Divergent Binding and Transactivation by Two Related Steroid Receptors at the Same Response Element*

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Transcription factor (TF) recruitment to chromatin is central to activation of transcription. TF-chromatin interactions are highly dynamic, which are evaluated by recovery half time (t1/2) in seconds, determined by fluorescence recovery experiments in living cells, and chromatin immunoprecipitation (ChIP) analysis, measured in minutes. These two states are related: the larger the t1/2, the longer the ChIP occupancy resulting in increased transcription. Here we present data showing that this relationship does not always hold. We found that histone deacetylase inhibitors (HDACis) significantly increased t1/2 of green fluorescent protein (GFP) fused androgen receptor (AR) on a tandem array of positive hormone response elements (HREs) in chromatin. This resulted in increased ChIP signal of GFP-AR. Unexpectedly, however, transcription was inhibited. In contrast, the GFP-fused glucocorticoid receptor (GR), acting through the same HREs, displayed a profile consistent with current models. We provide evidence that these differences are mediated, at least in part, by HDACs. Our results provide insight into TF action in living cells and show that very closely related TFs may trigger significantly divergent outcomes at the same REs.

Transcription is modulated by changes in the activity of transcription factors (TFs), which then steer general transcription machinery and the RNA polymerases. At the basis of these changes is the recruitment of TFs to regulatory elements (REs) that can be close to or far away from target genes. It is now well established that all aspects of transcription, from binding of TFs to activity of RNA polymerases, are very dynamic processes (1).

One level of dynamism for TF action is that observed in chromatin immunoprecipitation (ChIP) experiments where association of the TF with chromatin may cycle with a half-life measured in 10 s of minutes. For example, the estrogen receptor binding to its response element at the pS2 promoter is cyclical, which coincides with changes occurring at the chromatin template and cofactor interactions (2). The second level of dynamism has been studied by fluorescence recovery after photobleaching (FRAP) experiments in single cells that contain tandem gene arrays (e.g. (3–5)) or by the use of natural gene arrays found in some cell types (e.g. (6)). These studies enabled the calculation of binding kinetics of the green fluorescent protein (GFP)-labeled androgen receptor (GFP-AR) and glucocorticoid receptor (GFP-GR) to their target sites in living cells with a recovery half-time (t1/2) of ~5 s (4, 7). Dynamic receptor cycling was strongly ligand dependent where agonists rendered the receptor significantly less mobile compared with antagonists, which demonstrated a direct positive link between recovery time on the promoter and transcriptional outcome (7). This is consistent with the general mode of TF action: larger the t1/2, the more stably bound the TF (as observed in ChIP), and the stronger the transcriptional activation (e.g. Refs. 8 and 9; for a review, see Ref. 1).

While studying the dynamic interactions of the GFP-AR with the mouse mammary tumor virus (MMTV) promoter, we found that histone deacetylase inhibitors (HDACis) trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) dramatically increased t1/2 for GFP-AR binding to hormone response elements (HREs). This increase, measured in single living cells, resulted in a significant increase in the ChIP signals observed at the MMTV HREs. Surprisingly, increase in GFP-AR binding to MMTV HREs gave rise to inhibition of transcription instead of transcriptional activation, which challenges current models. This was not due to the tandem repeat nature of the gene target as HREs in some endogenous single copy AR/GR target genes displayed the same phenomenon. Interestingly, and in stark contrast to GFP-AR, the extent of GFP-GR binding to the same HREs positively correlated with the transcriptional outcome. We show that these differences may be due, at least in part, to the differential roles of HDAC1 and HDAC2 in serving as cofactors for the GFP-AR and GFP-GR.
Differential Activity of AR and GR on the Same HREs

Experimental Procedures

Chemicals—Methyltrienolone (R1881) was purchased from Dupont-NEN, hydroxyflutamide (OHF) was purchased from Schering-Plough Research Institute, Kenilworth, NJ, and dexamethasone (DEX) was purchased from Sigma. Ligands were dissolved in 100% ethanol and used at the following working concentrations: R1881 (10 nM), DEX (100 nM), or OHF (1 μM). Trichostatin A (TSA) was purchased from Sigma, dissolved in 100% ethanol and used at a final concentration of 10–100 nM as indicated. Suberoylanilide hydroxamic acid (SAHA) was purchased from Alexis Biochemicals, dissolved in DMSO and used at a final concentration of 5 μM.

Plasmids and Cell Lines—Reporter plasmid MMTV-Luc (10), GFP-AR (7), and GFP-GR (11) expression plasmids were described previously. The expression plasmids for HDAC1 and HDAC2 were kind gifts from Dr. Yi Qiu (University of Florida). The fusion proteins were detected by using a polyclonal anti-GFP antibody (Invitrogen). Images were acquired on an Olympus FluoView 1000 confocal laser scanning microscope, with an incubator maintained at 37 °C, and images were captured with a 60 × 1.4-numerical aperture oil immersion objective and argon laser. Five single prebleach images were acquired followed by a brief bleach pulse of 100 ms using 405-nm laser line at 100% laser power (laser output, 50%) without attenuation. Single optical sections were acquired at 500 msec intervals by using 488-nm laser line with laser power attenuated to 3%. Fluorescence intensities in the regions of interest were analyzed, and FRAP recovery curves were generated using Olympus FV10-ASW 1.7b software and Microsoft Excel as previously described (13). All of the quantitative data for FRAP recovery kinetics represent means ± S.E. from at least 25 cells imaged in three independent experiments. Time for half-maximum recovery (t1/2) was calculated as the time to reach 50% of fluorescence intensity at the end of FRAP experiment (80 s), and represent means ± S.E.

siRNA-mediated Knockdown—The cells were seeded in 6-well culture plates at 1 × 10^5 cells per well or 15-cm dishes at 1 × 10^6 cells per dish—24 h before transfection. Small interfering RNA (siRNA) pool directed against mouse HDAC1 and HDAC2 or a scrambled siRNA control (Santa Cruz Biotechnology) were transfected with Oligofectamine (Invitrogen) according to the manufacturer’s recommendations. Cells were then subjected to different treatments for indicated times and either harvested for RNA extraction or assayed for ChIP as described below.

