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Flightless I (*Drosophila*) homolog facilitates chromatin accessibility of the estrogen receptor α target genes in MCF-7 breast cancer cells

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ABSTRACT

The coordinated activities of multiple protein complexes are essential to the remodeling of chromatin structure and for the recruitment of RNA polymerase II (Pol II) to the promoter in order to facilitate the initiation of transcription in nuclear receptor-mediated gene expression. Flightless I (*Drosophila*) homolog (FLII), a nuclear receptor coactivator, is associated with the SWI/SNF-chromatin remodeling complex during estrogen receptor (ER)α-mediated transcription. However, the function of FLII in estrogen-induced chromatin opening has not been fully explored. Here, we show that FLII plays a critical role in establishing active histone modification marks and generating the open chromatin structure of ERα target genes. We observed that the enhancer regions of ERα target genes are heavily occupied by FLII, and histone H3K4me3 and Pol II binding induced by estrogen are decreased in FLII-depleted MCF-7 cells. Furthermore, formaldehyde-assisted isolation of regulatory elements (FAIRE)-quantitative polymerase chain reaction (qPCR) experiments showed that depletion of FLII resulted in reduced chromatin accessibility of multiple ERα target genes. These data suggest FLII as a key regulator of ERα-mediated transcription through its role in regulating chromatin accessibility for the binding of RNA Polymerase II and possibly other transcriptional coactivators.

1. Introduction

Chromatin remodeling complexes are multi-protein structures that utilize energy derived from ATP hydrolysis to alter the chromatin architecture at the nucleosomal level. The four major classes of ATP-dependent chromatin remodeling complexes, SWI/SNF, ISWI, Mi-2/NuRD, and INO80, are characterized by the identity of their ATPase subunits [1–3]. The SWI/SNF complex is one of the most studied and well-characterized remodeling complexes in nuclear receptor-mediated transcription [4,5].

The SWI/SNF families, originally identified by genetic screens in yeast, were initially considered to play an important role in mating-type switching [6,7]; thereafter, their functional role in transcriptional regulation has also been uncovered [8–10]. The human SWI/SNF complex contains either BRG1 (also known as SMARCA4) or hBRM (also known as SMARCA2) as the core ATPase and 10–12 BRG1-associated factors (BAFs) [11]. Four subunits, BRG1, BAF155, BAF170, and BAF47, are required for the minimal catalytic activity of the complex [12]. It appears that the BRG1- and the hBRM-containing complexes have similar activities *in vitro* [13], but distinct biological functions *in vivo* [14,15]. In addition, BRG1 knockout mice show early-stage embryonic death, whereas BRM null mice are viable [14,15]. Biochemical analysis has revealed that the SWI/SNF complex alters nucleosomal structures and increases the accessibility of proteins to their DNA templates by mediating open chromatin configuration in an ATP-dependent manner *in vitro* [1]. Recent studies show that the SWI/SNF complex is associated with the formation of DNase I hypersensitive chromatin regions [16–18] at potential glucocorticoid receptor (GR)-binding sites *in vivo*.

Flightless I (*Drosophila*) homolog (FLII) is an evolutionarily conserved member of the gelsolin family with actin binding and severing activities [19]. Analysis of tissue distribution showed ubiquitous expression of FLII in human tissues, especially high level in cervix and bone marrow [20]. The N-terminal region of FLII contains leucine-rich repeat (LRR) domains, which are implicated in protein–protein interactions; on the other hand, the C-terminal region of FLII is homologous to the gelsolin family of actin-binding proteins. It has been demonstrated that FLII interacts with nuclear receptors (e.g., ER, TR), coactivators (e.g., GRIP1 and CARM1), and BAF53, an actin-like protein [21,22]. FLII directly binds to ERα through an LXXLL motif in the gelsolin-like repeat-3 (G3) domain in a hormone-dependent manner, and this interaction is required for efficient hormonal induction of endogenous ERα target genes

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[21]. In addition, FLII interacts with BAF53, an actin-related component of the SWI/SNF chromatin-remodeling complex, implicating a potential role of FLII in maintaining the local chromatin structure in the promoter or enhancer regions. Although we previously showed that FLII is required for recruitment of the SWI/SNF complex to the promoter of Trefoil Factor 1 (*TFF1*), a well-known ERα target gene [21,23], the function of FLII in the chromatin accessibility of ERα target genes remains unclear.

