

# Histone H2A.Z is essential for estrogen receptor signaling

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**Incorporation of H2A.Z into the chromatin of inactive promoters has been shown to poise genes for their expression. Here we provide strong evidence that H2A.Z is incorporated into the promoter regions of estrogen receptor (ER $\alpha$ ) target genes only upon gene induction, and that, in a cyclic pattern. Moreover, members of the human H2A.Z-depositing complex, p400, also follow the same gene recruitment kinetics as H2A.Z. Importantly, cellular depletion of H2A.Z or p400 leads to a severe defect in estrogen signaling, including loss of estrogen-specific cell proliferation. We find that incorporation of H2A.Z within *TFF1* promoter chromatin allows nucleosomes to adopt preferential positions along the DNA translational axis. Finally, we provide evidence that H2A.Z is essential to allow estrogen-responsive enhancer function. Taken together, our results provide strong mechanistic insight into how H2A.Z regulates ER $\alpha$ -mediated gene expression and provide a novel link between H2A.Z–p400 and ER $\alpha$ -dependent gene regulation and enhancer function.**

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The integration of physiological and environmental cues into complex transcriptional responses is orchestrated by the coordination of numerous regulatory events and mechanisms that control both the repression and activation of genes. Such signals can be conveyed, for example, by nuclear steroid hormone receptors, such as the estrogen receptors (ERs). Estrogens are a class of steroid hormones that play central roles in female development and reproductive functions (Couse and Korach 1999; Nilsson et al. 2001). In addition, estrogens are directly involved in hormone-dependant diseases, such as breast cancers (Deroo and Korach 2006). The biological functions of estrogens are mediated through ER $\alpha$  and ER $\beta$ , which operate as ligand-dependent transcription factors that work in conjunction with coregulator proteins to regulate gene expression and activate transcription of target genes by two distinct, but not mutually exclusive, transcription activation functions (Green and Carroll 2007). More specifically, there is an N-terminal ligand-independent activation function (AF-1) and a C-terminal ligand-dependant activation function (AF-2) located within the ligand-binding domain. AF-1 and AF-2 function in a synergistic manner and are required for full transcriptional

activation by ER in most cell types (Tora et al. 1989; Berry et al. 1990; Pham et al. 1992; Tzukerman et al. 1994; Benecke et al. 2000). Little is known about the activation mechanism of AF-1, but several transcriptional coactivators for AF-2 have been found over the past few years. For example, ligand-bound AF-2 has been shown to interact with the LXXLL motif of proteins belonging to the important p160 family of coactivators (Onate et al. 1995; Anzick et al. 1997; Brzozowski et al. 1997; Hong et al. 1997). Furthermore, this domain has been linked to the recruitment of the general transcriptional machinery and ATP chromatin remodeling complexes such as Swi/Snf (Ichinose et al. 1997; DiRenzo et al. 2000).

Chromatin immunoprecipitation (ChIP) assays have revealed that ER $\alpha$  can direct ordered and cyclical recruitment of cofactors, such as histones modifying enzymes (CBP/p300, Tip60, CARM-1), the Swi/Snf ATP-dependant chromatin remodeling complex, and general transcription factors (GTF; RNA polymerase II [RNAPII], TBP, and TFIIB), to the *TFF1* locus (Shang et al. 2000; Metivier et al. 2003). Receptor cycling induces subtle changes in nucleosome positioning, which in turn plays a key role in the repression and activation of the *TFF1* gene (Metivier et al. 2003). These changes are characterized by an undefined translational nucleosomal position on the inactive gene to a preferred (stable) nucleosomal position on the active *TFF1* proximal promoter. Moreover, transcriptional

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regulation of *TFF1* and an important majority of ER $\alpha$ -regulated genes have been found dependent on the binding of the transcription factor FoxA1 (Carroll et al. 2005). Of note, this factor is thought to contribute to the recruitment of ER $\alpha$  and gene regulation due to its ability to remodel chromatin. However, it is unclear whether FoxA1 possesses intrinsic remodeling activity or recruits a protein with chromatin-modifying properties. FoxA1 also retains cell type-specific functions, which rely primarily on its differential recruitment to chromatin, predominantly at distant enhancers rather than proximal promoters (Lupien et al. 2008).

Due to the high level of DNA compaction established within chromatin, it is generally assumed that this condensed state is an obstacle to all metabolic transactions involving DNA, including ligand-dependent transcriptional regulation by the ER (Mellor 2005). Given that chromatin often has repressive effects on transcription, the ability of nucleosomes to be disrupted or displaced represents a critical step in gene regulation. One mechanism that generates a specialized chromatin environment is the incorporation of histone variants into specific nucleosomes. H2A.Z is one such histone variant, and it has been implicated principally in the regulation of gene expression. Much of what we know regarding the function of H2A.Z stems from studies performed in the yeast *Saccharomyces cerevisiae*, where it was shown to regulate genes both positively (Santisteban et al. 2000; Adam et al. 2001; Laroche and Gaudreau 2003) and negatively (Dhillon and Kamakaka 2000). In addition, genome-wide localization studies have shown that H2A.Z is preferentially localized within a few nucleosomes of the initiator region of a large proportion of inactive yeast genes (Guillemette et al. 2005; Li et al. 2005; Raisner et al. 2005; Zhang et al. 2005). In mammalian cells, H2A.Z loci have been found predominantly at sites occupied by RNAPII along with enhancer regions (Barski et al. 2007). Furthermore, our laboratory has shown that yeast H2A.Z is essential for the appropriate positioning of a nucleosome spanning the initiator region of the *GAL1* gene (Guillemette et al. 2005). Moreover, we observed that promoters that are enriched in H2A.Z have defined nucleosome locations compared with promoters that are not significantly enriched in H2A.Z, thereby arguing that H2A.Z may regulate gene expression by allowing nucleosomes to adopt preferred positions within promoter regions.

One seemingly unique feature of H2A.Z is that it can be incorporated within chromatin by an ATP-dependent chromatin remodeling mechanism, which exchanges H2A–H2B dimers for H2A.Z–H2B. In yeast, H2A.Z has been shown to be incorporated by the Swr1 complex, which shares essential subunits with the NuA4 histone acetyltransferase complex (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004). In mammals, there are two orthologs of Swr1 that have the ability to exchange H2A–H2B for H2A.Z–H2B within chromatin. SRCAP is the first complex to show an ability to catalyze the incorporation of H2A.Z into nucleosomes in vitro (Ruhl et al. 2006), and a recent report has demonstrated that SRCAP can be recruited to inactive and active promoters (Wong

