

# Enhancer Activation Requires *trans*-Recruitment of a Mega Transcription Factor Complex

Zhijie Liu,<sup>1,\*</sup> Daria Merkurjev,<sup>1,2</sup> Feng Yang,<sup>1</sup> Wenbo Li,<sup>1</sup> Soohwan Oh,<sup>1,3</sup> Meyer J. Friedman,<sup>1</sup> Xiaoyuan Song,<sup>1,4</sup> Feng Zhang,<sup>1</sup> Qi Ma,<sup>1,2</sup> Kenneth A. Ohgi,<sup>1</sup> Anna Krones,<sup>1</sup> and Michael G. Rosenfeld<sup>1,\*</sup>

<sup>1</sup>Howard Hughes Medical Institute, Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, CA 92093, USA

<sup>2</sup>Graduate Program in Bioinformatics and System Biology, University of California, San Diego, La Jolla, CA 92093, USA

<sup>3</sup>Graduate Program in Biological Sciences, University of California, San Diego, La Jolla, CA 92093, USA

<sup>4</sup>CAS Key Laboratory of Brain Function and Disease and School of Life Sciences, University of Science and Technology of China, Hefei 230027, China

\*Correspondence: z9liu@ucsd.edu (Z.L.), mrosenfeld@ucsd.edu (M.G.R.)

<http://dx.doi.org/10.1016/j.cell.2014.08.027>

## SUMMARY

Enhancers provide critical information directing cell-type-specific transcriptional programs, regulated by binding of signal-dependent transcription factors and their associated cofactors. Here, we report that the most strongly activated estrogen (E<sub>2</sub>)-responsive enhancers are characterized by *trans*-recruitment and *in situ* assembly of a large 1–2 MDa complex of diverse DNA-binding transcription factors by ERα at ERE-containing enhancers. We refer to enhancers recruiting these factors as mega transcription factor-bound in *trans* (MegaTrans) enhancers. The MegaTrans complex is a signature of the most potent functional enhancers and is required for activation of enhancer RNA transcription and recruitment of co-activators, including p300 and Med1. The MegaTrans complex functions, in part, by recruiting specific enzymatic machinery, exemplified by DNA-dependent protein kinase. Thus, MegaTrans-containing enhancers represent a cohort of functional enhancers that mediate a broad and important transcriptional program and provide a molecular explanation for transcription factor clustering and hotspots noted in the genome.

## INTRODUCTION

Functional specialization and precise patterning of different cell and tissue types are vital for all metazoans, which also generate cell- or tissue-specific gene expression patterns. Enhancers, initially defined as DNA elements that act over a distance to positively regulate expression of protein-encoding target genes, are the principle regulatory components of the genome that enable such cell-type-specific and signal-dependent patterns of gene expression (Banerji et al., 1981; Shlyueva et al., 2014). Each cell type harbors more than 100,000 candidate enhancers in

humans, vastly outnumbering protein-coding genes (Bernstein et al., 2012; Heintzman et al., 2009; Shlyueva et al., 2014). This makes it very important to be able to predict and understand which enhancers are actually functionally required for target coding gene transcriptional regulation.

Enhancer activation requires the presence of specific recognition sequences for the cooperative recruitment of DNA-binding transcription factors (TFs) and their cofactors that initially activate gene expression (Rosenfeld et al., 2006). While the role of a large number of coactivator complexes and their associated enzymatic activities is well established (Rosenfeld et al., 2006), the precise biochemical mechanisms by which so many coactivators are recruited and required for the different functional activities at specific enhancer sites remain incompletely understood. Global genomic technologies have uncovered characteristic markers of enhancers and have provided clues as to their activation. Features that have been used to predict enhancers that are likely to be functional include the levels of enhancer RNAs (eRNAs) transcribed from enhancer-like regions in the genome (Li et al., 2013), the presence of the histone acetyltransferase p300/CBP (Visel et al., 2009), the timing of RNA Pol II occupancy (Bonn et al., 2012), and levels of H3K4me2 and H3K27Ac (Chepelev et al., 2012; Heintzman et al., 2009). However, because enhancers identified using these features are not equally functional, additional methods are needed to distinguish the enhancers with different activation potential.

There are ~2,600 DNA-binding TFs encoded by the human genome (Babu et al., 2004), with ~200–300 TFs being expressed in each cell type (Vaquerizas et al., 2009). A long-standing question is how different TFs collaborate to regulate the enhancer network in a specific cell type. With the large expansion of genome-wide binding data, DNA-binding transcription factors were noted to co-bind to some so-called “hotspot” regions or to cooperatively cluster to some functional enhancers in various organisms or cell lines (Junion et al., 2012; Rada-Iglesias et al., 2012; Siersbæk et al., 2014a, 2014b; Wilson et al., 2010; Yan et al., 2013). However, the underlying mechanism(s) and functional significance of this phenomenon are not well understood.

Recently, the idea of clustered enhancers associated with critical developmental or cancer-associated transcription units has

been proposed (Hnisz et al., 2013; Lovén et al., 2013; Whyte et al., 2013). The initial definition of this super-enhancer model was described as clusters of enhancers spanning >8–10 kb, occupied by critical DNA-binding transcription factors at their cognate binding motifs (Lovén et al., 2013; Whyte et al., 2013). These clustered super-enhancers control key coding transcription units in stem cells or various disease states and exhibit high levels of co-activators, which are suggested to contribute to gene activation. Cancer cells were also noted to acquire super-enhancers regulating oncogene drivers (Hnisz et al., 2013; Lovén et al., 2013). While the super-enhancer model can explain the higher expression levels for a small number of genes in some environments, it also highlights the need for exploring the functional activities of single enhancers in the regulation of coding genes critical for development and disease and understanding the phenomenon of TF clustering in short-range genomic regions.

Here, we report a signature of the functionally active estrogen-regulated enhancers, particularly the 1,333 most active ER $\alpha$  enhancers linked to target coding gene activation. This signature is the selective recruitment in *trans* of an apparent complex of other DNA-binding TFs, including RAR $\alpha/\gamma$ , GATA3, AP2 $\gamma$ , STAT1, AP1, and FoxA1. By gel filtration, we found these TFs migrated with ER $\alpha$  as a 1–2 MDa complex(es), referred to as the MegaTrans complex. The MegaTrans complex is almost invariably recruited to functional ER $\alpha$ -bound enhancers, ~22% of which fit the criteria of being components of super-enhancers. Furthermore, the MegaTrans complex is required for activation of the functional enhancers, apparently based in part on specific recruitment of enzymes. This is exemplified by the functionally important recruitment of the DNA-dependent protein kinase to ER $\alpha$ -regulated enhancers by RARs. The MegaTrans complex, in turn, is also required for activation of eRNA transcription and recruitment of coactivators, including p300 and Med1, and thus exerts critical biological functions, conceptually parallel to what has been proposed for super-enhancers.

## RESULTS

### **trans**-Bound RARs on ER $\alpha$ Active Enhancers Regulate ER $\alpha$ Enhancer Function

ER $\alpha$  functions as a central transcription factor for gene programs that mediate cell growth and proliferation, and it accomplishes this role primarily through enhancer regulation. Among the total ~7,174 ER $\alpha$ -bound enhancers, a subset of 1,333 enhancers that are located in proximity (<200 kb) to their regulated coding transcription units have proved to be the most significantly activated upon estrogen stimulation according to levels of H3K27Ac and increased eRNA transcription and appear to constitute the most potent functional enhancer program (Li et al., 2013).

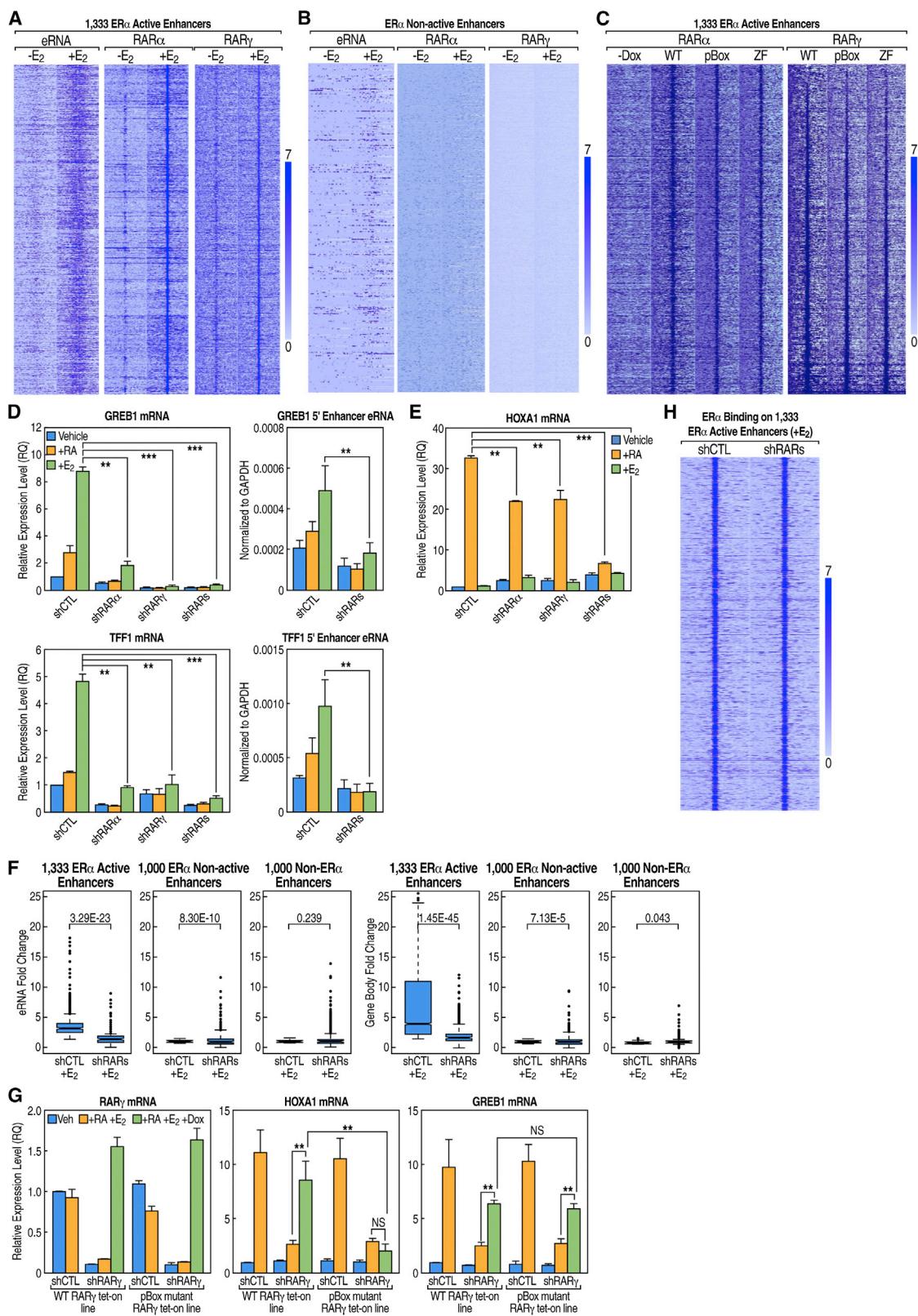
Our current study was initiated by investigating the possible functional mechanisms by which RARs on retinoic acid response element (RARE)-containing enhancers mediate RA-induced coding gene transcriptional programs, as well as the functional role(s) of RAR at enhancers that accommodate the effects of other signals, such as E $_2$ -induced coding gene transcriptional programs (Hua et al., 2009; Ross-Innes et al., 2010). To distinguish the possible binding in *cis* (the chromatin association of a transcription factor through direct DNA binding at its recognition sites)

and in *trans* (the chromatin association of a transcription factor through protein-protein interaction) functional models of RAR, we engineered MCF7 to express a bacterial biotin ligase (BirA) that can biotinylate a biotin ligase recognition peptide (BLRP)-tagged protein in vivo (Figure S1A available online). Under control of a Tet-On promoter, wild-type RAR and two DNA-binding domain mutants that cannot bind to RARE DNA sites (Figures S1B and S1C) were expressed at similar levels as the endogenous proteins upon doxycycline induction (Figure S1D). Using these lines, we first performed biotin chromatin immunoprecipitation sequencing (ChIP-seq) for wild-type (WT) and mutant RAR $\alpha/\gamma$  (RAR $\beta$  is not expressed in MCF7 breast cancer cells) upon RA and E $_2$  stimulation. Comparing wild-type and non-DNA-binding mutants, we found that ~15,000/18,000 of WT RAR $\alpha/\gamma$ -bound sites required the intact RAR DNA-binding ability because binding was lost with mutant RARs, and none of these sites were bound by ER $\alpha$  (Figures S1E–S1G). Among these 15,000 sites, 3,540 were enhancers that exhibited RA activation (Figure S1E), exemplified by the ~700 most active RAR *cis*-binding enhancers, which showed significant RA-induced eRNA and gene target activation by global run-on sequencing (GRO-seq) (Figure S1H).