Chromatin Immunoprecipitation Assay (ChIP)—ChIP experiments were carried out according to standard protocols (Upstate Biotechnology) with a crosslinking step (1% formaldehyde at 37 °C), followed by a quenching step with 125 mM glycine. Chromatin was sheared using the Bioruptor sonicator (Diagenode). Sonicated chromatin was immunoprecipitated with antibodies against GFP (Invitrogen), HDAC1 and HDAC2 (Abcam), acetylated histone H3 and H4 (Millipore), and nonspecific IgG (Vector Laboratories). Antibody-bound chromatin complexes were then captured with protein A-agarose beads, and eluted in SDS buffer. Formaldehyde crosslinking was reversed, followed by DNA purification using Phenol-Chloroform (Sigma). Eluted DNA, as well as input DNA, was quantified by quantitative polymerase chain reaction (qPCR) using primer sets specific for the MMTV-LTR nucleosome boundary region. Primer sequences are available upon request.

Quantitative Polymerase Chain Reaction (qPCR)—RT-PCR was carried out with purified RNA isolated using Trizol (Invitrogen) following the manufacturer’s instructions. cDNA was reverse transcribed using the SuperScript II system (Invitrogen). Ras, Lcn2, Suox, Fkbp5, HDAC1, HDAC2, and 36B4 mRNA expression levels were determined using the LightCycler 480 instrument (Roche) using the LightCycler 480 SYBR Green I Master mix (Roche). Standard curves were created by serial dilutions of cDNA to calculate the relative amount of the mRNAs for each sample. These values were then normalized to images processed with ImageJ (National Institutes of Health).

Fluorescence Recovery after Photobleaching (FRAP)—Cells were grown in MatTek plates for live cell imaging (Nunc) and treated with ligands for the desired time periods. FRAP analysis was carried out on an Olympus Fluoview 1000 confocal laser scanning microscope, with an incubator maintained at 37 °C, and images were captured with a 60 × 1.4-numerical aperture oil immersion objective and argon laser. Five single prebleach images were acquired followed by a brief bleach pulse of 100 ms using 405-nm laser line at 100% laser power (laser output, 50%) without attenuation. Single optical sections were acquired at 500 msec intervals by using 488-nm laser line with laser power attenuated to 3%. Fluorescence intensities in the regions of interest were analyzed, and FRAP recovery curves were generated using Olympus FV10-ASW 1.7b software and Microsoft Excel as previously described (13). All of the quantitative data for FRAP recovery kinetics represent means ± S.E. from at least 25 cells imaged in three independent experiments. Time for half-maximum recovery (t1/2) was calculated as the time to reach 50% of fluorescence intensity at the end of FRAP experiment (80 s), and represent means ± S.E.

RNA FISH and Immunofluorescence Analysis—The cells were grown on cover slips placed in 6-well culture plates. 18 h before hormone induction, cells were left untreated or treated with either 100 nM TSA or 5 μM SAHA and AR and GR ligands R1881 or DEX, respectively, were added to either pre-treated or untreated cells. Cells were further fixed with 3% paraformaldehyde and processed for immunofluorescence microscopy combined with RNA fluorescence in situ hybridization (FISH) to detect the MMTV-Ras transcript as described previously (12). The fusion proteins were detected by using a polyclonal anti-GFP antibody (Invitrogen) and anti-rabbit Alexa 488 secondary antibody (Invitrogen). Images were acquired on an Olympus FluoView 1000 upright confocal laser scanning microscope with a PlanApo 60X 1.4 NA oil immersion objective (Olympus, Hamburg, Germany). The RNA FISH signals were quantified using Olympus FV10-ASW 1.7b software after subtraction of the background nuclear fluorescence as previously described (12). The integrated total RNA FISH intensity was calculated for each condition and normalized to the levels in untreated cells to obtain relative RNA FISH intensity. Representative
the relative amount of the internal standard 36B4 in the same sample. Primer sequences are available upon request.

**Reporter Gene Assay**—COS-7 cells were grown in 6-well culture plates to ~50% confluence and transiently co-transfected with luciferase reporter (MMTV-Luc), GFP-AR or GFP-GR, HDAC1 or HDAC2, and pcDNA3 expression plasmids, as indicated, to a total of 1 μg of DNA per well using FuGene6 (Roche) according to the manufacturer’s recommendations. After 12–15 h, medium was changed to DMEM supplemented with 0.5% charcoal-stripped serum. One day after transfection, the cells were treated with R1881, or DEX for 24 h. The cells were harvested and lysed in luciferase lysis buffer (25 mM Tris-HCl, pH 7.8, 2 mM DTT, 10% glycerol, and 1% Triton-X-100). 100 μl of luciferin buffer containing 0.2 mM D-Luciferin (Anaspec) and 2 mM ATP, was added to 10 μl cell extracts, and luciferase activity was determined using a multi-plate reader (Wallac Victor2, Perkin Elmer). Protein concentrations were determined using the Bradford protein assay, and luciferase activity was normalized to total protein concentration.

**Results**

**HDAC Inhibitors Repress GFP-AR Transcriptional Activity at Positive HREs**—It was previously demonstrated that HDACis, such as TSA and SAHA, modulate AR transcriptional activity (14–17). In order to investigate the possible effect of HDACis on AR dynamics on a target promoter in living cells, we used a cell line with an integrated tandem repeat of the MMTV promoter stably expressing GFP fused to wild type AR (GFP-AR) (3108 cells) (7). The integrated MMTV array in 3108 cells contains common HREs for steroid receptors, including GR and AR, which enables the visualization of the GFP-tagged receptors when bound to their HRE in living cells and study of their binding kinetics (4, 7). We first checked the efficiency of the HDACis TSA and SAHA. 3108 cells were treated with TSA or SAHA and global histone acetylation was assessed at different time points. As expected, there was a significant increase in histone H3 and H4 acetylation confirming efficient HDAC inhibition by both compounds (data not shown).

