pancreatic islet cell promoters bound by pol II and HNF4α (54), and overexpression of Med1 in mouse hepatocytes results in a rapid cellular proliferation response, linking Mediator subunit activity to a concerted physiological response (55). Interestingly, a similar use of DNase hypersensitivity in mouse liver reveals enrichment in hypersensitive sites of H3K27ac markers that positively correlate with sex-biased binding of transcription factors but correlate inversely with H3K27me3 (56). These data are consistent with the hypothesis that for CYP2C9 activation, cross-talk between CAR and HNF4α involves local changes in chromatin architecture in which the promoter adopts a looped conformation (37) that may, in part, rely on the differential histone modifications described previously. Future FAIRE-seq analyses of genome-wide FAIRE data will reveal regions of MED25 regulation in genes that are important for diverse cellular processes (57).

In addition, we hypothesized that the mechanism of CYP2C9 inactivation involves an inverse correlation between MED25 and PRC2 function at the HNF4α binding region. Therefore, MED25 acts as a vital regulator for the derepression of epigenetically silenced CYP2C9. However, whereas HNF4α remains bound to the DNA, the interaction of the PIC with the binding region is reduced when MED25 is absent, thus allowing a reversion of the chromatin to an intrinsic and diminished state of expression that is maintained by PRCs. It is thought that PRCs may act at multiple steps during the transcriptional cycle (47), but further testing must occur to fully delineate the contribution of PRCs at regulatory regions that are under the control of MED25.

In support of increased recruitment of PRC members to the promoter when MED25 is silenced, some HA-tagged PRC proteins revealed a stronger association with HNF4α during co-immunoprecipitation experiments than in the presence of MED25 overexpression. The differences in PRC2 association with HNF4α observed here may only partially reflect the dynamics at the HNF4α binding region of CYP2C9 when HNF4α is bound to the chromatin as a homodimer. This is because the HNF4α ligand binding domain has been shown to be asymmetrically positioned upon binding to DNA, which reorients the protein for further interaction with transcriptional regulators (24, 56). Additionally, HNF4α displays multiple binding modes with cofactors that contain one or more LXXLL motifs such as PGC-1α (29) and MED25. However, PRC proteins do not contain a LXXLL motif that would interact with the AF2 domain of HNF4α and therefore are posited to utilize a binding mode separate from coactivators. For instance, the interaction of EED-HA with HNF4α may include allosteric regulation after DNA binding or an indirect rather than a direct interaction. PRC2 proteins have not been reported previously to associate with HNF4α, and our data are the first to identify a
putative mechanism of PRC2 recruitment to HNF4α target genes. Consistent with this finding, Arnold et al. (58) recently identified dynamic changes in chromatin modification by Polycomb recruitment to NR2F1/HNF4α consensus binding sites in murine embryonic stem cells. We present a novel paradigm of PRC2 association with a nuclear receptor that may help identify genes that are epigenetically silenced, but the mechanism of interaction between HNF4α and PRC proteins requires further clarification.

A previous study shows assembly of a functional PIC to be blocked by incubation of PRC1 with chromatin templates in vitro (59); The addition of PRC1 both before and after PIC formation was able to inhibit recruitment to a GAL4-responsive promoter. In the context of our ChIP data indicating an inverse correlation of MED25 with PRC binding to the HNF4α binding region, we confirmed the chromatin dynamics in vitro. The presence of MED25 expression supports the recruitment of PIC components to this site, including pol II phosphorylated at serine 5. In yeast, phosphorylated pol II is stronger at the 5′-end than the 3′-end for well expressed genes (60) and, with the H3K27ac marker, positively correlates with the gene activation in our studies. Transition from transcription initiation to elongation is also a hallmark associated with pol II Ser-5 phosphorylation (39). The underlying mechanism of pol II Ser-5 phosphorylation may involve the variable Mediator subunit CDK8, which has been shown to phosphorylate pol II at this residue and activate the p53 transcriptional network in HCT116 colon cancer cells (61). The order of recruitment of cofactors to the HNF4α binding region is unclear, but reduced recruitment of CREBBP associated acetyltransferase upon silencing of MED25 expression correlates with a similar level of reduction of H3K27ac in those samples. Importantly, transcription from in vitro chromatin encompassing the HNF4α binding region of the CYP2C9 promoter was achieved, but RNA production was reduced without the inclusion of MED25 protein. The results of our in vitro chromatin recruitment assays suggest a role for MED25 in PIC formation and epigenetic modification at the HNF4α binding region of CYP2C9.

In our initial observations, the change in H3K27 from methylated to acetylated, coupled to the activation of CYP2C9, could
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be explained to a certain extent by our ChIP analysis, where we clearly show that CREBBP recruitment is abolished in MED25 silencing experiments. This suggests that CREBBP-associated HAT activity might be involved in the H3K27 acetylation and that said HATs require identification and characterization. However, regulation of CYP2C9 cannot exclude PRC2-mediated H3K27 methylation. There is the possibility that the established mechanism of functional PRC2 disruption through phosphorylation of EZH2 (62) could still be achieved by the Mediator-associated CDK8 and cyclin C. EZH2 binds EED at the N-terminal, but phosphorylation of Thr-487 in the SUZ12 binding domain of EZH2 results in PRC2 dissociation. The CDK1 phosphorylation site (S/T)PKX(K/R) that is found in EZH2 can be phosphorylated by CDK8 of the Mediator complex (42). Phosphorylation of EZH2 removes the PRC2 complex from the promoter site, and in our case the absence of MED25 prevents CDK8-dependent phosphorylation of EZH2, thereby allowing PRC2 to remain continuously on the promoter to recognize and propagate the H3K27me3 mark for a repressive chromatin state. Our work raises the prospect that the mechanisms of HAT activation and PRC2 dissociation could be operating individually or in combination, taking place in the transition from activation to inactivation associated with the H3K27 status in the CYP2C9 promoter.

In summary, we have demonstrated that the interaction of MED25 with HNF4α confers a selective recruitment of cofactors as a function of PIC formation at HNF4α responsive promoters, which results in the epigenetic modification of chromatin. Our observations point toward a mechanism of deliberate targeting of HNF4α by MED25 for a subset of HNF4α-inducible genes, which ultimately leads to changes in histone modifications and alteration of the chromatin architecture for these genes. Our study provides novel insights into the underlying epigenetic mechanisms involved with regulation of the drug metabolism gene CYP2C9, including an inverse correlation between PRC2 activity and MED25 expression.

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