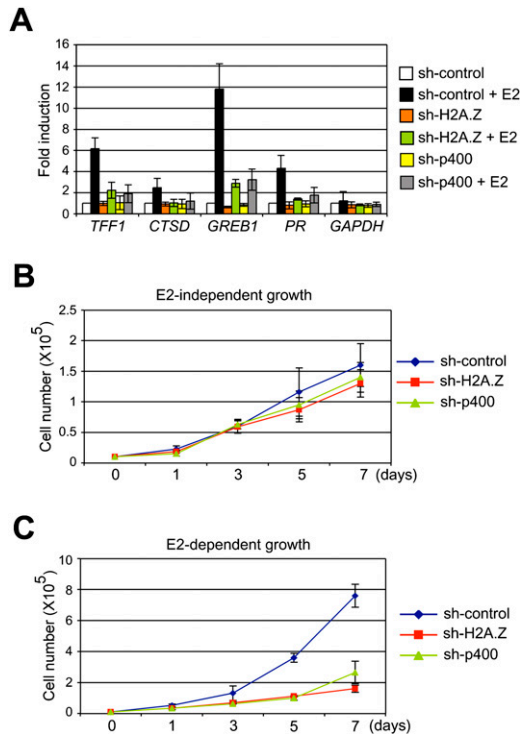
et al. 2007). Moreover, depletion of SRCAP in vivo affects the loading of H2A.Z within chromatin (Wong et al. 2007). In spite of being isolated as a CREB-binding protein partner (Johnston et al. 1999), little is known about how SRCAP is recruited to specific promoters. The second complex that has been shown to be able to incorporate H2A.Z into chromatin is p400 (Gevry et al. 2007). Our laboratory showed that H2A.Z, via p400, suppresses the activation of the *p21* gene by p53 and senescence responses. Furthermore, the presence of sequence-specific transcription factors, such as p53 and Myc, dictates the positioning of H2A.Z-containing nucleosomes within these promoters, thus suggesting that DNA-binding regulatory proteins may participate in targeting H2A.Z within specific chromatin loci (Gevry et al. 2007).

Here we show that both H2A.Z and p400 are essential regulators of ER $\alpha$ -dependent gene activation and cell proliferation. We also demonstrate that p400–H2A.Z is actively recruited to ER $\alpha$  target genes in a cyclic fashion with a period of  $\sim$ 60 min. We further show that incorporation of H2A.Z within the promoter region of *TFF1* allows nucleosomes to adopt preferential positions, a condition that is permissive to the recruitment of the general transcriptional machinery. Interestingly, ChIP–chip assays performed on human chromosome 17 illustrate that H2A.Z is also actively recruited to the proximal promoter of several genes upon treatment of cells with estradiol, thereby expanding the generality of our findings at *TFF1*. Finally, H2A.Z associates to ER $\alpha$ -responsive enhancers and is required for the association of the pioneer factor FoxA1. Collectively, our data provide strong evidence for a role of H2A.Z in ER $\alpha$  function.

## Results

### *H2A.Z and p400 are required for estrogen-mediated gene induction and cell proliferation*

In an effort to determine whether H2A.Z and p400 can regulate ER $\alpha$  target gene expression upon estradiol signaling, we used shRNA constructs that selectively deplete cellular levels of H2A.Z or p400 (Supplemental Fig. S1; Rangasamy et al. 2004; Chan et al. 2005; Gevry et al. 2007) and assayed whether the knockdown of H2A.Z or p400 affected ER $\alpha$ -mediated gene expression upon estradiol treatment of MCF7 cells. As shown in Figure 1A, cellular depletion of H2A.Z or p400 was found to significantly reduce the estradiol-dependent induction of *TFF1*, *CTSD*, *GREB1*, and *PR* to a similar extent, but not to affect the *GAPDH* housekeeping gene. Given that induction of ER $\alpha$  target genes was severely affected by H2A.Z or p400 knockdown, we next wanted to test whether this condition would affect estrogen-dependent cell proliferation. Knockdown of either H2A.Z or p400 had no significant effect on the proliferation of MCF7 cells grown in the absence of estrogen (Fig. 1B). However, cellular depletion of both H2A.Z and p400 dramatically reduced estrogen-dependent proliferation of MCF7 cells to levels comparable with those observed in the absence of any estrogen (Fig. 1C). Taken together, these results



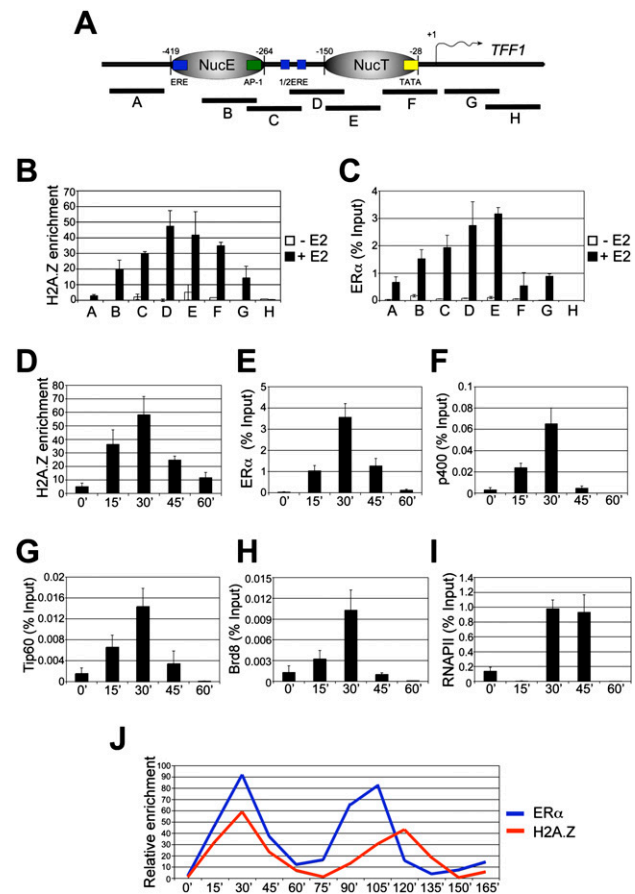
**Figure 1.** H2A.Z regulates estradiol-dependent gene transcription and cell growth. (A) Expression levels of *TFF1*, *CTSD*, *GREB1*, *PR*, and *GAPDH* in MCF7 cells depleted or not of H2A.Z and p400 using specific shRNA constructs, and in the absence (–E2) or presence (+E2) of estradiol. mRNA expression levels were determined by qPCR and normalized against expression levels of the *36B4* ribosomal gene. (B,C) Cell proliferation assays were performed using MCF7 cells depleted for p400 and H2A.Z. Ligand-independent (B) and ligand-dependent (C) proliferation were both monitored at different time points as indicated.

show that both H2A.Z and p400 are required for estrogen-dependent cell proliferation by acting as positive factors that modulate ER $\alpha$ -dependent transcription.