We then determined GFP-AR transcriptional activity at the MMTV array in response to HDAC is by RNA FISH (Fig. 1, A and B). R1881 induced a significant increase in the FISH signal at the MMTV array, as expected. Both TSA and SAHA treatment significantly decreased the FISH signals showing that transcription by GFP-AR was inhibited in 3108 cells.

**HDAC Inhibition Affects GFP-AR Mobility at HREs in Living Cells**—We next performed FRAP experiments to assess possible changes in GFP-AR mobility in response to HDACis in single cells. Since transcription was inhibited, consistent with previous findings reviewed above, including ours (7), we predicted that the $t_{1/2}$ will decrease (i.e. weaker interactions with the HREs). 3108 cells were treated with R1881 alone or R1881 + TSA for increased time, and FRAP was used to determine GFP-AR mobility on the MMTV array. Surprisingly, there was a significant delay of GFP-AR recovery at the MMTV array already after 45 min of TSA pre-treatment compared with R1881 alone indicating a significant increase in $t_{1/2}$ by 18 h. This effect was time-dependent with longer TSA-treatments resulting in slower GFP-AR recovery leading to an ~6-fold increase in $t_{1/2}$ by 18 h. This effect was also dose-dependent (data not shown). Similar results were obtained when SAHA...
was used (Fig. 2B). These results show that HDACis increase the GFP-AR $t_{1/2}$ at HREs in living cells.

We have previously shown that antagonist-bound AR was $\sim$10-fold more mobile (decreased $t_{1/2}$) at the MMTV array compared with an agonist-bound AR and that this correlated with decreased transcriptional activation (7). We therefore examined whether the nature of AR-bound ligand influences the AR dynamics in response to HDACis. When GFP-AR was bound to the antagonist hydroxyflutamide (OHF), TSA did not affect AR mobility (Fig. 2C) indicating that agonist-bound AR is required for HDACi-induced effects on chromatin. Taken together, these data demonstrate that HDAC inhibition increases the residence time of AR on its target promoter in living cells. This effect is time- and dose-dependent and is restricted to agonist-bound, transcriptionally active AR. Since HDACis inhibit AR transcriptional activity at the MMTV promoter (Fig. 1), these data demonstrate that an increase in $t_{1/2}$ of GFP-AR in the FRAP experiments should give rise to an increased stable binding detected by ChIP, which should in turn result in activation of transcription.

Changes in Binding Site Occupancy of GFP-AR and GFP-GR at HREs in Response to HDAC Inhibition—In trying to reconcile the FRAP data with those obtained by ChIP, it is expected that changes either in the frequency or duration of the TF exchange with chromatin would be reflected as changes in ChIP (1). Thus, an increase in $t_{1/2}$ of GFP-AR in the FRAP experiments should give rise to an increased stable binding detected by ChIP, which should in turn result in activation of transcription. To assess if this is the case, we performed ChIP experiments in 3108 cells. As shown in Fig. 4A (right panel), there was a robust increase in the ChIP signal in response to R1881 which was increased further in the presence of TSA or SAHA. These data show that, as expected, under conditions where there is significantly increased $t_{1/2}$ of GFP-AR at the MMTV array, there is a corresponding increase in GFP-AR ChIP signals (compare Fig. 2B). These results show that HDACis increase the GFP-AR $t_{1/2}$ at HREs in living cells.

We then performed FRAP experiments on the MMTV array in 3617 cells. Surprisingly, in contrast to GFP-AR, $t_{1/2}$ of GFP-GR was not significantly affected by TSA or SAHA (Fig. 3C). These data demonstrate that HDACis affect $t_{1/2}$ of GFP-GR and GFP-AR at the MMTV HREs differently, even though the two receptors are closely related structurally, bind exactly the same HREs, and regulate expression of the same gene.

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However, parallel qPCR analysis showed that, similar to the FISH results (Fig. 1), HDAC is significantly inhibited R1881-induced MMTV transcription by GFP-AR (Fig. 4A, left panel). In summary, contrary to current models, the outcome of increased \( t_{1/2} \) of GFP-AR at the MMTV array and corresponding increase in GFP-AR ChIP signals at the HRE is transcriptional repression.

Next, we performed parallel ChIP and qPCR experiments with GFP-GR in 3617 cells. Since there was no significant change in \( t_{1/2} \) for GFP-GR (Fig. 3C), ChIP signal and corresponding transcription was not expected to change in the presence of HDACIs. As shown in Fig. 4B (right panel), there was a strong increase in the ChIP signal upon DEX treatment, which decreased by \(~60–70\%\) in response to TSA or SAHA. These data show that, \( t_{1/2} \) of GFP-GR is not reflected in ChIP signals. Consistent with the FISH data (Fig. 3, A and B), DEX-induced GFP-GR transcription was significantly inhibited by HDACs (Fig. 4B, left panel). Therefore, for the GFP-GR, in contrast to GFP-AR, HDACIs did not affect \( t_{1/2} \) of GFP-GR, but the correlation between transcriptional outcome and ChIP signal was maintained. Taken together, these data show that HDACIs differentially affect GFP-AR and GFP-GR dynamics at the MMTV array, but still result in repression of transcription in both cases.

HDAC Inhibitors Differentially Affect Receptor Activity at HREs of Single Copy Genes—To assess the possible validity of these findings for endogenous, single copy genes, we examined a sample of genes that are directly regulated by both GFP-AR and B with Fig. 4A, right panel). However, parallel qPCR analysis showed that, similar to the FISH results (Fig. 1), HDAC is significantly inhibited R1881-induced MMTV transcription by GFP-AR (Fig. 4A, left panel). In summary, contrary to current models, the outcome of increased \( t_{1/2} \) of GFP-AR at the MMTV array and corresponding increase in GFP-AR ChIP signals at the HRE is transcriptional repression.

