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Recognition of enhancer element-specific histone methylation by TIP60 in transcriptional activation

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Abstract

Many coregulator proteins are recruited by DNA-bound transcription factors to remodel chromatin and activate transcription. However, mechanisms for coordinating actions of multiple coregulator proteins are poorly understood. We demonstrate that multiple protein-protein interactions by protein acetyltransferase TIP60 are required for estrogen-induced transcription of a subset of estrogen receptor (ER) α target genes in human cells. Estrogen-induced recruitment of TIP60 requires direct binding of TIP60 to ERα and the action of chromatin remodeling ATPase BRG1, leading to increased recruitment of histone methyltransferase MLL1 and increased monomethylation of histone H3 at Lys4. TIP60 recruitment also requires preferential binding of the TIP60 chromodomain to histone H3 containing monomethylated Lys4, which marks active and poised enhancer elements. After recruitment, TIP60 increases acetylation of histone H2A at Lys5. Thus, complex cooperation of TIP60 with ERα and other chromatin remodeling enzymes is required for estrogen-induced transcription.

INTRODUCTION

The ability to elicit transcription factor binding and transcriptional activation of specific genes by addition of a small molecular weight hormone to cell culture medium makes steroid hormone-regulated transcription an ideal system to study transcriptional activation. Steroid hormone receptors are hormone-regulated transcription factors belonging to the nuclear receptor family. Binding to their cognate hormone causes steroid hormone receptors to bind to and activate or repress transcription of specific target genes. Transcriptional activation by the DNA-bound receptor is accomplished by recruitment of a large number of coregulator proteins which remodel chromatin conformation and promote the assembly

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AUTHOR CONTRIBUTIONS

K.W.J. conceived and carried out the studies with the guidance of M.R.S. K.K. and W.A. carried out the experiments shown in Supplementary Figure 5h. A.J.S. and T.S.U. provided the expertise and instrumentation required for the experiment shown in Supplementary Figure 5g.

and/or activation of a transcription complex on the target gene promoter[1–5]. Chromatin immunoprecipitation (ChIP) studies on selected target genes of estrogen receptor (ER) α (e.g. the *TFF1* or *pS2* gene) during the first 60 min of hormone treatment revealed a hormone-initiated sequence of transient steady state occupancy of the promoter and associated ER binding sites by ERα and many coregulator proteins and histone modifications, culminated by enhanced occupancy by RNA polymerase II[4–6]. Among the earliest coregulator occupants is the ATP-dependent chromatin remodeling complex SWI/SNF containing ATPase subunit BRG1, followed closely in time by a succession of histone modifying enzymes, including the histone acetyltransferase TIP60. Subsequent target gene occupants include Steroid Receptor Coactivator proteins (SRC-1, SRC-2, and SRC-3), Mediator complex, and other coregulators.

TIP60 belongs to the MYST (MOZ, YBF2, SAS2, and TIP60) family of histone acetyltransferases, which participate in diverse cellular processes, such as transcriptional regulation, DNA damage repair and apoptosis[7–10]. Recombinant TIP60 acetylates core histones H2A, H3 and H4 *in vitro*[11,12]; in cells TIP60 is found in a stable multi-protein complex which can acetylate nucleosomes[9,12] and several non-histone proteins including the transcription factors p53 (ref. 13–15) and MYC[16]. TIP60 is also known as a nuclear receptor coactivator. It binds to the ligand binding domain of the androgen receptor and enhances hormone-dependent activation of transiently transfected reporter genes by several steroid hormone receptors, including ERα[17]. However, little is known about the mechanism by which TIP60 is recruited to endogenous target genes of steroid receptors upon hormonal activation or how TIP60 contributes to chromatin remodeling and transcriptional activation.

To further elucidate the mechanisms of chromatin remodeling and transcription complex assembly and activation, we set out to define the carefully coordinated sequence of physical and functional interactions among the participating coregulators during the early stages of these processes. In this study we demonstrate that TIP60 is required for efficient estrogen-induced expression of a subset of the target genes of ERα. We then use the combination of RNA interference and ChIP to define a cascade of chromatin remodeling events and protein-protein interactions occurring on target gene promoters during the first hour of estrogen treatment, including chromatin remodeling by SWI/SNF ATPase BRG1, subsequent methylation of histone H3 at lysine 4, and the ability of TIP60 to interact with ERα and the enhancer element-specific histone mark, monomethylation of histone H3 at Lys4.

RESULTS

Requirement of TIP60 for estrogen regulated transcription

TIP60 enhances hormone-stimulated expression of transiently transfected reporter genes of androgen receptor and ERα[17,18], but its requirement for endogenous ERα target genes has not been reported. When TIP60 was depleted from MCF-7 breast cancer cells by transiently transfected siRNA duplexes (Fig. 1a), 17β-estradiol (E2)-induced expression of several endogenous ERα target genes (*TFF1, GREB1, SGK3, and PKIB*) was compromised, compared with cells transfected with non-specific siRNA (Fig. 1b and Supplementary Fig. 1a). In contrast, the E2-induced levels of *MYC, CyclinD1 (CCND1) and CXCL12* mRNAs were not affected by TIP60 depletion, and *cathepsin D* (*CTSD*) expression was only

marginally impaired. As a measure of transcription rate we also measured the level of pre-mRNA for three ERα target genes, by using PCR primers that span an exon-intron junction. Consistent with the results observed for E2-induced mRNA levels, TIP60 depletion dramatically compromised the E2-induced increase in pre-mRNA levels for *TFF1* and *GREB1* but had no effect on the pre-mRNA levels for *CyclinD1* (Supplementary Fig. 1a,c). Thus TIP60 is required for E2-induced expression of some but not all ERα target genes, and the major effect of TIP60 appears to be on the rate of mRNA production.

TIP60 recruitment to ERα target genes in response to E2

Chromatin immunoprecipitation studies have defined an ordered and cyclical pattern of steady-state occupancy by ERα and various coactivators on ERα binding sites associated with ERα target genes in MCF-7 cells, with particular focus on the *TFF1* (also known as *pS2*) gene[4,19]. ERα binding sites associated with the *TFF1*, *GREB1*, and *CTSD* genes have been established[20–23]. BRG1 occupancy on the most promoter-proximal ERα binding site (ERE1) associated with the *TFF1* gene increases within 5 min after addition of E2, followed closely by TIP60 occupancy[4,19]. We observed two peaks of TIP60 occupancy at approximately 15–25 min and 40–60 min after addition of E2 to MCF-7 cells; TIP60 occupancy occurred at all major ERα binding sites associated with the *TFF1*, *GREB1*, and *CTSD* genes and was absent or weak in coding regions or at weak ERα binding sites (Fig. 1c and Supplementary Fig. 2a,b). The temporal peaks of TIP60 occupancy coincided approximately but not exactly with ERα binding. We also observed similar temporal patterns of hormone-dependent occupancy by TIP60 on ERα-occupied enhancer elements associated with the *CyclinD1* and *MYC* genes (Supplementary Fig. 2c). Since the hormone-induced expression of these genes was not affected by TIP60 depletion (Fig. 1b), we conclude that the selective requirement for TIP60 is due to gene-specific differences in the regulatory environment (i.e. DNA sequence and chromatin architecture at regulatory sites of specific target genes), not due to differences in the ability to recruit TIP60.

