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FOXA1 is a key determinant of estrogen receptor function and endocrine response

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Estrogen receptor-α (ER) is the key feature of most breast cancers and binding of ER to the genome correlates with expression of the Forkhead protein FOXA1 (also called HNF3α). Here we show that FOXA1 is a key determinant that can influence differential interactions between ER and chromatin. Almost all ER-chromatin interactions and gene expression changes depended on the presence of FOXA1 and FOXA1 influenced genome-wide chromatin accessibility. Furthermore, we found that CTCF was an upstream negative regulator of FOXA1-chromatin interactions. In estrogen-responsive breast cancer cells, the dependency on FOXA1 for tamoxifen-ER activity was absolute; in tamoxifen-resistant cells, ER binding was independent of ligand but depended on FOXA1. Expression of FOXA1 in non-breast cancer cells can alter ER binding and function. As such, FOXA1 is a major determinant of estrogen-ER activity and endocrine response in breast cancer cells.

Estrogen receptor is the defining feature of luminal breast cancers, where it functions as a transcription factor to regulate cell division. Luminal breast cancers comprise the majority of all breast cancers and are generally treated with endocrine therapies, including the antiestrogen tamoxifen[1], although resistance occurs in a substantial fraction of women[2]. Defining the basis of drug resistance requires an understanding of the molecular mechanisms of ER activity. Our knowledge of ER activity has advanced markedly in recent years and we now appreciate the multitude of factors that augment or inhibit the transcriptional activity of ER[3]. At the same time, a better understanding of the *cis*-regulatory elements and enhancers of ER target genes has emerged[4,5].

A number of studies have mapped ER binding events genome-wide in MCF-7 breast cancer cells, by combining chromatin immunoprecipitation (ChIP) with microarrays or high-throughput sequencing[6–8]. The general conclusions were that ER rarely binds to promoter proximal regions and in most studies Forkhead motifs were enriched at the ER binding sites. The Forkhead protein FOXA1 is found at many ER binding regions[9–11] and is required for a few tested ER binding events[9,10], where it probably functions as a pioneer factor[12,13]. The global importance of FOXA1 in mediating ER function and the underlying factors that determine FOXA1 specificity are not clear, although specific histone marks (H3K4me1 and H3K4me2) seem to demarcate FOXA1 regulatory regions[11]. Clinically, FOXA1 predicts outcome in patients with ER-positive breast cancer[14] and it is one of the minimal gene features of ER-positive luminal tumors[15,16].

The interaction between ER and FOXA1 has been limited to correlation of binding events in one cancer cell line[11] and mechanistic analysis on a genomic level is lacking. Whole genome analysis of ER-FOXA1 interactions is required to understand the molecular mechanisms of ER activity. Here we investigate the dependency of ER on FOXA1 on a global scale and in multiple cellular contexts. We show that FOXA1 dictated global chromatin structure and was necessary for ER-chromatin interaction in breast cancer cells under different ligand conditions, including the breast cancer treatment tamoxifen. We also show that FOXA1 was sufficient to permit ER-chromatin interactions and transcriptional activity in diverse target tissues.

RESULTS

Differential ER binding correlates with FOXA1 binding

We mapped FOXA1 binding events using ChIP with high-throughput sequencing (ChIP-seq) in asynchronous MCF-7, ZR75-1 and T-47D breast cancer cells (the three most commonly studied estrogen-responsive breast cancer cell lines)[17]. Binding events were called using MACS[18] and we found 79,651 FOXA1 binding events in MCF-7 cells, 80,327 in ZR75-1 cells and 43,336 in T-47D cells (**Fig. 1a**). The overlap among the three cell lines suggests that FOXA1 binding events can be dynamic and cell line–specific. We also conducted ChIP-seq experiments to map ER binding events in the three breast cancer cell lines and integrated the ER and FOXA1 binding information (**Fig. 1b** and **Supplementary Fig. 1**). We defined an ER-FOXA1 overlapping region as one where the binding events shared at least one base pair. The overlap between ER and FOXA1 binding within the same cell line was 52–58% (**Fig. 1b**), but there was substantially lower overlap between different cell lines (**Fig. 1c**). The cell line–unique ER binding events were also more likely to be cell line–unique FOXA1 binding regions (data not shown). Examples of cell line–specific ER and FOXA1 binding events are shown in **Figure 1d**. By selecting similar numbers of random genomic regions and overlapping with the FOXA1 binding events, we found that ER overlap was significantly more enriched ($P < 0.00001$) than expected by chance. By chance, the expected overlap (derived from hundreds of random comparisons),

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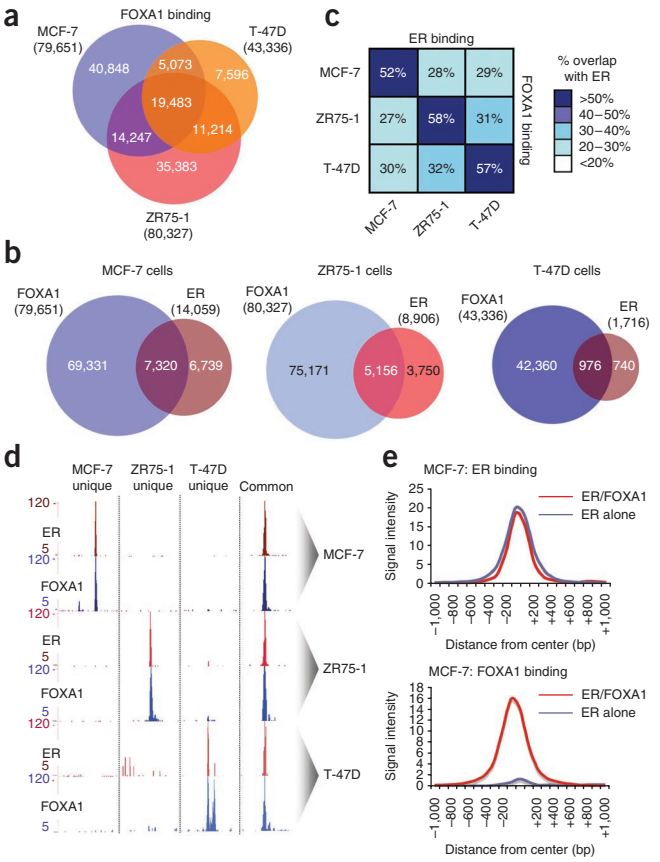