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HDAC Inhibitors Differentially Affect Receptor Activity at HREs of Single Copy Genes—To assess the possible validity of these findings for endogenous, single copy genes, we examined a sample of genes that are directly regulated by both GFP-AR
and GFP-GR in parallel gene expression profiling experiments (20). We picked three of these genes for which the HREs were identified in this cell background (20) and determined their response to HDACis. The genes examined were lipocalin 2 (Lcn2), sulfite oxidase (Suox), and FK506-binding protein 5 (Fkbp5). We assessed the effects of HDACis on both GFP-AR- and GFP-GR-mediated transcriptional activity using qPCR, as well as their binding to HREs by ChIP. As shown in Fig. 5 (left panels), Lcn2 and Suox expression were induced in response to HDACis and R1881, while the expression of Fkbp5 was inhibited. Despite this variable outcome for transcription, the ChIP signals of GFP-AR, for all three genes, were significantly increased in response to HDAC inhibition, similar to that at the MMTV HRE. Thus, whereas for Lcn2 and Suox characteristics of GFP-AR activity and chromatin association follow current models (increased binding and increased transcription), Fkbp5 displays a profile that is similar to that observed at the MMTV promoter (increased binding and decreased transcription). These data demonstrate that uncoupling of transcriptional activation and chromatin association is not specific to the MMTV array, but can also occur in single copy endogenous genes.

Similar analysis was performed in 3617 cells with GFP-GR and the same endogenous genes (Fig. 5, right panels). For all genes, there was a significant decrease in expression accompanied by a significant decrease in GFP-GR binding at the corresponding HREs, consistent with the current models. Taken together, these data show that, similar to the MMTV promoter, HDACis can exert diametrically opposite effects on the binding and transcriptional activity of two closely related steroid receptors at HREs of single copy genes.

We then determined whether TSA or SAHA affect RNA Polymerase II (Pol II) recruitment to the MMTV reporter and the endogenous genes. There are several steps at which Pol II can be regulated (for a review, see Refs. 21 and 22). Two of the most important steps in this regard are initiation and elongation, which are achieved by Ser-5 and Ser-2 phosphorylation, respectively, of the C-terminal domain (CTD) of Pol II. Using ChIP, we assessed possible changes in the loading of Ser-5- and Ser-2-phosphorylated Pol II to the MMTV array and endogenous target genes in response to HDACis. For Lcn2 and Suox whose expression increased in response to HDACis, there was an increase in Ser-5- and Ser-2-phosphorylated Pol II loading. Conversely, for MMTV and Fkbp5 that are inhibited by HDACis, there was a decrease in Ser-5- and Ser-2-phosphorylated Pol II loading (data not shown). These data suggest that the differential transcriptional effects of HDACis for the different genes tested are not due to direct effects on RNA-Pol II elongation step, but are related to events prior to the assembly of the transcriptional initiation complex.

**Differential Role of HDACs on Transcription Mediated by GFP-AR and GFP-GR**—Inhibition of transcription and differential effects on GFP-AR compared with GFP-GR at the MMTV HREs could be mediated, at least in part, by differential utilization of cofactors by the two receptors. As the name suggests, the primary targets of HDACs are HDACs. Interestingly, previous work has implicated various HDACs in regulating AR and GR activity (e.g. Refs. 17, 23, and 24). In particular, HDAC1
and HDAC2 have been shown to function as coactivators for GR at the MMTV HREs (25, 26), while HDAC1 was suggested to work as a corepressor for AR at the prostate-specific antigen (PSA) promoter (16).

To evaluate possible ligand-dependent association of HDAC1 and HDAC2 at the MMTV HREs, we used CHIP. In 3108 cells in response to R1881, there was a significant decrease in HDAC2 binding to the MMTV HREs suggesting that it may act as a corepressor for AR (Fig. 6A). In contrast, R1881 did not affect HDAC1 recruitment to MMTV. Similar results were obtained for the single copy genes Lcn2, Suox, and Fkbp5. In contrast, in 3617 cells there was a significant increase in recruitment of HDAC1 to the MMTV array in response to DEX, but not for HDAC2 (Fig. 6B). For the Lcn2, Suox, and Fkbp5 HREs, similar to MMTV, there was a significant increase in HDAC1 loading in response to DEX, whereas HDAC2 loading increased for Fkbp5, slightly increased for Lcn2, and modestly decreased for Suox HREs. These data show that association profiles of HDACs-1 and -2 at the same HREs are different for GFP-AR compared with GFP-GR suggesting that they differentially affect the two receptors.

We next determined the effects of HDAC1 or HDAC2 on GFP-AR or GFP-GR transcriptional activity at the MMTV promoter, using a reporter assay. A MMTV luciferase reporter construct (MMTV-Luc) was transfected into COS-7 cells in the presence of either GFP-AR or GFP-GR, and expression vectors for HDAC1 or HDAC2. Cells were either left untreated or treated with R1881 or DEX for 24 h, harvested and luciferase activity was determined. As shown in Fig. 6C, in the presence of HDAC1 or HDAC2, there was a significant decrease in R1881-induced MMTV-Luc activation. In contrast, DEX-induced MMTV-Luc activity was significantly increased in the presence of HDAC1, but was not affected by HDAC2 (Fig. 6D). These data are consistent with the findings from above (Fig. 6, A and B) and suggest that HDAC1 and HDAC2 differentially serve as cofactors for AR and GR at the MMTV promoter.