However, there were ~3,000 RAR $\alpha/\gamma$  binding sites that did not depend on RAR DNA-binding ability (Figures S1E and S2A). Remarkably, we found that both RAR $\alpha$  and RAR $\gamma$  were recruited to virtually all of the ER $\alpha$ -bound 1,333 active enhancers in response to E $_2$  (Figure 1A). This observation is consistent with previous evidence that RAR can bind to ER $\alpha$  binding sites, although conflicting conclusions were reached regarding its activating or repressive effects (Hua et al., 2009; Ross-Innes et al., 2010). However, ER $\alpha$  did not exhibit colocalization with RARs on ER $\alpha$  non-active enhancers (Figure 1B). By comparing the binding patterns of wild-type and two non-DNA-binding mutants, we found the binding of RARs on the 1,333 ER $\alpha$  active enhancers was in *trans* (Figures 1C and S2A).

Knockdown of either RAR $\alpha$  or RAR $\gamma$  caused a significant decrease in both E $_2$ -dependent induction of eRNAs and activation of target coding genes, while knockdown of both caused almost complete inhibition, as assessed by quantitative PCR (qPCR) of targets such as GREB1 and TFF1 (Figure 1D). The knockdown of RAR $\alpha$  and RAR $\gamma$ , which was confirmed for both RNA and protein levels (Figures S2B and S2C), inhibited RA induction of the HoxA1 gene target as expected (Figure 1E). Box-plot analysis of the GRO-seq experiments showed that the presence of RARs was required for effective induction of both eRNAs and target coding gene transcription units upon E $_2$  treatment (Figures 1F and S2D). RAR $\alpha/\gamma$  knockdown also inhibited classical RAR *cis*-bound enhancers and their target genes (Figures S1H and S2E). Thus, while RAR binding in *cis* activates a distinct RA-responsive transcriptional program, its recruitment in *trans* is also required for effective E $_2$ -dependent activation of ER $\alpha$ -bound functional enhancers.

Next, we utilized wild-type and pBox mutant RAR $\gamma$  to test their ability to rescue ER $\alpha$ -regulated enhancer function following endogenous RAR $\gamma$  knockdown (Figure S2F). Intriguingly, the non-DNA-binding mutant receptor continued to be effectively recruited to the ER $\alpha$ -bound regulatory enhancers at the GREB1 gene (Figure S2A) and was capable of restoring full E $_2$ -dependent GREB1 gene activation in rescue experiments (Figure 1G).



(legend on next page)

However, as expected, it failed to activate the *cis*-bound, RAR-regulated *HoxA1* gene (Figure 1G).

Administration of ICI 182780 to knockdown ER $\alpha$  caused a loss of RAR binding at the ER $\alpha$ -regulated enhancers (Figures 2D, S3C, and S3D), but did not alter the binding of RAR $\alpha$  or RAR $\gamma$  at activated enhancers harboring *cis* RAR binding sites (data not shown). Knockdown of RAR did not cause downregulation of ER $\alpha$  RNA or protein levels (Figures S2B and S2C) and did not affect the ER $\alpha$  binding pattern on ER $\alpha$  active enhancers (Figure 1H).

Collectively, our data indicate that ER $\alpha$  selectively recruits RAR $\alpha$  and RAR $\gamma$  in *trans* on the functional enhancers regulating the most robustly activated target coding genes and that this strong activation depends on the ER $\alpha$ -mediated *trans*-binding of RARs.

### ER $\alpha$ Recruits a Mega DNA-Binding Transcription Factor Complex In Situ at Functional ER $\alpha$ Enhancers

These findings prompted us to examine the behavior of additional DNA-binding TFs associated with ER $\alpha$ , based on previously reported mass spectrometry analysis of proteins that coimmunoprecipitated with ER $\alpha$  (Mohammed et al., 2013) as well as our own confirmatory data. From these ER $\alpha$  complex data, we noted a number of DNA-binding transcription factors associated with ER $\alpha$ , including RAR $\gamma$ , GATA3, AP2 $\gamma$ , STAT1, and, intriguingly, FoxA1. To complement these observations, we also examined the proteins associated with RAR following pull down from MCF7 cells stably expressing, at physiological levels, biotin-tagged RAR $\alpha$  (Figures S1D and S3A). In addition to RAR $\alpha$ , RXRs, and many well-known cofactors for nuclear receptors, GATA3 was also detected along with other DNA-binding proteins including AP2 $\gamma$ , STAT1, c-Fos, and FoxA1 (Figure 2A). We then performed gel filtration analysis on nuclear extracts prepared from MCF7 cells in the absence of DNase treatment and analyzed all fractions for ER $\alpha$ , RAR $\alpha/\gamma$ , GATA3, and the other DNA-binding transcription factors identified in the mass spectrometry analysis. This analysis revealed co-elution of ER $\alpha$ , RAR $\alpha$ , RAR $\gamma$ , GATA3, AP2 $\gamma$ , FoxA1, STAT1, c-Fos, and other proteins in an estimated 1–2 MDa complex(es) (Figure 2B). These components were all present in ER $\alpha$ -immunoprecipitates from nuclear extracts and their association was enhanced upon E<sub>2</sub> treatment (Figure S3B). Importantly, knockdown of nuclear ER $\alpha$  by administration of ICI 182780 caused a virtual loss of the entire complex associated with ER $\alpha$  by gel filtration analysis

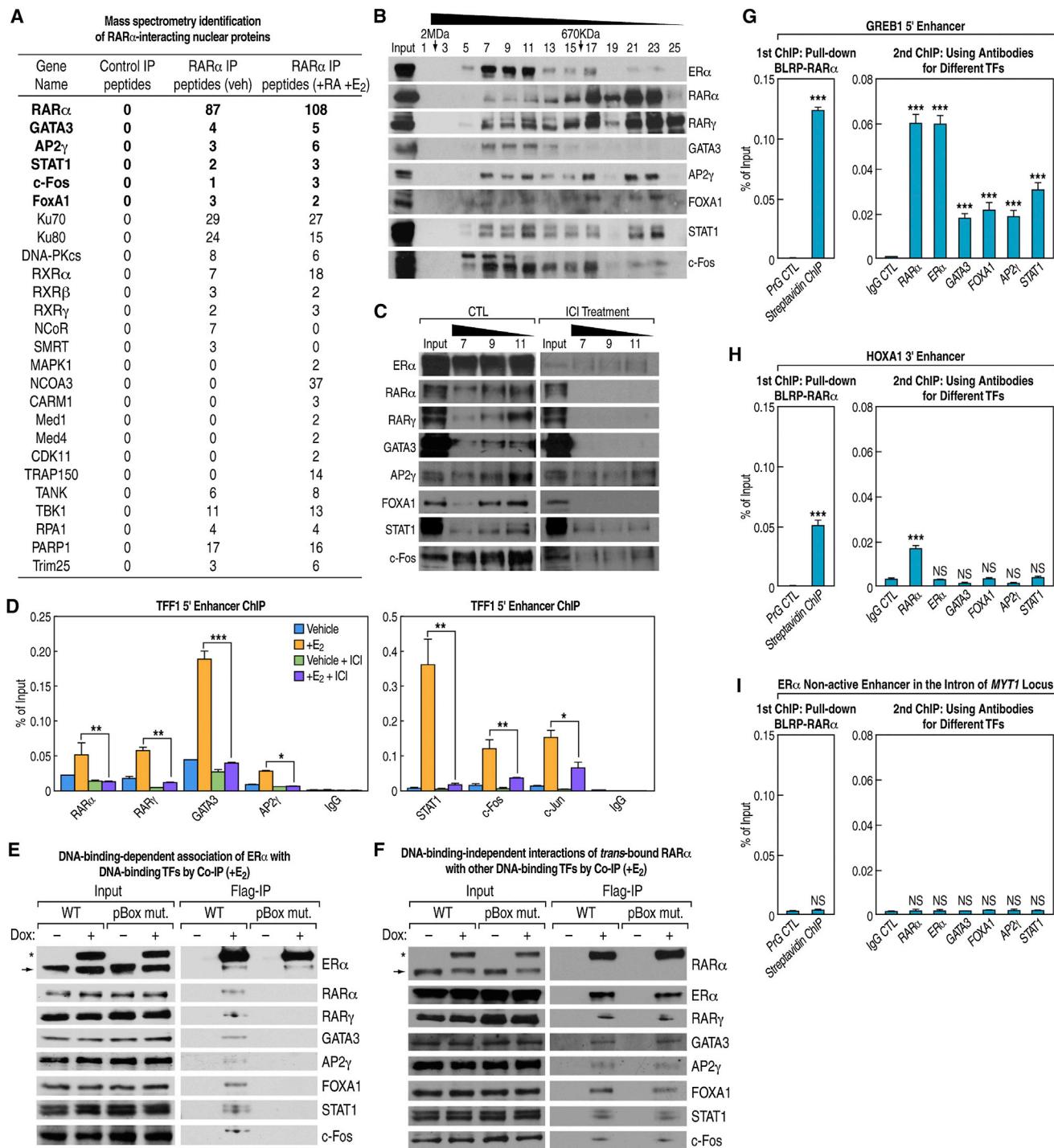
(Figures 2C and S3C) and recruitment of each factor to ER $\alpha$ -bound functional enhancers (Figures 2D and S3D). Thus, the material co-migrating in the gel filtration represented proteins interacting as a complex with ER $\alpha$  rather than artifacts. This complex remained intact in the presence of 250 mM NaCl, but was lost under 600 mM NaCl high-salt conditions (data not shown).

To further investigate the hypothesis that the ER $\alpha$ -dependent *trans*-recruitment/assembly of other DNA-binding transcription factors occurs only *in situ* at ER $\alpha$  active enhancers, we first confirmed that the interactions between ER $\alpha$  and the TFs were dependent on DNA (Figure S3E). Using a non-DNA-binding ER $\alpha$  pBox mutant, which is incapable of binding the estrogen response element (ERE) motif (Stender et al., 2010), we could show that this mutation abolished the interactions of ER $\alpha$  and these associated TFs (Figure 2E). As a control, a comparable RAR $\alpha$  pBox mutant did not affect its interaction with ER $\alpha$  and these TFs (Figure 2F). These data suggest that RAR $\alpha$  and other TFs are recruited by ERE-bound ER $\alpha$  to its activated enhancers; thus, the entire complex is assembled *in situ* on ER $\alpha$ -bound enhancers.