Interaction with ERα required for TIP60 recruitment

The correlation between ERα and TIP60 occupancy on ERα target genes suggested that ERα may facilitate TIP60 occupancy, especially since TIP60 contains a C-terminal NR box motif (LXXLL, where L is leucine and X is any amino acid) required for TIP60 binding to androgen receptor and enhancement of steroid hormone-stimulated expression of transient reporter genes[24]. The ERα ligand binding domain (LBD) fused to glutathione S-transferase (GST) bound *in vitro* to full length TIP60 in an E2-dependent manner, but not to the chromodomain or histone acetyltransferase regions of TIP60 (Supplementary Fig. 3a). TIP60 did not bind to the N-terminal AF1 region of ERα (Supplementary Fig. 3b). Mutation of the C-terminal NR box of full length TIP60 to LXXAA (Leu492 and Leu493 changed to Ala) eliminated E2-dependent binding to ER LBD *in vitro* (Fig. 2a) and *in vivo* (Supplementary Fig. 3c), although a small amount of hormone independent binding was still observed *in vivo*. In transient ERα reporter gene assays the LXXLL motif is required for TIP60 coactivator function (Fig. 2b), in agreement with previous androgen receptor studies[24]. Furthermore, when FLAG-tagged wild type and mutant TIP60 were over-expressed at similar levels in MCF-7 cells by transient transfection, quantitative ChIP assays performed with anti-FLAG antibody demonstrated E2-dependent occupancy by wild type

TIP60, but not mutant TIP60, on ERα binding sites associated with the *TFF1* and *GREB1* genes (Fig. 2c). Thus interaction of ERα LBD with the TIP60 NR box is required for E2-induced occupancy by Tip 60 on endogenous ERα target genes.

SWI/SNF action required for TIP60 recruitment

Chromatin remodeling complex SWI/SNF containing BRG1 as ATPase subunit is the earliest coregulator (at 5 min) observed to occupy the *TFF1* ERE1 site after E2 treatment, followed closely by TIP60 (ref. 4), suggesting a possible functional relationship between BRG1 and TIP60. Depletion of BRG1 by transfection with two different siRNA duplexes dramatically decreased E2-induced expression of *TFF1* and *GREB1* mRNA (Supplementary Fig. 1b) and reduced but did not eliminate occupancy of TIP60 on the proximal and distal enhancer elements (ERE1 and ERE3) of the *TFF1* gene (Fig. 3a and Supplementary Fig. 4). However, BRG1 depletion had no effect on *TIP60* mRNA level (Supplementary Fig. 4a) or occupancy of *TFF1* ERE1 or ERE3 regions by ERα (Fig. 3a and Supplementary Fig. 4b). Thus BRG1, presumably as part of SWI/SNF, is required for part but not all of the E2-induced pattern of TIP60 occupancy on the *TFF1* gene. Since interaction of TIP60 with ERα is required for TIP60 occupancy on the *TFF1* gene (Fig. 2c), we speculate that the remaining TIP60 occupancy after BRG1 depletion is due to TIP60 interaction with ERα, and that chromatin remodeling by SWI/SNF is required to further stabilize occupancy of TIP60 on the gene.

To further examine the SWI/SNF-TIP60 relationship, we performed ERα-mediated reporter gene assays in SW13 cells which lack a SWI/SNF ATPase subunit (BRG1 or hBRM) but express the other SWI/SNF subunits[25,26]. Without BRG1, ERα and TIP60 failed to activate transcription of the transient reporter gene in response to E2 (Fig. 3b, lanes 1–5). However, co-expression of BRG1, but not an enzymatically dead BRG1 mutant, restored the ability of TIP60 to activate the reporter gene (lanes 6–13). Thus some type of chromatin remodeling by SWI/SNF is required for recruitment and coactivator function of TIP60.

TIP60 chromodomain binds histone H3 monomethylated at Lys 4

We considered that SWI/SNF action might facilitate subsequent posttranslational histone modifications required for TIP60 recruitment. Methylation of Lys4 of histone H3 (H3K4me) is often associated with transcriptional activation[27–31]; moreover, TIP60 contains an N-terminal chromodomain, and chromodomains of other proteins have been shown to bind preferentially to histones containing methylated versus unmethylated lysine residues[10,32–35]. In assays using biotinylated histone tail peptides bound to Streptavidin-agarose beads, TIP60 specifically bound to the histone H3 tail peptides containing monomethylated K4 (H3K4me1), slightly to peptides with H3K4me2, but not to peptides with H3K4me0 or H3K4me3 (Fig. 4a and Supplementary Fig. 5a). TIP60 also failed to bind or bound very weakly to histone tails with a variety of other methylation modifications, including H3R17me2, H4R3me2, H3K27me1/2, and H4K20me1/3 (Supplementary Fig. 5b–d). In contrast, the PHD region of MLL1 bound strongly to H3K4me3 and bound more weakly to H3K4me2, but did not bind to H3K4me1 or H3K4me0 (Supplementary Fig. 5e), as previously reported[36].

The N-terminal chromodomain of TIP60 was responsible for binding H3K4me1 (Supplementary Fig. 5a). Preferential binding of TIP60 chromodomain to histone H3K9me3 was recently shown to activate TIP60 acetyltransferase activity and facilitate DNA double-strand break repair[37]. We also observed specific binding of H3K9me3 by purified, recombinant TIP60 chromodomain, but this binding was weaker than the binding to H3K4me1 (Supplementary Fig. 5f), which was not tested in the previous study[37]. Isothermal titration calorimetry confirmed the preferential binding of TIP60 chromodomain to the H3K4me1 peptide, with a dissociation constant in the micromolar range (Supplementary Fig. 5g). Recombinant TIP60 also bound preferentially to reconstituted nucleosomes containing H3K4me1 versus H3K4me0 (Supplementary Fig. 5h). Chromodomains of some other proteins use a hydrophobic cage formed by three aromatic amino acids to bind methylated lysine[33,34]. Sequence alignment demonstrated that TIP60 contains aromatic amino acids at equivalent positions (W26, F43, and Y47) in its chromodomain (Fig. 4b). Mutation of Tyr47 in the chromodomain of TIP60 abolished its binding to H3K9me3 (ref. 37). We found that mutation of any of the conserved aromatic amino acids in TIP60 chromodomain to alanine completely abolished binding to H3K4me1 (Fig. 4a). In MCF-7 cells wild type TIP60 associated preferentially with crosslinked, micrococcal nuclease-generated mononucleosomes containing H3K4me1, and this association was inducible by E2 (Supplementary Fig. 5i). However, the Y47A mutation abolished most of this interaction.

Finally, in ChIP assays analyzing the *TFF1* and *GREB1* genes in MCF-7 cells, the Y47A mutation almost eliminated E2-induced occupancy by FLAG-TIP60, indicating that the interaction of TIP60 chromodomain with H3K4me1 is critical for TIP60 occupancy (Fig. 4c). FLAG-TIP60 wild type and Y47A mutant were expressed at similar levels (Fig. 4c), and the Y47A mutant retained its ability to bind ERα *in vitro* in a hormone-dependent manner (Supplementary Fig. 3e).

MLL1 and SET1A methylate histone H3K4 to recruit TIP60

The requirement of H3K4 methylation for TIP60 recruitment (Fig. 4) and the previously reported requirement of H3K4 methylation for ERα-mediated transcriptional activation[38,39] led us to investigate the involvement of SET1 family H3K4-specific methyltransferases[40–43]. Depletion of MLL1 or SET1A in MCF-7 cells had no effect on the levels of *TIP60*, *ER*α, or *BRG1* mRNA (Supplementary Fig. 6a), but global mono-, di-, and tri-methylation of H3K4 were dramatically reduced (Fig. 5a), as were the induction of *TFF1* and *GREB1* pre-mRNAs by E2 treatment (Fig. 5b). ChIP studies found a high level of MLL1 occupancy at ERE3 of the *TFF1* gene and a much lower but positive level at ERE1 (compared to a non-specific genomic site); and MLL1 occupancy of these sites existed before hormone treatment but increased almost 2-fold after hormone treatment (Fig. 5c, upper panels). Depletion of BRG1 eliminated the E2-enhanced portion of the ChIP signal for MLL1, indicating that BRG1 is required for the hormonal enhancement of MLL1 occupancy but not the basal pre-hormone level of MLL1 occupancy (Fig. 5c, lower panels). Depletion of MLL1 or SET1A reduced TIP60 occupancy at both the promoter-proximal and distal ERα binding sites of the *TFF1* and *GREB1* genes (Supplementary Fig. 6b), but it also dramatically diminished hormone-dependent binding of ERα to the same EREs

(Supplementary Fig. 6c), suggesting that H3K4 methylation (basal and/or hormone-induced) by these enzymes is required to facilitate ERα binding.