To explore this further, we used siRNAs against HDAC1 or HDAC2 in 3108 and 3617 cells and assessed GFP-AR and GFP-GR binding activity and corresponding transcription at the MMTV array. Interestingly, upon efficient knockdown of HDAC1 mRNA, there was an increase in HDAC2 mRNA expression and vice versa, albeit to a varying extent, suggesting that compensatory mechanisms are at play when HDAC expression is manipulated (data not shown). HDAC1 or HDAC2 depletion increased MMTV expression by GFP-AR as well as its HRE binding assessed by ChIP. These data show that both HDAC1 and HDAC2 act as corepressors for AR, at least in part, by inhibiting AR occupancy at the MMTV HREs (data not shown). In contrast to GFP-AR, HDAC1, but not HDAC2 depletion resulted in a significant decrease in MMTV expression by GFP-GR (data not shown). This is consistent with previous findings suggesting that HDAC1 serves as a coactivator for GR (26). Interestingly, GFP-GR binding at the MMTV HREs was significantly increased upon HDAC2 knockdown, but not that of HDAC1, suggesting that HDAC2 facilitates GR binding to MMTV HRE. These data provide another line of evidence for the differential cofactor role of HDACs for GFP-AR and GFP-GR.
Dual Inhibition of HDAC1 and HDAC2 Expression Differentially Affect GFP-AR and GFP-GR Activity and Recapitulates Effects of HDACis—Since we observed compensatory increases in HDAC1 or HDAC2 mRNA levels when the expression of one was inhibited, we used siRNAs to simultaneously target both HDAC1 and HDAC2. Levels of HDAC1 and HDAC2 mRNA expression decreased by more than 80% in both cell lines in response to double siRNA knock-down (data not shown). We then determined the consequence of dual HDAC1 and HDAC2 knockdown on GFP-AR and GFP-GR association with the MMTV HREs and the transcriptional outcome. In contrast to selective knockdown of HDAC1 or HDAC2, dual depletion of the two HDACs significantly decreased both GFP-AR- and GFP-GR-mediated MMTV transcription (Fig. 7). In parallel, dual HDAC knockdown increased GFP-AR, but decreased GFP-GR binding at the MMTV HREs in ChIP experiments. These results recapitulated the effect of HDACis TSA and SAHA on MMTV expression by both GFP-AR and GFP-GR suggesting that the divergent effects of HDACis on steroid receptor activity at the MMTV HREs are mediated through HDACs -1 and -2.

Next, we also explored GFP-AR and GFP-GR binding activity and corresponding target gene expression of selected endogenous single copy genes in response to dual HDAC1/2 knockdown. As shown in Fig. 7 (left panels), dual HDAC1/2 depletion increased expression of all studied genes by GFP-AR. Under these conditions, there was a significant increase in GFP-AR loading to Lcn2 and Fkbp5 HREs, whereas the loading to Suox HRE was decreased. Since dual inhibition of HDACs recapitulated the effect of HDACis only for MMTV and Lcn2, there may be other HDACs, or additional HDACi targets, which are important in regulating GFP-AR activity on Suox and Fkbp5, depending on the promoter context. In contrast to GFP-AR, dual HDAC1/2 knockdown resulted in significant decrease in GFP-GR binding as well as a decrease in GFP-GR-mediated expression of all tested genes (Fig. 7, right panels). Altogether, these results demonstrate that there are distinct differences in the way HDAC1 and HDAC2 affect GFP-AR compared with GFP-GR-mediated transcription.

Since the most widely studied direct targets of HDACs are histones, we evaluated if HDAC1 and HDAC2 regulate GFP-AR and GFP-GR binding or transcriptional activity through changes in acetylation state of histones. We thus determined possible changes in histone H3 and H4 acetylation in the presence and absence of both HDAC1 and HDAC2 by ChIP (Fig. 8). Upon R1881 induction there was a decrease in basal acetylation...
levels of both histones H3 and H4 for MMTV and all genes tested in GFP-AR cells, with the exception of Suox which was slightly increased (Fig. 8, left panels). In addition, dual HDAC1/2 knockdown did not significantly change this decrease in H3 and H4 acetylation, demonstrating that HDAC1 and HDAC2 are not involved in AR-induced histone deacetylation. The picture was quite different in GFP-GR cells. Similar to R1881, DEX stimulation decreased histone H3 and H4 acetylation for all tested genes (Fig. 8, right panels). However, in response to dual HDAC1/2 knockdown, histone acetylation markedly increased in all cases, indicating that HDAC1 and/or HDAC2 are responsible for histone acetylation upon GR activation. These data suggest that, there is no link between histone acetylation, GFP-AR binding and GFP-AR transcriptional activity. Conversely, HDAC1- and HDAC2-mediated histone deacetylation accompanies GFP-GR activation of target promoters.

Discussion

One of the central tenets in regulation of transcription is that the duration and strength of TF association at a RE is directly correlated to the magnitude of the transcriptional response. Thus, ChIP data are routinely used to document physical association of a TF with a target gene and the changes therein are used to assess the transcriptional outcome (e.g. Refs. 1, 2, and 27). Here we have presented data which show that this scheme does not always hold. For HREs of the MMTV promoter, under conditions where GFP-AR binding is significantly increased, measured by ChIP, transcription is inhibited. This suggests that there are promoter specific determinants of TF association and the transcriptional outcome. We have also presented data, which show that the activity and binding kinetics of two closely related hormone receptors at the same HREs can be completely different. These data are summarized in Fig. 9.

FRAP versus ChIP—The starting point of our analysis was to compare the dynamics of GFP-AR and GFP-GR interactions with the same HREs in chromatin in living cells. There has been a significant debate as to how one can reconcile the rapid exchange of TFs with chromatin, studied in single living cells with FRAP, and the longer term interactions which are detected in cell populations using ChIP (for reviews, see Refs. 1 and 28). Two potential explanations have emerged: First, it is possible that the residence time of the TF on its target RE, normally
in the order of seconds, increases during transcriptional activation such that the TF can be more easily ‘captured’ during the crosslinking step in the ChIP protocol. Second, although the residence time of the TF on chromatin does not significantly change, the frequency of interactions with the chromatin template, induced by modifications to the template and/or the TF, may be increased. This would give rise to an increase in the local concentration of bound TFS at any moment, which will then result in an increased ChIP signal.

We have found that HDACis significantly increased the $t_{1/2}$ of GFP-AR at the MMTV array (Fig. 24), which resulted in a significant enhancement of the ChIP signals (Fig. 4A, right panel), thus providing direct evidence for the first model. However, at the same HRE, HDACis did not affect the $t_{1/2}$ of GFP-GR, but nevertheless resulted in a significant decrease in the ChIP signals. These findings suggest that, at least in some cases, the association of a TF with chromatin, measured by ChIP, may not reflect changes in the TF mobility with its RE. Changes to the chromatin template or the transcription initiation complex may affect the frequency of TF-chromatin interactions and thus may be at the basis for this observation. Further work on other HREs in tandem arrays where both FRAP and ChIP analyses can be carried out is required to obtain additional insight into these observations. In addition, there have been recent advances in experimental and modeling possibilities to observe single-molecule TF binding events (29) and single gene transcription units (30) in living cells; these can be applied in the future for better understanding of GFP-AR and GFP-GR transcriptional dynamics.