Considering that FLII recruits the SWI/SNF chromatin remodeling complex to the *TFF1* gene and regulates the expression of multiple ERα target genes, we hypothesized that FLII might play a critical role in establishing active histone modification marks and generating an open chromatin structure at multiple ERα target genes. We observed that the enhancer regions of various ERα target genes were heavily occupied by FLII. Using formaldehyde-assisted isolation of regulatory elements (FAIRE)-quantitative polymerase chain reaction (qPCR) to assess chromatin accessibility, we investigated how the depletion of FLII affects the formation of open chromatin structure at the enhancer or promoter regions of *TFF1* and other ERα target genes. These results provide substantial evidence for the functional role of FLII in mediating SWI/SNF-dependent chromatin remodeling to maintain chromatin accessibility for the binding of RNA Polymerase II (Pol II) and possibly other transcriptional coactivators.

2. Materials and methods

2.1. Cell culture and immunoblotting

MCF-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Immunoblotting was performed as described previously [24], using the following antibodies: anti-ERα and anti-FLII from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-β-actin from Thermo Scientific (Pittsburgh, PA).

2.2. RNA interference and reverse transcription (RT)-qPCR

MCF-7 cells were transfected with siRNA against FLII (siFLII) or non-specific siRNA (siNS) as described previously [25]. The sequences for siRNAs used were: siFLII, 5′-CAACCUGACCACGCUUCAUdTdT-3′ (sense) and 5′-AUGAAGCGUGGUCAGGUUGdTdT-3′ (anti-sense); siNS, 5′-UUCUCCGAACGUGUCACGUdTdT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAAdTdT-3′ (anti-sense). Total RNA was isolated from MCF-7 cells with Trizol from Invitrogen (Grand Island, NY) after hormone treatment and subjected to reverse transcription using the iScript cDNA Synthesis Kit from Bio-Rad (Hercules, CA) in a total volume of 20 μL. After a 10-fold dilution with water, 2 μL of each sample was used for qPCR analysis with the following primers: *TFF1* pre-mRNA, 5′-GGTCCTGGTGTCCATGCT-3′ (forward) and 5′-CAGGAAGAAGCACGCCTTAC-3′ (reverse); *FLII* mRNA, 5′-CCTCCTACAGCTAGCAGGTTATCAAC-3′ (forward) and 5′-GCATGTGCTGGATATATACCTGGCAG-3′ (reverse); *18S* mRNA, 5′-GAGGATGAGGTGGAACGTGT-3′ (forward) and 5′-TCTTCAGTCGCTCCAGGTCT-3′ (reverse). Relative expression levels were obtained by normalization to *18S* mRNA levels. Results shown depict the mean and range of variation of duplicate PCR reactions performed on the same cDNA sample.

2.3. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed according to previously described protocols [21,26]. Briefly, MCF-7 ells were transfected with siRNA and cultured for 3 days in phenol red-free DMEM supplemented with 5% charcoal-dextran-stripped FBS. At approximately 90% confluency, the cells were treated with 100 nM estradiol (E2) or ethanol for the indicated time periods. After cross-linking with formaldehyde, cell extracts were prepared from control and E2-treated MCF-7 cells. Immunoprecipitation of sonicated chromatin solutions was conducted by overnight incubation at 4 °C with anti-ERα, anti-FLII from Santa Cruz Biotechnology (Santa Cruz, CA), anti-H3K4me1 from Abcam (Cambridge, MA), anti-H3K4me2, or anti-H3K4me3 from Active Motif (Carlsbad, CA) antibodies. Cross-linking was reversed by heating, and the immunoprecipitated DNA was purified by phenol–chloroform extraction and ethanol precipitation. The purified DNA was dissolved in 100 μL of TE buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA) and analyzed by qPCR using a Roche LightCycler 480 II system with SYBR Green dye. The primers used were: *ERE1*, 5′-CCGGCCATCTCTCACTATGAA-′3 (forward) and 5′-CCTTCCCGCCAGGGTAAATAC-3′ (reverse); *ERE2*, 5′-CCTCCCCAGCTCACGTTGT-3′ (forward) and 5′-GGGTTGCATTTAAGGGACCTT-3′ (reverse); *ERE3*, 5′-GTCGTTGCCAGCGTTTCC-3′ (forward) and 5′-CTTCTCCACGCCCTGTAAATTT-3′ (reverse). Results shown depict the mean and range of variation of duplicate PCR reactions performed on the same DNA sample. Results were derived from a single experiment that is representative of at least two independent experiments.