To further confirm that these factors were, indeed, co-recruited to the same transcription units, rather than the consequence of differential recruitment behavior in different cell populations, we performed serial pairwise two-step ChIP analyses to assess the co-recruitment of RAR $\alpha$  with ER $\alpha$ , GATA3, FoxA1, AP2 $\gamma$ , and STAT1 on the same ER $\alpha$ -bound enhancers. Using a BLRP-tagged RAR $\alpha$  stable cell line, two-step ChIP was performed with biotin-streptavidin pull-down of RAR $\alpha$  in the first round followed by immunoprecipitation with antibodies for RAR $\alpha$  (as positive control), ER $\alpha$ , GATA3, FoxA1, AP2 $\gamma$ , and STAT1. In each case, we found that these proteins were present on the interrogated active enhancers, including the *GREB1* enhancer (Figure 2G). In contrast, as a control, this was not the case for the RAR *cis*-bound enhancer regulating the *HoxA1* transcription unit (Figure 2H). Thus, the MegaTrans complex was co-recruited to ER $\alpha$ -bound active enhancers but not to functional enhancers that directly bind RAR $\alpha$  in *cis*. RAR $\alpha$  and the other TFs also were not present at ER $\alpha$ -bound, non-active enhancers (Figure 2I). Double-ChIP experiments performed with a BLRP-tagged GATA3 stable line similarly demonstrated the co-binding of GATA3 with ER $\alpha$  and all of the other TFs at ER $\alpha$  active enhancers but not at either the *HoxA1* enhancer or ER $\alpha$  non-active enhancers (Figure S3F). Together, these data indicate that a feature

**Figure 1. trans-Bound RARs on ER $\alpha$  Active Enhancers Regulate E<sub>2</sub>-Ligated Transcription Activation**

- (A) Heatmaps of GRO-seq and ChIP-seq data ( $\pm$ E<sub>2</sub>) for 1,333 ER $\alpha$  active enhancers showing strong E<sub>2</sub>-induced eRNA transcription and E<sub>2</sub>-enhanced binding of both RAR $\alpha$  and RAR $\gamma$ , respectively.
  - (B) Heatmaps of GRO-seq and ChIP-seq for a control group of ER $\alpha$  non-active enhancers exhibiting no RAR $\alpha/\gamma$  binding and no significant E<sub>2</sub>-induced eRNA transcription.
  - (C) For the 1,333 ER $\alpha$  active enhancers, heatmaps of ChIP-seq data for the wild-type and two DNA-binding mutants of RAR $\alpha/\gamma$  (+RA and E<sub>2</sub>) show that their association with these enhancers is DNA binding independent.
  - (D) Knockdown of either RAR $\alpha$  or RAR $\gamma$  by shRNA inhibits ER $\alpha$  target gene induction by E<sub>2</sub>, as demonstrated by qPCR analysis.
  - (E) Knockdown of either RAR $\alpha$  or RAR $\gamma$  using shRNA inhibits expression of the RAR *cis*-binding target *HoxA1* gene in response to RA, as shown by qPCR analysis.
  - (F) RARs are required for the E<sub>2</sub>-liganded activation of ER $\alpha$  active enhancers and their targets, as shown by GRO-seq boxplots. No significant effects were found for either ER $\alpha$  non-active enhancers or non-ER $\alpha$  enhancers.
  - (G) The pBox mutant RAR $\gamma$  fails to rescue expression of its *cis*-binding target *HoxA1* after knockdown of endogenous RAR $\gamma$ . In contrast, both wild-type and pBox mutant RAR $\gamma$  can rescue expression of the *trans*-binding target *GREB1*. For details regarding rescue experiments see Extended Experimental Procedures.
  - (H) Heatmap showing that knockdown of RARs does not affect ER $\alpha$  binding at the 1,333 active enhancers.
- Data are represented as mean  $\pm$  SEM. NS, not significant. \*\*p < 0.01, \*\*\*p < 0.001. See also Figures S1 and S2.



**Figure 2. ER $\alpha$  Interacts with a Mega Complex of DNA-Binding Transcription Factors at ERE-Containing Active Enhancers**

(A) RAR $\alpha$  associates with several DNA-binding TFs, as shown by mass spectrometry analysis after pull down of biotin-tagged RAR $\alpha$  and elution with TEV protease digestion. The same inducible BLP-tagged RAR $\alpha$  stable cell line without doxycycline induction was used as a control.

(B) Western blots of gel filtration samples from MCF7 nuclear lysates (+E<sub>2</sub>) show various DNA-binding TFs associate with ER $\alpha$  in 1–2 MDa fractions.

(C) Knockdown of ER $\alpha$  by ICI 182780 causes loss of the DNA-binding TFs in 1–2 MDa ER $\alpha$ -containing complex, as revealed by immunoblotting of gel filtration fractions from the 1–2 MDa range (fractions 7, 9, and 11).

(D) DNA-binding TFs in the ER $\alpha$  complex bind to an ER $\alpha$  active enhancer at *TFF1* locus upon E<sub>2</sub> signal, and knockdown of ER $\alpha$  reduces their binding. ChIP signals are presented as percentage of input.

(legend continued on next page)

of the active, regulatory ER $\alpha$ -bound enhancers, in addition to their increased levels of eRNA transcription, is the selective recruitment of this MegaTrans complex.

### **trans-Bound GATA3 Also Regulates Functional ER $\alpha$ Enhancers**

To explore the possible functional consequences of the additional ER $\alpha$ -interacting transcription factors, we next explored the potential recruitment and function of GATA3 on ER $\alpha$  active and non-active enhancers. ChIP-seq experiments revealed, as in the case of RAR $\alpha$  and RAR $\gamma$ , that GATA3 was recruited in an E<sub>2</sub>-dependent fashion to active enhancers (Figure 3A) but not non-active enhancers (Figure 3B). Because we found the presence of GATA3 on functional ER $\alpha$ -bound enhancers that did not harbor apparent GATA3 *cis*-binding elements by motif analysis, we again assessed the possibility that GATA3 was recruited in *trans* to these ER $\alpha$ -bound active enhancers. Knockdown of ER $\alpha$  by administration of ICI 182780 inhibited GATA3 recruitment to ER $\alpha$  active enhancers (Figures 2D and S3D). Because direct or indirect ER $\alpha$  and GATA3 interactions were suggested by immunoprecipitation experiments (Figure S3B), we investigated the consequences of disrupting the ability of GATA3 to bind to cognate DNA sites by two different mutations of the second zinc finger that is required for *cis*-binding of GATA3 (Nesbit et al., 2004) (Figure S4A). We generated inducible BLRP-tagged stable lines expressing wild-type and the two DNA-binding mutants at physiological levels (Figure S4B), and biotin ChIP-seq revealed they were equivalently recruited, apparently in *trans*, to these ER $\alpha$ -bound active enhancers (Figures 3B and S4C). By comparing the ChIP-seq data for wild-type and DNA-binding mutants, we found that among ~18,000 wild-type GATA3 binding peaks ~5,000 were retained in the two GATA3 mutants, and these *trans*-binding sites featured ERE as the top motif by Homer analysis (Figure S4D). For the ~13,000 *cis*-binding peaks, GATA motifs were enriched and a heatmap of the non-ER $\alpha$  enhancers containing a GATA motif was used to confirm a total loss of binding of the two non-DNA-binding GATA3 mutants (Figures 3C and S4D).

Using qPCRs or GRO-seq analysis, we explored the consequences of specific siRNA-mediated knockdown of GATA3 on E<sub>2</sub>-dependent induction of eRNAs. We found a dramatic inhibition of the eRNA activation events on active enhancers (Figures 3D, 3E, and S4E) but no effect on ER $\alpha$ -bound non-active enhancers or non-ER $\alpha$ -bound enhancers (Figure 3E). The same inhibition effects were also found for gene body expression of the targets of these 1,333 ER $\alpha$  active enhancers (Figures 3D, 3E, and S4E). Knockdown of GATA3 did not affect ER $\alpha$  gene expression at either the RNA or protein level (Figures S4F and S4G) or ER $\alpha$  binding at active enhancers (Figure 3F). Thus, GATA3 and RARs, as components of a complex of DNA-binding TFs associated in

*trans* with ER $\alpha$  on active enhancers, are required for E<sub>2</sub>-dependent enhancer activation.

### **ER $\alpha$ Active Enhancers Are Regulated by the MegaTrans Complex**

We next investigated whether other DNA-binding transcription factors in the MegaTrans complex co-migrating with ER $\alpha$  were also recruited to E<sub>2</sub>-activated enhancers even in the absence of their cognate DNA-binding elements. We reviewed our own and published ChIP-seq data from MCF7 cells for other DNA-binding TFs present in the MegaTrans complex (Joseph et al., 2010; Theodorou et al., 2013). E<sub>2</sub>-regulated active enhancers were found to harbor AP2 $\gamma$ , FoxA1, c-Jun, and c-Fos, along with RAR $\alpha/\gamma$  and GATA3 (Figures 4A and 4B), but these TFs were not present on non-active enhancers (Figures 4A and S5A). Similar to RAR $\alpha/\gamma$  and GATA3, the recruitment of the other TFs was also increased by E<sub>2</sub> and abolished by knockdown of nuclear ER $\alpha$  using ICI 182780 (Figures 4C, 2D, and S3D).

In order to investigate whether, in fact, all DNA-binding transcription factors present in the MegaTrans complex were recruited in *trans* to ER $\alpha$  functional enhancers, a series of DNA-binding domain mutations were generated for AP2 $\gamma$ , c-Fos, c-Jun, and STAT1. ChIP-qPCR data on the *GREB1* and *TFF1* enhancers showed that the binding of the non-DNA-binding mutants at these two ER $\alpha$  active enhancers was comparable to that of the wild-type proteins (Figure 4D), which confirms the *trans*-recruitment of these TFs by ER $\alpha$ .

Based on the roles of RARs and GATA3 on ER $\alpha$  active enhancers, we evaluated the functional effects of other recruited transcription factors. Beginning with AP2 $\gamma$ , we found that, in addition to its recruitment in response to E<sub>2</sub> on ER $\alpha$  regulatory enhancers (Figure 4C), knockdown of AP2 $\gamma$  caused a dramatic inhibition of eRNA and target coding gene expression, as assayed by both qPCR and GRO-seq (Figures 4E, S5B, and S5C). Similarly, as STAT1 was also recruited to ER $\alpha$ -bound enhancers (Figures 2D and S3D), we evaluated its effect on two well-described ER $\alpha$  bound/regulated enhancers. Again, we found a functional contribution to the outcome of E<sub>2</sub>-induced activation of enhancer transcription and target coding gene expression (Figure 4F). The same regulatory effects were also demonstrated upon knockdown of two AP1 components, c-Jun and c-Fos (Figures S5D and S5E), that were present in the MegaTrans complex (Figures 4A and 4B).