**GFP-AR versus GFP-GR: Differential Transcriptional Response to HDACis at the Same HRE**—The differential effects of HDACis in $t_{1/2}$ and ChIP signals of GFP-AR and GFP-GR prompted us to assess the corresponding changes in transcription. Contrary to expectations, we found that under conditions where GFP-AR $t_{1/2}$ and binding to the MMTV was increased, there was a significant decrease in transcription. In contrast, the GFP-GR response to HDACis at the MMTV was as expected from the classical view of TF action: there was significant inhibition of GFP-GR-mediated transcription with a concomitant decrease in GFP-GR ChIP signals. When single copy genes that are common targets for both GFP-AR and GFP-GR were studied, similar results were obtained for Fkbp5. For Lcn2 and Suox, whereas the same picture was obtained for GFP-GR, HDACi treatment significantly activated GFP-AR-mediated transcription, concurrently increasing GFP-AR ChIP signals at the corresponding HREs. In addition, the observed effect of HDACis on GFP-AR- and GFP-GR-mediated transcriptional activity at the MMTV and endogenous target genes was accompanied by loading of Pol II phosphorylated at Ser-5 and Ser-2. For example, for Lcn2 and Suox whose expression increased in response to HDACis, there was an increase in Ser-5 and Ser-2 phosphorylated Pol II; conversely, decreased expression of Fkbp5 was accompanied by a decrease in Pol II binding. These data clearly demarcate the normally assumed relationship between binding site occupancy and transcription, and suggest that in certain cases additional mechanisms may be at play. For example, it is possible that a stimulus can increase the affinity of a TF for its RE, but may give rise to a conformational change in the TF such that it no longer fruitfully interacts with its cofactors and the transcriptional initiation complex and thus transcription is inhibited. Another possibility is that under similar conditions changes to the chromatin template render the TF inactive even though it remains bound to its RE. Future studies are required to assess these and other possibilities.

**HDAC1 and/or HDAC2: Conduits for Differential Transcriptional Response to HDACis**—Two of the possible targets of HDACis relevant to our experiments are GFP-AR and coactivators such as HDACs. AR has been shown to be acetylated which can differentially affect its transcriptional properties depending on the promoter (31, 32). However, a mutant AR that cannot be acetylated efficiently activated transcription from the MMTV promoter (31) suggesting that GFP-AR is not the target of HDACis that we observed here. Of the HDACs, we focused on HDAC1 and HDAC2 that have been most widely studied before and were previously found to function as coactivators for GR (25, 33). HDAC1 and HDAC2 are associated with the MMTV promoter in the absence of ligand and determine the activity of the promoter, possibly through regulating histone acetylation and blocking the access to TF binding sites. During AR-mediated transcription, the level of HDAC2 occupancy at the MMTV HRE was decreased while HDAC1 association did not significantly change. The same effect was observed at the HREs of endogenous genes, suggesting that HDAC2 functions as a coactivator for AR. Inhibitory function
of HDAC1 and HDAC2 on GFP-AR binding to MMTV and transcription activation was further confirmed by knockdown and reporter assay studies. In contrast, we found that the association of HDAC1 with the MMTV HRE was increased in response to GR-mediated transcription, consistent with the previously documented role of HDAC1 as a coactivator for GFP-GR (25, 33). The coactivator function of HDAC1 for GR was also confirmed on single copy endogenous genes. We did not observe a coactivator role of HDAC2 for GR that was previously reported in mouse NIH3T3 cells with the same MMTV reporter (25) suggesting that there may be cell type determinants for HDAC2 effects on GFP-GR.

These data suggest that HDACs may have independent roles in regulating both HRE binding and transactivation properties of GFP-AR and GFP-GR. Since HDAC1 and HDAC2 differentially affect GFP-AR compared with GFP-GR and their activity is inhibited by HDACis, HDAC1 and/or HDAC2 knockdown should at least in part recapitulate HDACis effects we observed at the MMTV HREs. This was in fact the case: whereas single HDAC1/2 depletion resulted in mixed results, possibly due to compensatory mechanisms, dual HDAC1/2 knockout qualitatively recapitulated outcome of HDACis for all genes regulated by GFP-GR, as well as for GFP-AR and Lcn2 in the case of GFP-GR (Figs. 4, 5, and 7, or Fig. 9). These results have mechanistic implications. First, these data clearly suggest that HDAC1 and HDAC2 serve as cofactors for GFP-AR and GFP-GR. Second, this indicates that other factors, which differentially interact with HDAC1 and HDAC2 (or their downstream targets) may be involved in regulating GFP-AR and GFP-GR binding kinetics and activity.

One of the well-established functions of HDACs is the removal of acetyl groups from both histone and non-histone proteins that result in positive or negative regulation of transcription (for a review, see Refs. 34 and 35). Thus, we also evaluated the effects of dual HDAC1/2 depletion on histone acetylation by ChIP, to check if the acetylation of histones H3 and H4 can be related to receptor binding and/or transcriptional activity. We found that both histone H3 and H4 acetylation levels were significantly increased by dual HDAC1/2 knockdown upon GFP-GR activation (Figs. 8 and 9). However, increased histone acetylation did not correlate with GR-mediated decrease in transcriptional activity. This could be due to depletion of HDAC1 that serves as a coactivator for GR; alternatively, mechanisms involving other factors may be responsible for these observations. In contrast to findings in GFP-GR cells, there was no significant change in the acetylation status of histones H3 or H4 upon dual HDAC1/2 knockdown in the presence of androgen for all genes studied in GFP-AR cells. This suggests that there is no direct link between histone acetylation, AR binding and AR transcriptional activity on these AR target genes. It is possible, however, that continual turnover of histone acetylation by additional HDAC(s) may be involved in regulating GFP-AR. In addition, HDAC1 and/or HDAC2 may collectively regulate deacetylation of a non-histone protein(s) that is a critical determinant in AR-mediated transcription. For example, recently identified cofactors for AR, such as lysine demethylase, KDM4B, cell cycle and apoptosis regulator 1 (CCAR1), and the hairy/enhancer-of-split-related with YRPW-like motif (HEY) family of proteins that can affect AR function may interact with, or are affected by, HDACs (36–38). Similarly, modifications of AR, such as histone methyltransferase SET9-mediated methylation, that have been shown to affect AR function, may be influenced by HDACs (39). Further work on HDACs, other AR/GR associated proteins, as well as additional aspects of the HRE template in response to HDAC inhibition are required to tease out the full molecular details of these observations.