Scanning the *TFF1* gene region by ChIP, we observed progressive increases in H3K4me1 at ERE3 and ERE1 and in H3K4me3 at the transcription start site (TSS) during the first hour of E2 treatment, and a further increase after 4 hours (Fig. 6a and Supplementary Fig. 7). Depletion of either MLL1 or SET1A caused major reductions in the basal and E2-induced levels of mono-, di-, and trimethylation of H3K4 at the EREs and TSS. Like the *TFF1* gene, there was also a small E2-induced increase in H3K4me1 at the ERα binding sites for the *CyclinD1* and *MYC* genes (Supplementary Fig. 2d), which recruit TIP60 but have TIP60-independent expression in response to E2. Whether MLL1 and SET1A are directly responsible for TIP60 recruitment is difficult to judge, since their depletion diminished ERα binding (Supplementary Fig. 6c), which is also required for TIP60 recruitment (Figs. 1c and 2c).

Depletion of BRG1 had no effect on the H3K4 methylation patterns of the *TFF1* gene before E2 treatment (Fig. 6b). However, E2-induced increases in H3K4me1 at the EREs and the increase in H3K4me3 at the TSS were eliminated by BRG1 depletion, indicating a role for SWI/SNF in facilitating enhanced H3K4 methylation after E2 treatment.

Role of TIP60 protein acetyltransferase activity

Next we explored the role of TIP60 acetyltransferase activity in transcriptional activation by ERα. TIP60 acetylates histones, including Lys5 of H2A (H2AK5ac)[11,44]. Indeed, at the *TFF1* gene we observed an E2-stimulated 2-fold increase in H2AK5ac at ERE3 and ERE2 (but not at ERE1) which was nearly eliminated by depletion of TIP60 (Fig. 7a). For transient E2-responsive reporter gene assays, we used SW13 cells which lack BRG1 and hBRM, the ATPase subunits of the SWI/SNF complex. When ERα and BRG1 were expressed in these cells by transient transfection, co-expression of wild type TIP60 enhanced the E2-dependent reporter gene expression, but TIP60 containing two point mutations that eliminate acetyltransferase activity[9,45] had little or no coactivator function in this assay (Fig. 7b). The mutant TIP60 retained the ability to bind to ERα LBD in a hormone-dependent manner (Supplementary Fig. 3d) and to bind selectively to H3K4me1 (Supplementary Fig. 5j), indicating that the mutation caused only selective loss of protein function. To inquire whether TIP60 acetyltransferase activity is required for recruitment of TIP60 or for the downstream action of TIP60 on transcription complex formation, we performed ChIP on FLAG-tagged TIP60 in MCF-7 cells. The wild type and mutant TIP60 were recruited at equivalent levels to EREs on the *TFF1* and *GREB1* genes (Fig. 7c). Thus, the role of TIP60 acetyltransferase activity is for E2-induced acetylation of H2A (and possibly other protein substrates) leading to transcriptional activation, after TIP60 is recruited to the target gene.

DISCUSSION

Selective requirement of TIP60 for ERα target gene expression

In contrast to its function in the DNA double strand break repair process through acetylation of ATM and activation of p53-dependent genes[15,46–48], little is known about the role of

TIP60 in the nuclear receptor signaling pathway. We found that TIP60 is required for efficient hormonal induction of some (but not all) endogenous ERα target genes (Fig. 1b). It is noteworthy that selective requirement of a specific coregulator (TIP60 in this case) on different target genes of ERα provides the cell with a potential mechanism for fine-tuning the response to E2 by modulating E2-induced expression of a subset of ERα target genes. For example, this could be accomplished by using posttranslational modifications or protein-protein interactions to regulate the availability or activity of TIP60. Thus, the reduced expression of TIP60 previously observed in some types of tumors (e.g. lymphomas, head and neck tumors, and breast carcinomas) and the over-expression of TIP60 in other tumor types (e.g. prostate cancer)[49,50], may cause selective effects on the expression of the TIP60-dependent subset of steroid hormone regulated genes; this selective modulation of the overall steroid hormone responses could contribute to tumor formation or progression.

Novel methyl-histone binding activity of TIP60

We identified a novel methyl-histone binding activity within the TIP60 chromodomain, which plays an important role in the E2-induced occupancy by TIP60 on target genes of ERα (Fig. 4 and Supplementary Fig. 5). Preferential binding of the TIP60 chromodomain to H3K4me1 versus unmodified histone H3 occurred with free histone H3, with reconstituted nucleosomes, and in MCF-7 cells (Supplementary Fig. 5). Chromodomains of Polycomb and HP1 interact preferentially with specific di- and trimethylated lysine residues in histones, and a trio of conserved aromatic residues form a hydrophobic cage which binds the methyl moeities of the methylated lysine residue[32,33,51]. The TIP60 chromodomain retains the conserved aromatic amino acid trio, and these residues were required for binding to H3K4me1 *in vitro* and *in vivo* and for the E2-induced occupancy of TIP60 on the *TFF1* and *GREB1* genes (Fig. 4 and Supplementary Fig. 5). We believe that TIP60 is the first identified protein which preferentially recognizes H3K4me1. Not only does this finding contribute to our understanding of TIP60 action, but it also is noteworthy because enhancer elements associated with active or potentially active genes are characteristically marked by H3K4me1, while TSS are marked by H3K4me3 (ref. 27,28,52). Thus, we speculate that TIP60 belongs to a class of heretofore undefined enhancer recognition proteins which read and interpret into action the characteristic H3K4me1 enhancer mark.

Role of H3K4 methyltransferases in E2-induced transcription

While there are several other H3K4-specific methyltransferases[53,54], MLL1 has been shown to interact with ERα directly or through Menin (a component of MLL1 complex) and to regulate the expression of ER-responsive genes[38,39]. In addition, MLL1 was shown to be recruited to an ERα target gene through its interaction with the hSNF5 core subunit of SWI/SNF[55,56], consistent with our finding that some action of SWI/SNF is required for TIP60 occupancy (Fig. 3). Here, we showed that MLL1 and SET1A are required for E2-induced transcription of *TFF1* and *GREB1* genes (Fig. 5b). Our results suggest that there are multiple contributions of MLL1 and/or SET1A to the process of E2-induced transcriptional activation. While previous studies reached varying conclusions about the cellular roles of MLL1 and SET1A in generating mono-, di-, and trimethylation of H3K4 ref. 36,42,57), we found that each of these enzymes is important for maintaining global levels of H3K4 mono-, di-, and trimethylation in MCF-7 cells (Fig. 5a). It is yet unclear whether these enzymes

generate all three degrees of global methylation directly or in combination with specific demethylases. We observed pre-existing levels of MLL1 occupancy (Fig. 5c) and H3K4me1 (Fig. 6) at ERE3, the primary ERα binding site and enhancer element for the *TFF1* gene, and E2 further enhanced the levels of MLL1 and H3K4me1 at this site. Depletion of BRG1 eliminated the E2-induced increases in MLL1 occupancy and H3K4me1 at this site but did not affect the pre-existing levels there (Fig. 5c and 6b). This helps to explain the specific mechanistic contribution of BRG1 to the recruitment of TIP60 and the activation of transcription; it is also noteworthy that BRG1 depletion did not affect the E2-dependent binding of ERα to this site (Fig. 3a and Supplementary Fig 4b). In contrast, depletion of MLL1 or SET1A caused a dramatic reduction in the pre-existing as well as the E2-induced peak of H3K4me1 at ERE3 of the *TFF1* gene (Fig. 6a and Supplementary Fig. 7), consistent with the severe global reduction of all levels of H3K4 methylation in the cells (Fig. 5a). MLL1 depletion also eliminated ERα binding to ERE3 of the *TFF1* gene (Supplementary Fig. 6c), suggesting that MLL1 and the pre-existing peak of H3K4 methylation at this site maintain a local chromatin structure at EREs in the absence of hormonal stimulation which is required for the ERα binding that occurs after E2 treatment. Because of the global effect of depleting MLL1 or SET1A on cellular H3K4 methylation, it would not be surprising if the binding of many other transcription factors to their enhancer elements were also compromised by depletion of either of these two H3K4 methyltransferases.