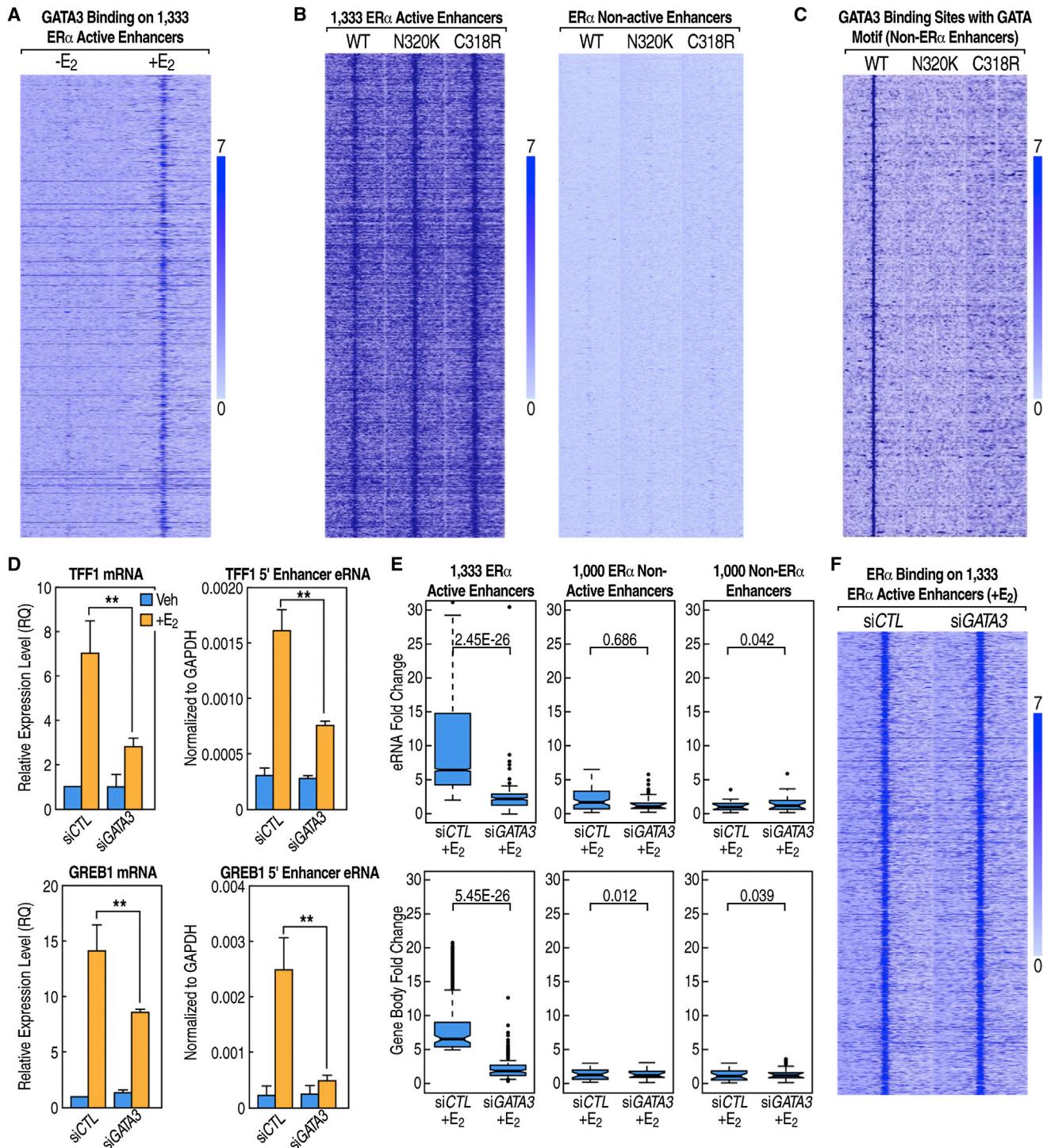
To begin to assess the interdependency of the components of the MegaTrans complex on recruitment to ER $\alpha$ -bound functional enhancers, we tested the consequences of knockdown of RAR $\alpha/\gamma$ , GATA3, and AP2 $\gamma$  on *GREB1* and *TFF1* enhancer occupancy. We found a marked inhibition of recruitment of other MegaTrans components upon knockdown of RAR $\alpha/\gamma$  and GATA3 (Figures 4G and 4H) but not by knockdown of AP2 $\gamma$ .

(E) The interaction of ER $\alpha$  with other DNA-binding TFs is dependent on its DNA-binding ability, as shown by coimmunoprecipitation using BLRP-tagged WT or pBox mutant ER $\alpha$ . The asterisk marks BLP-tagged ER $\alpha$ , and the arrow marks endogenous ER $\alpha$ .

(F) The interaction of RAR $\alpha$  with other DNA-binding TFs is independent of its DNA-binding ability, as demonstrated by coimmunoprecipitation of BLP-tagged WT or pBox mutant RAR $\alpha$  and other TFs. The asterisk marks BLP-tagged RAR $\alpha$ , and the arrow marks endogenous RAR $\alpha$ .

(G–I) ChIP-reChIP analysis confirms the co-binding of RAR $\alpha$ , ER $\alpha$ , and other DNA-binding TFs on ER $\alpha$  active enhancers but not on the ER $\alpha$  non-active enhancers or RAR-bound *HoxA1* enhancer. ChIP signals are presented as percentage of input and are compared to negative controls.

Data are represented as mean  $\pm$  SEM. NS, not significant. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. See also Figure S3.



**Figure 3. trans-Bound GATA3 on ER $\alpha$  Active Enhancers Regulates ER $\alpha$  E<sub>2</sub>-Ligated Transcription Activation**

(A) Heatmap displaying GATA3 binding at the 1,333 ER $\alpha$  active enhancers is enhanced by E<sub>2</sub>.

(B) Heatmaps of ChIP-seq data for wild-type and two DNA-binding mutants of GATA3 (+E<sub>2</sub>) show the binding of GATA3 to these ER $\alpha$  active enhancers is not dependent on its DNA-binding ability. There is no binding of either wild-type or mutant GATA3 to ER $\alpha$  non-active enhancers.

(C) Heatmap of ChIP-seq data for wild-type and two DNA-binding mutants of GATA3 (+E<sub>2</sub>) shows the binding of GATA3 to these non-ER $\alpha$  enhancers that contain the GATA motif requires its DNA-binding ability.

(legend continued on next page)

(Figure 4I), consistent with interdependency of at least some components of the complex for recruitment of other components. RAR $\alpha/\gamma$  and GATA3 may serve as key functional components, along with ER $\alpha$ , in recruitment/assembly of the MegaTrans complex on functional ER $\alpha$ -bound enhancers.

### FoxA1 Is Required for ER $\alpha$ Recruitment and MegaTrans Complex Stabilization on ER $\alpha$ Active Enhancers

These experiments raised a question about potential differences in DNA sequence features between ER $\alpha$  active enhancers that bound the MegaTrans complex and ER $\alpha$  non-active enhancers that did not. Comparison of the EREs between these two groups revealed that its frequency and the primary consensus sequences were essentially identical (Figure 5A). In contrast, and in accord with the well-known importance of FoxA1 as a pioneer factor for ER $\alpha$  binding (Hurtado et al., 2011), we noted that the functional, MegaTrans-bound 1,333 ER $\alpha$  active enhancers generally harbor a FoxA1 binding motif within 200 bp of the ERE sites, while the FoxA1 motif was virtually absent on the nonfunctional, non-MegaTrans-bound ER $\alpha$  enhancers (Figure 5B). Indeed, the affinity for ER $\alpha$  is >90% lower on the nonfunctional than functional ER $\alpha$ -bound enhancers (Figure 5C). Consistent with FoxA1 functioning as a key determinant of ER $\alpha$  binding (Hurtado et al., 2011), our data showed greatly reduced binding of ER $\alpha$  at the 1,333 ER $\alpha$ -bound active enhancers upon FoxA1 knockdown (Figure 5D).

Because FoxA1 appears to be a component of the MegaTrans complex based on gel filtration and coimmunoprecipitation (co-IP) data (Figures 2B and S3B) and also exhibits E<sub>2</sub>-induced binding at the 1,333 ER $\alpha$  active enhancers (Figure 5E), we speculate that FoxA1 potentially plays dual roles in the binding of ER $\alpha$  to functional enhancers and in ER $\alpha$ -dependent recruitment of the MegaTrans complex. Indeed, knockdown of FoxA1 caused a dramatic impairment of ER $\alpha$  binding on the functional ER $\alpha$  enhancers (Figure 5D), which was accompanied by a loss of recruitment of the MegaTrans complex on this functional enhancer cohort (Figure 5F) and inhibition of both eRNA and gene body activation (Figure 5G). Thus, FoxA1 is distinct from the other DNA-binding TFs in the MegaTrans complex that apparently do not affect ER $\alpha$  binding upon knockdown (Figures 1H and 3F).

### Roles of MegaTrans Complex in Coactivator Recruitment and in Super-Enhancer Function

A basic aspect of the mechanism by which MegaTrans components function is their requirement for effective activation of E<sub>2</sub>-induced eRNAs on the functional enhancers. Accordingly, we assessed the recruitment of the coactivator p300 by qPCR and ChIP-seq upon knockdown of RAR $\alpha/\gamma$  or GATA3. All of these knockdowns inhibited the E<sub>2</sub>-induced accumulation of p300 on activated enhancers (Figures 6A–6C, and S6A), consistent with a previous report of a role for RAR $\alpha$  in p300 recruitment (Ross-In-

nes et al., 2010). Based on the importance of Mediator complex for enhancer function, putatively due to its roles in enhancer:promoter looping events (Kagey et al., 2010), we also evaluated the effects of RAR $\alpha/\gamma$  and GATA3 knockdown on E<sub>2</sub>-dependent recruitment of Med1 to functional enhancers by qPCR, finding a dramatic inhibition following these knockdowns (Figures 6A and 6B). This result was confirmed genome-wide by ChIP-seq (Figures 6D and S6B).

Based on the criteria developed in the initial description of super-enhancers (Hnisz et al., 2013; Whyte et al., 2013), we assessed the number of super-enhancers in MCF7 cells by Med1 ChIP-seq under both -E<sub>2</sub> and +E<sub>2</sub> conditions. While there are only ~122 super-enhancers under the -E<sub>2</sub> condition, E<sub>2</sub> treatment increases the total to ~320 such enhancers (Figure 6E), of which ~212 contained at least one ER $\alpha$ -bound functional enhancer, including one at the c-Myc gene locus (Figure S6C). Thus, only ~300 of the 1,333 ER $\alpha$ -bound functional enhancers characterized by MegaTrans complex fulfill the current definition of being located in super-enhancers. The efficacy of this subset of 300 ER $\alpha$ -bound active enhancers was only slightly better than the other 1,033 ER $\alpha$ -bound active enhancers with respect to eRNA induction (Figure 6F). Thus, the functional strength of the ER $\alpha$ -bound enhancers, irrespective of their presence in a super-enhancer, is predicted by the presence of the MegaTrans complex.

Actually, for the 212 super-enhancers that contain ER $\alpha$  active enhancers, their Med1 levels were also dependent on the E<sub>2</sub> signal (Figure S6C). Interestingly, we observed greatly reduced levels of Med1 at these 212 super-enhancers following knockdown of RARs (Figure 6G), suggesting that MegaTrans enhancers are important constituents in the function of these clustered super-enhancers.

