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Original Articles

CUEDC1 is a primary target of ERα essential for the growth of breast cancer cells

Rui Lopes[a,1], Gozde Korkmaz[a,**,1], Sonia Aristin Revilla[a], Romy van Vliet[a], Remco Nagel[a], Lars Custers[a], Yongsoo Kim[a], Pieter C. van Breugel[a], Wilbert Zwart[a], Behzad Moumbeini[a], Zohar Manber[c], Ran Elkon[c], Reuven Agami[a,b,*]

[a] Division of Oncogenomics, Oncode Institute, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands
[b] Department of Molecular Genetics, Erasmus University Medical Center, Wytemaweg 80, 3015 CN, Rotterdam, The Netherlands
[c] Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv, 69978, Israel

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ABSTRACT

Breast cancer is the most prevalent type of malignancy in women with ~1.7 million new cases diagnosed annually, of which the majority express ERα (ESR1), a ligand-dependent transcription factor. Genome-wide chromatin binding maps suggest that ERα may control the expression of thousands of genes, posing a great challenge in identifying functional targets. Recently, we developed a CRISPR-Cas9 functional genetic screening approach to identify enhancers required for ERα-positive breast cancer cell proliferation. We validated several candidates, including CUTE, a putative ERα-responsive enhancer located in the first intron of *CUEDC1* (CUE-domain containing protein). Here, we show that CUTE controls CUEDC1 expression, and that this interaction is essential for ERα-mediated cell proliferation. Moreover, ectopic expression of CUEDC1, but not a CUE-domain mutant, rescues the defects in CUTE activity. Finally, CUEDC1 expression correlates positively with ERα in breast cancer. Thus, CUEDC1 is a functional target gene of ERα and is required for breast cancer cell proliferation.

1. Introduction

Breast cancer is the most prevalent type of malignancy in women, and each year ~1.7 million new cases are diagnosed [1]. Approximately 70% of breast tumors express ERα (ESR1), which is a transcription factor (TF) that plays a critical role in cell proliferation [2]. ERα is activated by estradiol (E2), which is its natural ligand, or through phosphorylation events mediated by kinases such as MAPK/PI3K [2]. ERα is a ligand-dependent TF that is recruited directly or indirectly to the chromatin, to activate the expression of target genes [3]. Currently, ERα is considered as a major drug target in breast cancer for hormonal therapy, either by inhibiting its association with E2 using tamoxifen [4] or by preventing estrogen synthesis through aromatase inhibitors [5]. Despite the extensive use of these drugs, a substantial number of tumors relapse after initial treatments and patients develop resistance to therapy [6].

ERα regulates the expression of several genes that play a central role in the development of breast cancer, including *CCND1*, *E2F1* and *Myc* [7]. Chromatin immunoprecipitation and sequencing (ChIP-seq) experiments revealed that the vast majority of ERα binding events map to regions that have features of enhancers and are distantly located from protein-coding genes [8,9]. Enhancers are non-coding DNA elements that control gene expression in space and time, which is critical for specifying different cell-lineages during the development of organisms [10]. Subsequent studies identified ~1200 enhancers that express enhancer-associated RNAs (eRNAs) upon activation of ERα in breast cancer cells [4,11]. eRNA expression is a hallmark of active enhancer elements [12] and several studies suggest that they are required for the activation of their target genes [4,11,13]. The studies mentioned above contributed to elucidate the mode of action of ERα and provided a comprehensive map of its binding sites throughout the genome [4,8,11]. However, they are inherently descriptive and do not fully explain the function and mechanisms of ERα-regulated enhancers. Moreover, it is not clear which are the primary target genes of ERα and how they contribute to the proliferation of breast cancer cells.

Traditionally, it is challenging to study the function of enhancers

* Corresponding author. Division of Oncogenomics, Oncode Institute, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands.
** Corresponding author.
E-mail address: r.agami@nki.nl (R. Agami).
[1] These authors contributed equally.

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due to a lack of genetic tools to manipulate these DNA elements. The development of CRISPR-Cas9 systems opened exciting possibilities for targeted genome editing. Of note, Cas9 can be directed to virtually any genomic sequence by a single guide RNA (sgRNA), provided that there is a protospacer adjacent motif (PAM) downstream of the target sequence [14]. Cas9 is a nuclease that efficiently induces double-strand breaks (DSBs), which give rise to small insertions and deletions (indels) when repaired by non-homologous end joining (NHEJ). The CRISPR-Cas9 system is capable of cleaving multiple target sequences in parallel [15,16], making it particularly suitable to perform genome-wide genetic screens [17,18]. Recently, we and others pioneered the application of CRISPR-Cas9 to map functional enhancer elements in human cells [19–22]. In our work, we performed genetic screens to identify enhancers that are required for the growth of ERα-dependent breast cancer cells [20]. We validated three candidate hits from the screen (enh588, enh1830 and enh1986). Enh588 controls the expression of *CCND1* in ERα-positive breast cancer cells, whereas the function of enh1986 is not clear to date. Enh1830 is a putative enhancer located in the first intron of *CUEDC1* that we named here CUTE (CUEDC1 Transcriptional Enhancer). CUEDC1 is a ubiquitously expressed protein that contains a CUE domain, and so far is not associated with breast cancer. Very little is known about the function of CUEDC1 in humans, though CUE-containing proteins were shown to bind ubiquitin chains to facilitate K48 and K63-linked polyubiquitin and protein turnover in yeast [23]. In particular, a recent large-scale mass spectrometry study confirmed the interaction between K63-linked diubiquitin and human CUEDC1 [24]. Furthermore, publicly available protein-protein interaction data indicated an experimental interaction of human CUEDC1 with TOM1 (target of Myb protein 1), a cellular trafficking protein [25].

Here, we show that CUTE is a *bona-fide* enhancer that activates *CUEDC1* expression in response to ERα signaling. The inactivation of CUTE significantly decreases the expression of *CUEDC1* and hampers cell proliferation. Ectopic expression of *CUEDC1* wild-type, but not a CUE-domain mutant, rescues this phenotype, indicating a key functional role of CUEDC1 within the ERα signaling network. Finally, we found that expression of *CUEDC1* is significantly increased in ERα-positive tumors. All this evidence corroborates that *CUEDC1* is a primary target gene of the estrogen pathway that is required for the proliferation of breast cancer cells *in vivo*.

2. Materials and methods

2.1. Cell lines and chemical reagents

MCF-7, MDA-MB-231 and HEK293T cells were cultured in DMEM (Gibco), supplemented with 10% FCS (Hyclone), and 1% penicillin/streptomycin (Gibco). For the estrogen-stimulation experiments, MCF-7 cells were washed 3 times with PBS and cultured in phenol red–free DMEM medium (Gibco) supplemented with 5% charcoal stripped serum (Gibco) for 72 h prior to E2 treatment (10^{-8} M). E2 (17β-estradiol) was purchased from Sigma. All cell lines were obtained from the American Type Culture Collection, and they were tested for mycoplasma.