#### *H2A.Z and members of the p400 complex are transiently recruited to an ER $\alpha$ -dependent promoter upon estrogen signaling*

Since H2A.Z has been shown to be associated with several promoters in both yeast (Guillemette et al. 2005; Li et al. 2005; Raisner et al. 2005; Zhang et al. 2005) and human cells (Farris et al. 2005; Barski et al. 2007; Gevry et al. 2007; Jin and Felsenfeld 2007; Wong et al. 2007), and as H2A.Z is an essential positive regulator of a subset of ER $\alpha$  target genes, we wanted to verify whether the histone variant could also associate with ER $\alpha$  target genes. To address this issue, we performed ChIP experiments at the *TFF1* gene using antibodies directed against H2A.Z (Gevry et al. 2007). Figure 2A depicts the *TFF1* promoter regions surveyed by ChIP. Figure 2B first shows that in the absence of estradiol, H2A.Z is not significantly associated with the *TFF1* promoter. On the other hand, upon addition of estradiol, robust recruitment of H2A.Z is

observed at the promoter region, also occupied by ER $\alpha$  during the activation process (see Fig. 2C). Note that ER $\alpha$  association with the *TFF1* promoter covers a region of  $\sim$ 400 base pairs (bp), which is expected given the presence of ER predictive binding elements (either a full ERE or two half ERE sites) as well as an AP-1 predictive site that is known to recruit ER $\alpha$  (Barkhem et al. 2002). Given that ER $\alpha$  appears to orchestrate recruitment cycles of the transcriptional machinery, as well as chromatin remodeling activities, at certain responsive promoters including the *TFF1* gene, we wanted to determine whether H2A.Z could also associate with the *TFF1* promoter in a cyclic fashion. To test this, we carried out ChIP experiments at the *TFF1* promoter region using amplicons corresponding to regions C and D of Figure 2A. As shown in Figure 2D,

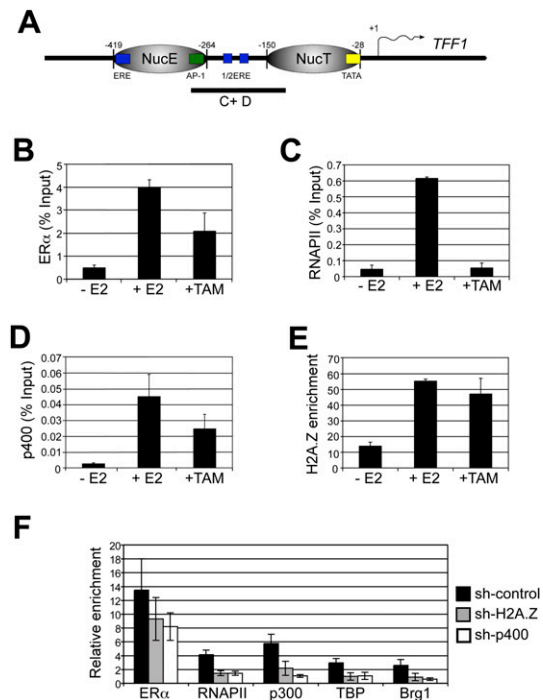


**Figure 2.** H2A.Z colocalizes with ER $\alpha$  at the *TFF1* promoter. (A) Diagram of the *TFF1* promoter region and its regulatory elements (ERE, AP1, and TATA box), phased nucleosomes, and segments (columns A–H) used for qPCR. (B,C) ChIP analysis of H2A.Z enrichment (percent input of H2A.Z/H3 to account for nucleosome density) and ER $\alpha$  at the *TFF1* promoter in MCF7 upon estradiol (E2) treatment. (D–I) ChIP assay showing the kinetics of H2A.Z (D), ER $\alpha$  (E), p400 (F), Tip60 (G), Brd8 (H), and RNAPII (I) occupancy during *TFF1* promoter activation after E2 treatment in MCF7 cells. (J) Kinetics of recruitment of H2A.Z and ER $\alpha$  at the *TFF1* promoter after E2 treatment. The primers used in these experiments correspond to the regions C and D of the *TFF1* promoter.

maximal H2A.Z incorporation at the *TFF1* promoter was achieved after 30 min of estradiol treatment. In addition, the association of H2A.Z with *TFF1* was found to diminish after 30 min of estradiol treatment, with H2A.Z enrichment nearly reduced to uninduced levels after 60 min. This binding profile of H2A.Z is very similar to that observed with ER $\alpha$  (Fig. 2E), p400 (Fig. 2F), and two other members of the p400 complex, Tip60 and Brd8 (Fig. 2, G,H, respectively). We also performed a ChIP experiment to investigate RNAPII binding to *TFF1* and found that RNAPII has a similar association profile to what is observed with ER $\alpha$ , but with a slight delay in its ability to cycle, as previously described (see Fig. 2I; Metivier et al. 2003). We next sought to determine whether H2A.Z incorporation into *TFF1* promoter chromatin could engage in more than one cycling event upon estradiol treatment. To compare H2A.Z association with the *TFF1* promoter with that of ER $\alpha$ , we thus repeated the time-course experiment taking samples for ChIP every 15 min for a duration of up to 165 min. A graphical representation of H2A.Z and ER $\alpha$  binding to *TFF1* for two ER $\alpha$  complete binding cycles is shown in Figure 2J. While H2A.Z could engage in at least two binding cycles, a lag was present in the second cycle when compared with ER $\alpha$ . It remains to be determined whether this lag is biologically significant. Taken together, our results show that histone H2A.Z and members of the p400 complex are actively recruited to *TFF1* in a cyclic fashion that resembles patterns previously documented for other ER $\alpha$ -recruited factors (Shang et al. 2000; Metivier et al. 2003). This association pattern of p400–H2A.Z with *TFF1* does not appear to be unique as similar results could also be obtained at another promoter (see Supplemental Fig. S2).

#### H2A.Z is incorporated into *TFF1* promoter chromatin independently of the ligand-dependent activating function of ER $\alpha$

In order to gain mechanistic insight into how H2A.Z is actively recruited to the *TFF1* promoter chromatin, we examined the recruitment of p400 and incorporation of H2A.Z into chromatin at the *TFF1* promoter following treatment with tamoxifen, an antagonist of AF-2, but not AF-1, activity (Brzozowski et al. 1997; Shiau et al. 1998; Celik et al. 2007). Tamoxifen is a selective ER modulator (SERM) used to treat hormone-responsive breast cancer that acts by competing with estradiol as a ligand for ER $\alpha$  and by inducing an AF-2 conformational change that blocks the interaction of ER $\alpha$  with coactivators including SRC-1, GRIP1, and CBP/p300 (Shang et al. 2000). As was observed with estradiol, tamoxifen treatment of MCF7 cells induced ER $\alpha$  binding at the *TFF1* promoter (Fig. 3B). As expected, the tamoxifen–ER $\alpha$  complex did not recruit RNAPII to the promoter (Fig. 3C). In contrast, recruitment of p400 and incorporation of H2A.Z at the *TFF1* promoter was detected following tamoxifen treatment (Fig. 3D,E). These results suggest that recruitment of p400 and incorporation of H2A.Z are dependent on AF-1, but independent of the ligand-binding domain. To gain further evidence that these events are AF-1-dependent, we



**Figure 3.** AF-2-independent incorporation of H2A.Z into chromatin. (A) Diagram of the *TFF1* promoter region with the amplicon used for qPCR. (B–E) ChIP analysis of ER $\alpha$  (B), RNAPII (C), H2A.Z enrichment (D), and p400 (E) recruitment before and after treatment with E2 or tamoxifen (TAM) for 30 min in MCF7 cells. (F) ChIP assay showing the effect of H2A.Z and p400 depletion on the recruitment of ER $\alpha$ , RNAPII, p300, TBP, and Brg1 at the *TFF1* promoter after E2 activation.