### DNA-Binding TFs of the MegaTrans Complex Might Recruit Specific Functionally Required Components for Enhancer Activation

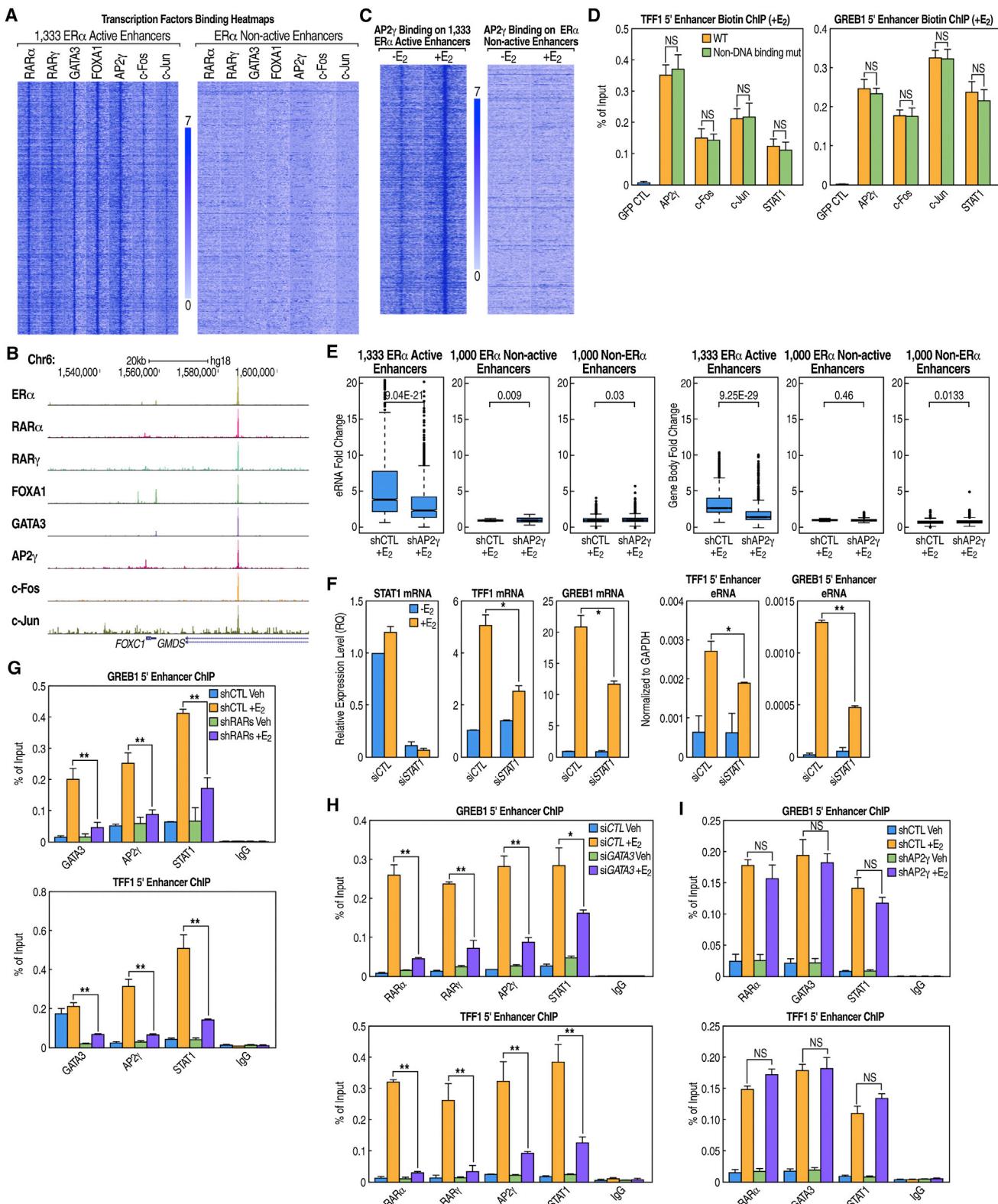
Based on the presence of specific non-transcription factor components in the mass spectrometry analysis of RAR $\alpha$ -associated proteins (Figure 2A), we evaluated the functional significance of these additional proteins. We elected to focus on DNA-dependent protein kinase (DNA-PK), comprising the catalytic subunit DNA-PKcs, Ku70, and Ku80, because all three DNA-PK subunits were present in the RAR $\alpha$  pull down as revealed by mass spectrometry. We confirmed these associations by co-IP and western blot analysis (Figure 7A). DNA-PKcs has previously been reported as a component of the ER $\alpha$  complex that directly phosphorylates S118 of ER $\alpha$  (Foulds et al., 2013), and we confirmed that knockdown of DNA-PKcs partially impacted phosphorylation of ER $\alpha$  S118 without affecting ER $\alpha$  binding at ER $\alpha$  active enhancers (Figure 7B). Using a specific antibody against DNA-PKcs for ChIP analysis, we first evaluated the temporal kinetics of its potential recruitment on the GREB1 and TFF1 enhancers,

(D) Knockdown of GATA3 affects ER $\alpha$ -dependent activation of eRNA transcription and coding gene expression for GREB1 and TFF1 genes. Mean ± SEM based on three independent qPCR experiments (\*\*p < 0.01).

(E) GRO-seq boxplots showing that GATA3 is required for the E<sub>2</sub>-liganded activation of ER $\alpha$  active enhancers and their coding gene targets.

(F) Heatmap showing that knockdown of GATA3 does not affect ER $\alpha$  binding at the 1,333 active enhancers.

See also Figure S4.



**Figure 4. ER $\alpha$  Active Enhancers Are MegaTrans Enhancers Regulated by DNA-Binding TFs**

(A) Heatmaps of ChIP-seq data for different TFs (+E<sub>2</sub>) displaying strong binding of these DNA-binding TFs at the 1,333 ER $\alpha$  active enhancers but not at ER $\alpha$  non-active enhancers.

(legend continued on next page)

finding recruitment at ~10 min following E<sub>2</sub> treatment of MCF7 cells (Figure 7C). In addition, a specific antibody against phosphorylated ER $\alpha$  S118 revealed strong enrichment that peaked at 20 min, slightly after the recruitment of ER $\alpha$  and DNA-PKcs (Figure 7C). Based on these observations, we conducted ChIP-seq analysis of DNA-PKcs in MCF7 cells after 10 min of E<sub>2</sub> treatment, which revealed 12,629 peaks that mostly located in intergenic regions (Figure S7A). Of the detected peaks, 971 were on the ER $\alpha$ -bound, MegaTrans-containing active enhancers but few were present on non-active enhancers (Figures 7D and S7B). A second antibody for DNA-PKcs yielded similar ChIP-seq results, confirming the specificity of the signal (data not shown).

In order to determine whether *trans*-bound RAR is required for the recruitment of DNA-PKcs at ER $\alpha$  active enhancers, we performed ChIPs for both DNA-PKcs and pER $\alpha$ S118 after knockdown of RAR $\alpha/\gamma$ . We found that RAR $\alpha/\gamma$  knockdown substantially reduced the levels of both DNA-PKcs and pE-R $\alpha$ S118 at ER $\alpha$  active enhancers (Figures 7E–7G, S7C, and S7D), suggesting that *trans*-bound RARs may be required for the functionally relevant recruitment of DNA-PKcs at these ER $\alpha$  enhancers.

Knockdown of DNA-PKcs significantly inhibited E<sub>2</sub>-induced activation of ER $\alpha$ -bound functional enhancers and their target coding gene expression but did not affect RA-induced *HoxA1* activation (Figure 7H). Consistently, the treatment of MCF7 cells with the DNA-PK kinase inhibitor NU7441 also inhibited ER $\alpha$ -dependent target activation (Figure S7E). Thus, at least one role of RARs that are recruited to ER $\alpha$ -bound functional enhancers may be to facilitate the concomitant recruitment of a specific protein kinase. It is possible that, analogous to this role of RARs in recruitment of DNA-PK, other DNA-binding TFs components in the MegaTrans complex also contribute to the recruitment of additional enzymatic factors that are required for functional enhancer activation.

## DISCUSSION

### The MegaTrans Complex Is a Signature of ER $\alpha$ Functional Enhancers

Here, we suggest that, in addition to the critical recruitment of an ever-increasing number of well-characterized coactivator complexes, many with specific enzymatic functions, activation of the most robust subset of ER $\alpha$  enhancers by E<sub>2</sub> is dependent upon, and can be predicted by, their ability to recruit a complex of established DNA-binding transcription factors, referred to as the MegaTrans complex (Figure 7I). This complex appears to

be recruited/assembled in *trans* on ER $\alpha$ -bound functional enhancers and requires the presence of ER $\alpha$ . In addition to the requirement for ER $\alpha$ , certain other components of the complex appear to be necessary for its assembly on functional enhancers; for example, knockdown of RAR $\alpha/\gamma$  and GATA3 abolishes recruitment of other components of the complex and inhibits enhancer/target coding gene activation. Although the precise biochemical interactions that underlie the formation of the MegaTrans complex remain incompletely defined, our data on the effects of DNase I treatment and DNA-binding domain mutation suggest that the MegaTrans complex assembles *in situ* at ER $\alpha$ -bound, ERE-containing enhancers, which also typically harbor nearby FoxA1 *cis*-binding sites.

While the idea that DNA-binding transcription factors can be recruited in *trans* to either activate or repress specific target coding genes is well established (Langlais et al., 2012; Pascual et al., 2005; Reichardt et al., 1998), this study provides an initial description of a ligand-dependent recruitment in *trans* of a complex of DNA-binding transcription factors that proves important for ER $\alpha$  function. Using the published criteria for defining super-enhancers (Hnisz et al., 2013; Whyte et al., 2013), only ~22% of the functional MegaTrans enhancers can be classified as components of super-enhancers, and we note that there is only a very slight distinction in the levels of eRNA induction in response to E<sub>2</sub> on the functional MegaTrans enhancers associated with super-enhancers compared to those not associated with the super-enhancers. Thus, recruitment of the MegaTrans complex serves as a mark that distinguishes the most active enhancers of the estrogen-regulated transcriptional program.

These observations raise several corollary questions. First, does this MegaTrans complex serve on all active or activated enhancers, irrespective of the DNA-binding transcription factors bound in *cis* to those enhancers? It appears that the RARE-containing functional enhancers, which recruit RAR $\alpha/\gamma$  in *cis*, do not recruit this complex or ER $\alpha$  (Figure S1G). Therefore, we speculate that there may be a number of distinct MegaTrans complexes that are recruited only by certain regulatory DNA-binding factors, and these complexes, analogous to events for ER $\alpha$ -regulated enhancers, serve to mark and initiate other specific enhancer activation events. Second, how is the MegaTrans complex selectively recruited only to the functional ER $\alpha$ -bound enhancers? Based on our initial data, we suggest that the answer likely involves the apparent dual roles of the “pioneer factor” FoxA1, which is selectively recruited to the functional, MegaTrans-dependent enhancers at <200 bp from the ERE but is also required for the binding of ER $\alpha$  to these enhancers. In

(B) UCSC browser snapshot of an ER $\alpha$  active enhancer for *FoxC1*, which exemplifies a MegaTrans-bound enhancer (+E<sub>2</sub>).

(C) Heatmap showing AP2 $\gamma$  binding at ER $\alpha$  active enhancers, but not at ER $\alpha$  non-active enhancers, in response to E<sub>2</sub>.