2.2. Cloning of sgRNAs

Custom sgRNAs were designed using CRISPR Design tool (http://crispr.mit.edu/) and cloned into lentiCRISPRv2 (gift from Feng Zhang (Addgene plasmid #52961)) according to the protocol described by the Zhang lab [17]. The oligos were purchased from IDT and their sequences are listed on Table 1.

2.3. Transduction of human cell lines

To produce lentivirus, 4×10^6 HEK293T cells were seeded in 100 mm dishes one day prior to transfection. For each dish, we mixed 10 μg of the target lentiviral construct, 3.5 μg of pVSV-G, 5 μg of pMDL RRE and 2.5 μg of pRSV-REV in a total volume of 450 μl, added 50 μl of CaCl2 and incubated 5 min at RT. Plasmid DNA was precipitated by adding 500 μl 2× HBS to the solution while vortexing at full speed. Lentivirus-containing supernatants were collected 48 h post-transfection, filtered through a 0.45 μm membrane (Milipore Steriflip HV/PVDF) and stored at −80 °C. All cell types and lentivirus batches tested were titrated in order to achieve a multiplicity of infection (MOI) of ~0.3. Cell lines were infected with lentivirus supernatants supplemented with 8 μg/ml polybrene (Sigma). At 24 h post-infection, medium was replaced and cells were selected with 2 μg/ml puromycin (Gibco) until there were no cells surviving on the negative control plate (non-transduced cells).

Table 1
Sequences of sgRNAs.

Name	Oligo 1 (forward)	Oligo 2 (reverse)
sg1830	caccgtttacagcattggtaaggtc	aaacgaccttaccaatgctgtaaac
sgCUEDC1 exon#1	caccgaccacatgcacgtgttcgac	aaacgtcgaacacgtgcatgtggtc
sgCUEDC1 exon#2	caccgtaggctggcggggagtacac	aaacgtgtactccccgccagcctac
sgCUEDC1 intron#1	caccgattcccattgaaaccccta	aaactaggggtttcaatgggaatc
sgCUEDC1 intron#2	caccgtaaggcttgaggtcaacgat	aaacatcgttgacctcaagccttac

2.4. DNA-seq

Cell pellets were collected and gDNA was isolated with DNeasy Blood and Tissue kit (Qiagen). For each sample, we performed two separate reactions (max. 500 ng of gDNA per reaction) using Phusion DNA polymerase (Thermo Scientific) and combined the resulting amplicons. Amplification was carried out with 18 cycles for both first and second PCR. In the first PCR, we used the following primer sequences:

PCR1_F1
ACACTCTTTCCCTACACGACGCTCTTCCGATCTXXXXXXGGCTTTATATATCTTGTGGAAAGGACG (XXXXXX represents a 6 bp barcode)
PCR1_R1
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTGACGGGCACCGGAGCCAATTCC

A second PCR was performed to attach Illumina adaptors and index samples. The second PCR was done in 50 μl reaction volume, including 5 μl of the product from the first PCR, and using the following primers:

PCR2_P5
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCR2_P7
CAAGCAGAAGACGGCATACGAGATXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT (XXXXXX represents a 6 bp index)

After the second PCR, the amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter), quantified in a Bioanalyzer 2100 (Agilent), and sequenced using a HiSeq 2500 (Illumina).

2.5. RNA isolation, reverse transcription and qPCR

RNA was harvested using TriSure (Bioline) reagent for cell lysis and Rneasy mini kit (Qiagen) to isolate total RNA according to the manufacturer's protocols. cDNA was produced from RNA using Superscript III (Invitrogen). qPCR experiments were performed in a Lightcycler 480 (Roche) using SYBR green I Master mix (Roche) according to the manufacturer's protocol. The primers used for this experiment are listed on Table 2.

Table 2
Primers used for qPCR experiments.

CUEDC1 Forward	aaggaactgcaacggaacc
CUEDC1 Reverse	ggattcgtatttcaatcgatctct
CUTE Forward	acaccagcttcctggttcc
CUTE Reverse	ctgaggtccttccctgcac
TBP Forward	ggagagttctgggattgtac
TBP Reverse	cttatcctcatgattaccgcag

2.6. Western blot

Whole-cell extracts were prepared using RIPA buffer (NaCl 150 mM; NP-40 1%; Sodium deoxycholate 0.5%; SDS 0.1%; Tris pH 7.4 25 mM), separated on 10% SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore). Membranes were immunoblotted with the following antibodies: V5 (ab27671, Abcam); HSP90 (H-114, Santa Cruz Biotechnology). Protein bands were visualized using corresponding secondary antibodies (Dako) and ECL reagent (GE Healthcare).

2.7. Cell proliferation assay

MCF-7 cells were transduced with lentiCRISPRv2 containing sgRNAs of interest (listed on Table 1). Separately, we generated MCF-7 cells stably expressing GFP using pLX304-GFP (gift from David Root; Addgene plasmid #25890). GFP-expressing cells were mixed with cells containing individual lentiviral constructs in a 1:3 ratio. The percentage of GFP-expressing cells was assessed by flow cytometry at the beginning of the experiment (T = 0) and at subsequent time-points. We recorded at a minimum of 10,000 events for each condition, and the data were analyzed using FlowJo software.

2.8. Luciferase reporter assays

A DNA fragment (~2 kb) containing CUTE was amplified by PCR from gDNA of MCF-7 cells and cloned into pGL3-promoter using KpnI/NheI. The constructs were transfected into MCF-7 cells and treated with 10^{-8} M E2 or vehicle (DMSO) for 24 h. Reporter activity was measured 40 h after transfection using Dual-Luciferase system (Promega) according to the manufacturer's instructions.

2.9. RNA-seq

RNA-seq samples were processed with TruSeq RNA library prep kit v2 (Illumina) and sequenced in a HiSeq 2500 (Illumina). Sequenced reads were aligned to the human genome (hg19) using TopHat2 [26] and gene expression counts were calculated using HTseq34 based on Ensembl's human gene annotations (v69) [27]. Gene expression levels were normalized by quantiles.