Figure 1 Differential binding of FOXA1 and ER overlaps in a cell context–dependent manner. (**a**) Overlap in FOXA1 binding events among MCF-7, ZR75-1 and T-47D cells. (**b**) Overlap between binding of ER and FOXA1 in the three ER-positive breast cancer cell lines. (**c**) Relative overlap of ER and FOXA1 binding events within and between the three cell lines. The percentages represent the fraction of ER binding events in that cell line. An overlap was considered if the peaks shared at least one base pair. (**d**) Examples of regions showing cell line–specific binding of ER and FOXA1. An example of a region bound by ER and FOXA1 in all three cell lines is also shown. (**e**) Average signal intensity of ER binding sites that are or are not shared with FOXA1 binding regions. The signal intensity of ER binding events that are not shared with FOXA1 is similar to that of those that overlap with FOXA1. Also included is the average signal intensity for FOXA1 binding at these two ER binding categories.

is ~2%, as compared to the >50% overlap that we found. Together, these data show that differential ER binding correlated with FOXA1 binding, suggesting that there is genome-wide, context-specific co-operativity between these factors.

The signal intensity of ER binding events in MCF-7 cells that overlapped with FOXA1 was equivalent to that of events that did not overlap with FOXA1 (**Fig. 1e**), implying that both categories represent direct ER binding events. Similarly, when we overlapped the ER-only or ER-FOXA1 shared binding regions with genomic regions involved in active chromatin loops (ChIP-PET data)[5], we found comparable overlaps. Approximately 25% of the ER-FOXA1 shared regions correlated with active chromatin loops versus 26% of the ER-alone binding events, suggesting that there is no difference in functionality between ER binding events that are shared with FOXA1 and those that are not.

FOXA1 mediates ER binding and transcriptional activity

To investigate the effect of FOXA1 on ER-chromatin interactions on a genome-wide scale, we transfected hormone-depleted MCF-7 cells with a control small inhibitory RNA (siControl) or an siRNA against FOXA1 (siFOXA1; **Fig. 2a**). This had no appreciable effect on ER protein (**Fig. 2a**) or mRNA (**Supplementary Fig. 2**). After silencing of FOXA1 (or control), we mapped ER binding events by ChIP-seq. In control transfected MCF-7 cells, we found 13,631 high-confidence estrogen-induced ER binding events, in line with our previous genome-wide ER map[19]. When we mapped ER binding events after silencing FOXA1, ER binding decreased substantially (an example is shown in **Fig. 2b**). The signal intensity of more than 90% of ER binding events decreased by at least 50% (**Fig. 2c**,**d**), despite the fact that the amount of ER protein did not change (**Fig. 2a**). This was validated by an independent siRNA, and re-expression of FOXA1 rescued the decreased binding (**Supplementary Fig. 3**). Only 595 ER binding events did not decrease by more than 50% after we silenced FOXA1 (**Supplementary Fig. 2**). However, these 595 regions were also the strongest ER binding regions; as silencing was not complete, the strong binding at these probably decreased, but not by 50%. There was no specificity or distinct feature to these 595 regions. We validated these findings by silencing FOXA1 and protein blotting for ER on the chromatin fraction (**Fig. 2e**). Binding of ER to unchromatinized, naked DNA was not influenced by silencing FOXA1, as an oligonucleotide pull-down using a 40-bp double-stranded ER binding region (from an experimentally identified ER binding event)[19] was still bound by ER even in the absence of FOXA1 (**Fig. 2f**). These data suggest that binding of ER to non-chromatinized DNA does not require FOXA1 (**Fig. 2f**), but that the association of ER with chromatin depends on FOXA1. It is unclear why most ER binding events require FOXA1, yet only ~50% of the ER binding events overlap exactly with a FOXA1 binding event. Two possibilities exist to explain this: either ER binding is stabilized by a FOXA1 binding event that does not occur at the same region, or FOXA1 stabilizes a secondary factor that subsequently regulates ER binding.

When we performed the reciprocal experiment, inhibiting ER and mapping FOXA1 binding, we found no appreciable difference in FOXA1 binding in the presence or absence of ER (**Supplementary Fig. 4**), confirming that FOXA1 is upstream of ER-chromatin interactions.

We assessed the effects of FOXA1 on global gene expression. We transfected hormone-deprived MCF-7 cells with siControl or siFOXA1 and treated them with vehicle or estrogen for 6 h. We performed gene expression microarray analysis and identified genes that were differentially regulated. Specific silencing of FOXA1 globally affected the estrogen-mediated transcriptome (**Fig. 2g**), with more than 95% of all estrogen-regulated genes requiring FOXA1 for estrogen regulation. This suggests that the primary role for FOXA1 in breast cancer cells is to facilitate ER-mediated transcription. We validated these findings with an independent siRNA and by FOXA1 rescue (**Supplementary Fig. 3**). Furthermore, silencing of FOXA1 resulted in significant ($P = 2.6 \times 10^{-6}$) growth arrest of MCF-7 cells (**Supplementary Fig. 2**), confirming an absolute requirement of FOXA1 for the estrogen response in breast cancer cells. We validated this result using an independent measure of cell proliferation (% S-phase; data not shown). This finding was recapitulated in the ZR75-1 ER-positive breast cancer cell line (**Supplementary Fig. 3**). The growth arrest mimics the effects of inhibition of ER in MCF-7 cells (data not shown).