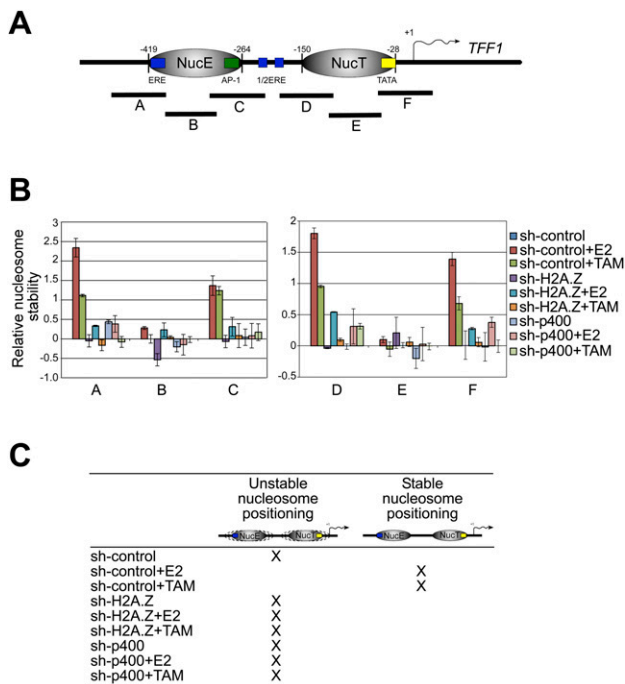
next determined whether p400 and ER $\alpha$  could physically interact. Protein–protein interaction experiments using the glutathione S-transferase (GST) system, as well as immunoprecipitation assays, revealed that p400 could indeed interact with the AF-1 domain of ER $\alpha$ , as well as with its DNA-binding domain (DBD) in a ligand-independent manner (Supplemental Figs. S3, S4). Reciprocally, ER $\alpha$  was found to interact with the C-terminal region of p400 (amino acids 2033–2062), eliminating the possibility of an interaction with the two potential LXXLL motifs present in p400 (Supplemental Fig. S3). The fact that ER $\alpha$  interacts with p400 and is able to perform this task independently of AF-2 suggests strongly that both p400 and H2A.Z are required to allow recruitment of other chromatin remodelers as well as the transcriptional machinery to the *TFF1* promoter. To test this, we performed ChIP experiments in cells depleted for either p400 or H2A.Z using antibodies directed against ER $\alpha$ , RNAPII, the p300 histone acetyltransferase, TBP, and Brg1 (catalytic subunit of Swi/Snf). Our data, shown in Figure 3F, indicate that ER $\alpha$  can be efficiently recruited to the *TFF1* promoter, even upon acute depletion of p400 or H2A.Z. In contrast, p400–H2A.Z appears to be essential to allow efficient recruitment of RNAPII, p300, TBP, and Swi/Snf to *TFF1* upon estradiol treatment. Taken together, our results suggest that ER $\alpha$  recruits the p400 complex

independently of its hormone-dependent functions, which then participates in H2A.Z incorporation within specific nucleosomes at *TFF1*, an essential mechanism that allows chromatin remodelers and the transcriptional machinery to be recruited to the gene.

*Replacement of H2A with H2A.Z allows nucleosomes to adopt preferential positions within the TFF1 promoter*

An earlier publication by Metivier et al. (2003) reported the striking discovery that nucleosomes within the *TFF1* promoter (NucE and NucT) (see Fig. 4A) adopt rather random positions along the translational axis of the promoter, a consequence that may well prevent appropriate recruitment of chromatin remodelers and the transcriptional machinery to the gene. However, activating ER $\alpha$  by estradiol treatment of cells allowed both NucE and NucT to adopt preferential sites within the promoter, thereby allowing ER $\alpha$  to interact and recruit its partners to the promoter. It is noteworthy to consider, however, that NucE and NucT stabilization upon *TFF1* induction does not involve gross nucleosome eviction or remodeling that could be observed by conventional nucleosome mapping assays such as indirect end-labeling (Sewack and Hansen 1997). For this reason, Metivier et al.

(2003) developed a highly sensitive and high-resolution assay that enables visualization and quantification of subtle nucleosome movement that occurs along the DNA translational axis. Since we showed that p400–H2A.Z is essential to the recruitment of remodelers–transcriptional machinery, we hypothesized that incorporation of H2A.Z into *TFF1* promoter chromatin would be the triggering element that would allow NucE–NucT stabilization along the translational axis of the promoter. To test this, we analyzed NucE and NucT positioning at the *TFF1* promoter in the presence or absence of p400–H2A.Z. We thus used a similar strategy as described by Metivier et al. (2003) by performing quantitative PCR (qPCR) analyses on mononucleosomal DNA prepared from untreated and estradiol-treated  $\alpha$ -amanitin synchronized cells. In considering the high sensitivity of this method, synchronization of cells using  $\alpha$ -amanitin is essential in order to clear the *TFF1* promoter of the residual general transcription factors that are associated with DNA and that are responsible for the basal expression of the *TFF1* gene (Metivier et al. 2003). This is expected because it is nearly impossible to completely eliminate estrogen levels within our culture medium. The general idea of the nucleosome stability assay is if PCR products can be detected within our defined nucleosome boundaries, then there has to be a certain degree of movement along the translational axis. Conversely, if no PCR product is detected, it is indicative of low nucleosome movement. The results, shown in Figure 4B, are represented as a relative nucleosome stability index, where a high score is assigned for nucleosomes that show a preferred translational position, thus corresponding to weak PCR amplifications. Conversely, a low score is attributed to an unstable translational nucleosomal position corresponding to a high PCR amplification. Figure 4A provides a graphical representation of NucE and NucT with the corresponding amplicons used for this assay. The positions of NucE and NucT vary during gene activation from an indefinite translational position on the inactive gene to a well-positioned nucleosome on the active gene (as revealed with the amplicons A, C, D, and F) following 30 min of estradiol treatment (see Fig. 4B, wherein amplicons B and E were used as an internal control for each MNase-digested sample). In order to determine the participation of H2A.Z and p400 in the stabilization of these nucleosomes, we depleted H2A.Z and p400 by shRNA and analyzed the effect on the positional status of both nucleosomes. Interestingly, depletion of both H2A.Z and p400 prevented the stabilization of NucE and NucT following gene induction by estradiol. These results could be taken to suggest that H2A.Z incorporation into *TFF1* promoter chromatin actually stabilizes NucE and NucT. Alternatively, it could also be a consequence of actively recruiting other chromatin remodelers that act through AF-2. To verify this possibility we again used tamoxifen in order to separate the recruitment of ER $\alpha$  and incorporation of H2A.Z from the actual gene activation process, as translational stabilization of nucleosomes could be a consequence of remodeling events that occur during activation. As previously demonstrated with estradiol,



**Figure 4.** Incorporation of H2A.Z defines nucleosome position at the *TFF1* promoter. (A) Schematic representation of the *TFF1* promoter with phased nucleosomes and amplicons used for qPCR. (B) Analysis of NucE and NucT positions on the *TFF1* promoter using qPCR amplification of mononucleosome-sized DNA. Mononucleosomes were prepared from MCF7 cells depleted for H2A.Z or p400 and treated with vehicle, E2 or TAM for 30 min. (C) Table summarizing nucleosomal position within the *TFF1* promoter following different treatments as indicated.