2.4. Formaldehyde-assisted isolation of regulatory elements (FAIRE)-qPCR

FAIRE-qPCR was performed as previously described [26]. Briefly, MCF-7 cells were transfected with siRNA and cultured for 3 days in phenol red-free DMEM supplemented with 5% charcoal-dextran-stripped FBS. At approximately 90% confluency, the cells were treated with 100 nM E2 or ethanol for 60 min. After cross-linking with formaldehyde, cell extracts were prepared from control and E2-treated MCF-7 cells. Cross-linked chromatin was sonicated and the open chromatin DNA (FAIRE DNA) was purified from the aqueous phase by phenol–chloroform extraction and ethanol precipitation. For input control (Input DNA), cross-linking was reversed by heating before extraction. The purified DNA was dissolved in 100 μL of TE buffer and analyzed using the Roche LightCycler 480 II system with SYBR Green dye. Results shown depict the mean and range of variation of duplicate PCR reactions performed on the same DNA sample. Results are expressed as the percent of input chromatin (Input DNA) and were derived from a single experiment that is representative of at least two independent experiments.

2.5. Cell proliferation assay

MCF-7 cells were plated into 6-well plates (2×10^5 cells/well). The next day, the cells were transfected with siRNA and cultured for 3 days in DMEM supplemented with 10% FBS. Cells were trypsinized on indicated days and total number of live cells was counted after Trypan blue staining.

3. Results

3.1. FLII is recruited to the promoter and enhancer regions of the TFF1 gene

Our previous ChIP results showed that the promoter region of the *TFF1* gene (ERE1, −0.3 kb) was occupied by FLII in a hormone-dependent manner and that recruitment of FLII to this region was required for the transcription of *TFF1* [21]. To test whether FLII also binds to the enhancer region of *TFF1*, we performed a ChIP scanning assay to survey the sequences located 12 kb upstream (−12 kb) through 5 kb downstream of the

transcription start site (TSS) of *TFF1* (Fig. 1A). We observed an E2-mediated increase in FLII binding at the proximal ER binding sites after 60 min of E2 treatment. Interestingly, the distal enhancer region of *TFF1* (ERE2, −9.1 kb and *ERE3*, −9.9 kb) was also found to be heavily occupied by FLII in the presence of E2.

It has been shown that FLII binds to ERα in a hormone-dependent manner via the LXXLL motif [21], and that both ERα and FLII are recruited to the enhancer and promoter region of *TFF1* (Fig. 1A). To test whether FLII is required for the recruitment of ERα to *TFF1* gene, we performed an ERα–ChIP assay in FLII-depleted MCF-7 cells (Fig. 1B, upper panel). Depletion of FLII did not affect the levels of ERα binding at the promoter and enhancer regions, both in the absence and presence of E2. However, E2-induced binding of Pol II to the promoter region was reduced by FLII depletion in MCF-7 cells (Fig. 1B, lower panel), suggesting that FLII may function downstream of the ERα binding event.

To test whether FLII regulates the expression of *TFF1* gene at the transcriptional level, we measured the pre-mRNA levels of *TFF1* gene using PCR primers spanning an exon/intron junction. This method provides more reliable results of the rapid changes in transcriptional rates. E2 treatment increased the pre-mRNA levels of *TFF1* after 8 h, and this increase was ablated by the depletion of FLII (Fig. 1C), indicating that FLII is critically involved in the transcriptional regulation of the endogenous *TFF1* gene.