FOXA1 is required for tamoxifen action

The effectiveness of tamoxifen, one of the most successful cancer drugs[20], is primarily due to its ability to inhibit estrogen-ER activity in breast cancer cells, where tamoxifen-ER is recruited to chromatin[21] to repress transcription. Previous data implied that in MCF-7 cells,

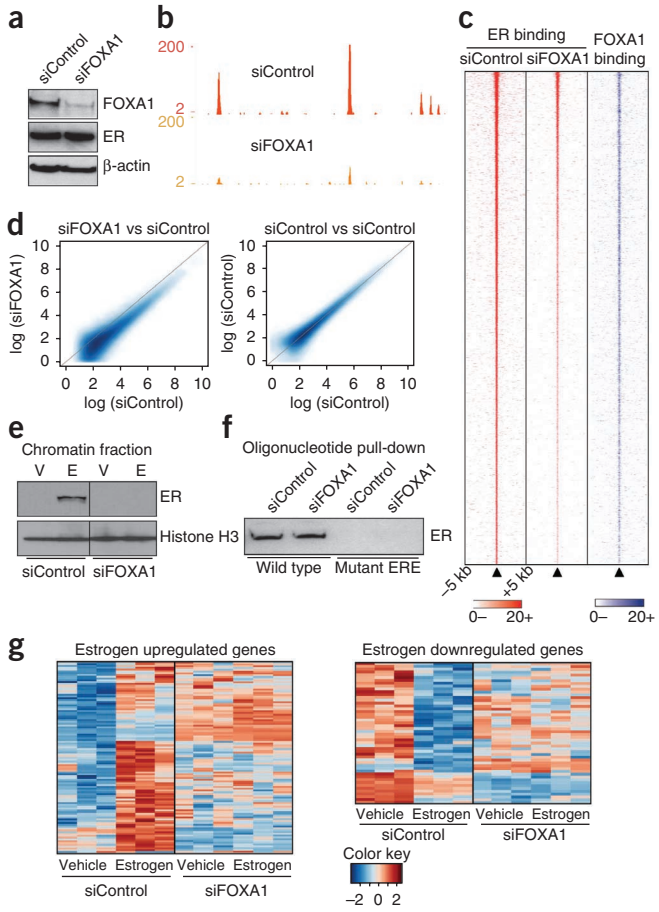

Figure 2 Binding of ER to chromatin and transcriptional activity requires FOXA1. (**a**) Protein blot of cells transfected with siControl or siFOXA1. (**b**) An example of ER binding in cells transfected with siControl or siFOXA1. (**c**) Heatmap showing the signal intensity of ER binding in cells transfected with siControl or siFOXA1 in a window of ±5 kb. Signal intensity for FOXA1 at the equivalent genomic region is also shown. The heatmap represents binding events ranked from the strongest to weakest ER binding (in the siControl condition), and the adjacent columns represent the signal from the corresponding genomic region but under the different experimental conditions. (**d**) Smoothened scatterplot comparing ER binding intensity in cells transfected with siControl and those transfected with siFOXA1. As a control, a scatterplot representing two different siControl experiments is shown. (**e**) Cells were transfected with siControl or siFOXA1, treated with vehicle (V) or estrogen (E) and were fractionated to enrich for the chromatin fraction, which was protein blotted. Histone H3 was used as a loading control. The uncropped protein blot is in **Supplementary Figure 2**. (**f**) Oligonucleotide pull-down using total protein from cells transfected with siControl or siFOXA1. A double-stranded, biotin-labeled oligonucleotide containing a perfect ERE sequence or a mutant sequence was used and protein enriched by the oligonucleotide was protein blotted. (**g**) Gene expression microarray analysis following transfection of siControl or siFOXA1 and treatment with vehicle or estrogen for 6 h.

estrogen and tamoxifen could induce significant differences in ER binding profiles[8], suggesting that mechanisms must exist for regulating ligand-specific ER binding events. We replicated the ER ChIP-seq data in MCF-7 cells following estrogen or tamoxifen treatment and considered the consensus of the triplicate experiments. We also included ER ChIP-seq of vehicle-treated cells. Around 93% of the tamoxifen-ER binding events were also estrogen-ER binding events (**Fig. 3a**), representing natural variation between identical experiments. We therefore did not find a substantial number of unique tamoxifen-ER binding events (**Fig. 3a**), although these have been reported[8], possibility owing to differences in replicate numbers (**Supplementary Fig. 5**). Examples of binding are shown in **Figure 3b**. However, the intensity of tamoxifen-ER binding was substantially less than that of estrogen-ER binding (**Supplementary Fig. 5**), so there were fewer binding events in total (**Fig. 3c**). We also performed gene expression microarray analysis in hormone-deprived MCF-7 cells treated with vehicle, estrogen, tamoxifen or estrogen plus tamoxifen for 6 h. This analysis confirmed that tamoxifen mostly antagonized estrogen-mediated gene expression (**Supplementary Fig. 6**), with only 27 genes being regulated by tamoxifen but not estrogen. Of these 27 genes, almost all have been shown in previous studies to be regulated by estrogen[6,22], suggesting that they are not truly unique tamoxifen gene targets. At later time points tamoxifen may be able to regulate unique genes[22], but at the early time point, tamoxifen almost exclusively antagonizes estrogen function. Therefore, tamoxifen induces similar ER binding events to estrogen and estrogen and tamoxifen regulate common genes.

As tamoxifen induced similar ER binding profiles to estrogen, tamoxifen-ER might use the same mechanisms to interact with chromatin. We found that silencing of FOXA1 inhibited tamoxifen-mediated binding of ER to chromatin (**Fig. 3d**). Our experimental conditions preclude the possibility of determining whether FOXA1 is required for the tamoxifen-mediated growth arrest of breast cancer cells (cells transfected with siRNA to FOXA1 could not circumvent tamoxifen-ER growth arrest and acquire increased proliferation, as estrogen activity was also inhibited). To assess the requirement for FOXA1 in the tamoxifen-mediated growth of breast cancer cells, we focused on a tamoxifen-resistant (Tam-R) MCF-7 breast cancer cell line[23], as this would allow us to determine whether endocrine-resistant cells still require FOXA1. Initially, we mapped ER binding events in hormone-deprived Tam-R cells that were treated with vehicle or tamoxifen. The ER binding profile in the Tam-R cells was substantially different from that of wild-type MCF-7 cells and ER binding occurred independently of tamoxifen treatment (**Fig. 3e** and **Supplementary Fig. 7**). The ER binding in vehicle-treated Tam-R cells was similar to that in tamoxifen-treated cells (**Fig. 3e**). We sequenced ER in the Tam-R cells and found no mutations, showing that the differences in ER binding were not due to mutations in ER. We mapped FOXA1 binding events by ChIP-seq in tamoxifen-treated Tam-R cells and found that the FOXA1 binding events were markedly different in Tam-R cells when compared to those in wild-type MCF-7 cells (**Supplementary Fig. 7**). However, the FOXA1 binding events that were specific to Tam-R cells correlated with the Tam-R–specific ER binding regions (~55% of the Tam-R ER binding events overlapped with a FOXA1 region; **Supplementary Fig. 7**). Furthermore, silencing of FOXA1 in the Tam-R cells resulted in decreased ER binding (**Supplementary Fig. 7**) and significant growth inhibition ($P < 0.001$; **Fig. 3f**), confirming that the altered ER binding and ligand-independent growth of the Tam-R cells required FOXA1.