2.10. ChIP-seq experiments and data analysis

ChIP-seq of ERα, FOXA1 and H3K27ac in WT and mutant MCF-7 cells were performed as described before [28] using the following antibodies: ERα (SC-543; Santa Cruz Biotechnology); FOXA1 (SC-6554; Santa Cruz Biotechnology); H3K27ac (39133; Active Motif). Sequencing reads were aligned to the human genome (hg19) using bwa v 0.7.5 with default parameters. Peaks in control MCF-7 cells were called with MACS36 (default parameters) and DFilter37 (parameters: −bs = 50 −ks = 30 −refine −nonzero) algorithms. In-house MCF-7 mixed input was used for peak calling of MCF-7 cell line. Intersect of the two peak calling algorithms was used for further analysis. ChIP-seq data sets of ERα-Vehicle and ERα-E2 in MCF-7 cells was obtained from GEO. The raw files were aligned to hg19 using Bowtie [29]. Unique reads were converted into bigWig files using BEDTools [30] for visualization in the UCSC Genome Browser.

2.11. ChIA-PET data

ChIA-PET data (publicly available on the Washington University Epigenome browser) of ERα and RNAPII in MCF-7 cells was reanalyzed to identify long-range chromatin interactions at the CUTE locus.

2.12. DNase-seq data

DNase-seq data sets from the ENCODE project were reanalyzed to identify open chromatin regions at the CUTE locus. Available tracks were uploaded to the UCSC genome browser for visualization.

2.13. GRO-seq experiments and data analysis

GRO-seq experiments were performed as described before [20]. We also used publicly available GRO-seq data of MCF-7 cells treated with E2 that was obtained from Ref. [12]. Data points were downloaded from the UCSC genome browser using table browser. Transcription levels were quantified by calculating of the sum of data points per gene and normalizing to their length.

2.14. Gene expression analysis of TCGA samples

The Regulome Explorer tool (explorer.cancerregulome.org) was used to interrogate the TCGA database to find correlations of gene expression in breast cancer samples (ID: BRCA). The identification of genes significantly ($p < 0.05$) correlating with *ESR1* expression was restricted to genes located within 1 Mb of CUTE.

3. Results

We previously conducted a CRISPR-Cas9 genetic screen for mitogenic estrogen-responsive elements (EREs), and identified sgRNA1830 as a guide RNA affecting the proliferation of ERα-positive breast cancer cells [20]. Therefore, enh1830 (the target of sgRNA1830) is a potential transcriptional enhancer that is essential for the mitogenic function of ERα. To study the function of enh1830 in detail, we first analyzed the landscape surrounding its genomic locus. enh1830 is located within the first intron of *CUEDC1* gene and is predicted to be an enhancer in different human cell types due to high H3K27Ac and low H3K4me3 marks indicative of enhancers [31] (Fig. 1A). In line with this, we detected transcription of enhancer RNAs (eRNAs) from enh1830 by global run on sequencing (GRO-seq) in MCF-7 cells (Fig. 1A). Importantly, elevated levels of eRNA expression were measured upon activation of ERα by estradiol (E2) in MCF-7 cells, whereas in MDA-MB-231 (ERα-negative) they were not detectable (Fig. 1A, **red arrows**). Finally, we observed that the binding of ERα at enh1830 overlaps with that of p300, a known coactivator protein associated with enhancers [32]. We therefore named the region enh1830 as CUTE (CUEDC1 Transcriptional Enhancer) and conclude that it is a putative enhancer regulated by ERα in breast cancer cells.

Next, we examined the function of CUTE in heterologous reporter assays. Enhancers are known to activate gene expression regardless of their orientation relative to the target gene [33]. To address this point, we cloned a DNA fragment of ~2 kb containing CUTE in either forward or reverse orientation upstream of a luciferase reporter gene of a pGL3-promoter plasmid. We transfected these constructs into MCF-7 cells and observed a strong activation of luciferase expression (~8 fold; *P*-value < 0.001) independently of the orientation of CUTE (Fig. 1B). Next, we tested the responsiveness of CUTE to estrogen activation by treating transfected MCF-7 cells with estradiol (E2). We observed a strong increase (~30 fold; *P*-value < 0.001) in the expression of luciferase pGL3 vectors containing CUTE (Fig. 1C), indicating that the transcriptional enhancer activity of CUTE is dependent of ERα. To confirm this hypothesis, we generated mutations in the estrogen-responsive element (ERE) of CUTE by CRISPR-Cas9 and cloned the