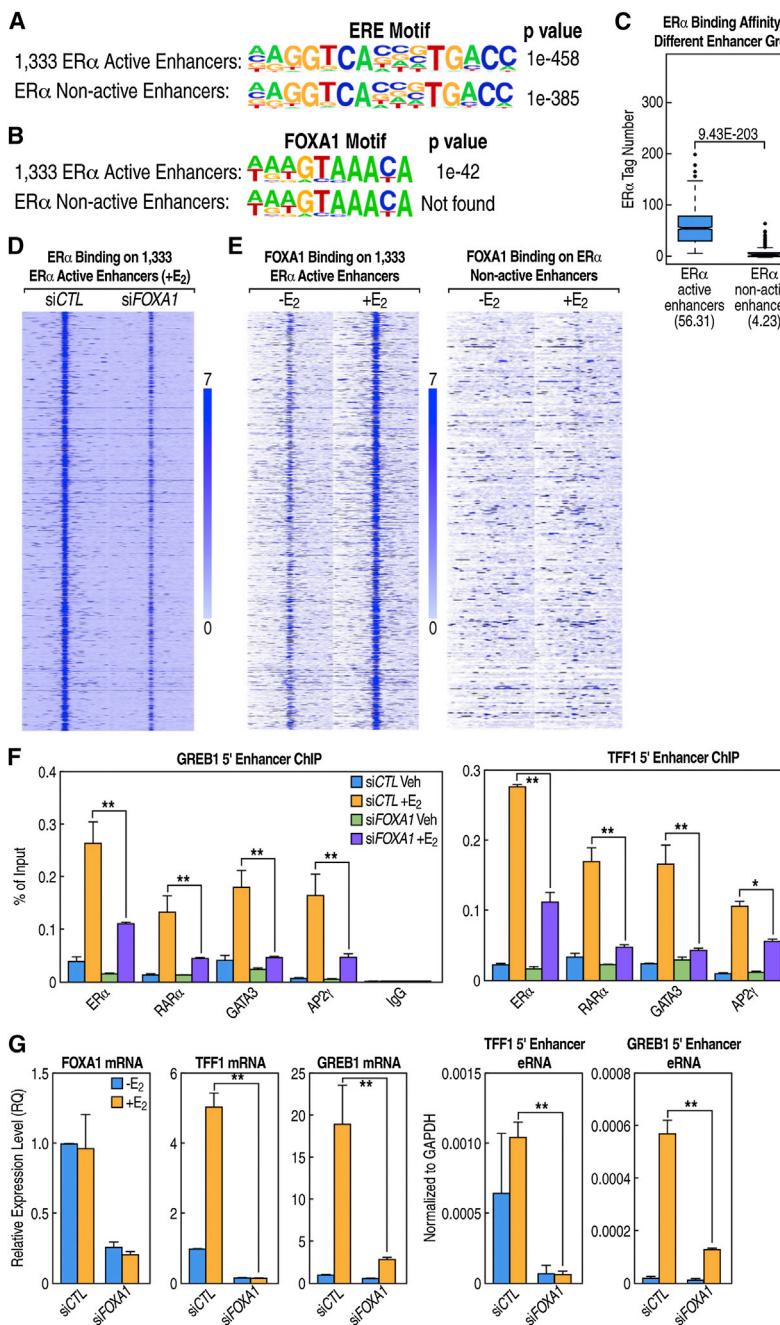
(D) WT and non-DNA-binding mutants of MegaTrans TF components bind equivalently to two ER $\alpha$  active enhancers of *TFF1* and *GREB1*, as demonstrated by biotin ChIP using BLRP-tagged TFs (GFP served as control). For details regarding DNA-binding domain mutagenesis see Extended Experimental Procedures. ChIP signals are presented as percentage of input.

(E) GRO-seq boxplots showing that AP2 $\gamma$  is required for ligand-dependent activation of both eRNA and target gene body transcription for ER $\alpha$  active enhancers.

(F) STAT1 is required for the activation of ER $\alpha$  active enhancers and coding gene expression by E<sub>2</sub> for *GREB1* and *TFF1* genes, as demonstrated by knockdown and qPCR analysis.

(G–I) Knockdown of RARs or GATA3, but not AP2 $\gamma$ , greatly reduces the E<sub>2</sub>-enhanced occupancy of DNA-binding TFs on ER $\alpha$  active enhancers of *TFF1* and *GREB1*. ChIP signals are presented as percentage of input.

Data are represented as mean  $\pm$  SEM. NS, not significant. \*p < 0.05, \*\*p < 0.01. See also Figure S5.



**Figure 5. FoxA1 Performs Dual Roles on ER $\alpha$  Active Enhancers**

(A and B) ER $\alpha$  and FoxA1 motif analyses using Homer program for 1,333 ER $\alpha$  active enhancers and ER $\alpha$  non-active enhancers (see Extended Experimental Procedures for analysis details).

(C) Boxplot based on ER $\alpha$  ChIP-seq data (+E $_2$ )

showing higher binding affinity of ER $\alpha$  at 1,333

ER $\alpha$  active enhancers than at ER $\alpha$  non-active

enhancers.

(D) Heatmap showing that knockdown of FoxA1

greatly reduces ER $\alpha$  binding at the 1,333 active

enhancers.

(E) Heatmap showing that knockdown of FoxA1

greatly reduces ER $\alpha$  binding at the 1,333 active

enhancers, is enhanced in response to E $_2$ .

(F) Conventional ChIP assays for TFF1 and GREB1

enhancers showing knockdown of FoxA1 sub-

stantially reduced binding of ER $\alpha$  and the Mega-

Trans components following E $_2$  treatment. ChIP

signals are presented as percentage of input.

(G) FoxA1 is required for the activation of ER $\alpha$

active enhancers in response to E $_2$ , as exemplified

by the effects of FoxA1 knockdown on coding

gene expression and eRNA transcription for

GREB1 and TFF1 genes.

Data are represented as mean  $\pm$  SEM. \*p < 0.05,

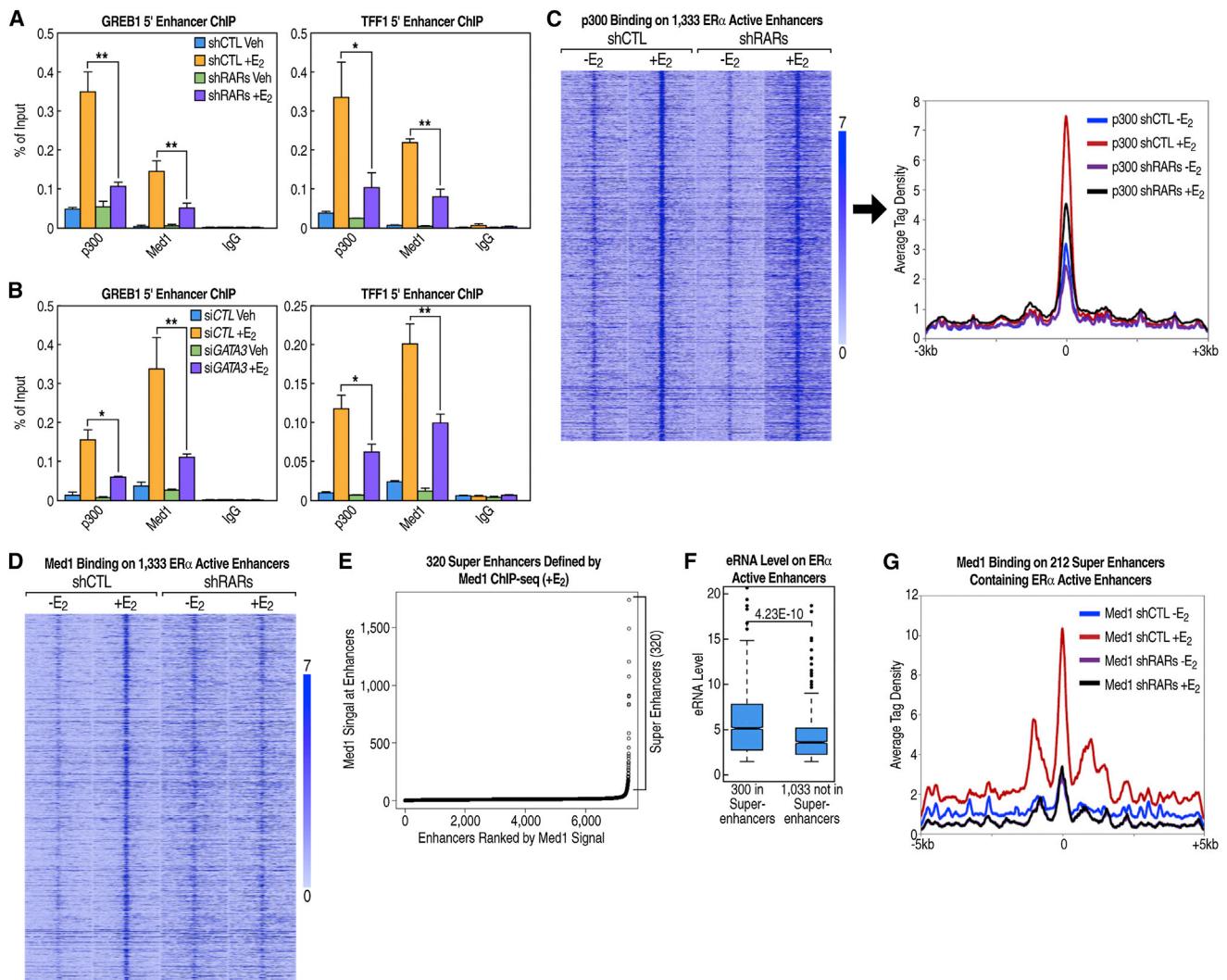
\*\*p < 0.01.

### The MegaTrans Complex as a Platform for Regulatory Enzymes

In light of the already large number of important coactivator complexes, why would these additional DNA-binding transcription factors, most of which are recruited to the active enhancers by the ERE-bound ER $\alpha$ , be required? First, we have found that they play important “early” roles in enhancer function as they are important for eRNA induction and the ligand-dependent increase of p300 and Med1 occupancy on the enhancers. Thus, components of the MegaTrans complex are required to license the recruitment of well-known, important coactivators, as exemplified by p300 and Mediator subunits. In this regard, the DNA-binding transcription factors summoned to bind in *trans* through

addition to its established pioneering role, FoxA1 may also make an important contribution to the recruitment/stabilization of the MegaTrans complex. We are tempted to speculate that, in addition to promoting cooperative binding of ER $\alpha$  to enhancers, FoxA1 may cause a conformational alteration in the ER $\alpha$  receptor, either directly or via altered enhancer DNA architecture, that facilitates the recruitment of the MegaTrans complex; however, it is formally possible that the increased affinity of ER $\alpha$  for the enhancer alone determines binding of the MegaTrans complex. These questions and other undefined aspects of the MegaTrans complex represent fascinating issues for future investigation.

ER $\alpha$  are subserving functions that are quite analogous to those of the recognized coactivator complexes, many of which feature associated/intrinsic enzymatic activities. Similarly, we note that RARs are capable of interacting with many known or potential coactivators, and we have focused on one such potential regulator. The enzyme DNA-PKcs binds to RARs and is recruited with rapid temporal kinetics to ER $\alpha$ -bound functional enhancers. Additionally, knockdown of DNA-PKcs partially phenocopies the functional consequences of RAR $\alpha/\gamma$  knockdown in MCF7 cells. Therefore, we are tempted to speculate that components of the MegaTrans complex individually



**Figure 6. trans-Bound TFs on MegaTrans Enhancers Are Required for Recruitment of ER $\alpha$  Coactivators and Super-Enhancer Function**

(A and B) Knockdown of RARs or GATA3 greatly reduces the E<sub>2</sub>-enhanced binding of p300 and Med1 to ER $\alpha$  active enhancers. ChIP signals are presented as percentage of input.

(C) Heatmap and tag density plot of p300 ChIP-seq data for four different conditions demonstrating that knockdown of RARs by shRNA reduces E<sub>2</sub>-enhanced p300 recruitment on 1,333 ER $\alpha$  active enhancers.

(D) trans-bound RARs are required for E<sub>2</sub>-enhanced recruitment of the coactivator Med1 to ER $\alpha$  active enhancers, as shown by a heatmap of Med1 ChIP-seq data on 1,333 ER $\alpha$  active enhancers.

(E) A Med1 tag density plot based on Med1 ChIP-seq (+E<sub>2</sub>) data and clustering of enhancers identifies ~320 super-enhancers in MCF7 cells (see [Extended Experimental Procedures](#) for analysis details).