FOXA1 can render ER functional in non-breast cancer cells

Although FOXA1 is necessary for binding of ER to the genome in a breast cancer cell line, it is not known whether FOXA1 is sufficient for ER binding to occur. To address this, we used the U20S oesteosarcoma cell line, which stably expresses exogenous ER[24] but expresses low levels of FOXA1 (**Fig. 4a**). We transfected U20S-ER cells with control plasmid or with a FOXA1 expression construct (**Fig. 4a**) and assessed ER binding at known breast cancer *cis*-regulatory elements

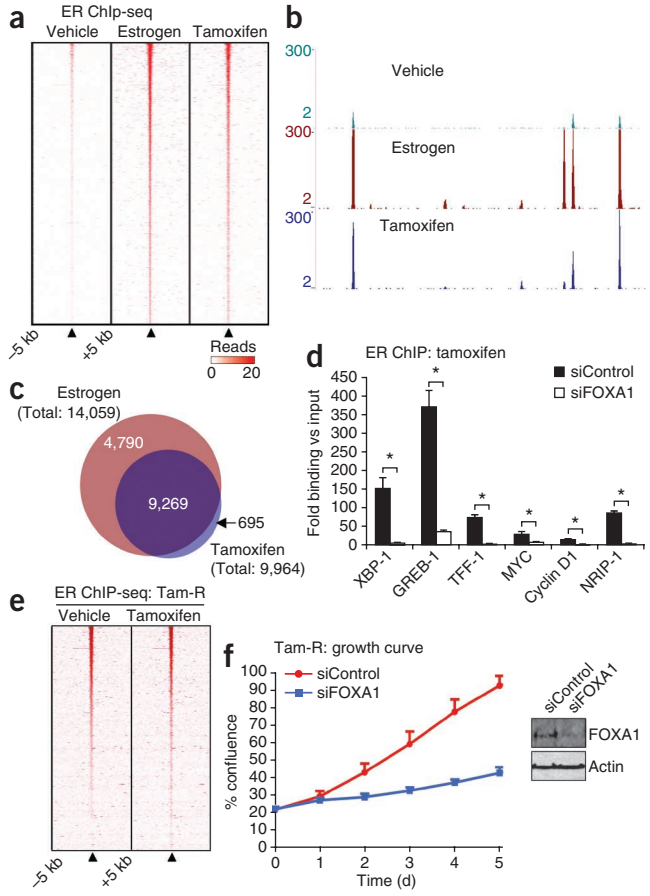

Figure 3 Tamoxifen induces similar ER binding events to estrogen in a FOXA1-dependent manner. (**a**) Heatmap representing the signal intensity of ER binding in hormone-deprived MCF-7 cells treated with vehicle, estrogen or tamoxifen for 45 min. The window represents ±5 kb. The heatmap represents binding events ranked from the strongest to weakest ER binding (in the estrogen condition). (**b**) Example of ER binding under the different ligand conditions. (**c**) Venn diagram representing the overlap in ER binding events between estrogen- and tamoxifen-treated cells. Comparisons with published data are provided in **Supplementary Figure 5**. (**d**) Hormone-deprived MCF-7 cells were transfected with siControl or siFOXA1 and treated with tamoxifen. ER ChIP was performed followed by real-time PCR of known ER binding regions. The data are the fold enrichment over input. The data are the mean of independent replicates ± s.d. (**e**) ER ChIP-seq binding data from hormone-deprived, tamoxifen-resistant MCF-7 cells (Tam-R) treated with vehicle or tamoxifen. (**f**) Tam-R cells were hormone deprived, transfected with siControl or siFOXA1 and treated with tamoxifen. Total cell growth was assessed. The data are the mean of independent replicates ± s.d.

by ChIP. In U20S-ER cells transfected with FOXA1, ER binding was increased at every tested region (**Fig. 4b**).

We assessed global FOXA1-mediated enrichment of chromatin-associated ER in transfected U20S-ER cells. Protein blot analysis for ER on the chromatin fraction showed a FOXA1-mediated increase in chromatin-bound ER (**Fig. 4c**). Binding of ER to unchromatinized DNA occurred in U20S-ER cells independently of FOXA1 and FOXA1 expression did not increase ER binding to naked DNA (**Fig. 4d**). Therefore, in osteosarcoma cells ER binding is dictated by unknown factors[25], but the expression of FOXA1 can alter ER binding.

U20S-ER cells that express ER possess a gene expression program and binding profile that is different from that of breast cancer cells[25]. We transfected U20S-ER cells with vector or FOXA1, after which we assessed the expression of known ER target genes that are found in breast cancer. In the presence of FOXA1, estrogen-rich complete medium could induce the expression of a number of breast cancer genes and tamoxifen could downregulate all the tested genes (**Fig. 4e**). U20S-ER cells do not show growth arrest in the presence of tamoxifen, but instead show increased growth[26]. We hypothesized that the expression of FOXA1 might alter the response of U20S-ER cells to tamoxifen. In control transfected U20S-ER cells, there was no anti-proliferative response to tamoxifen treatment[26], but expression of FOXA1 was sufficient to impart tamoxifen sensitivity ($P < 0.05$; **Fig. 4f**). This tamoxifen-mediated growth repression of U20S-ER cells did not occur in control transfected cells (**Fig. 4f**) and was not