Model for TIP60 recruitment and contribution to transcription

The mechanisms for coordination of the highly choreographed sequence of events leading to chromatin remodeling and transcription complex assembly are largely unknown and represent an area of important future research. Here, we studied how the loss of individual coregulators affects E2-induced histone modifications and recruitment of TIP60 and other coactivators to the *TFF1* gene and other target genes of ERα. We demonstrated how the activities of ERα, an ATP-dependent chromatin remodeling complex (SWI/SNF), histone lysine methyltransferases (MLL1 and SET1A), and a histone and protein acetyltransferase (TIP60) are coordinated. Our results suggest the following sequence of events in the early stages of E2-induced chromatin remodeling and transcription complex assembly (Fig. 8): 1) recruitment of SWI/SNF by ERα with the help of coregulator FLII[19]; 2) remodeling of chromatin and recruitment of histone methyltransferases MLL1 and/or SET1A by SWI/SNF; 3) increased methylation of H3K4 by MLL1 and/or SET1A; 4) direct interaction of TIP60 LXXLL motif with ERα on the ERE; 5) stabilization of TIP60 occupancy on the gene by binding of TIP60 chromodomain to H3K4me1 on nucleosomes associated with the enhancer element; and 6) acetylation of H2AK5 by TIP60, leading to further chromatin remodeling. This model applies to ERα target genes that require TIP60 for E2-induced expression but not to the TIP60-independent target genes. Our results begin to explain how specific chromatin remodeling, histone modifications, and protein-protein interactions are coordinated to accomplish critical early steps in the formation of an active transcription complex on a target gene in response to a cellular signal.

Supplementary Material

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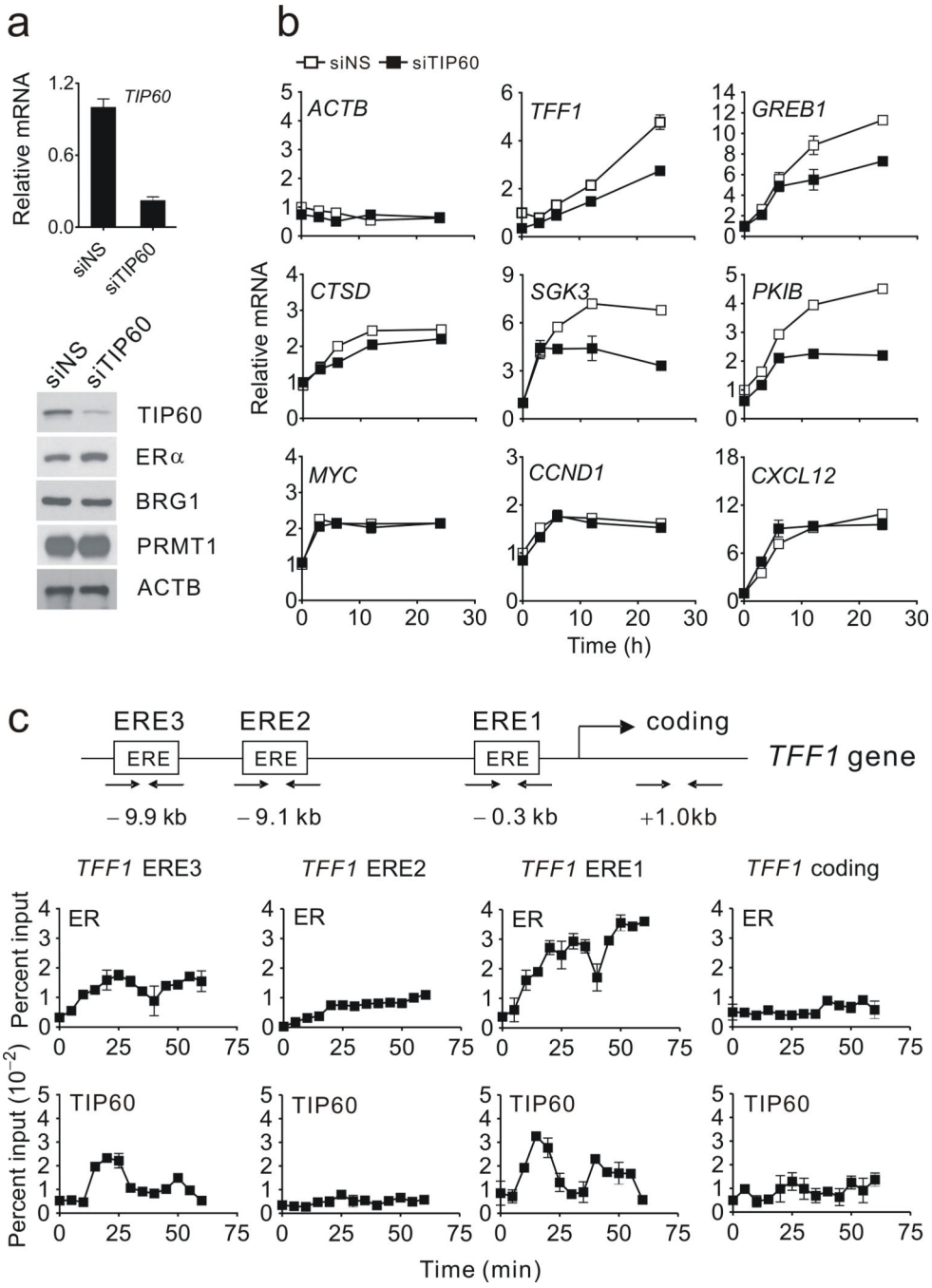

Figure 1.

Requirement of endogenous TIP60 for expression of endogenous ERα target genes. **(a)** Depletion of *TIP60* mRNA and protein by siRNA transfection. MCF-7 cells were transfected with siRNA against TIP60 (siTIP60) or non-specific siRNA (siNS). Total RNA was analyzed for TIP60 mRNA by qRT-PCR and normalized to *GAPDH* mRNA. Levels of the indicated proteins were assessed by immunoblot. **(b)** Effect of reduced TIP60 on the expression of estrogen-responsive genes. MCF-7 cells were transfected with siTIP60 or siNS and treated with E2 for the indicated time before harvest. Total RNA was analyzed by

qRT-PCR. Levels of all mRNAs were normalized to that of *GAPDH* mRNA; *ACTB* mRNA served as a control that was unaffected by E2 or by siTIP60. Results shown are mean and range of variation of duplicate PCR reactions performed on the same cDNA sample; the results are from a single experiment which is representative of at least two independent experiments. **(c)** Estrogen-dependent occupancy of ERα target genes by TIP60 in MCF-7 cells. ChIP assays were performed with MCF-7 cells treated with 100 nM E2 or vehicle for the indicated time. The amount of the indicated regions (see diagram) of the *TFF1* gene precipitated by antibodies against ERα or TIP60 was determined by qPCR. Results shown are mean and range of variation of duplicate PCR reactions performed on the same DNA sample and are from a single experiment which is representative of two independent experiments.