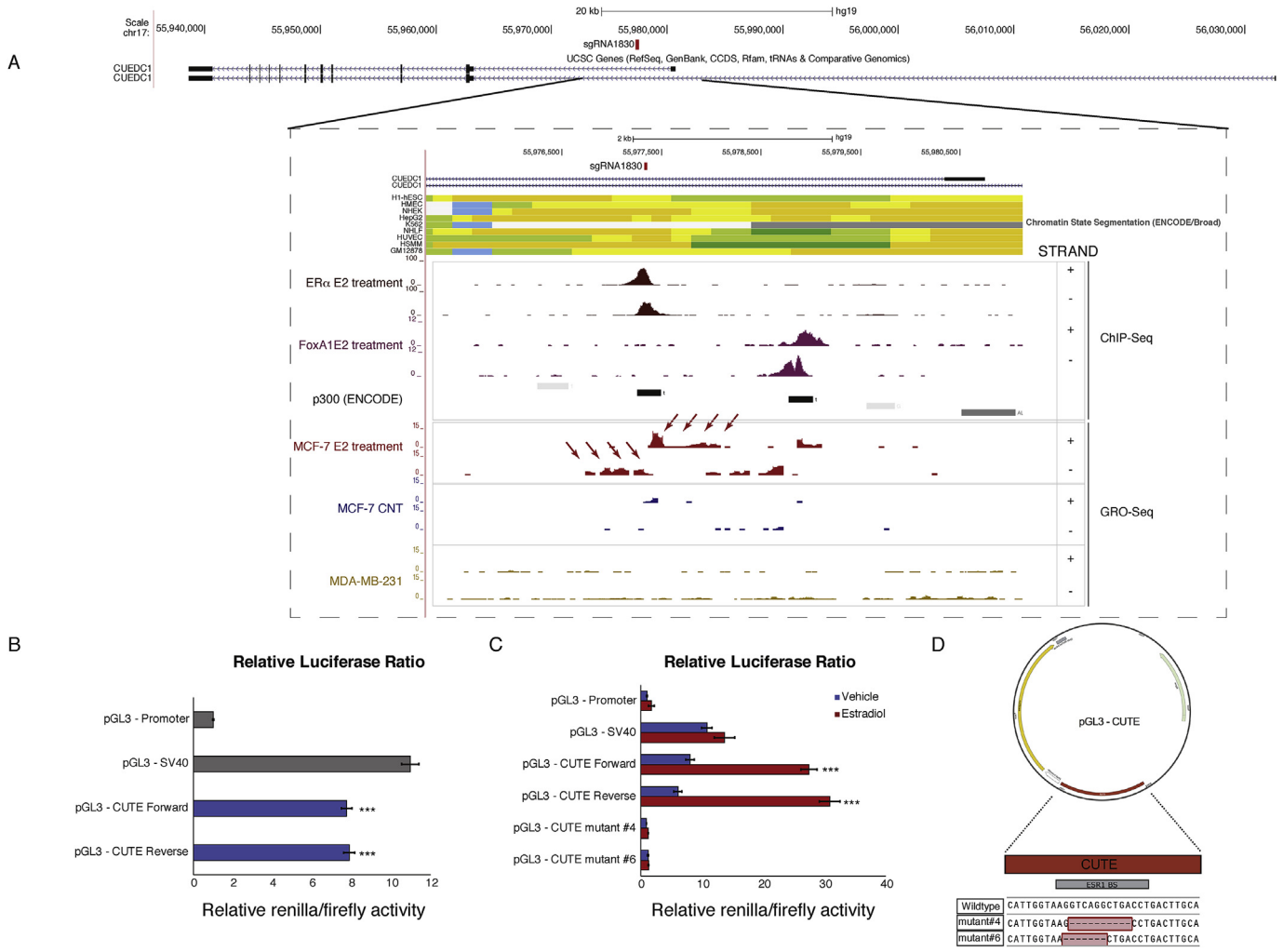

Fig. 1. CUTE is a putative enhancer element regulated by ERα. (**A**) RefSeq genes track from NCBI showing the genomic location of sgRNA1830 (red vertical line) and CUTE (zoomed in window). Chromatin State Segmentation by a hidden Markov model from ENCODE/Broad in nine human cell-types. Color code: yellow/orange - enhancer; green - active transcription; blue - insulator; grey - low signal. ChIP-seq of ERα and FOXA1 in MCF-7 cells treated with E2. The lower track corresponds to ChIP-seq data of p300 in MCF-7 cells. GRO-seq of MCF-7 cells treated with E2 (in red) and normal medium (in blue). The bottom track corresponds to GRO-seq data in MDA-MB-231 cells. (**B**) MCF-7 cells were co-transfected with *Renilla* and pGL3-based vectors and luciferase activities were measured after 48 h. The SV40 enhancer (pGL3-SV40) was used as a positive control in this assay. The relative luciferase activities (*Renilla*/firefly) were normalized to pGL3-empty. Data represent mean ± s.d of $n = 3$. ***$P < 0.001$, relative to pGL3-empty. Fw, forward. RV, reverse. (**C**) MCF-7 cells were co-transfected with *Renilla* and pGL3-based vectors and treated with vehicle or E2 for 24 h. The relative luciferase activities (*Renilla*/firefly) were normalized to pGL3-empty vehicle. Data represent mean ± s.d of $n = 3$. ***$P < 0.001$, relative to pGL3-empty vehicle. (**D**) Schematic representation of pGL3-CUTE constructs used in the reporter assays. The mutant sequences of CUTE (mut. #4 and #6) were generated by CRISPR-Cas9 gene editing in MCF-7 cells, amplified by PCR and cloned into pGL3-promoter. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mutant sequences in pGL3. Luciferase reporter assays showed a drastic reduction in their transcriptional activity (Fig. 1D), which confirms that CUTE is as a *bona fide* enhancer regulated by ERα.

To further assess the effect of sgRNA1830 on its target (the ERE of CUTE), we transduced MCF-7 cells with sgRNA1830 and performed next-generation DNA sequencing in this region (Fig. 2A, Supplementary Fig. S1A). We observed that the vast majority of deletions generated by Cas9 are small (≤15 bp) and map to the expected cleavage site (Supplementary Fig. S1B). Next, we performed ChIP-seq for ERα in MCF-7 cells transduced with sgRNACtrl and sgRNA1830 (MCF-7^{sgCtrl} and MCF-7^{sg1830}, respectively) to assess the specificity of CRISPR-Cas9 targeting. Reassuringly we found that the binding of ERα is significantly decreased only at the CUTE locus in MCF-7^{sg1830} cells (Pearson = 0.91; *P*-value = 0) (Fig. 2B, Supplementary Fig. S1D). More importantly, two other hits from our previous screen [20] (enh588 and enh1986) remained unchanged, confirming specificity of the effect on the phenotype. It is known that the binding of ERα is frequently associated with FOXA1, which is thought to be required for the activation of target genes of estrogen [34]. Intriguingly, FOXA1 binding to CUTE was also specifically decreased in MCF7^{sg1830} cells (Pearson = 0.94; *P*-value = 0) (Fig. 2C, and Supplementary Fig. S1E). These results suggest that the disruption of ERα binding causes loss of FOXA1, which can in turn affect the transcriptional activity of CUTE. Consistent with this hypothesis, we also found that the activating histone mark H3K27Ac is significantly decreased at the CUTE locus in MCF-7^{sg1830} cells (Pearson = 0.96; *P*-value = 0) (Fig. 2D). So far our results indicate that sgRNA1830 specifically impairs the binding of ERα to CUTE, which leads to reduced transcriptional activity and loss of enhancer identity.

The identification of functional enhancers and their target genes is a major challenge in the field of transcriptional research [35]. Therefore, we combined multiple experimental techniques to identify the target gene of CUTE in breast cancer cells. First, we reanalyzed chromatin interactions identified by ChIA-PET and found that the CUTE locus

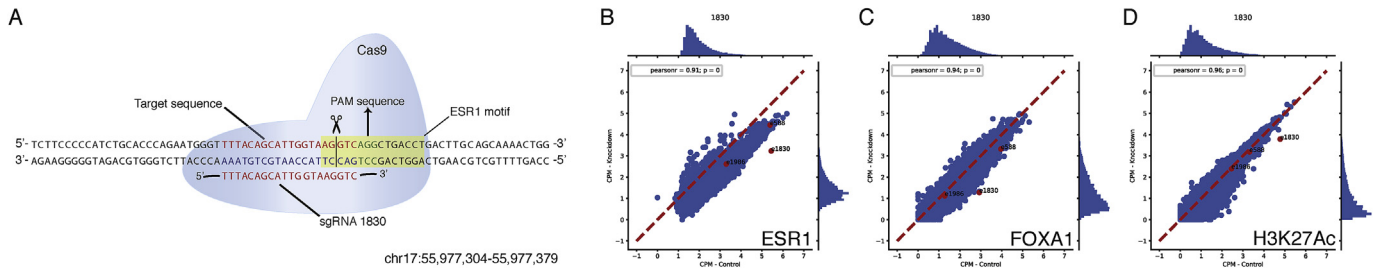

Fig. 2. The disruption of ERα binding at the CUTE locus is associated with loss of FOXA1 and H3K27Ac. (**A**) Schematic representation of the targeting of CUTE by CRISPR-Cas9 (sgRNA1830). (**B-D**) ChIP-seq of ERα (**C**), FOXA1 (**D**) and H3K27Ac (**E**) in MCF-7^{CNT} and MCF-7^{sg1830} cells. e588 - ERE 588. e1830 - ERE 1830. e1986 - ERE 1986. CPM, counts per million. Pearson, Pearson number. P, *P*-value.