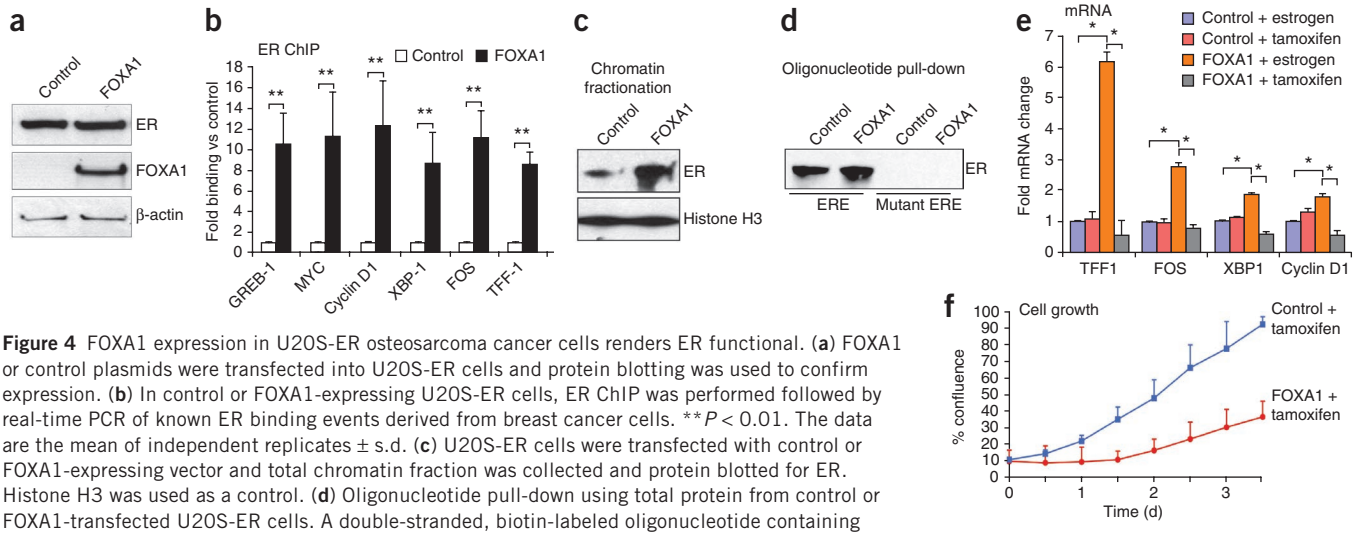

Figure 4 FOXA1 expression in U20S-ER osteosarcoma cancer cells renders ER functional. (**a**) FOXA1 or control plasmids were transfected into U20S-ER cells and protein blotting was used to confirm expression. (**b**) In control or FOXA1-expressing U20S-ER cells, ER ChIP was performed followed by real-time PCR of known ER binding events derived from breast cancer cells. $^{**}P < 0.01$. The data are the mean of independent replicates ± s.d. (**c**) U20S-ER cells were transfected with control or FOXA1-expressing vector and total chromatin fraction was collected and protein blotted for ER. Histone H3 was used as a control. (**d**) Oligonucleotide pull-down using total protein from control or FOXA1-transfected U20S-ER cells. A double-stranded, biotin-labeled oligonucleotide containing a perfect ERE or a mutant sequence was used and protein enriched by the oligonucleotide was protein blotted. (**e**) Control or FOXA1-expressing U20S ER cells were treated with estrogen or tamoxifen and mRNA levels of known breast cancer–associated target genes were assessed. The data are the mean of independent replicates ± s.d. $^{*}P < 0.05$. Specifically for TFF1, the fold change is ×10 of the *y*-axis values. (**f**) Cell proliferation was performed and cell confluence assessed in U20S-ER cells transfected with control or FOXA1 and treated with tamoxifen. The data are the mean of independent replicates ± s.d.

due to FOXA1 expression alone. We reproduced these findings in ovarian cancer cells, where expression of FOXA1 altered ER binding and target gene expression (**Supplementary Fig. 8**).

FOXA1 maintains euchromatic conditions

FOXA1 is required for maintaining euchromatic conditions at specific *cis*-regulatory elements[11,27]. To gain insight into the global impact of FOXA1 on the regulation of chromatin structure, we performed formaldehyde-assisted isolation of regulatory elements (FAIRE), a method for identifying nucleosome-free, euchromatic regions of the genome[28,29]. We coupled FAIRE with high-throughput sequencing. Hormone-deprived cells were transfected with siControl or siFOXA1 and treated with vehicle or estrogen for 1 h. FAIRE was performed and processed for high-throughput sequencing. In vehicle-treated, control-transfected cells we found 71,342 FAIRE regions and we found 48,881 in estrogen-treated, control-transfected cells. We found significant overlap between vehicle- and estrogen-treated cells, with 31,447 regions being found in a ligand-independent manner (**Fig. 5a**). We found similar overlap in an independent FAIRE replicate (data not shown). Examples of FAIRE signal, representing nucleosome-depleted chromatin regions, are shown in **Figure 5b**. When we overlapped the FAIRE data (FAIRE signal from vehicle and estrogen treatment combined) with ER binding data, we found substantial, but not complete overlap (**Fig. 5c**). Regions that were both ER binding events and FAIRE regions were likely to bind FOXA1 ~35% of the time (**Fig. 5c**). The ER binding events that occured in nucleosome-rich chromatin regions (no FAIRE signal) were more likely to be shared ER and FOXA1 binding regions. This suggests that binding of ER to nucleosome-free, euchromatic regions of the genome is less likely to occur at regions already bound by FOXA1, whereas binding of ER to more condensed, nucleosome-rich chromatin requires the pioneering function of FOXA1. When we compared the FAIRE signal in the presence or absence of FOXA1, we found substantial decreases in global FAIRE signal in siFOXA1-transfected cells (**Fig. 5c**). At specific regions, therefore, FOXA1 is required for effective maintenance of nucleosome-depleted 'euchromatic' conditions within the genome, in many cases in a ligand-independent manner.

Given the observation that promoter regions are traditionally represented by nucleosome-free chromatin, we integrated the FAIRE data with estrogen-mediated gene expression data[30]. Most of the genes that were induced or repressed by estrogen contained FAIRE signal at their promoter regions (**Fig. 5d**). The global FAIRE signal was decreased by ~50% at the transcription start sites of genes in cells transfected with siFOXA1 (**Fig. 5d**), confirming that FOXA1 is required to maintain optimal chromatin conditions at promoters of estrogen target genes.