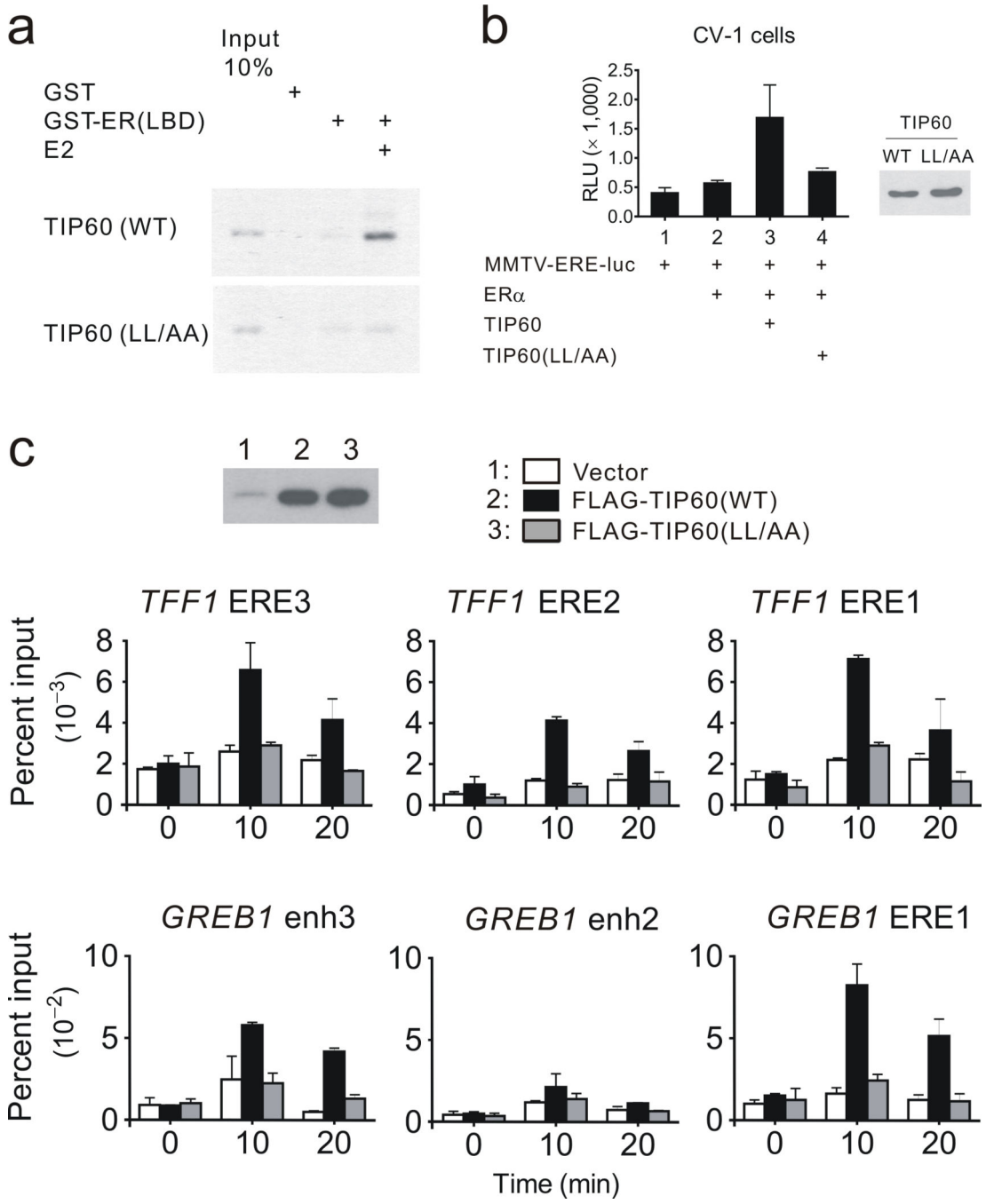

Figure 2.

Interaction with ERα is critical for occupancy of ERα target genes by TIP60. **(a)** GST pull-down assays were performed with the indicated *in vitro* translated FLAG-tagged wild type or mutant TIP60 incubated in the absence or presence of E2 with GST-fused ER-LBD bound to glutathione-Sepharose beads. Bound proteins were analyzed by immunoblot with anti-FLAG antibody. **(b)** CV-1 cells were transfected with MMTV-ERE luciferase reporter plasmid (200 ng) and expression plasmids encoding ERα (0.2 ng), and pCDNA-FLAG-TIP60 (wild type or LXXAA mutant, 10 ng), as indicated, and grown with E2 before

harvesting and measuring luciferase activities (bar graph; n = 3 biological replicates, error bars represent s.d.). Expression of wild type and mutant TIP60 was monitored by immunoblot using antibodies against FLAG epitope. **(c)** FLAG-tagged TIP60 wild type or LXXAA mutant was transiently expressed in MCF-7 cells, which were then treated with E2 for the indicated time before ChIP was performed with anti-FLAG antibody. Precipitated DNA was analyzed by qPCR with primers representing the ERα binding sites of the *TFF1* and *GREB1* genes. Results shown are mean and s.d. of three PCR reactions performed on the same DNA sample and are from a single experiment which is representative of two independent experiments. Expression of wild type and mutant TIP60 was monitored by immunoblot using antibodies against FLAG epitope.

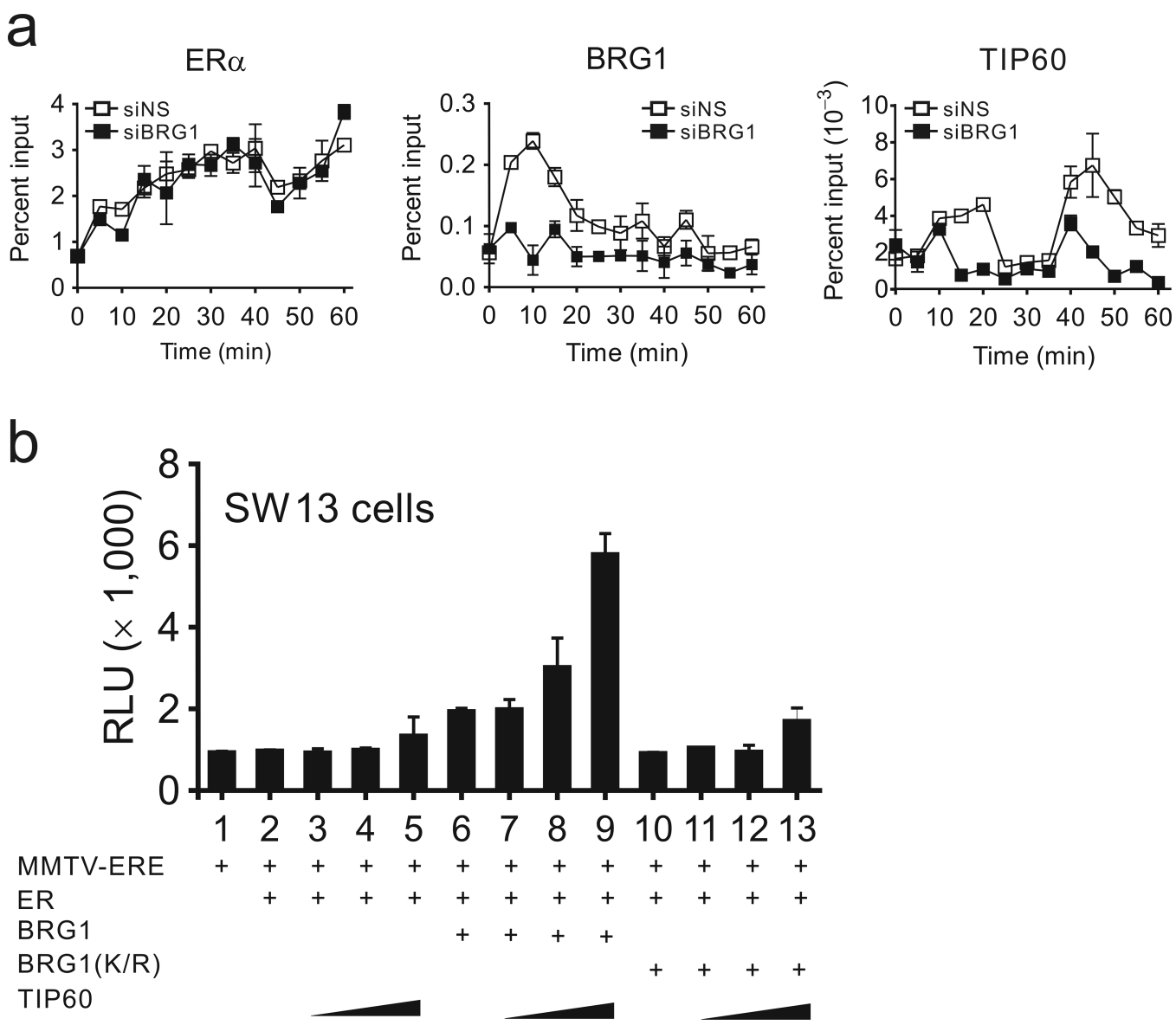

Figure 3.