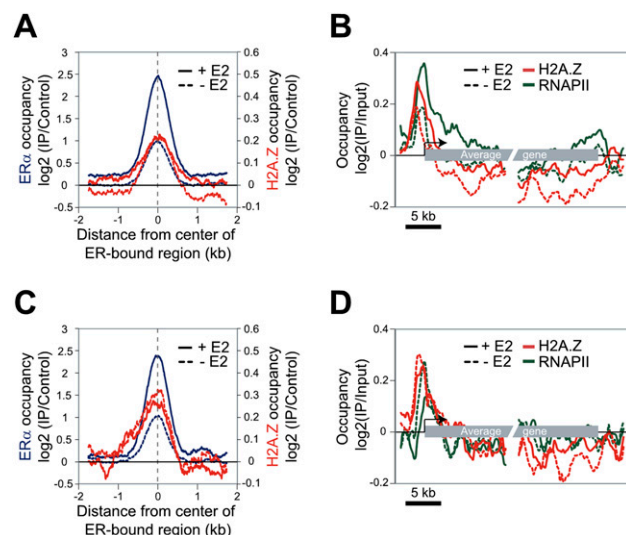
tamoxifen induces the stability of both nucleosomes, suggesting that the function of AF-2 in the recruitment of coactivators is not implicated in this preferential nucleosome positioning phenomenon. We further used the RNAi approach to determine the effect of p400 and H2A.Z on this translational stability in tamoxifen-treated cells. As demonstrated with estradiol, H2A.Z and p400 knockdown prevented the stabilization of NucE and NucT during gene-inducing conditions. Figure 4C shows a summary of the results obtained in Figure 4B. Taken together, these results indicate that incorporation of H2A.Z within *TFF1*, orchestrated by ER $\alpha$ , organizes the positions of the proximal nucleosomes to permit the recruitment of other factors, thus allowing transcriptional activation to occur.

#### *H2A.Z is present at both the distal and proximal promoter elements of ER $\alpha$ -regulated genes*

The data presented above clearly demonstrate a role of H2A.Z in the regulation of *TFF1* and a small subset of ER $\alpha$  target genes. In order to test the contribution of H2A.Z to ER $\alpha$  signaling at other genes, we performed ChIP-chip experiments with ER $\alpha$ , RNAPII, and H2A.Z in MCF7 cells in the presence or absence of estradiol (see Fig. 5). The ChIP samples were hybridized on a tiling microarray covering the nonrepetitive parts of chromo-

some 17 with an average of 4 probes per kilobase. We identified 389 genomic regions associated with ER $\alpha$  in the presence of estradiol in these experiments (at  $P < 10^{-5}$ ). Interestingly, statistically enriched H2A.Z-bound sites overlap with  $\sim 16\%$  of these ER $\alpha$ -bound regions, representing a highly significant overlap ( $P < 10^{-80}$ ) (Supplemental Fig. S7A). This suggests that the variant histone affects ER $\alpha$  function at many other genes in addition to *TFF1* (see also Supplemental Fig. S5). Figure 5A shows the average ER $\alpha$  (blue) and H2A.Z (red) signals over the fraction of these distal regions that are associated with an estradiol-activated gene. A certain level of ER $\alpha$  can be detected at the majority of these regions even in the absence of estradiol, although the presence of the hormone greatly stimulates ER $\alpha$  binding (Fig. 5A, cf. dashed blue line and solid blue line). Interestingly, H2A.Z accumulates at these regions, in agreement with what was observed at the *TFF1* and *CTSD* promoters. However, the level of H2A.Z tends to remain constant at those regions regardless of the presence of estradiol. At the proximal promoters associated with these regions (these are presumably the promoters regulated by these ER $\alpha$ -targeted sites), the level of H2A.Z increases in the presence of estradiol in a manner that correlates with RNAPII occupancy (Fig. 5B). Taken together, these data suggest that H2A.Z occupies distal regulatory regions also occupied by ER $\alpha$ , even prior to estradiol signaling and ER $\alpha$  binding, but is recruited to proximal promoters upon gene activation. The recruitment of H2A.Z to proximal promoters upon gene activation is also observed at other estradiol-regulated genes not associated with ER $\alpha$ -bound regions (see Supplemental Fig. S6B,C), suggesting that the recruitment of H2A.Z to proximal promoters is not unique to ER $\alpha$  but may be a general scheme for gene activation in mammalian cells.

Some of the genes associated with ER $\alpha$ -bound sites are down-regulated upon estradiol treatment, which is consistent with the known role of ER $\alpha$  as a repressor at some genes (Zubairy and Oesterreich 2005; Kininis et al. 2007). Interestingly, while the level of RNAPII decreases upon estradiol treatment at the promoter of these genes, the level of H2A.Z remains constant both at the promoters and the ER $\alpha$ -bound sites (Fig. 5C,D). This suggests that while H2A.Z recruitment at promoters correlates with gene activation, H2A.Z eviction is not a general scheme in gene repression.



**Figure 5.** Chromosome-wide localization of H2A.Z, RNAPII, and ER $\alpha$  in MCF7 cells. (A) The average ER $\alpha$  (blue) and H2A.Z (red) occupancy, as determined by ChIP-chip, at 231 genomic regions selected based on (1) the binding of ER $\alpha$  in the presence of E2 ( $P < 10^{-5}$ ) and (2) the fact that they are associated with genes “induced” by E2 (based on RNAPII occupancy). The closest gene within the same chromatin domain defined by CTCF binding was used here to assign genes to ER $\alpha$ -bound regions. The data are shown for cells treated with E2 (solid lines) or not (dashed lines). (B) The average RNAPII (green) and H2A.Z (red) occupancy at the 144 genes associated with the 231 ER $\alpha$ -bound regions analyzed in A. (C) Same as A but for 93 regions associated with genes that are “repressed” by E2. (D) Same as B but for the 63 genes associated with the 93 regions analyzed in C.

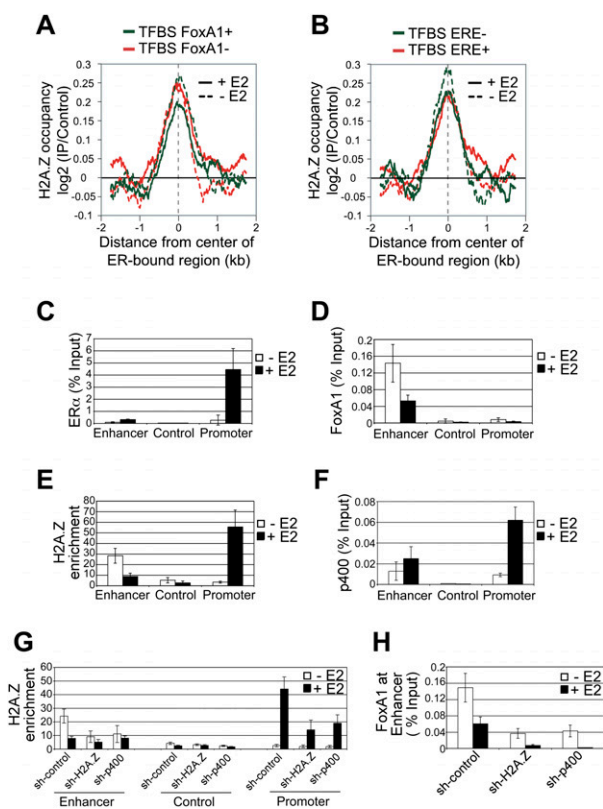
#### *H2A.Z is required for FoxA1 association to the enhancers of ER $\alpha$ -regulated genes*