Author Contributions—M. T., X. D., and H. Z. N. designed and performed the experiments. T. I. K. performed some of the FRAP experiments. F. M. analyzed the FRAP data. G. L. H. and F. S. provided important intellectual input and reagents. F. S. supervised the study. M. T., X. D., H. Z. N., and F. S. wrote the manuscript.

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References
1. Hager, G. L., McNally, J. G., and Misteli, T. (2009) Transcription dynamics. Mol. Cell. 35, 741–753
2. Métivier, R., Penot, G., Hübner, M. R., Reid, G., Brand, H., Kos, M., and Gannon, F. (2003) Estrogen receptor-α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 115, 751–763
3. Bosio, D., Marazzi, I., Agresti, A., Shimizu, N., Bianchi, M. E., and Natoli, G. (2006) A hyper-dynamic equilibrium between promoter-bound and nucleoplasmic dimers controls NF-κB-dependent gene activity. EMBO J. 25, 798–810
4. McNally, J. G., Müller, W. G., Walker, D., Wolford, R., and Hager, G. L. (2000) The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. Science 287, 1262–1265
5. Sharp, Z. D., Mancini, M. G., Hinojos, C. A., Dai, F., Berno, V., Szafran, A. T., Smith, K. P., Lele, T. T., Ingber, D. E., and Mancini, M. A. (2006) Estrogen-receptor-α exchange and chromatin dynamics are ligand- and domain-dependent. J. Cell Science 119, 4101–4116
6. Karpova, T. S., Kim, M. J., Spriet, C., Nalley, K., Stasevich, T. J., Kherrouche, Z., Heliot, L., and McNally, J. G. (2008) Concurrent fast and slow cycling of a transcriptional activator at an endogenous promoter. Science 319, 466–469
7. Klok, T. I., Kurys, P., Elbi, C., Nagaich, A. K., Hendarwanto, A., Slagsvold, T., Chang, C. Y., Hager, G. L., and Saatcioglu, F. (2007) Ligand-specific dynamics of the androgen receptor at its response element in living cells. Mol. Cell. Biol. 27, 1823–1843
8. Maston, G. A., Evans, S. K., and Green, M. R. (2006) Transcriptional regulatory elements in the human genome. Annu. Rev. Genom. Human Genet. 7, 29–59
9. Stavreva, D. A., Müller, W. G., Hager, G. L., Smith, C. L., and McNally, J. G. (2004) Rapid glucocorticoid receptor exchange at a promoter is coupled to transcription and regulated by chaperones and proteasomes. Mol. Cell. Biol. 24, 2682–2697
10. Lefebvre, P., Berard, D. S., Cordingly, M. G., and Hager, G. L. (1991) Two regions of the mouse mammary tumor virus long terminal repeat regulate the activity of its promoter in mammary cell lines. Mol. Cell. Biol. 11, 2529–2537
11. Walker, D., Htun, H., and Hager, G. L. (1999) Using inducible vectors to study intracellular trafficking of GFP-tagged steroid/nuclear receptors in living cells. Methods 19, 386–393
12. Rayasam, G. V., Elbi, C., Walker, D. A., Wolford, R., Fletcher, T. M., Edwards, D. P., and Hager, G. L. (2005) Ligand-specific dynamics of the progesterone receptor in living cells and during chromatin remodeling in vitro. Mol. Cell. Biol. 25, 2406–2418

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13. Elbi, C., Walker, D. A., Romero, G., Sullivan, W. P., Toft, D. O., Hager, G. L., and DeFranco, D. B. (2004) Molecular chaperones function as steroid receptor nuclear mobility factors. Proc. Natl. Acad. Sci. U.S.A. 101, 2876–2881

14. Korkmaz, C. G., Frønsdal, K., Zhang, Y., Lorenzo, P. I., and Saatcioglu, F. (2004) Potentiation of androgen receptor transcriptional activity by inhibition of histone deacetylation—rescue of transcriptionally compromised mutants. J. Endocrinol. 182, 377–389

15. List, H. J., Smith, C. L., Rodriguez, O., Danielsen, M., and Riegel, A. T. (1999) Inhibition of histone deacetylation augments dihydrotestosterone induction of androgen receptor levels: an explanation for trichostatin A effects on androgen-induced chromatin remodeling and transcription of the mouse mammary tumor virus promoter. Exp. Cell Res. 252, 471–478

16. Shang, Y., Myers, M., and Brown, M. (2002) Formation of the androgen receptor transcription complex. Mol. Cell 9, 601–610

17. Websie, D. S., Xu, J., Chen, Y., Borsui, L., Scher, H. I., Rosen, N., and Sawyers, C. L. (2009) Histone deacetylases are required for androgen receptor function in hormone-sensitive and castrate-resistant prostate cancer. Cancer Res. 69, 958–966

18. Nenseth, H. Z., Dezitter, X., Tesikova, M., Mueller, F., Klok, T. I., Hager, G. L., and Saatcioglu, F. (2014) Distinctly different dynamics and kinetics of two steroid receptors at the same response elements in living cells. PloS one 9, e105204

19. Bresnick, E. H., John, S., Berard, D. S., LeFebvre, P., and Hager, G. L. (1990) Glucocorticoid receptor-dependent disruption of a specific nucleosome on the mouse mammary tumor virus promoter is prevented by sodium butyrate. Proc. Natl. Acad. Sci. U.S.A. 87, 3977–3981