Differential FOXA1 binding and function is influenced by CTCF

Given that differential FOXA1 binding depends on context (**Fig. 1a**), we investigated possible factors that might influence the binding of FOXA1, including the insulator protein CTCF—a factor that has been shown to demarcate and influence ER transcriptional activity within specific regions of the genome[31,32]. We mapped CTCF binding events in MCF-7 cells by ChIP-seq, and found 62,153 binding regions in multiple replicates (**Fig. 6a**). Although a substantial number of FOXA1 sites overlapped with CTCF sites (~15.5%), only ~1% of FOXA1 binding regions overlapped with regions that bound both CTCF and ER (**Fig. 6a**). Binding events that were shared between ER and FOXA1 were almost exclusively not bound by CTCF (~88% of the ER- and FOXA1-bound regions did not bind CTCF). These ER and FOXA1 (but not CTCF) binding domains were enriched near most of the estrogen-regulated genes (data not shown), suggesting

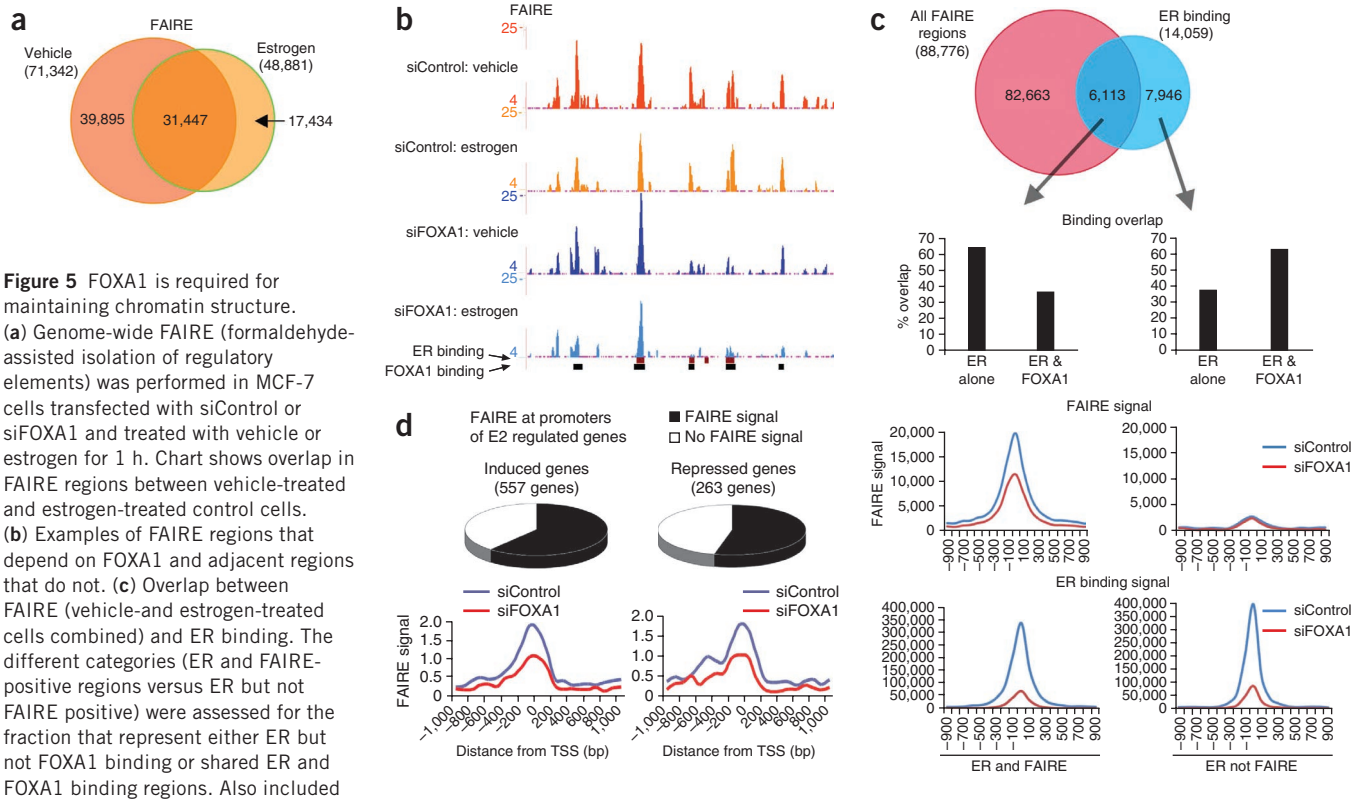

Figure 5 FOXA1 is required for maintaining chromatin structure. (**a**) Genome-wide FAIRE (formaldehyde-assisted isolation of regulatory elements) was performed in MCF-7 cells transfected with siControl or siFOXA1 and treated with vehicle or estrogen for 1 h. Chart shows overlap in FAIRE regions between vehicle-treated and estrogen-treated control cells. (**b**) Examples of FAIRE regions that depend on FOXA1 and adjacent regions that do not. (**c**) Overlap between FAIRE (vehicle-and estrogen-treated cells combined) and ER binding. The different categories (ER and FAIRE-positive regions versus ER but not FAIRE positive) were assessed for the fraction that represent either ER but not FOXA1 binding or shared ER and FOXA1 binding regions. Also included are the changes in FAIRE and ER binding signal within the two categories. (**d**) Fraction of promoter proximal regions of genes induced or repressed by 6 h estrogen treatment that possess FAIRE signal. The relative difference in FAIRE signal in cells transfected with siControl or siFOXA1 is shown.