interacts with several regions nearby *CUEDC1* (Fig. 3A). Interestingly, the interactions with the promoter of *CUEDC1* involve both ERα and RNAPII, suggesting that they might be functionally relevant. Then, we performed RNA-seq experiments in MCF-7^{sgCtrl} and MCF-7^{sg1830} cells in order to identify genes regulated by CUTE. We analyzed changes in gene expression in the vicinity of CUTE (± 500 kb) and found that *CUEDC1* was the most downregulated gene in MCF-7^{sg1830} cells (Fig. 3B). We confirmed by qPCR analysis that the expression of *CUEDC1* is significantly decreased in MCF-7^{sg1830} cells (~70%; *P*-value < 0.01) (Fig. 3C). On the other hand, sgRNA1830 had no significant effect on *CUEDC1* expression in MDA-MB-231 cells, despite having sgRNA1830-induced mutations (Fig. 3D, Supplementary Fig. S1C), which suggests that CUTE is an active enhancer only in ERα-positive cells. In line with this, we found a significant decrease in CUTE expression in MCF-7^{sg1830} (Fig. 3C) but not in MDA-MB-231^{sg1830} cells (Fig. 3D). Next, we reanalyzed publicly available GRO-seq data of MCF-7 cells treated with E2 in order to identify target genes of ERα. We focused our attention on the CUTE locus (± 500 kb) and identified *CUEDC1* as the most upregulated gene upon ERα activation (Fig. 3E). Then, we tested whether the expression of CUTE and *CUEDC1* depend on ERα function by removing E2 from the culture medium of MCF-7 and MDA-MB-231 cells. We found that both CUTE and *CUEDC1* expression were significantly downregulated in MCF-7 cells, but not in MDA-MB-231 cells upon 72 h of E2 depletion (Fig. 3F–G). We validated this result by performing a time-course experiment in MCF-7 and MDA-MB-231 cells treated with E2 after estrogen depletion. Indeed, *CUEDC1* expression was significantly increased (~2.5 fold) already 4 h after E2 treatment and remained high over the course of 24 h (Fig. 3H), indicating that *CUEDC1* is a primary target gene of ERα. The expression of eRNAs transcribed from CUTE followed a similar pattern in MCF-7 cells (Fig. 3H), supporting the notion that CUTE is responsive to ERα. Importantly, neither CUTE nor *CUEDC1* expression showed any significant alteration in MDA-MB-231 cells (Fig. 3I). These results suggest that CUTE mediates the activation of *CUEDC1* in response to ERα stimulation. In order to test this hypothesis, we treated estrogen-depleted MCF-7^{sgCtrl} and MCF-7^{sg1830} cells with E2 (24 h) and measured the expression of CUTE and *CUEDC1* by qPCR. Reassuringly, we found that the induction of CUTE and *CUEDC1* expression by E2 was severely compromised in MCF-7^{sg1830} cells (Fig. 3J), whereas in MDA-MB-231 cells no significant change in their expression was observed (Fig. 3K). Altogether, our experiments establish CUTE as an ERα-dependent enhancer mediating *CUEDC1* activation in breast cancer cells.

In a previous work, we showed that the disruption of CUTE by CRISPR-Cas9 (sgRNA1830) is associated with decreased proliferation of MCF-7 cells [20]. Given that CUTE is an enhancer of *CUEDC1*, we hypothesized that this gene is required for ERα-mediated cell proliferation. Accordingly, we designed two sgRNAs targeting the coding sequence of *CUEDC1* (sgRNACUEDC1 exon#1 and exon#2), and as a control we used two sgRNAs targeting introns nearby CUTE (sgRNACUEDC1 intron#1 and intron#2) (Fig. 4A). We observed a significant decrease in the proliferation of MCF-7 cells transduced with the sgRNAs targeting the exons of *CUEDC1*, but not with those targeting the introns (Fig. 4B). This effect on proliferation is similar to the one caused by inactivating CUTE (Fig. 4B), indicating that CUEDC1 is required for optimal cell proliferation and is likely to be the mediator of CUTE-induced cell proliferation phenotype. To assess the specificity of the impact of CUEDC1-exon targeting, we measure RNA levels by qRT-PCR. Out-of-frame mutations induced by CRISPR-Cas9 are expected to trigger reduced levels of mRNA expression due to the nonsense mediated mRNA decay pathway [36]. Indeed, *CUEDC1* levels were markedly reduced by sgRNAs targeting its exons (2.5–3 fold, Fig. 4C). This effect was specific since targeting the introns did not change significantly the expression of *CUEDC1* (Fig. 4D). Moreover, no effect was observed on the expression of eRNAs (Fig. 4C–D), indicating that the transcriptional activity of CUTE was not affected. Next, we ectopically expressed *CUEDC1* in MCF-7^{sg1830} and observed a complete rescue of the proliferative defect phenotype (Fig. 4E). To test specificity of the rescue mediated by CUEDC1 ectopic expression, we mutated the CUE domain (CUEDC1^{mut}) which is the only functional domain currently known for this protein (Fig. 4E). The ectopic expression of CUEDC1^{mut} in MCF-7^{sg1830} cells was not able to rescue the proliferation defect (Fig. 4E), suggesting that the CUE domain is required for the proper function of CUEDC1 in breast cancer cells. It was previously reported that CUEDC1 interacts with TOM1 - a protein suggested to be involved in cellular trafficking [25]. In co-IP experiments, we observed that CUEDC1^{mut} binds to TOM1 in a similar extent as wild type CUEDC1 (Fig. 5A), which indicates that the CUE domain is dispensable for the interaction with TOM1. Altogether, our results demonstrate that activation of CUEDC1 expression is essential for ERα-mediated stimulation of cellular proliferation.

Finally, we examined the expression levels of ERα and CUEDC1 in a publicly available breast cancer cohort (TCGA, http://xenabrowser.net) and we expected to have a positive correlation according to our findings above. Indeed, CUEDC1 expression was positively associated with ERα (R = 0.29; *P*-value = 10^{-199}, Fig. 5B), suggesting that *CUEDC1* is also a target of the estrogen pathway *in vivo*.