BRG1-dependent coactivator activity and occupancy of ERα target genes by TIP60. **(a)** Time course of TIP60 occupancy of ERE1 of the *TFF1* gene in BRG1-depleted cells. MCF-7 cells were transfected with siNS or siRNA against BRG1 (siBRG1). After treatment of cells with E2 for the indicated times, ChIP assays were performed as in Figure 1c with antibodies against the indicated proteins. Results shown are mean and range of variation of two PCR reactions performed on the same DNA sample and are from a single experiment which is representative of two independent experiments. **(b)** SWI/SNF complex plays an important role in TIP60 coactivator function. SW13 cells were transfected with MMTV-ERE luciferase reporter plasmid (200 ng) and expression plasmids encoding ERα (0.1 ng), BRG1 or BRG1(K/R) mutant (10 ng), and TIP60 (1, 10, or 100 ng). Transfected cells were grown with E2, and luciferase activities of the transfected-cell extracts were determined.

Results shown are mean and s.d. of triplicate biological replicates and are from a single experiment which is representative of 3 independent experiments.

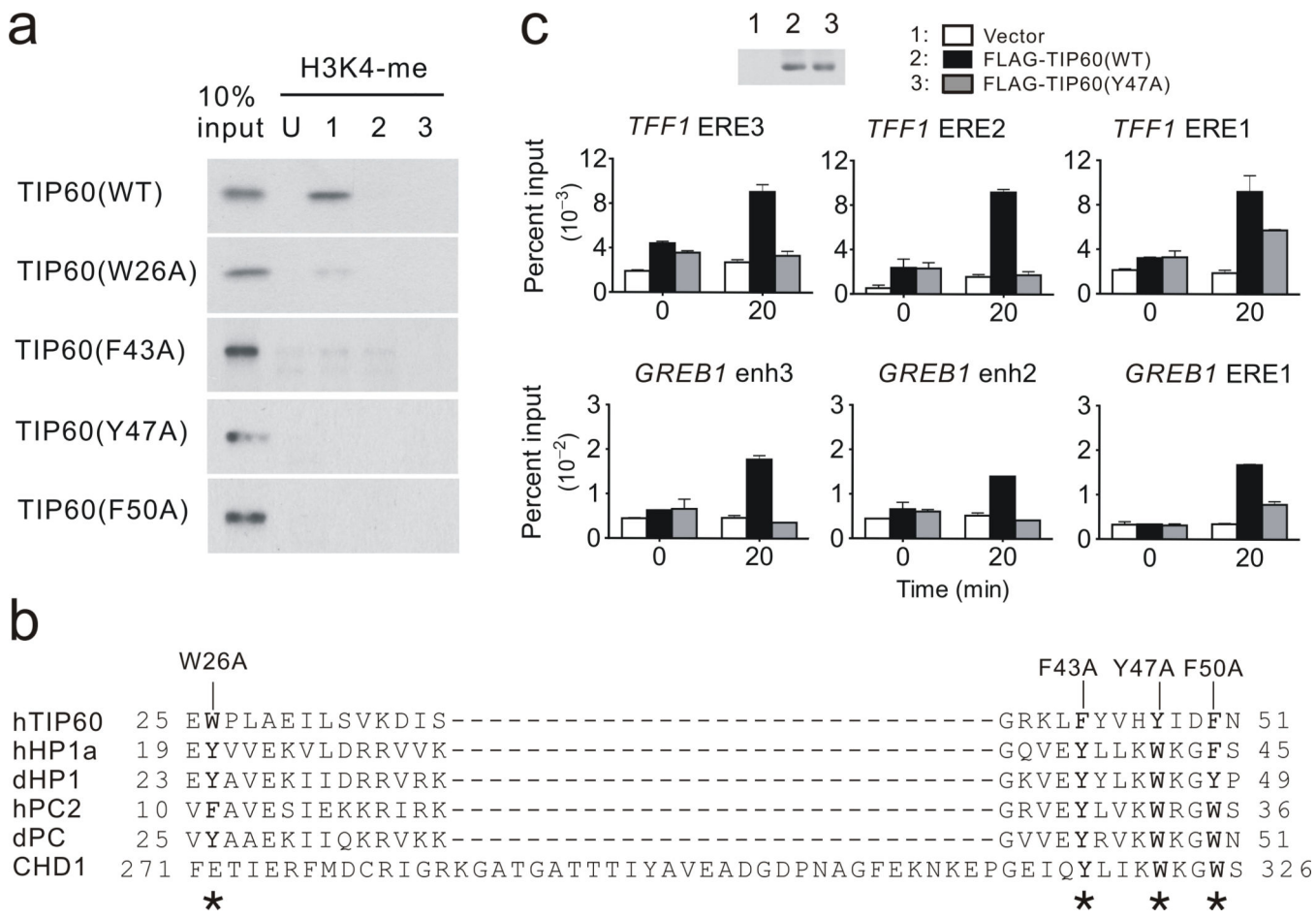

Figure 4.

Binding of TIP60 to H3K4me1 is required for TIP60 recruitment to ERα target genes. **(a)** *In vitro* translated FLAG-tagged full length TIP60 or TIP60 proteins with mutations in the chromodomain were incubated with beads pre-bound with biotinylated peptides representing unmodified histone H3 (amino acids 1–23) (U), or histone H3 (amino acids 1–21) containing monomethyl H3K4, dimethyl H3K4, or trimethyl H3K4. Bound TIP60 was detected by SDS-PAGE and immunoblot using anti-FLAG antibody. **(b)** Conserved aromatic residues (*) in the chromodomains of TIP60 and other proteins are indicated along with the TIP60 mutations used here. **(c)** Interaction with H3K4me1 is critical for estrogen-dependent occupancy of ERα target genes by TIP60. Empty plasmid or plasmid encoding FLAG-tagged wild type or Y47A mutant TIP60 was transiently transfected into MCF-7 cells as in Figure 2c; cells were then treated with E2 for the indicated time, and ChIP was performed as in Figure 2c using anti-FLAG antibody. Results shown are mean and s.d. of three PCR reactions performed on the same DNA sample and are from a single experiment which is representative of two independent experiments. Expression of FLAG-TIP60 in whole cell extracts was measured by immunoblot (upper panel).