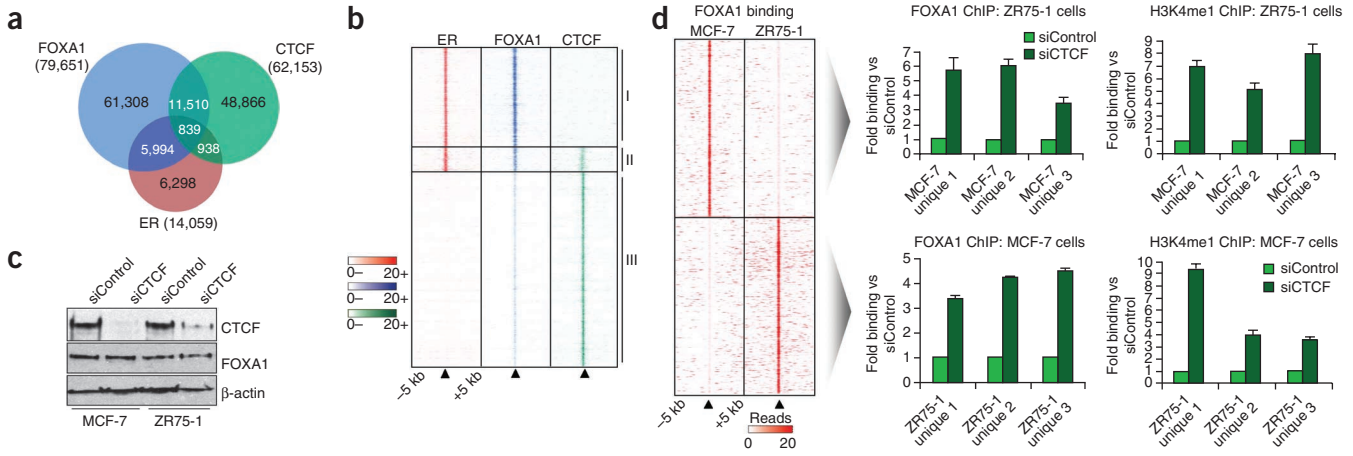

Figure 6 Binding events that are shared between ER and FOXA1 are exclusively independent of CTCF, and CTCF can repress the binding and activity of FOXA1. (**a**) Overlap between FOXA1, ER and CTCF binding events in MCF-7 cells. (**b**) Heatmap representing binding signal from regions where ER, FOXA1 and CTCF overlap. The categories are: I, ER and FOXA1 shared (but not CTCF) binding events; II, regions bound by ER, FOXA1 and CTCF; III, FOXA1 and CTCF shared (but not ER) binding regions. The window represents ±5 kb. The FOXA1 binding events that overlap with CTCF tend to be the weakest FOXA1 binding events. (**c**) Protein blot of MCF-7 and ZR75-1 cells transfected with siControl or siCTCF. (**d**) Heatmap showing FOXA1 binding regions that are unique to MCF-7 or ZR75-1 cells. ZR75-1 and MCF-7 cells were transfected with siControl or siCTCF. FOXA1 and H3K4me1 ChIP was performed, followed by real-time PCR of three regions that bound FOXA1 exclusively in the other cell line. The data are the mean of independent replicates ± s.d.

that the presence of CTCF might negatively influence transcriptional activity. It is unclear whether this is a direct effect or whether CTCF can alter chromatin state and indirectly influence binding of ER and FOXA1. Although some FOXA1 binding events correlated with CTCF binding, these tended to be weak (non-stabilized) FOXA1 binding events (**Fig. 6b**) and were are almost always devoid of ER binding and transcriptional potential.

We hypothesized that CTCF binding might negatively influence binding of FOXA1 or its ability to associate with additional transcription factors such as ER. We specifically silenced CTCF in MCF-7 or ZR75-1 cells (**Fig. 6c**) and performed ChIP of FOXA1 at regions that were unique FOXA1 binding regions in the other cell line. Surprisingly, we found that specific silencing of CTCF in ZR75-1 cells allowed FOXA1 to bind to regions in ZR75-1 cells that previously bound FOXA1 only in MCF-7 cells (**Fig. 6d**). The silencing of CTCF also resulted in an increase at these regions of H3K4me1, a histone mark that is found at distal ER *cis*-regulatory elements idependently of FOXA1[11]. Similarly, silencing of CTCF altered the chromatin in MCF-7 cells such that FOXA1 and H3K4me1 could be detected in regions that previously bound FOXA1 only in ZR75-1 cells (**Fig. 6d**). These data confirmed that CTCF imposes pressures that inhibit the stability of FOXA1 binding.

DISCUSSION

FOXA1 is one of the minimal signature genes that defines ER-positive luminal breast cancers[15] and can predict outcome in breast cancers[14]. Our data show that mechanistically, FOXA1 was required for almost all ER binding events in breast cancer cells. This was unexpected, as only about half of ER binding events directly overlapped with a FOXA1 binding event[11] (**Fig. 1**). However, FOXA1 may indirectly stabilize the additional ER binding events (potentially by regulating an additional factor that influences ER-chromatin interactions) or may stabilize ER from a distance, possibly through chromatin loops between distinct ER binding regions.

Our data also showed that tamoxifen recruited ER to a subset of the regions that were bound by estrogen-ER, contrary to previous findings[8] (**Supplementary Fig. 5**). The tamoxifen-'unique' regions described in ref. 8 were mostly estrogen-ER binding events, precluding the possibility that differences in reagents contributed to the discrepancy. The most likely explanation is the lack of replicates in ref. 8. Furthermore, the authors of ref. 8 suggested that Forkhead motifs were not enriched in ER binding events, which is contradicted by our mechanistic data and by the discovery of Forkhead motifs in other ER mapping investigations[6,7,10,11,33]. Our data suggest that FOXA1 is required for the functions of both estrogen-ER and tamoxifen-ER. This might explain why high FOXA1 levels predict a positive response to tamoxifen in ER-positive patients[14]; functional tamoxifen-ER requires the same pioneering machinery as estrogen-ER and the absence of FOXA1 impairs the chromatin-binding capacity of tamoxifen-ER and the efficacy of tamoxifen. Another possibility is that tumors with low FOXA1 also have low transcriptional activity of ER and that their growth is less dependent on ER. It is unclear whether additional breast cancer endocrine therapies inhibit growth through similar mechanisms or whether resistance to these treatments also depends on FOXA1. As aromatase inhibitors deplete tumors of estrogen, resistance to aromatase inhibitors could be due to the acquisition of hypersensitivity to low levels of estrogen, as previously suggested[34]. If this is the case, ER may simply switch on the endogenous transcriptional pathways, either in low levels of estrogen or in a ligand-independent manner, both of which may still require FOXA1 for ER binding to chromatin and for effective transcriptional potential.