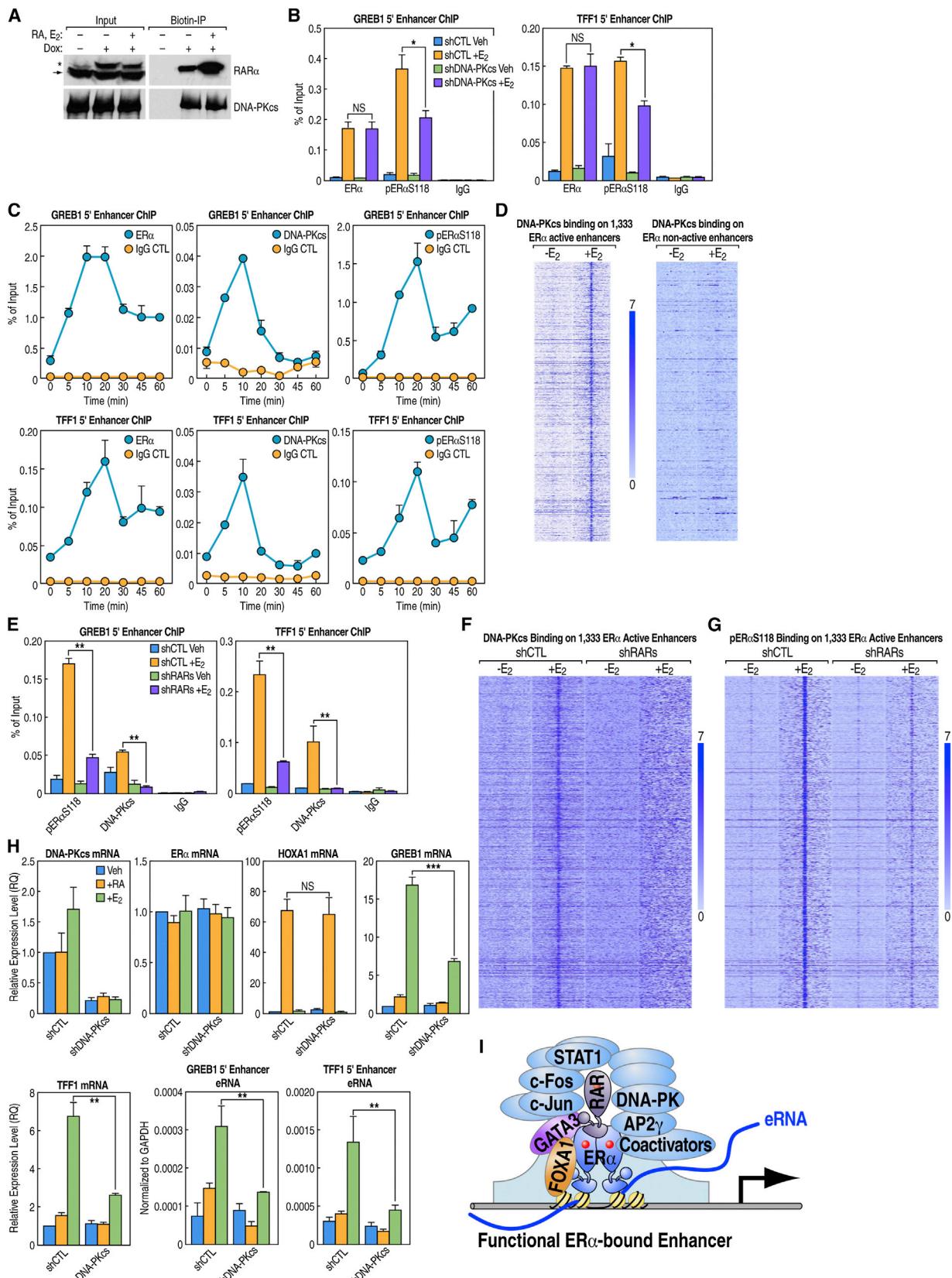
(F) A boxplot analysis based on GRO-seq data (+E<sub>2</sub>) of eRNA expression levels for two groups of ER $\alpha$  active enhancers: the 300 ER $\alpha$  active enhancers located in super-enhancers (median: 5.14) and 1,033 ER $\alpha$  active enhancers that are not located in super-enhancers (median: 3.59).

(G) Tag density plot showing knockdown of trans-bound RARs, which affects the function of ER $\alpha$  active enhancers, reduces the E<sub>2</sub>-enhanced Med1 signal at 212 super-enhancers that contain ER $\alpha$  active enhancers.

Data are represented as mean  $\pm$  SEM. \*p < 0.05, \*\*p < 0.01. See also [Figure S6](#).

recruit various enzymes/factors that collectively are mechanistically important in initial activation of the functional enhancer program. DNA-PK is a kinase with multiple targets, including ER $\alpha$  on Ser118 ([Foulds et al., 2013](#)), which we find occurs on the active ER $\alpha$ -bound enhancers, dependent on the presence of RARs on these functional enhancers. It is particularly intriguing that DNA-PKcs is associated with the Ku80 complex,

classically considered to be involved in DNA damage repair ([Hartley et al., 1995](#); [Jin and Weaver, 1997](#)), which may in fact be pertinent to its functions in transcriptional control events. The rapid appearance of DNA-PKcs on the ligand-regulated enhancers is analogous to other examples of recruited protein kinases in gene regulation events ([Perissi et al., 2008](#); [Tee et al., 2014](#)).



(legend on next page)

Thus, investigation of the ER $\alpha$ -regulated enhancers has revealed that an additional and critical component of the most active enhancers is the ER $\alpha$ -dependent recruitment of the MegaTrans complex, which promotes combinatorial recruitment of additional coactivators/enzymes that increase enhancer activation and target coding gene transcription. Analogous to the hypothesis that super-enhancers regulate critical developmental or disease-associated coding gene transcriptional programs, MegaTrans complex recruitment appears to serve as a mechanism of marking/empowering enhancers to control key aspects of the regulatory transcriptional programs in a specific cell type. The super-enhancer model defines the combinatorial effects of multiple, clustered enhancers spanning >8–10 kb, while the MegaTrans enhancer model explains the differential functional activity of single enhancers.

### MegaTrans Enhancers as a Commonly Utilized Strategy?

The uncovering of another layer of machinery involved in the effective activation of ER $\alpha$ -regulated enhancers raises the possibility that distinct MegaTrans enhancers exist for other classes of DNA-binding TFs that are responsible for activation of unique transcriptional programs. We note that ChIP-seq analyses for many established DNA-binding TFs have revealed their binding on enhancers that do not harbor any known cognate binding sequences. This raises the possibility that these TFs might exert roles, in *trans*, on other transcription programs analogous to the effects of the MegaTrans complex on the ER $\alpha$ -regulated functional enhancers. The “hotspot” or “clustering” phenomenon of DNA-binding TFs has recently been reported in several different organisms (Junion et al., 2012; Rada-Iglesias et al., 2012; Siersbaek et al., 2014a; 2014b; Wilson et al., 2010; Yan et al., 2013). However, the underlying molecular mechanism(s) and functional significance are not well understood. Our results provide a functional model to explain at least many cases of the clustering phenomena. Specifically, our data suggest that the DNA-dependent binding of ER $\alpha$  and FoxA1 at ER $\alpha$  functional enhancers establishes a platform for recruiting a MegaTrans complex of other DNA-binding TFs by protein-protein interactions (*in trans*). MegaTrans complex-bound enhancers function as more

robust enhancers by recruiting certain unique factors and enzymes, such as DNA-PK. Thus, our study provides insights into understanding the phenomenon of TF clustering. Our data also simultaneously help to explain why ChIP-seq analyses reveal ~50% of the regions occupied by many of the DNA-binding TFs assayed in the ENCODE project do not harbor cognate DNA-binding motifs.

### EXPERIMENTAL PROCEDURES

A detailed description of all methods and any associated references is provided in the *Extended Experimental Procedures* (including Tables S1, S2, and S3), which can be found in the supplemental data section.

#### Cell Culture and BLRP-Tagged Stable Cell Lines

MCF7 cells, initially obtained from ATCC, were maintained in culture and treated as described (Li et al., 2013). To study binding patterns for wild-type and non-DNA-binding mutants of RAR $\alpha/\gamma$ , GATA3, ER $\alpha$ , and other TFs, we first established a parental MCF7 stable cell line that expressed BirA enzyme and Tet-Repressor. We then used this parental cell line to make doxycycline-inducible stable cell lines expressing BLRP-tagged proteins at close to endogenous levels. BLRP-tagged proteins were biotinylated *in vivo* by BirA enzyme, allowing for pull downs to be performed with NanoLink streptavidin magnetic beads (Solulink) under very stringent washing conditions.

#### Chromatin Immunoprecipitation and Global Run-on Sequencing

ChIP-qPCRs, ChIP-seqs, and GRO-seqs were performed as previously reported (Li et al., 2013). Immunoprecipitated DNA was recovered by purification on QIAquick spin columns (QIAGEN) after decrosslinking and then analyzed by qPCR using primers listed in Table S1. The qPCR-validated DNA samples were used to make libraries for deep sequencing. The details of ChIP-seq and GRO-seq data analysis are included in the *Extended Experimental Procedures*.

### ACCESSION NUMBERS

The Gene Expression Omnibus databank accession number for all deep sequencing data reported in this paper is GSE60272, which includes ChIP-seq data sets (GSE60270) and GRO-seq data sets (GSE60271).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2014.08.027>.

### Figure 7. *trans*-Bound RAR May Contribute to the Recruitment of DNA-PK Kinase as a Coactivator for ER $\alpha$ Active Enhancers

- (A) Western blots demonstrating interaction of doxycycline-induced BLRP-RAR $\alpha$  protein with DNA-PKcs after pull down by streptavidin magnetic beads. The asterisk marks BLRP-tagged RAR $\alpha$ , and the arrow marks endogenous RAR $\alpha$ .
  - (B) Conventional ChIP assays for *TFF1* and *GREB1* enhancers showing DNA-PKcs is not required for the occupancy of ER $\alpha$  but is partially required for the presence of S118-phosphorylated ER $\alpha$  (pER $\alpha$ S118). ChIP signals are presented as percentage of input.
  - (C) The kinetics of ER $\alpha$ , DNA-PKcs, and pER $\alpha$ S118 occupancy at ER $\alpha$  active enhancers. ChIP signals are presented as percentage of input.
  - (D) Heatmaps, based on ChIP-seq data, showing DNA-PKcs binding at 1,333 ER $\alpha$  active enhancers is enhanced by E<sub>2</sub>, while its binding is not apparent at ER $\alpha$  non-active enhancers.
  - (E) Knockdown of RARs by shRNA greatly reduces DNA-PKcs binding to ER $\alpha$  active enhancers and affects enrichment of pER $\alpha$ S118. ChIP signals are presented as percentage of input.
  - (F) Heatmap of DNA-PKcs ChIP-seq data showing loss of E<sub>2</sub>-enhanced DNA-PKcs binding to ER $\alpha$  active enhancers upon knockdown of both RARs.
  - (G) Heatmap of pER $\alpha$ S118 ChIP-seq data demonstrating partial reduction of E<sub>2</sub>-enhanced pER $\alpha$ S118 binding to ER $\alpha$  active enhancers upon knockdown of both RARs.
  - (H) Knockdown of DNA-PKcs by shRNA affects E<sub>2</sub>-liganded activation of gene body and eRNA transcription for *GREB1* and *TFF1* genes but does not affect ER $\alpha$  levels or RA induction of the *HoxA1* gene, as demonstrated by qPCR.
  - (I) Working model of a MegaTrans enhancer. At ER $\alpha$  active enhancers that contain ERE and FoxA1 motifs, DNA-bound ER $\alpha$  and FoxA1 dynamically recruit *in situ* the functionally required MegaTrans complex of DNA-binding TFs, including RAR, GATA3, AP2 $\gamma$ , STAT1, and AP1. The *trans*-bound components of the MegaTrans complex may recruit specific, functional enzymatic machinery, exemplified by the recruitment of DNA-PK.
- Data are represented as mean  $\pm$  SEM. NS, not significant. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. See also Figure S7.