3.2. Role of FLII in activation-associated histone H3K4me marks on the TFF1 gene

The observation that FLII depletion causes decreased Pol II recruitment to the promoter region, thereby reducing the transcription initiation of *TFF1* (Fig. 1), prompted us to investigate the effects of FLII depletion on H3K4 methylation at the regulatory sites of the *TFF1* gene. H3K4 monomethylation (H3K4me1) peaks are generally observed at enhancer elements of active or potentially active genes, while H3K4 trimethylation (H3K4me3) peaks are commonly associated with the TSS regions of such genes [27–31]. As expected, high levels of H3K4me1 at ERE3 as well as of H3K4me3 at the promoter region were observed before E2 treatment, compared to +3.1 kb control region. Sixty minutes after E2 treatment, a further significant increase of H3K4me3 at ERE1 was observed (Fig. 2). This E2-induced trimethylation was found to decrease after FLII depletion.

These results indicate that FLII plays a critical role in generating E2-induced H3K4me3 mark at the promoter region of the *TFF1* gene (Fig. 2), supporting the fact that E2-induced Pol II binding and transcription initiation is decreased by silencing of FLII (Fig. 1B and C).

3.3. FLII is required for chromatin accessibility of the enhancer of ERα target genes

Given that both the enhancer and promoter regions of the *TFF1* gene are occupied by FLII (Fig. 1), and that FLII facilitates the recruitment of the SWI/SNF chromatin remodeling complex in MCF-7 cells [21], we used FAIRE-qPCR to investigate whether FLII influences the chromatin accessibility of the *TFF1* promoter and enhancer regions. Hormone-deprived MCF-7 cells were transfected with siNS or siFLII and treated with ethanol or E2 for 60 min. While siNS-transfected and ethanol-treated MCF-7 cells showed weak FAIRE signals at the proximal and distal ERα-binding sites (Fig. 3A), an increased FAIRE signal at the ERα-binding sites (ERE1 and ERE3) was observed after E2 treatment. FAIRE signals remained unchanged for all other regions examined in this assay. Surprisingly, specific silencing of FLII by siRNA dramatically decreased the E2-induced FAIRE signals at the enhancer and promoter regions of *TFF1* that were previously observed in