4. Discussion

The identification of direct target genes of the estrogen pathway is a fundamental question in current breast cancer research [11]. Genome-wide studies using GRO-seq identified ~3000 protein-coding genes that are regulated by estrogen in breast cancer cells [11]. This number is substantially higher than what was previously determined using expression microarrays and corresponds to ~33% of all expressed RefSeq genes in MCF-7 [12]. Despite these advances, it is still not clear which are the direct functional targets of this pathway as ERα binds mostly to distal enhancer elements [8,9]. Recently, we showed that functional enhancers can be annotated in an unbiased manner by CRISPR-Cas9 screens [20,37]. Moreover, we demonstrated that combining genome editing technologies with gene expression analysis is a powerful method to identify the target genes of enhancers. Here, we extended our

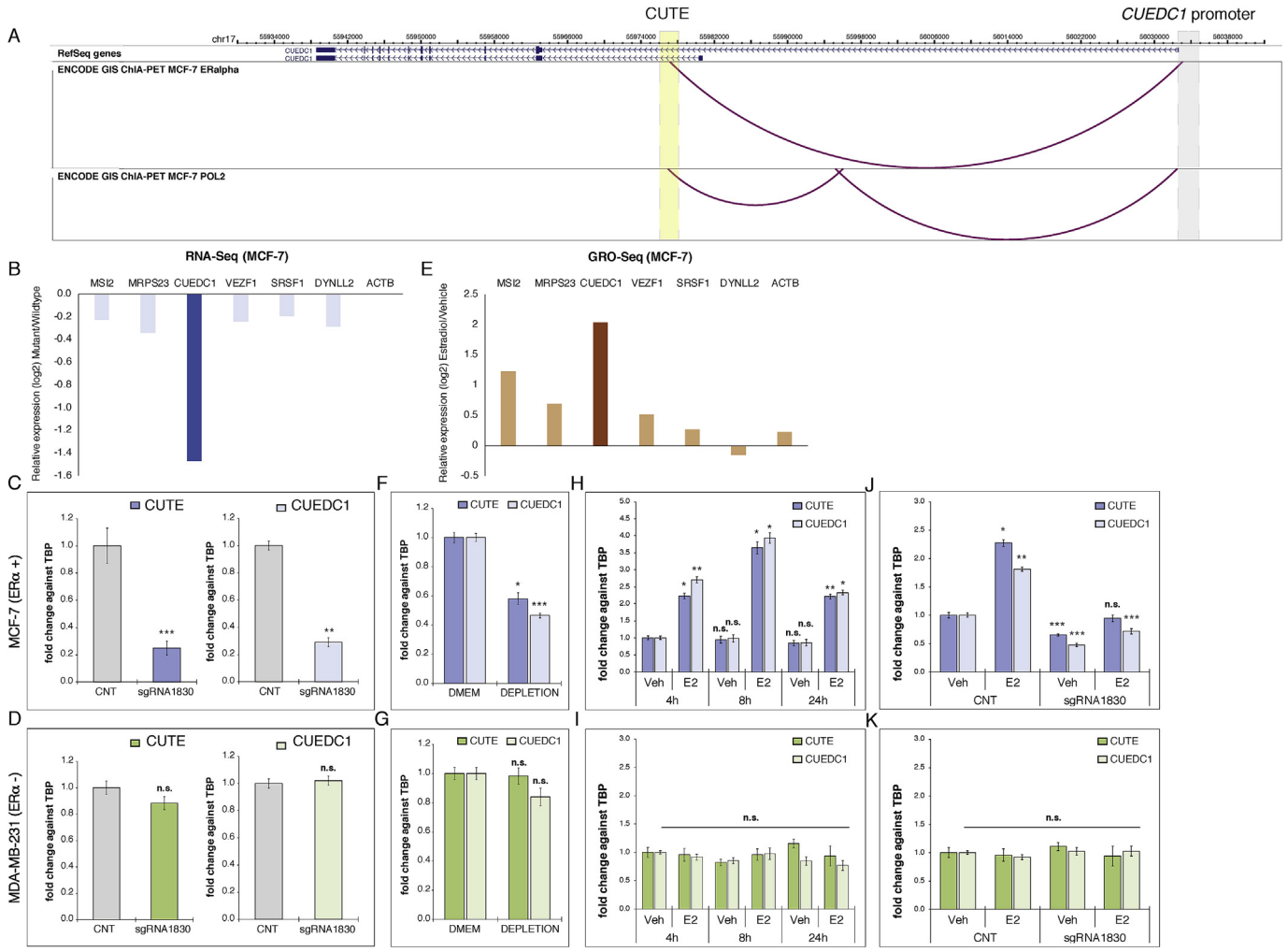

Fig. 3. CUTE is an ERα-responsive enhancer that regulates the expression of *CUEDC1* (**A**) ChIA-PET data of ERα and RNAPII in MCF-7 cells. Violet arcs represent long-range chromatin interactions that are statistically significant. (**B**) RNA-seq was performed in MCF-7^{CNT} and MCF-7^{sg1830} cells. Differential expression analysis was restricted to genes located −/+ 500 Kb of CUTE. ACTB was used as a negative control. (**C**) Expression analysis of CUTE eRNA and *CUEDC1* mRNA in WT (CNT) and mutant (sgRNA1830) MCF-7 cells. Data represent mean ± s.d of $n = 3$. $^{**}P < 0.01$. $^{***}P < 0.001$. (**D**) Expression analysis of CUTE eRNA and *CUEDC1* mRNA in WT (CNT) and mutant (sgRNA1830) MDA-MB-231 cells. Data represent mean ± s.d of $n = 3$. n. s., not significant. (**E**) Relative mRNA expression measured by GRO-seq in MCF-7 cells treated with vehicle (Veh) and estradiol (E2). Differential gene expression analysis was restricted to genes located −/+ 500 Kb of CUTE. *ACTB* was used as a negative control. (**F**) Expression analysis of CUTE eRNA and *CUEDC1* mRNA in MCF-7 cells treated with complete (DMEM) and conditioned medium (E2 depletion). Data represent mean ± s.d of $n = 3$. $^{*}P < 0.05$, $^{***}P < 0.001$. (**G**) Expression analysis of CUTE eRNA and *CUEDC1* mRNA in MDA-MB-231 cells treated with complete (DMEM) and conditioned medium (E2 depletion). Data represent mean ± s.d of $n = 3$. n. s., not significant. (**H**) Analysis of CUTE eRNA and *CUEDC1* mRNA expression by qPCR in MCF-7 cells treated with vehicle (Veh) or estradiol (E2). Gene expression levels are normalized to *TBP*. Data represent mean ± s.d of $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$., n. s., not significant. Two-tailed Student's t-test relative to Vehicle-4h (**I**) Analysis of CUTE eRNA and *CUEDC1* mRNA expression by qPCR in MDA-MB-231 cells treated with vehicle (Veh) or estradiol (E2). Gene expression levels are normalized to *TBP*. Data represent mean ± s.d of $n = 3$. n. s., not significant. Two-tailed Student's t-test relative to Vehicle-4h. (**J**) Analysis of CUTE eRNA and *CUEDC1* mRNA expression by qPCR in MCF-7^{CNT} and MCF-7^{sg1830} cells treated with vehicle (Veh) or estradiol (E2). Expression levels are normalized to *TBP* and compared to CNT-Veh. Data represent mean ± s.d of $n = 3$. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. n. s., not significant. (**K**) Analysis of CUTE eRNA and *CUEDC1* mRNA expression by qPCR in MDA-MB-231^{CNT} and MDA-MB-231^{sg1830} cells treated with vehicle (Veh) or estradiol (E2). Expression levels are normalized to *TBP* and compared to CNT-Veh. Data represent mean ± s.d of $n = 3$. n. s., not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