## AUTHOR CONTRIBUTIONS

Z.L. and M.G.R. conceived the original ideas, designed the project and wrote the paper. Z.L. performed the majority of the experiments with participation from F.Y., W.L., S.O., M.J.F., X.S., F.Z., K.A.O., and A.K.. D.M. performed most bioinformatic analyses with the assistance from Q.M.

## ACKNOWLEDGMENTS

The authors are grateful to Janet Hightower for assistance with figure preparation, to Rachel Pardee for proofreading, and to Dr. Majid Ghassemian (University of California, San Diego [UCSD]) for assistance in mass spectrometry. We thank Dr. Dimple Notani (UCSD) for critically reading the manuscript, and Drs. Namit Singh and Kevin Corbett (UCSD) for their experimental contributions on gel filtration studies. We thank Drs. Dorota Skowronska-Krawczyk and Bogdan Tanasa (UCSD) for critical comments and discussions on this paper. M.G.R. is an investigator with the Howard Hughes Medical Institute. This work was supported by NIH grants (DK018477, HL065445, NS034934, DK039949, and CA173903).

Received: April 3, 2014

Revised: July 2, 2014

Accepted: August 14, 2014

Published: October 9, 2014

## REFERENCES

- Babu, M.M., Luscombe, N.M., Aravind, L., Gerstein, M., and Teichmann, S.A. (2004). Structure and evolution of transcriptional regulatory networks. *Curr. Opin. Struct. Biol.* 14, 283–291.
- Banerji, J., Rusconi, S., and Schaffner, W. (1981). Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. *Cell* 27, 299–308.
- Bernstein, B.E., Birney, E., Dunham, I., Green, E.D., Gunter, C., and Snyder, M.; ENCODE Project Consortium (2012). An integrated encyclopedia of DNA elements in the human genome. *Nature* 489, 57–74.
- Bonn, S., Zinzen, R.P., Girardot, C., Gustafson, E.H., Perez-Gonzalez, A., Delhomme, N., Ghavi-Helm, Y., Wilczynski, B., Riddell, A., and Furlong, E.E. (2012). Tissue-specific analysis of chromatin state identifies temporal signatures of enhancer activity during embryonic development. *Nat. Genet.* 44, 148–156.
- Chepelev, I., Wei, G., Wangsa, D., Tang, Q., and Zhao, K. (2012). Characterization of genome-wide enhancer-promoter interactions reveals co-expression of interacting genes and modes of higher order chromatin organization. *Cell Res.* 22, 490–503.
- Foulds, C.E., Feng, Q., Ding, C., Bailey, S., Hunsaker, T.L., Malovannaya, A., Hamilton, R.A., Gates, L.A., Zhang, Z., Li, C., et al. (2013). Proteomic analysis of coregulators bound to ER $\alpha$  on DNA and nucleosomes reveals coregulator dynamics. *Mol. Cell* 51, 185–199.
- Hartley, K.O., Gell, D., Smith, G.C., Zhang, H., Divecha, N., Connelly, M.A., Admon, A., Lees-Miller, S.P., Anderson, C.W., and Jackson, S.P. (1995). DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell* 82, 849–856.
- Heintzman, N.D., Hon, G.C., Hawkins, R.D., Kheradpour, P., Stark, A., Harp, L.F., Ye, Z., Lee, T.I., Stuart, R.K., Ching, C.W., et al. (2009). Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature* 459, 108–112.
- Hnisz, D., Abraham, B.J., Lee, T.I., Lau, A., Saint-André, V., Sigova, A.A., Hoke, H.A., and Young, R.A. (2013). Super-enhancers in the control of cell identity and disease. *Cell* 155, 934–947.
- Hua, S., Kittler, R., and White, K.P. (2009). Genomic antagonism between retinoic acid and estrogen signaling in breast cancer. *Cell* 137, 1259–1271.
- Hurtado, A., Holmes, K.A., Ross-Innes, C.S., Schmidt, D., and Carroll, J.S. (2011). FOXA1 is a key determinant of estrogen receptor function and endocrine response. *Nat. Genet.* 43, 27–33.
- Jin, S., and Weaver, D.T. (1997). Double-strand break repair by Ku70 requires heterodimerization with Ku80 and DNA binding functions. *EMBO J.* 16, 6874–6885.
- Joseph, R., Orlov, Y.L., Huss, M., Sun, W., Kong, S.L., Ukil, L., Pan, Y.F., Li, G., Lim, M., Thomsen, J.S., et al. (2010). Integrative model of genomic factors for determining binding site selection by estrogen receptor- $\alpha$ . *Mol. Syst. Biol.* 6, 456.
- Junion, G., Spivakov, M., Girardot, C., Braun, M., Gustafson, E.H., Birney, E., and Furlong, E.E. (2012). A transcription factor collective defines cardiac cell fate and reflects lineage history. *Cell* 148, 473–486.
- Kagey, M.H., Newman, J.J., Bilodeau, S., Zhan, Y., Orlando, D.A., van Berkum, N.L., Ebmeier, C.C., Goossens, J., Rahl, P.B., Levine, S.S., et al. (2010). Mediator and cohesin connect gene expression and chromatin architecture. *Nature* 467, 430–435.
- Langlais, D., Couture, C., Balsalobre, A., and Drouin, J. (2012). The Stat3/GR interaction code: predictive value of direct/indirect DNA recruitment for transcription outcome. *Mol. Cell* 47, 38–49.
- Li, W., Notani, D., Ma, Q., Tanasa, B., Nunez, E., Chen, A.Y., Merkurjev, D., Zhang, J., Ohgi, K., Song, X., et al. (2013). Functional roles of enhancer RNAs for oestrogen-dependent transcriptional activation. *Nature* 498, 516–520.
- Lovén, J., Hoke, H.A., Lin, C.Y., Lau, A., Orlando, D.A., Vakoc, C.R., Bradner, J.E., Lee, T.I., and Young, R.A. (2013). Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell* 153, 320–334.
- Mohammed, H., D'Santos, C., Serandour, A.A., Ali, H.R., Brown, G.D., Atkins, A., Rueda, O.M., Holmes, K.A., Theodorou, V., Robinson, J.L., et al. (2013). Endogenous purification reveals GREB1 as a key estrogen receptor regulatory factor. *Cell Reports* 3, 342–349.
- Nesbit, M.A., Bowl, M.R., Harding, B., Ali, A., Ayala, A., Crowe, C., Dobbie, A., Hampson, G., Holdaway, I., Levine, M.A., et al. (2004). Characterization of GATA3 mutations in the hypoparathyroidism, deafness, and renal dysplasia (HDR) syndrome. *J. Biol. Chem.* 279, 22624–22634.
- Pascual, G., Fong, A.L., Ogawa, S., Gamliel, A., Li, A.C., Perissi, V., Rose, D.W., Willson, T.M., Rosenfeld, M.G., and Glass, C.K. (2005). A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* 437, 759–763.
- Perissi, V., Scafoglio, C., Zhang, J., Ohgi, K.A., Rose, D.W., Glass, C.K., and Rosenfeld, M.G. (2008). TBL1 and TBLR1 phosphorylation on regulated gene promoters overcomes dual CtBP and NCoR/SMRT transcriptional repression checkpoints. *Mol. Cell* 29, 755–766.
- Rada-Iglesias, A., Bajpai, R., Prescott, S., Brugmann, S.A., Swigut, T., and Wysocka, J. (2012). Epigenomic annotation of enhancers predicts transcriptional regulators of human neural crest. *Cell Stem Cell* 11, 633–648.
- Reichardt, H.M., Kaestner, K.H., Tuckermann, J., Kretz, O., Wessely, O., Bock, R., Gass, P., Schmid, W., Herrlich, P., Angel, P., and Schütz, G. (1998). DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* 93, 531–541.
- Rosenfeld, M.G., Lunyak, V.V., and Glass, C.K. (2006). Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev.* 20, 1405–1428.
- Ross-Innes, C.S., Stark, R., Holmes, K.A., Schmidt, D., Spyrou, C., Russell, R., Massie, C.E., Vowles, S.L., Eldridge, M., and Carroll, J.S. (2010). Cooperative interaction between retinoic acid receptor-alpha and estrogen receptor in breast cancer. *Genes Dev.* 24, 171–182.
- Shlyueva, D., Stampfel, G., and Stark, A. (2014). Transcriptional enhancers: from properties to genome-wide predictions. *Nat. Rev. Genet.* 15, 272–286.
- Siersbæk, R., Baek, S., Rabiee, A., Nielsen, R., Traynor, S., Clark, N., Sandelin, A., Jensen, O.N., Sung, M.H., Hager, G.L., and Mandrup, S. (2014a). Molecular architecture of transcription factor hotspots in early adipogenesis. *Cell Reports* 7, 1434–1442.
- Siersbæk, R., Rabiee, A., Nielsen, R., Sidoli, S., Traynor, S., Loft, A., La Cour Poulsen, L., Rogowska-Wrzesinska, A., Jensen, O.N., and Mandrup, S.

- (2014b). Transcription factor cooperativity in early adipogenic hotspots and super-enhancers. *Cell Reports* 7, 1443–1455.
- Stender, J.D., Kim, K., Charn, T.H., Komm, B., Chang, K.C., Kraus, W.L., Benner, C., Glass, C.K., and Katzenellenbogen, B.S. (2010). Genome-wide analysis of estrogen receptor alpha DNA binding and tethering mechanisms identifies Runx1 as a novel tethering factor in receptor-mediated transcriptional activation. *Mol. Cell. Biol.* 30, 3943–3955.
- Tee, W.W., Shen, S.S., Oksuz, O., Narendra, V., and Reinberg, D. (2014). Erk1/2 activity promotes chromatin features and RNAPII phosphorylation at developmental promoters in mouse ESCs. *Cell* 156, 678–690.
- Theodorou, V., Stark, R., Menon, S., and Carroll, J.S. (2013). GATA3 acts upstream of FOXA1 in mediating ESR1 binding by shaping enhancer accessibility. *Genome Res.* 23, 12–22.
- Vaquerizas, J.M., Kummerfeld, S.K., Teichmann, S.A., and Luscombe, N.M. (2009). A census of human transcription factors: function, expression and evolution. *Nat. Rev. Genet.* 10, 252–263.
- Visel, A., Blow, M.J., Li, Z., Zhang, T., Akiyama, J.A., Holt, A., Plajzer-Frick, I., Shoukry, M., Wright, C., Chen, F., et al. (2009). ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* 457, 854–858.
- Whyte, W.A., Orlando, D.A., Hnisz, D., Abraham, B.J., Lin, C.Y., Kagey, M.H., Rahl, P.B., Lee, T.I., and Young, R.A. (2013). Master transcription factors and mediator establish super-enhancers at key cell identity genes. *Cell* 153, 307–319.
- Wilson, N.K., Foster, S.D., Wang, X., Knezevic, K., Schütte, J., Kaimakis, P., Chilaraska, P.M., Kinston, S., Ouwehand, W.H., Dzierzak, E., et al. (2010). Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. *Cell Stem Cell* 7, 532–544.
- Yan, J., Enge, M., Whitington, T., Dave, K., Liu, J., Sur, I., Schmierer, B., Jolma, A., Kivioja, T., Taipale, M., and Taipale, J. (2013). Transcription factor binding in human cells occurs in dense clusters formed around cohesin anchor sites. *Cell* 154, 801–813.