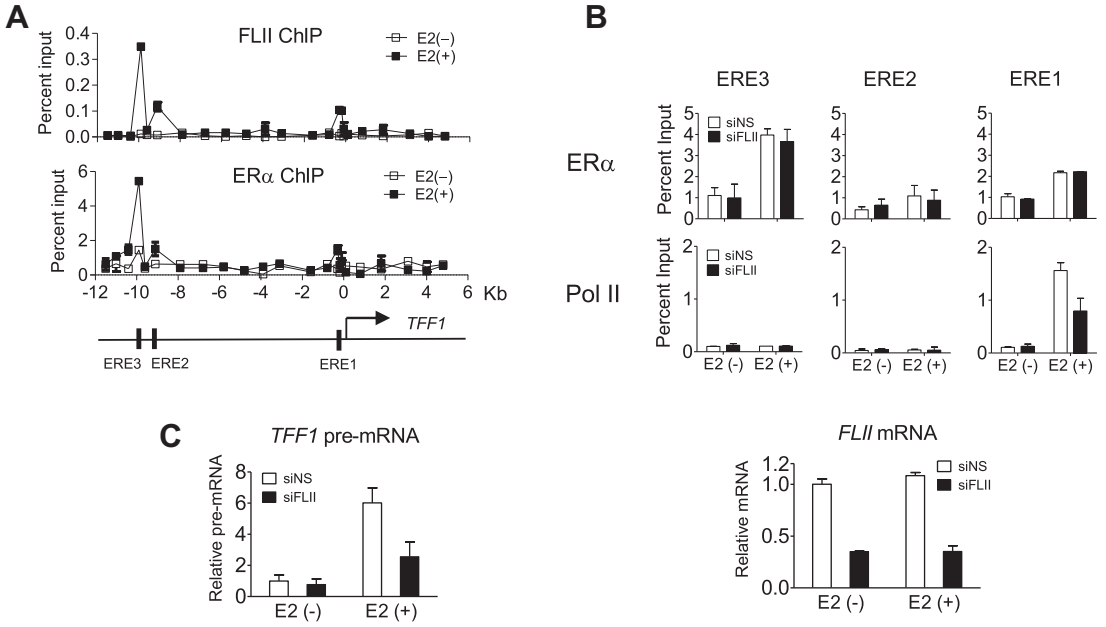

Fig. 1. Recruitment of FLII to the enhancer region of the *TFF1* gene. (A) ChIP scanning of the *TFF1* locus. DNA precipitated with the indicated antibodies was analyzed by qPCR with primer sets spaced at 1-kb intervals and spanning the region from −12 kb to +5 kb, relative to the TSS (denoted by a horizontal arrow in the diagram) of the *TFF1* gene. Primer sequences for ChIP scanning are available upon request. (B) The occupancy of EREs of the *TFF1* gene by ERα or Pol II in MCF-7 cells transfected with siRNA against FLII (siFLII) or non-specific siRNA (siNS). MCF-7 cells were transfected with siNS or siFLII, grown in hormone-free medium for 72 h, and then treated with E2 (100 nM) for 0 or 60 min before performing ChIP with antibodies against ERalpha or RNA Pol II. Precipitated DNA was analyzed by qPCR (upper panel). The mRNA levels of *FLII* were measured by qRT-PCR (bottom panel). (C) Effect of reduced FLII on the expression of *TFF1* gene. MCF-7 cells were transfected with siNS or siFLII, and treated with E2 (10 nM) for 8 h before harvest. Expression levels of mRNAs were normalized to that of *18S* mRNA. The pre-mRNA levels of the *TFF1* gene were measured by qRT-PCR using primers spanning the 3′ end of exon 1 and the 5′ end of intron 1 of the *TFF1* gene. Results shown depict the mean and range of variation of duplicate PCR reactions performed on the same cDNA sample; the results are from a single experiment that is representative of at least two independent experiments.

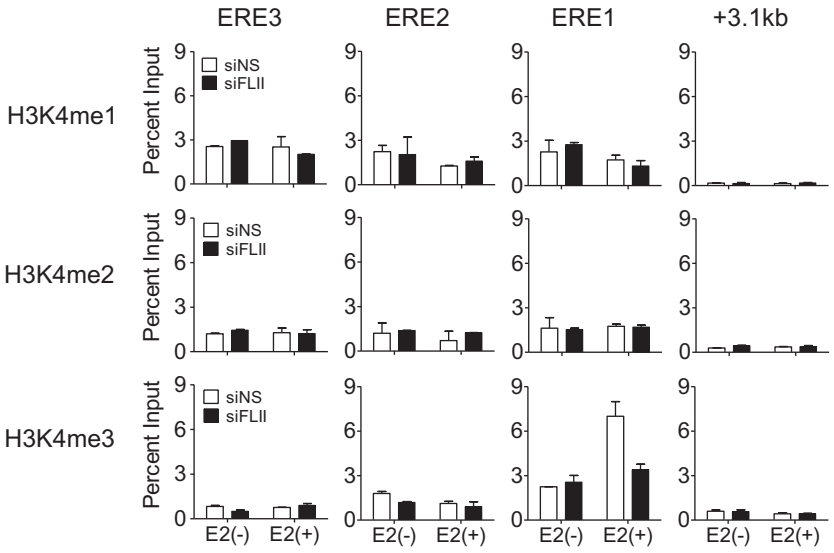

Fig. 2. Histone H3K4 methylation status in FLII-depleted MCF-7 cells. ChIP assays were performed as shown in Fig. 1B after transfection with siNS or siFLII. The extent of the indicated region of *TFF1* genes precipitated by H3K4 methyl-specific antibodies was determined by qPCR. Results are expressed as the percent of input chromatin (before immunoprecipitation).