strategy to characterize CUTE, an ERα-bound enhancer previously identified in our screen [20]. Several lines of evidence suggest that CUTE controls the expression of *CUEDC1* in response to estrogen signaling, and that this interaction is essential for ERα-mediated stimulation of cell proliferation. First, ERα binds robustly to CUTE in MCF-7 cells as detected by ChIP-seq experiments (Fig. 1A). Second, binding of ERα to CUTE is significantly decreased by mutations in the ERE (Fig. 2B). Third, chromatin interactions between CUTE and the promoter of *CUEDC1* involve ERα (Fig. 3A). Fourth, activation of *CUEDC1* expression by estrogen relies entirely on the ERE of CUTE (Fig. 3J). Finally, the expression of *CUEDC1* correlates positively with ERα expression in breast tumors (Fig. 5B). Altogether, our results strongly imply that *CUEDC1* is a direct functional target of estrogen in breast cancer cells (Fig. 5C).

Our results suggest that CRISPR-Cas9 has a high specificity of targeting at enhancer elements. This is supported by ChIP-seq data showing that ERα binding is specifically decreased at the CUTE region by sgRNA1830. The fact that a single locus is affected by CRISPR-Cas9 is quite remarkable since ERα binds to tens of thousands of EREs across the genome [38]. We observed similar effects at additional ERα-regulated enhancers in multiple breast cancer cell lines [20], further supporting the application of CRISPR-Cas9 to study transcriptional

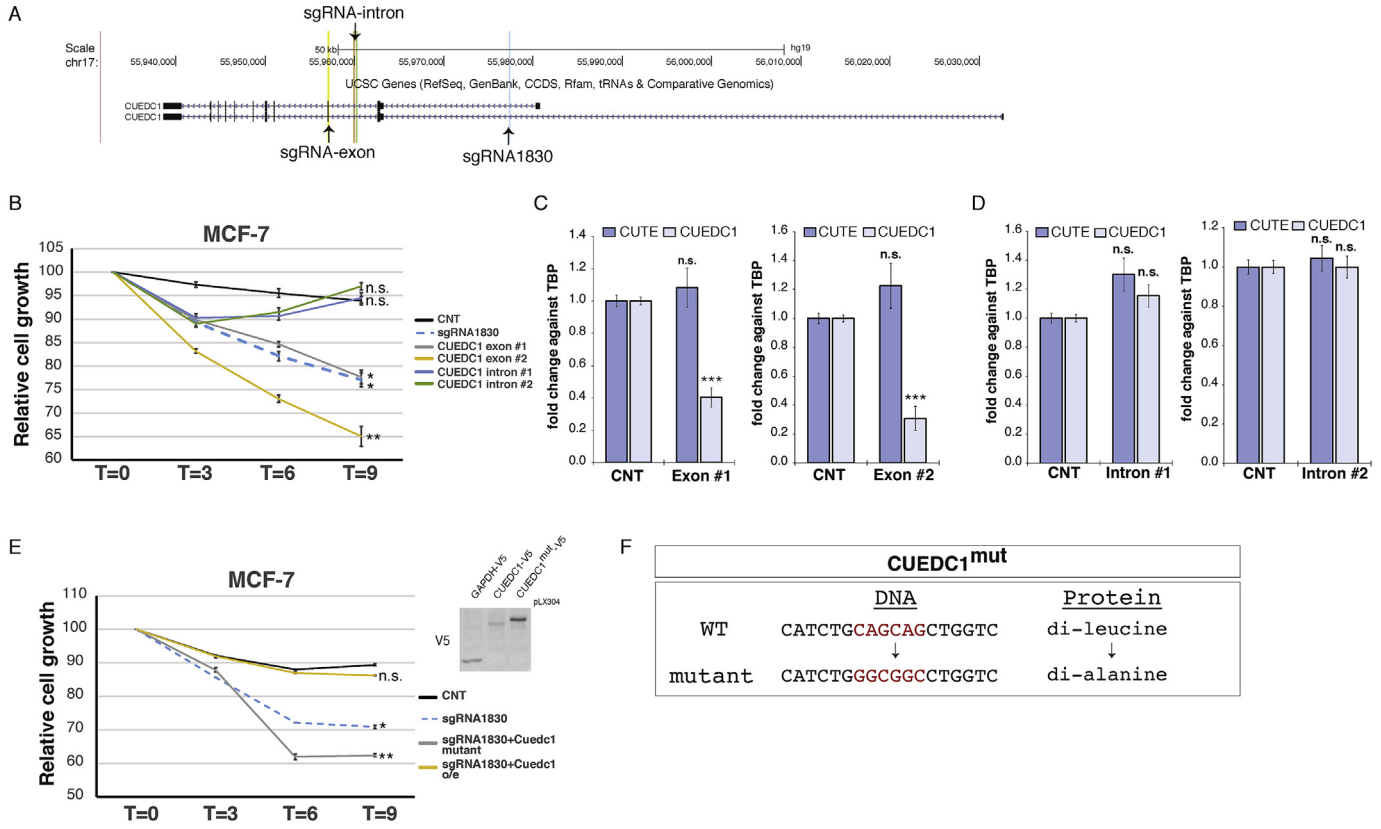

Fig. 4. *CUEDC1* is required for the growth of MCF-7 cells. (**A**) Schematic representation of the CUEDC1 mRNA and the sgRNAs targeting it (**B**) MCF-7 cells were transduced with the indicated sgRNAs and allowed to proliferate for nine days. Cell growth is represented as percentage of GFP, which is normalized to the beginning of the experiment (T = 0). (**C**) Expression analysis of CUTE eRNA and *CUEDC1* mRNA by qPCR in MCF-7 cells transduced with sgRNAs targeting an exon of *CUEDC1*. Data represent mean ± s.d of $n = 3$. ***$P < 0.001$. n. s., not significant. (**D**) Expression analysis of CUTE eRNA and *CUEDC1* mRNA by qPCR in MCF-7 cells transduced with sgRNAs targeting an intron of *CUEDC1*. Data represent mean ± s.d of $n = 3$. n. s., not significant. (**E**) MCF-7 cells were sequentially transduced with the indicated sgRNAs and with pLX304-GAPDH, pLX304-CUEDC1 or pLX304-CUEDC1^{mut}. The cells were allowed to proliferate for nine days and their growth is represented as percentage of GFP (normalized to T = 0). Data represent mean ± s.d of $n = 3$. Western blot analysis of MCF-7 cells expressing the different constructs is shown on the side. The V5 tag was used to detect expression of the proteins. (**F**) Schematic representation of the mutations generated in the CUE domain, which convert a di-leucine to a di-alanine motif.