20. John, S., Sabo, P. J., Johnson, T. A., Sung, M. H., Biddie, S. C., Lightman, S. L., Voss, T. C., Davis, S. R., Meltzer, P. S., Stamatoyannopoulos, J. A., and Hager, G. L. (2008) Interaction of the glucocorticoid receptor with the chromatin landscape. Mol. Cell 29, 611–624

21. Brookes, E., and Pombo, A. (2009) Modifications of RNA polymerase II are pivotal in regulating gene expression states. EMBO Reports 10, 1213–1219

22. Fuda, N. J., Ardehali, M. B., and Lis, J. T. (2009) Defining mechanisms that pivotal in regulating gene expression states. EMBO Reports 10, 1213–1219

23. Fu, M., Rao, M., Wang, C., Sakamaki, T., Wang, J., Di Vizio, D., Zhang, X., Albanese, C., Balk, S., Chang, C., Fan, S., Rosen, E., Palumbo, J. J., Jänecke, O. A., Muratoglu, S., Avantaggiati, M. L., and Pestell, R. G. (2003) Acetylation of androgen receptor enhances coactivator binding and promotes prostate cancer cell growth. Mol. Cell. Biol. 23, 8563–8575

24. Yang, M., Liu, M., Sauve, A. A., Jiao, X., Zhang, X., Wu, X., Powell, M. J., Yang, T., Gu, W., Avantaggiati, M. L., Pattahiranman, N., Pestell, T. G., Wang, F., Quong, A. A., Wang, C., and Pestell, R. G. (2006) Hormonal control of androgen receptor function through SIRT1. Mol. Cell. Biol. 26, 8122–8135

25. Yang, Y., Tse, A. K., Li, P., Ma, Q., Xiang, S., Nicosia, S. V., Seto, E., Zhang, X., and Bai, W. (2011) Inhibition of androgen receptor activity by histone deacetylase 4 through receptor SUMOylation. Oncogene 30, 2207–2218

26. Luo, Y., Jian, W., Stavreva, D., Fu, X., Hager, G., Bungert, J., Huang, S., and Qiu, Y. (2009) Trans-regulation of histone deacetylase activities through acetylation. J. Biol. Chem. 284, 34901–34910

27. Bourdeau, V., Deschênes, J., Méthivier, R., Nagai, Y., Nguyen, D., Bretschneider, N., Gannon, F., White, J. H., and Mader, S. (2004) Genome-wide identification of high-affinity estrogen response elements in human and mouse. Mol. Endocrinol. 18, 1411–1427

28. Méthivier, R., Reid, G., and Gannon, F. (2006) Transcription in four dimensions: nuclear receptor—directed initiation of gene expression. EMBO Rep. 7, 161–167

29. Elf, J., Li, G. W., and Xie, X. S. (2007) Probing transcription factor dynamics at the single-molecule level in a living cell. Science 316, 1191–1194

30. Larson, D. R., Zenklusen, D., Wu, B., Chao, J. A., and Singer, R. H. (2011) Real-time observation of transcription initiation and elongation on an endogenous yeast gene. Science 332, 475–478

31. Faus, H., and Haendler, B. (2008) Androgen receptor acetylation sites differentially regulate gene control. J. Cell. Biochem. 104, 511–524

32. Fu, M., Rao, M., Wang, C., Sakamaki, T., Wang, J., Di Vizio, D., Zhang, X., Albanese, C., Balk, S., Chang, C., Fan, S., Rosen, E., Palumbo, J. J., Jänecke, O. A., Muratoglu, S., Avantaggiati, M. L., and Pestell, R. G. (2003) Acetylation of androgen receptor enhances coactivator binding and promotes prostate cancer cell growth. Mol. Cell. Biol. 23, 8563–8575

33. Qiu, Y., Zhao, Y., Becker, M., John, S., Parekh, B. S., Huang, S., Hendarwanto, A., Martinez, E. D., Chen, Y., Lu, H., Adkins, N. L., Stavreva, D. A., Wiench, M., George, L. P., Schultz, R. L., and Hager, G. L. (2006) HDAC1 acetylation is linked to progressive modulation of steroid receptor-induced gene transcription. Mol. Cell 22, 669–679

34. Delucave, G. P., Khan, D. H., and Davie, J. R. (2012) Roles of histone deacetylases in epigenetic regulation: emerging paradigms from studies with inhibitors. Clin. Epigenetics 4, 5

35. Smith, C. L. (2008) A shifting paradigm: histone deacetylases and transcriptional activation. Bioessays 30, 15–24

36. Coffey, K., Rogerson, L., Ryan-Munden, C., Alkharaif, D., Stockley, J., Heer, R., Sahadevan, K., O’Neill, D., Jones, D., Darby, S., Staller, P., Mantilla, A., Gaughan, L., and Robson, C. N. (2013) The lysine demethylase, KDM4B, is a key molecule in androgen receptor signalling and turnover. Nucleic Acids Res. 41, 4433–4446

37. Lavery, D. N., Villaronga, M. A., Walker, M. M., Patel, A., Belandia, B., and Bevan, C. L. (2011) Repression of androgen receptor activity by HeyL, a third member of the Hairy/Enhancer-of-split-related family of Notch effectors. J. Biol. Chem. 286, 17796–17808

38. Seo, W. Y., Jeong, B. C., Kim, J. H., Kim, S. H., Lee, H. M., and Kim, J. H. (2013) CCAR1 promotes chromatin loading of androgen receptor (AR) transcription complex by stabilizing the association between AR and GATA2. Nucleic Acids Res. 41, 8526–8536

39. Gaughan, L., Stockley, J., Wang, N., McCracken, S. R., Treumann, A., Armstrong, K., Shaheen, F., Watt, K., McEwan, I. J., Wang, C., Pestell, R. G., and Robson, C. N. (2011) Regulation of the androgen receptor by SET9-mediated methylation. Nucleic Acids Res. 39, 1266–1279