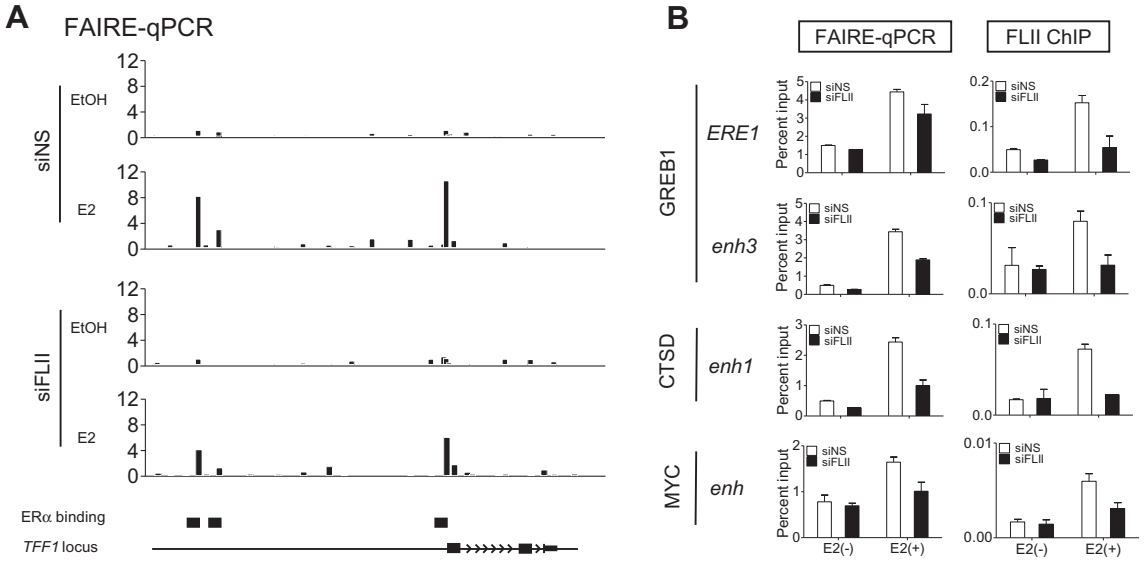

Fig. 3. FLII is required for chromatin accessibility of the promoter and enhancer of ERα target genes. (A) Chromatin accessibility at the *TFF1* locus was assessed by FAIRE-qPCR analysis using chromatin samples prepared from the MCF-7 cells transfected with siNS or siFLII. Data are normalized against non-cross-linked genomic DNA for each primer pair. (B) FLII is required for the chromatin remodeling of multiple ERα target genes. FAIRE-qPCR analysis was performed at the promoter or enhancer regions of *GREB1*, *CTSD*, and *MYC* in FLII-depleted or control MCF-7 cells treated with E2 (100 nM) or ethanol for 60 min. ChIP assays for FLII were performed as detailed in Fig. 1 after transfection with siNS or siFLII. The extent of the indicated region of the *TFF1* gene precipitated by anti-FLII antibodies was determined by qPCR.

siNS-transfected cells (Fig. 3A), suggesting that FLII depletion causes reduced chromatin accessibility to EREs.

We have previously demonstrated by RT-qPCR that FLII also plays a critical role in the regulation of other ERα target genes, such as *GREB1*, *MYC*, and *CTSD* (Cathepsin D). To assess whether FLII is also required for chromatin accessibility of these genes, we performed FAIRE-qPCR of their promoter or enhancer regions (Fig. 3B). We found strong recruitment of FLII to the promoter as well as enhancer regions of *GREB1* (ERE1 and enh3), and the enhancer regions of *CTSD* (enh1) and *MYC* (enh). The extent of FLII retention coincided approximately with that of ERα binding [26]. For example, high FLII binding was observed at the sites that were also highly occupied by ERα. Similar to*TFF1*, depletion of FLII altered the chromatin accessibility of the regulatory regions of other ERα target genes (*GREB1*, *CTSD*, and *MYC*) (Fig. 3B). These results indicate that FLII is required for the maintenance of optimal chromatin configuration at the enhancers of not only *TFF1*, but also other estrogen target genes.