FoxA1 plays a functional role in defining ER $\alpha$  binding to chromatin and subsequent transcriptional activation of target genes (Carroll et al. 2005; Eeckhoutte et al. 2006; Lupien et al. 2008). To gain further insight to the implication of H2A.Z in the regulation of ER $\alpha$ -dependent genes, we investigated the presence and the function of H2A.Z at enhancers. We focused on the ChIP-chip data at ER $\alpha$ -bound sites located at  $>4$  kb from any transcriptional start site (TSS) to avoid promoter contamination signal (hereafter referred to as “enhancers”), and grouped them

based on the presence or the absence of FoxA1 or ERE predicted binding sites. As mentioned above, H2A.Z occupies ER $\alpha$  enhancers even in the absence of estradiol. However, the addition of estradiol induces H2A.Z depletion at enhancers where the binding of ER $\alpha$  is potentially indirect (namely, at enhancers containing a FoxA1 predictive binding site or enhancers without ERE prediction) (see green curves in Fig. 6A,B), whereas it has no effect on H2A.Z levels at enhancers where the binding of ER $\alpha$  is potentially direct (see red curves in Fig. 6A,B; Supplemental Fig. S8). Admittedly, H2A.Z depletion at those enhancers is not dramatic but is nevertheless significant. One could imagine that the potential cross-

linking of increasing H2A.Z at promoters via a looping effect could counterbalance the depletion of H2A.Z at enhancers. This is also supported by the increase of RNAPII in the presence of estradiol at the enhancers (data not shown). Since the binding of FoxA1 has been shown to decrease following estrogen stimulation (Carroll et al. 2005), this suggests an interplay between H2A.Z and FoxA1. To further characterize the relationship between H2A.Z and FoxA1, we performed a detailed analysis of the *TFF1* distal enhancer. This enhancer is located ~11 kb upstream of the TSS and contains a FoxA1 predicted binding site. Using ChIP assays, we analyzed the binding of H2A.Z and p400 at this FoxA1-binding site, at the proximal promoter, and at a control region located between the proximal and the enhancer regions. As shown in Figure 2, ER $\alpha$  is recruited to the proximal promoter upon estrogen treatment as well as to the enhancer region of *TFF1*, but to a lower level (Fig. 6C). On the other hand, FoxA1 is prebound to the *TFF1* enhancer region in the absence of estrogen and the binding decreased following estrogen treatment (Fig. 6D). Interestingly, the incorporation of H2A.Z at the *TFF1* gene follows the binding of FoxA1 at the enhancer and ER $\alpha$  at the proximal promoter (Fig. 6E). p400 shows the same distribution pattern of recruitment as ER $\alpha$  at proximal promoter and enhancer regions. However, when compared with the control region, the enhancer still shows a significant level of p400 before estrogen treatment (Fig. 6F). We thus next investigated if this level of p400 is sufficient to incorporate H2A.Z at the FoxA1-bound site of *TFF1*. We combined shRNA-mediated depletion of p400 with ChIPs of H2A.Z in MCF7 cells treated or not with estrogen. As expected, acute depletion of p400 reduces the level of H2A.Z incorporation at the promoter comparable with the knockdown observed by the depletion of H2A.Z (Fig. 6H). Moreover, p400 depletion also reduces the incorporation of H2A.Z at the enhancer before estrogen treatment. This result suggests that p400 is also responsible for the incorporation of H2A.Z around the FoxA1-bound site at the enhancer region of the *TFF1* gene.

In order to further study the implication of H2A.Z in the control of the enhancer region, we investigated by ChIP assays the binding of FoxA1 at the *TFF1* enhancer region in H2A.Z- and p400-depleted MCF7 cells. Surprisingly, depletion of H2A.Z and p400 considerably decrease the binding of FoxA1 at the *TFF1* enhancer compared with the shRNA control (Fig. 6H). The levels observed after H2A.Z and p400 depletion is comparable with the level of FoxA1 after estrogen treatment. Since we showed that H2A.Z is important for FoxA1 association with the *TFF1* distal enhancer, and since Carroll et al. (2005) have shown that FoxA1 is important for ER $\alpha$  to associate with the *TFF1* distal enhancer and proximal promoter regions, it would imply that H2A.Z would also be important for ER $\alpha$  to associate with the *TFF1* proximal promoter upon gene induction. However, our results shown in Figure 3F clearly show that H2A.Z depletion does not affect ER $\alpha$  recruitment to the *TFF1* proximal promoter. Thus, in order to attempt to solve this discrepancy, we performed



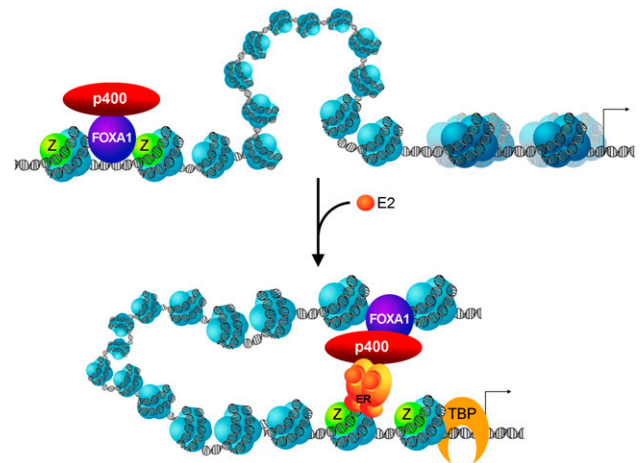
**Figure 6.** H2A.Z defines FoxA1 recruitment at enhancers of ER $\alpha$ -regulated genes. (A,B) Mapping of H2A.Z as in Figure 5A, except that genomic regions bound by ER $\alpha$  in the presence of E2 ( $P < 10^{-4}$ ) at >4 kb of any TSS were grouped by the presence/absence of a predictive binding site of FoxA1 (A: 205 and 199 enhancers, respectively, for the green and red curves), or the absence/presence of a ERE prediction site (B: 227 and 177 enhancers, respectively, for the red and green curves). The predictions for each region are available in Supplemental Table S4. (C–F) ChIP analysis showing the localization of ER $\alpha$  (C), FoxA1 (D), H2A.Z (E), and p400 (F) at the proximal promoter region and enhancer of the *TFF1* gene. The control region corresponds to a region located between the proximal promoter and the enhancer. (G) H2A.Z enrichment at the enhancer and proximal promoter region of *TFF1* after H2A.Z and p400 knockdown treated or not with E2. (H) ChIP experiments showing the effect of H2A.Z and p400 depletion on the binding of FoxA1 at the enhancer region.

an shRNA-mediated knockdown of FoxA1 in MCF7 cells to test whether it would affect ER $\alpha$  recruitment at the *TFF1* proximal promoter. The results of Supplemental Figure S8 show that, as expected, depletion of FoxA1 does indeed affect recruitment of ER $\alpha$  to the *TFF1* distal enhancer, but it does not affect ER $\alpha$  binding to the proximal promoter region. Moreover, we are unable to detect FoxA1 binding in the surrounding region where ER $\alpha$  binds the *TFF1* proximal promoter (Fig. 6D; Supplemental Fig. S8A). These results may suggest that H2A.Z is only required for ER $\alpha$  association with DNA when FoxA1 action is required. Taken together, these results suggest that H2A.Z defines or helps the recruitment of FoxA1 at enhancers of ER $\alpha$ -regulated genes, thus contributing to the activation of those genes.