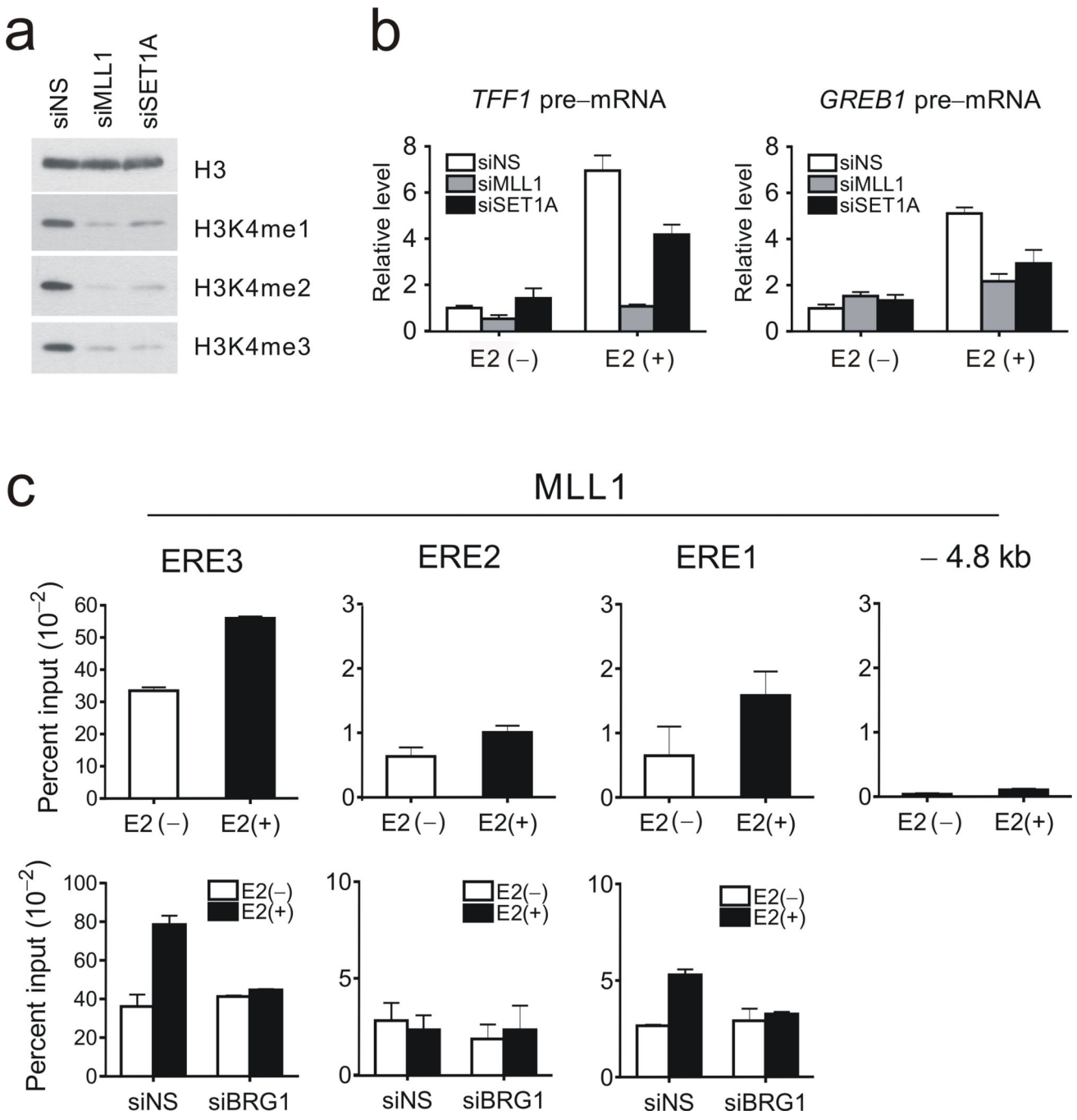

Figure 5.

Requirement of H3K4 methyltransferases for E2-induced expression of ERα target genes. **(a)** MCF-7 cells were transfected with siNS or siRNA against MLL1 (siMLL1) or SET1A (siSET1A). Histone H3 methylation levels were examined in total cell extracts by immunoblot. **(b)** Effect of MLL1 or SET1A depletion on estrogen-induced levels of *TFF1* and *GREB1* pre-mRNAs. MLL1 or SET1A was depleted from MCF-7 cells as in **a**, and pre-mRNA levels for *TFF1* and *GREB1* were determined by qRT-PCR as in Supplementary Figure 1c, after 0 or 8 hours of treatment with E2. **(c)** Occupancy of EREs of *TFF1* gene by

MLL1 in BRG1-depleted MCF-7 cells. ChIP was performed in MCF-7 cells as in Figure 1c with antibodies against MLL1 after no transfection (upper panels) or transfection with siNS or siBRG1 (lower panels), followed by treatment with E2 for 0 or 60 min. qPCR was performed on the precipitated DNA using primers against the indicated ERE of the *TFF1* gene or against a non-specific region at − 4.8 kb relative to the TSS (− 4.8 kb). Results shown are mean and s.d. of three PCR reactions performed on the same DNA sample and are from a single experiment which is representative of two independent experiments.

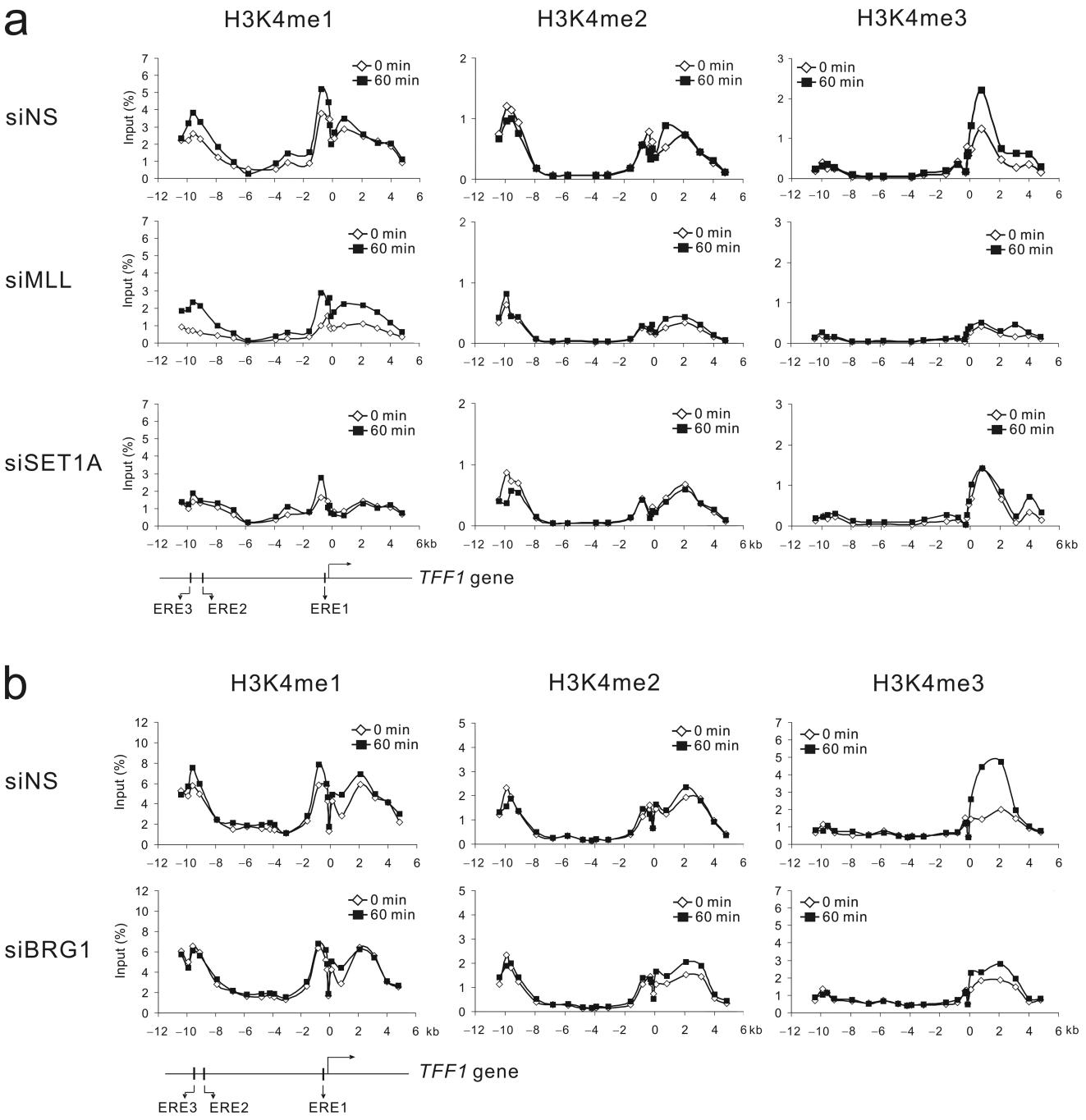

Figure 6.