Our data support the idea that FOXA1 mediates ER function in a non–breast cancer context. We observed a FOXA1-dependent response to estrogen and tamoxifen in both ovarian and osteosarcoma cell lines, suggesting that the estrogen-mediated effects in these cellular contexts may also be influenced by FOXA1. As ovarian and bone tissue can be ER-positive and genetic ablation of ER can alter the physiology of these tissues[35], ER binding and activity might be dictated by FOXA1 in these target tissues. The transcriptional activity of ER in non–breast cancer tissues, including osteosarcoma and ovarian cells, might therefore be influenced by the presence or absence of FOXA1.

FOXA1 can mimic linker histone and can bind directly to compacted chromatin[12,13], thereby providing the opportunity for other

transcription factors to associate with chromatin. We found that sites that bound both ER and FOXA1 were more likely to contain condensed chromatin (chromatin lacking in FAIRE signal), whereas ER binding events that were not shared with FOXA1 binding were more likely to be nucleosome-depleted and accessible (positive FAIRE signal). As such, ER *cis*-regulatory elements at condensed chromatin are more dependent on FOXA1 to maintain chromatin interactions. However, FOXA1 binding is not a stable event and seems to possess some context specificity. Our data suggest that one factor that can influence FOXA1 activity is the insulator protein CTCF, although it is unclear whether CTCF can directly influence FOXA1 binding or simply alters chromatin state and thereby has an indirect effect on FOXA1. CTCF has been reported to form demarcated domains within which ER functionality is promoted, and loss of CTCF binding in a different cell line may contribute to decreased ER transcriptional activity[32]. One clear role for CTCF is in mediating chromatin loops[36], presumably different chromatin loops than those that involve ER and FOXA1 during estrogen-mediated transcription in breast cancer cells[5]. Our data suggest that a number of FOXA1 binding events occur adjacent to CTCF sites, but these tend to be the weakest FOXA1 binding events and rarely recruit ER. As such, the presence of CTCF at these genomic regions might destabilize FOXA1 binding and render these sites non-functional or might change chromatin conditions that indirectly influence FOXA1 accessibility.

These findings suggest that FOXA1 is the primary determinant of ER binding and transcriptional activity in breast cancer cells, under both estrogenic and tamoxifen-treated conditions. Furthermore, FOXA1 expression in non-breast cancer cells can enhance ER-chromatin interactions and may be the defining feature that renders ER functional in other cellular contexts.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession numbers. All ChIP-seq and FAIRE data are deposited under ArrayExpress number E-MTAB-223. All microarray data are deposited under GEO number GSE25316.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

All experiments were conceived by A.H., K.A.H. and J.S.C. Experiments were conducted by A.H., K.A.H. and C.S.R.-I. Computational analysis was conducted by A.H. and D.S. The manuscript was written by A.H., K.A.H. and J.S.C. with help from C.S.R.-I. and D.S.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cell lines. MCF-7 cells were grown as described[37]. U20S-ER cells (a gift from T. Spelsberg, Mayo Clinic, Minnesota) were grown in DMEM supplemented with 10% (v/v) FCS. Tam-R cells (a gift from I. Hutcheson, Tenovus Centre, Cardiff University) were grown in phenol red–free DMEM as described[23]. OVCAR3 cells were obtained from ATCC and grown in RPMI with 10% (v/v) FCS. Estrogen was used at a final concentration of 100 nM and tamoxifen at a final concentration of 1 μM.

Chromatin immunoprecipitation. ChIP experiments were performed as described[38]. Antibodies used were anti-ER (sc-543) from Santa Cruz Biotechnologies, FOXA1 (ab5089 and ab23738) and H3K4me1 (ab8895) from Abcam and CTCF from Millipore (07-729). Primers used for ChIP are given in **Supplementary Table 1**.

ChIP-sequencing. ChIP DNA was amplified as described[38]. Sequences generated by the Illumina GAIIx genome analyzer (using 36-bp reads) were aligned against NCBI Build 36.3 of the human genome using MAQ (http://maq.sourceforge.net/) with default parameters. For each biological replicate, a corresponding set of input sequence reads of similar size was obtained by random sampling from the full set of input sequence reads. Peaks were called using model-based analysis for ChIP-Seq (MACS)[18], run using default parameters.

RT-PCR. Cells were deprived of hormones as described[39]. Total RNA was collected and RT-PCR was performed as described[6]. Primer sequences are given in **Supplementary Table 1**.

siRNA. siRNA experiments were as described[6]. The sequence of the siRNAs can be found in **Supplementary Table 1**. All siRNAs were from Thermo Scientific Dharmacon RNAi Technologies.

Plasmids. FOXA1 expression was from pcDNA3.1-FOXA1 (a gift from J. Eeckhoute, University of Rennes, France). ER expression was from pcDNA3.1-ER. As a control, empty pcDNA3.1 was used.

Protein blotting. Cells were deprived of hormones as described[39]. For chromatin fractionation experiments, the chromatin fraction was collected as described[40]. Antibodies used were: ERα (sc-543) from Santa Cruz Biotechnologies, FOXA1 (ab23738), β-Actin (ab6276) and histone H3 (ab1791) from Abcam and CTCF from Millipore (07-729).

Oligonucleotide pull-down. The oligonucleotide pull-down and sequences used were as described[19].

Formaldehyde assisted isolation of regulatory elements. MCF-7 cells were transfected with siControl or siFOXA1 and treated with vehicle or estrogen for 1 h. FAIRE was performed as described[29]. The enriched chromatin regions were processed for Illumina sequencing as described[38].

Microarray analysis. MCF-7 cells were transfected with siControl or siFOXA1 and treated with vehicle or estrogen for 6 h. For tamoxifen and estrogen microarray experiments, hormone-deprived MCF-7 cells were treated with vehicle, estrogen (100 nM), tamoxifen (1 μM) or estrogen plus tamoxifen for 6 h. Gene expression analysis was conducted as described[19].

Cell growth assay. Cells were plated at equal confluence, grown in hormone-depleted DMEM medium and treated with vehicle, estrogen (100 nM) or tamoxifen (1 μM). Confluence of cells was analyzed using the live-cell imaging Incucyte Analyzer (Bucher Biotec AG). This approach has been validated as a robust method for assessing cell growth (http://www.essenbioscience.com/).

Statistics. Statistical analysis was performed using two tailed paired *t*-tests. *P*-value cut-offs are indicated in the relevant figures.

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