enhancers [37]. The loss of ERα binding is associated with a concomitant decrease in the signal of FOXA1 and H3K27Ac at the CUTE locus of targeted cells (MCF-7^{sg1830}). FOXA1 is a pioneer factor that is required for ERα-mediated gene regulation [34], whereas H3K27Ac is a mark frequently associated with active enhancers [39,40]. Additionally, we found that the expression of eRNAs is decreased in MCF-7^{sg1830} cells, suggesting that RNA polymerase II (RNAPII) activity is impaired at the CUTE locus. We conclude that ERα is an essential component of CUTE since the disruption of its binding leads to loss of enhancer-associated marks and decreased transcriptional activity.

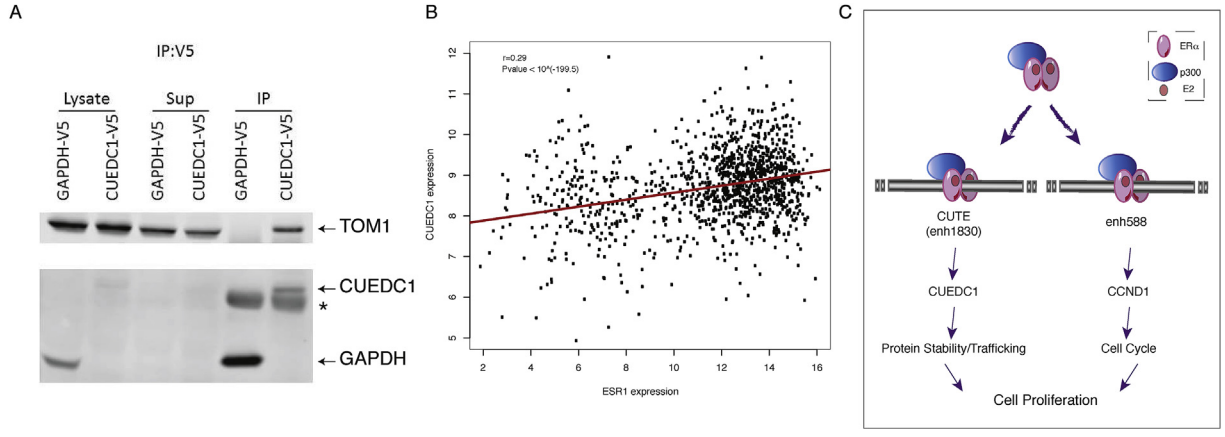

Fig. 5. *CUEDC1* expression is significantly increased in ERα-positive tumors (**A**) HEK-293 T cells were transfected with V5 tagged CUEDC1 and control GAPDH vectors. Cells were extracted and subjected to co-immuno-precipitation and blotting analysis with anti-TOM1 and anti-V5 antibodies. (**B**) The expression of *CUEDC1* and ESR1 was compared in 1219 samples from the TCGA (R = 0.29; *P*-value = 10^{-199}). (**C**) Graphic model depicting the role of CUTE (enh1830) and enh588 [20] in controlling cell proliferation.

However, it is possible that there are additional sequences, besides the ERα binding site, which are critical for the activity of CUTE. This question can be addressed by saturation mutagenesis experiments using CRISPR-Cas9, which allow to identify critical regulatory elements at near-nucleotide resolution [19,20,22].

The concept of "super-enhancers" has been recently proposed to describe regulatory elements that drive exceptionally high levels of transcription [41–43]. Super-enhancers typically comprise a cluster of regulatory elements, spanning up to 12.5 kb, which exhibit highly synergistic activities [44]. Our results suggest that CUTE is a strong enhancer in human cells: in reporter assays, it is able to activate gene expression in a robust manner (~30 fold upon E2 stimulation); genetic alterations in its sequence cause a dramatic reduction in *CUEDC1* expression (~70%). Interestingly, we noted that the CUTE locus fulfils the bioinformatic criteria of super-enhancers: extended signal of H3K27Ac (~8 kb), multiple DNAse Hypersensitive sites (DHSs) located in close proximity (~4 kb) and binding of RNAPII [41]. Moreover, the two DHSs in the vicinity of CUTE are also bound by cell-type-specific TFs (e.g. FOXA1, GATA3) and co-activator p300. Given the available evidence, it is tempting to speculate that these DHSs are enhancers that collaborate with CUTE to regulate *CUEDC1* expression in breast cancer cells. However, further experiments (e.g. deletion of each constituent element) are required to clarify whether they are functional elements and whether they act as a super-enhancer of gene expression [45,46].

The disruption of CUTE by CRISPR-Cas9 is associated with decreased cell proliferation, and our results strongly suggest that this is due to decreased expression of *CUEDC1*. To date, not much is known about the molecular mechanisms that underpin the function of CUEDC1, though available evidence points in the direction of the ubiquitin pathway. CUE domains are sequences of ~40 amino acids that bind monoubiquitin in yeast and human [47] and regulate chain formation by E3 ligases [23]. Interestingly, CUEDC1 was recently identified in a proteome-wide screen for ubiquitin interactors [24]. Zhang and colleagues showed that CUEDC1 binds to K33 and K63 diubiquitin in different human cell-types, suggesting that it might be involved in protein trafficking, signal transduction and degradation pathways [48]. This hypothesis is also supported by our results showing an interaction between CUEDC1 and TOM1. The connection of *CUEDC1* with cancer is thin; however, its expression is positively correlated with ERα in breast tumors (Fig. 5B) and is significantly upregulated in metastatic cervical tumors compared to primary tumors [49]. These results support our findings and suggest that *CUEDC1* is a downstream target of ERα mitogenic function, although additional studies are required to clarify its role in cell proliferation and tumor development (Fig. 5C).

Conflicts of interest

All authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2018.08.018.

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