H3K4 methylation status of the *TFF1* locus in MCF-7 cells depleted of MLL1, SET1A or BRG1. **(a)** E2-dependent H3K4 methylation across the *TFF1* locus. MCF-7 cells were transfected with siNS, siMLL1 or siSET1A and then treated with E2 for 0 or 60 min before performing ChIP with antibodies against mono-, di-, or trimethyl H3K4. Precipitated DNA was analyzed by qPCR with primer sets spaced at about 1-kb intervals and spanning the region from − 10 kb to + 5 kb relative to the TSS (designated by horizontal arrow in the diagram) of the *TFF1* gene. Primer sequences for ChIP scanning are available upon request.

(b) Role of BRG1 in H3K4 methylation at the *TFF1* locus. MCF-7 cells were transfected with siNS or siBRG1 and then treated with E2 for 0 or 60 min before performing ChIP with antibodies against mono-, di-, or trimethyl H3K4, as in **a**. Results shown are mean and range of variation of duplicate PCR reactions performed on the same DNA sample and are from a single experiment which is representative of two independent experiments.

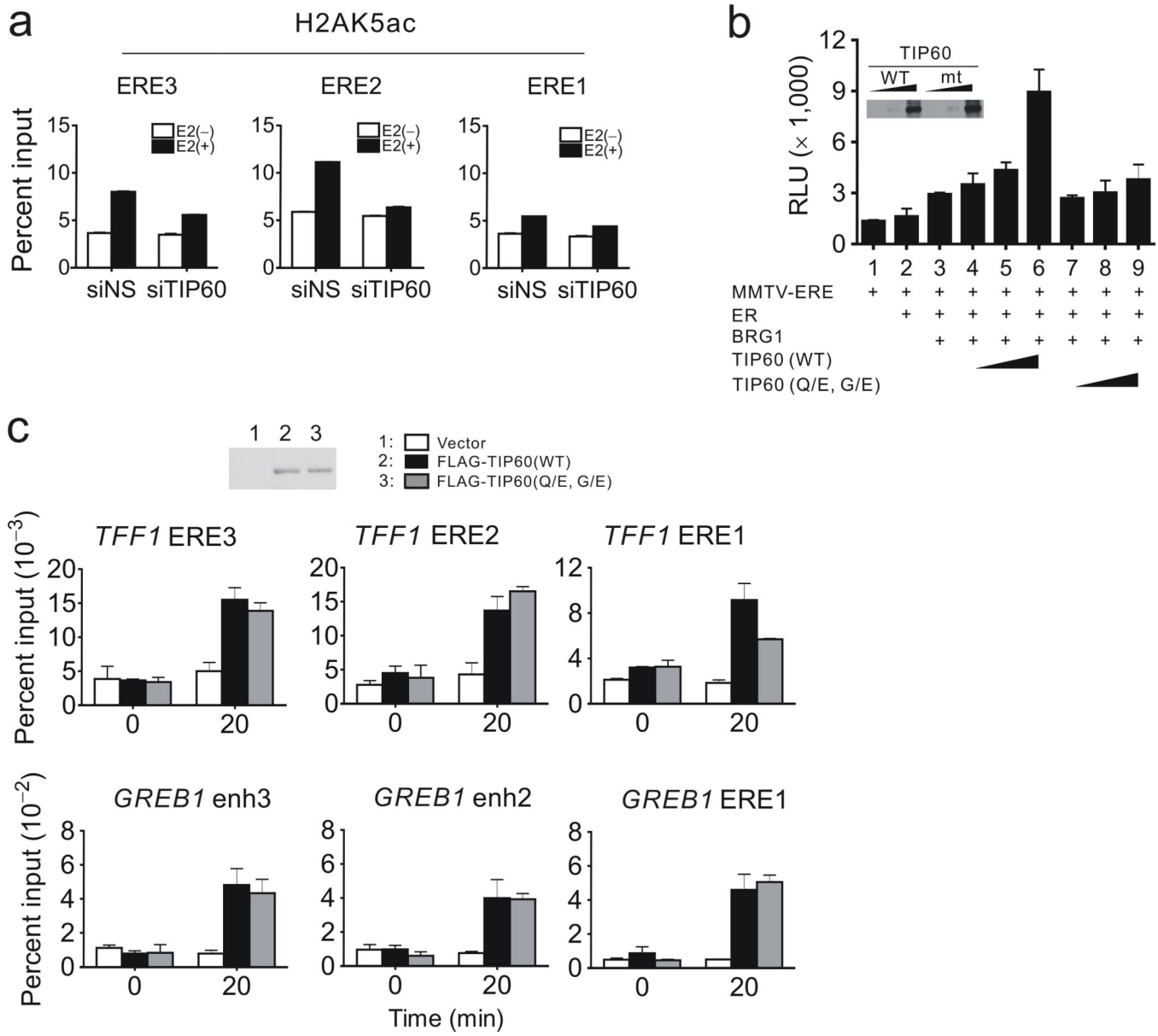

Figure 7.

Role of TIP60 histone acetyltransferase activity in TIP60 coactivator function. **(a)** E2-induced acetylation of histone H2A Lys5 by TIP60 at EREs of the *TFF1* gene. ChIP was performed as in Figure 1c with antibodies against H2AK5ac, using MCF-7 cells transfected with siTIP60 or siNS and treated with E2 for 0 or 60 min. **(b)** Histone acetyltransferase activity is required for TIP60 coactivator function. Luciferase assay in SW13 cells using wild type or enzymatically dead mutant TIP60 was performed as in Figure 3b. Results shown are mean and s.d. of triplicate biological replicates and are from a single experiment which is representative of 3 independent experiments. Expression of wild type and mutant (mt or Q/E, G/E) TIP60 was monitored by immunoblot using antibodies against the FLAG epitope (inset). **(c)** Histone acetyltransferase activity of TIP60 is not required for recruitment of TIP60 to target genes of ERα. Empty plasmid or plasmid encoding FLAG-tagged wild

type or enzymatically dead mutant TIP60 was transiently transfected into MCF-7 cells, and ChIP and immunoblot were performed as in Figure 2c using anti-FLAG antibody. Results shown are mean and s.d. of three PCR reactions performed on the same DNA sample and are from a single experiment which is representative of two independent experiments.

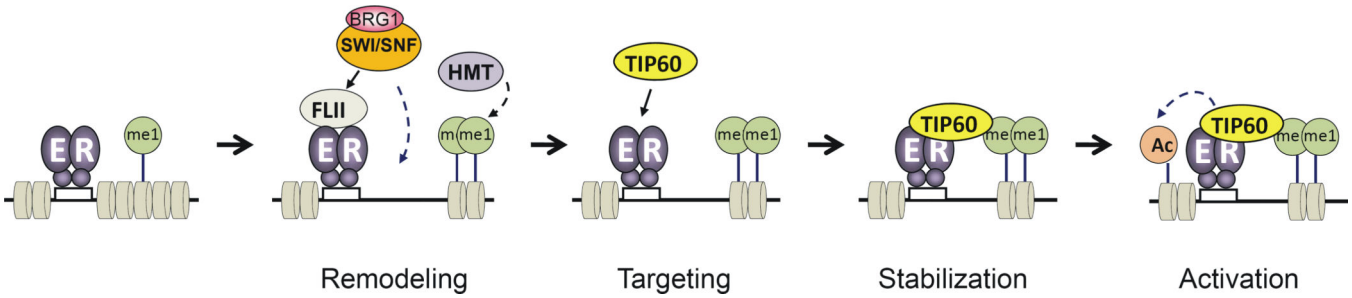

Figure 8.

Proposed model for recruitment of TIP60 to target genes of ERα. After E2-induced binding of ERα to the ERE, coactivator FLII binds to ERα and recruits SWI/SNF chromatin remodeling complex[19]. SWI/SNF complex remodels chromatin structure to allow histone methyltransferases, such as MLL1 or SET1A, to be recruited and increase the methylation of histone H3K4. Initially, TIP60 is recruited by the interaction with ERα via LXXLL motif at C-terminus of TIP60, then TIP60 binds to monomethylated histone H3K4 via its chromodomain to prolong its occupancy on the ERα target gene. Acetylation of H2AK5 by TIP60 contributes to chromatin remodeling, leading eventually to transcription complex assembly.