## Discussion

Incorporation of histone H2A.Z into promoter chromatin has been shown to prepare genes for expression upon appropriate physiological signals (Guillemette et al. 2005; Updike and Mango 2006; Gevry et al. 2007; March-Diaz et al. 2008). In all of these cases, H2A.Z association with target promoter regions occurs when genes are not active, and upon activation, the variant histone appears to be remodeled, as visualized by the loss of its association with the promoter region. Here we describe a case in which H2A.Z incorporation into ER $\alpha$  target proximal promoter chromatin only occurs at the onset of gene induction by estrogen. Moreover, H2A.Z also associates with estrogen-responsive enhancers, but in contrast to what we observe at proximal promoters, H2A.Z is associated with these enhancer regions prior to estrogen signaling, and this association is reduced upon hormone treatment of cells. These findings allow for the dissection of mechanisms by which H2A.Z can facilitate gene transcription: first by allowing translationally unstable nucleosomes to adopt preferential positions within a promoter region, and second by allowing FoxA1 to be recruited to target enhancers. In both cases, this is essential to allow the transcriptional machinery and other chromatin remodelers to be recruited to target genes as well as perhaps permit long-range communication between distal regulatory elements and proximal promoter regions (see Fig. 7).

Regulation of estrogen signaling by ER $\alpha$  is a complex process that requires the function of a plethora of coactivators and chromatin remodelers (Metivier et al. 2003; Perissi and Rosenfeld 2005). Adding to this innate complexity is the fact that several ER $\alpha$  target genes appear to exhibit expression cycles that last ~60 min. The apogees of these transcription cycles are timed with the concerted recruitment of positive factors, including ER $\alpha$  and the RNAPII machinery. Conversely, the onset of the non-productive cycles correlates with the recruitment of negative transcription cofactors as well as the ubiquitination of ER $\alpha$  and several other positive factors (Metivier et al. 2003; Reid et al. 2003). The fact that H2A.Z can also engage in these cycles, at least at the *TFF1* and *CTSD* target genes, suggests that it must first be incorporated



**Figure 7.** Summarizing model of the role of p400 and H2A.Z in the regulation of the nucleosomal organization and ER $\alpha$ -dependant target gene activation.

at the promoter in an ER $\alpha$ -dependent fashion, and must then also be transiently remodeled by a yet-unidentified mechanism, involving possibly histone ubiquitination and degradation or the action of other chromatin-modifying activities. We also entertain the possibility that the p400 complex could also be involved in remodeling H2A.Z-containing nucleosomes. The use of tamoxifen in our experiments, taken together with the protein-protein interaction assays, has allowed us to establish the following order of assembly of factors at the *TFF1* proximal promoter as well as propose a mechanism of action for p400–H2A.Z in this scenario (see Fig. 7). First, the ER $\alpha$  activator recruits p400 by virtue of interactions with AF-1 (and possibly the DBD). The p400 complex then incorporates H2A.Z into promoter chromatin, which allows nucleosomes (NucE and NucT) to adopt preferred positions along the DNA translational axis. These preferred positions, of NucT in particular, would render the TATA element accessible for TBP to bind, as well as permit the recruitment of the transcriptional machinery. This notion is also reinforced by our finding that both H2A.Z and p400 are required for p300, Brg1 (Swi/Snf), TBP, and RNAPII to be efficiently recruited to the *TFF1* promoter upon estradiol treatment of MCF-7 cells. In addition, our proposed model of action is also in accordance with previous reports that show that when *TFF1* is inactive and free of RNAPII, both NucE and NucT slide along the translational axis of the promoter, whereas upon activation of the gene, both nucleosomes are forced to engage their preferred positions (Metivier et al. 2003; Reid et al. 2003). We emphasize that our experiments do not address the issue of whether or not nucleosomes containing H2A.Z are more or less stable than nucleosomes bearing canonical H2A. Rather, our studies show that nucleosomes bearing H2A.Z tend to force nucleosome positioning at defined sites on DNA. In fact, we also observed a similar scenario in yeast in that promoters enriched in H2A.Z tend to have nucleosomes that are better positioned around the TSS as compared with gene



promoters that are depleted of H2A.Z (Guillemette et al. 2005).

The mechanistic role of H2A.Z at estrogen-responsive distal enhancer regions still remains somewhat elusive. However, since FoxA1 is essential for the full activity of ER $\alpha$  target gene expression, presumably by allowing the enhancer region to loop and thus interact with the basal promoter region (Carroll et al. 2005), we surmise that since H2A.Z is required for FoxA1 recruitment to enhancers, it must also be essential for long-range interactions of these distal DNA elements. Our results are also in line with a report by the Hager laboratory (John et al. 2008), which showed that H2A.Z localized to DNase I-hypersensitive sites—which are presumably enhancer regions—and that upon GR recruitment to those regions, H2A.Z binding decreased from these hypersensitive sites.

Our study also demonstrates that p400 is an important regulator of ER $\alpha$  signaling. We have shown in previous work that p400 can directly incorporate H2A.Z–H2B dimers into a regular nucleosome *in vitro*, and does so in an ATP-dependent fashion (Gevry et al. 2007). That same study also demonstrated that p400 was important for the incorporation of H2A.Z at several p53 target genes, but that the other Swr1 ortholog SRCAP did not appear to be as essential. Thus, we surmise that, as in the case of p53 target genes, p400 would also be directly involved in incorporating H2A.Z into chromatin at ER $\alpha$  target genes. In support of this notion, we observe that acute depletion of p400 severely impedes ER $\alpha$ -mediated gene activation. On the other hand, our results do not exclude the possibility that SRCAP might also participate in the regulation of ER $\alpha$  signaling, a possibility that still remains to be tested.

Our data also support a link between H2A.Z–p400 and ER $\alpha$ -dependent breast tumor proliferation. A few lines of evidence argue for such a possibility: First, both H2A.Z and p400 are essential components of estradiol-dependent breast cancer cell growth. Second, H2A.Z and ER $\alpha$  levels have been shown to correlate in several breast cancer samples tested (A Svtelis, N Gévry, and L Gaudreau, *in prep.*). Furthermore, a recent report has also associated H2A.Z expression with metastasis and decreased breast cancer survival (Hua et al. 2008). Thus, breast tumors whose growth depends on ER $\alpha$  would also require p400–H2A.Z. In general, patients with ER $\alpha$ -positive breast cancers receive a positive prognosis due to the efficiency of anti-estrogenic treatments (Normanno et al. 2005; Riggins et al. 2007). However, a serious problem arises when certain tumors become resistant to the anti-estrogen treatment, leading to a decrease in the possibility of recovery (Normanno et al. 2005; Riggins et al. 2007). This resistance phenomenon may be attributed to the activity of AF-1, which in turn has been suggested to be turned on by MAP kinase signaling pathways (Riggins et al. 2007). Since the p400–H2A.Z interaction with ER $\alpha$  occurs through AF-1 and the DBD, it will be interesting to determine whether AF-1 activity, under circumstances in which AF-2 is inactivated by anti-estrogens, also requires the action of p400–H2A.Z. Should this be true, it would then be foreseeable that the p400–H2A.Z path-

way would represent an attractive therapeutic target for both anti-estrogen-responsive and nonresponsive ER $\alpha$ -positive breast cancers.

## Materials and methods

### *Cell culture, transfection, and retroviral infection*

MCF7 and MDA-MB-231 cell lines were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (FBS) and antibiotics. 17 $\beta$ -Estradiol (Sigma) and tamoxifen were used at concentrations of 100 nM and 1  $\mu$ M, respectively. shRNAs directed against H2A.Z and p400 were transfected into MCF7 using Fugene6 or by retroviral infections as described previously (Chan et al. 2005; Gevry et al. 2007).

### *ChIP assays*

MCF7 cells were hormone-deprived for at least 3 d and then treated with 100 nM 17 $\beta$ -estradiol for the indicated time. ChIP assays were performed essentially as described previously (Gevry et al. 2007) with a panel of specific polyclonal antibodies generated in-house or from commercial sources, as well as preimmune, and no antibody controls. Samples were sonicated to generate DNA fragments <500 bp. qPCR was performed using a set of primers relevant to the promoter regions of the *TFF1* and *CTSD* genes. The primers used in qPCR are listed in Supplemental Table S1. Results are shown as percent input except in cases with H2A.Z, where results are represented as “enrichment” and where H3 binding was also considered to account for nucleosome density. Thus, in this case, H2A.Z percent input was divided to H3 percent input.

### *Micrococcal nuclease digestion and nucleosome mapping*

Chromatin was isolated as described by Kim et al. (2004) from formaldehyde-cross-linked MCF7 cells synchronized with  $\alpha$ -amanitin and treated with 17 $\beta$ -estradiol for 30 min. Chromatin samples were subjected to DNA digestion with increasing amounts (0, 5, 10, and 15 U) of micrococcal nuclease (MNase). The reactions were stopped by addition of EDTA and EGTA (50 mM final concentration). Cross-linking was reversed overnight at 65°C. DNA samples were extracted after RNase A and proteinase K digestion using PCR Qiaquick spin columns (Qiagen). Mononucleosome-sized DNA was analyzed following the qPCR method derived from Metivier et al. (2003). Relative nucleosome stability index was calculated as  $\log_2(1/CT)$  where  $CT = (CT \text{ mononucleosome size of specific DNA amplification}) - (CT \text{ Input})$ . The primers used are listed in Supplemental Table S3.

### *ChIP–chip*

Details are available in the Supplemental Material.

### *Statistical methods for ChIP–chip and ChIP expression analyses*

The data were normalized and analyzed as previously (Rufiange et al. 2007) using the limma's loess function (Yang et al. 2002) in BioConductor (from the ArrayPipe Analysis Pipeline) (Hokamp et al. 2004), and replicates were combined using a weighted average method as described previously (Ren et al. 2000). All the experiments were carried out in duplicate. The combined data sets are available in Supplemental Material. To interpolate between probes, a standard Gaussian filter (SD = 200 bp) was

applied twice to the data as described previously (Guillemette et al. 2005). This will be referred to as the “smoothed data.”

#### Effect of estradiol on genes of chromosome 17

In order to evaluate the impact of estradiol on RNAPII occupancy, we first calculated from the smoothed data, for each of the 2004 unique TSS using UCSC known genes hg18, the mean  $\log_2(\text{RNAPII IP/Input})$  ratio in a window of 1 kb centered on the TSS. Genes were then sorted by their difference on mean RNAPII occupancy in the presence or absence of estradiol.

#### Mapping of H2A.Z, ER $\alpha$ , and RNAPII on ER $\alpha$ -bound regions and along genes (Figs. 5, 6A,B; Supplemental Figs. S6, S8)

To automatically identify ER $\alpha$ -bound regions, the algorithm developed in the Young laboratory (Boyer et al. 2005) was applied to the ER $\alpha$  data set in the presence of estradiol with a *P*-value cutoff of  $P < 10^{-5}$ . These 389 regions were then associated with the proximal TSS using the CTCF-bound regions of Ren laboratory (Kim et al. 2007) as boundary regions (65 regions were not associated with any gene). Regions and their associated gene were then separated based on the RNAPII occupancy difference in presence of estradiol (as described above). The nonsmoothed data were mapped on the middle of the ER $\alpha$ -bound regions (Fig. 5B,D) or on the 5' and 3' boundaries of the associated genes (Fig. 5C,E) into 50-bp windows, and a sliding window of 300 bp was applied to the average ratios. Independently of the ER $\alpha$ -bound regions, genes were binned into five groups according to their RNAPII occupancy difference described above (each group contains, respectively, 153, 658, 506, 560, and 127 genes). The same mapping procedure was applied on the differential data set (with/without estradiol) of RNAPII and H2A.Z occupancy (Supplemental Fig. S6B,C), and on distal ER $\alpha$ -bound regions identified at  $P < 10^{-4}$  and grouped by the presence/absence of a predictive binding site for FoxA1 and ERE (Fig. 6A,B; Supplemental Fig. S7). Binding site predictions were obtained in regions of at least 600 bp centered at the middle point of identified ER $\alpha$ -bound regions with the “FoxA1 shared” matrix from the Brown laboratory (Lupien et al. 2008) and the half-ERE matrix from TransFac version 9.2 (Matys et al. 2006) using a PWM fast-matching approach developed in the Blanchette laboratory (JS Galan and M Blanchette, in prep.).

#### Antibodies and shRNA

The ER $\alpha$  polyclonal (HC20), ER $\alpha$  monoclonal (F10), FoxA1 polyclonal (H-120), and RNAPII antibodies (N20) were purchased from Santa Cruz Biotechnologies. Polyclonal antibodies for H2A (07-146), H2A.Z (07-594), and Tip60 (07-038) were from Upstate Biotechnologies. Histone H3 (ab 1791) and H2A.Z (ab 4174) were purchased from Abcam. Brd8 (Ab-2) and p400 (A300-541A) polyclonal antibodies were from Bethyl Laboratories. The H2A.Z and p400 antibodies used for ChIP assays were raised against an N-terminal H2A.Z peptide (CSLIGKKGQKQT) and C-terminal p400 peptide (MRVPAVRLKTPKPPCQ). The pSuper-retro-puro shRNA-H2A.Z and p400 were described previously (Gevry et al. 2007).

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