3.4. Requirement for FLII in the proliferation of MCF-7 cells

To investigate the possible role of FLII in the proliferation of breast cancer cells, we examined the growth of MCF-7 cells transfected with siFLII or siNS (Fig. 4). Silencing of endogenous FLII by siRNA in MCF-7 cells (Fig. 4C and D) cultured in normal media containing FBS, which is equivalent to their growth in E2, resulted in significant inhibition of cell proliferation ($P < 0.01$) (Fig. 4A and B), indicating that FLII plays a pivotal role in the growth of breast cancer cells.

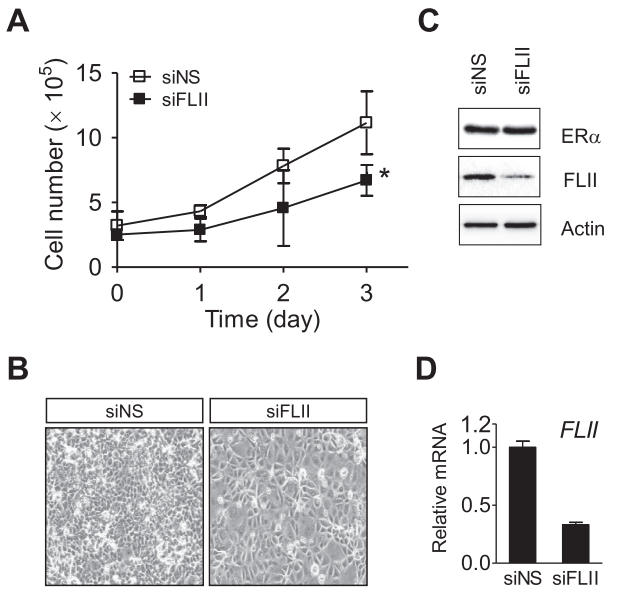

Fig. 4. Depletion of FLII causes inhibition of proliferation of MCF-7 cells. (A&B) MCF-7 cells were transfected with siNS or siFLII and cultured for 3 days in normal media supplemented with 10% FBS, for the proliferation assay. The data depict the mean ± s.d. of independent replicates. $^{*}P < 0.05$, significantly different from siNS. (C&D) Depletion of FLII protein by siRNA transfection. Levels of β-actin, ERα, and FLII were assessed by immunoblotting. The mRNA expression level of *FLII* was determined by RT-qPCR.

4. Discussion

A yeast two-hybrid screen for CARM1-binding proteins identified FLII, an actin-binding protein that plays essential roles in *Drosophila* and mouse development [22]. Since the identification of cofilin as the first actin-binding protein in 1987 [32], several other actin-binding proteins have been discovered, including profilin, thymosin β_4, CapG, gelsolin, and FilaminA. Most of the actin-binding proteins are predominantly cytoplasmic, but can translocate into the nucleus under conditions of stress, differentiation, or hormone-mediated cell stimulation. Recent studies have shown that FLII is involved in nuclear receptor-mediated signaling, interacts with ERα in an estrogen-dependent manner, and enhances ERα-mediated expression of transient reporter genes [21,22]. Although the mechanism of transcriptional activation by FLII is still unclear, the functional role of FLII in nuclear receptor-mediated transcription is well accepted [21,22]. In this study, we have defined the role of FLII in maintaining the local chromatin structure of the *TFF1* enhancer region that is required for transcription initiation.

We showed that E2 caused a progressive increase in FLII binding at the proximal ER-binding sites over a period of 1 h. We also found that the distal enhancer region of *TFF1* (ERE2, −9.1 kb and ERE3, −9.9 kb) was highly occupied by FLII in the presence of E2 (Fig. 1A). FLII is critically involved in the transcriptional regulation of endogenous ERα target genes without altering ERα binding to EREs at the enhancer and promoter regions (Fig. 1). Depletion of FLII in MCF-7 cells resulted in the decrease of estrogen-induced H3K4me3 and Pol II binding at the promoter region (Figs. 1B and 2). These results support our hypothesis that FLII is responsible for maintaining active histone marks at the promoter regions of *TFF1*as well as subsequent E2-induced Pol II binding and transcription initiation of the *TFF1* gene.

FLII makes multiple contacts and synergistically enhances transcription with other coactivators such as CARM1 and GRIP1 [22]. Previously, we have demonstrated that FLII interacts with BAF53, an actin-like protein in the SWI/SNF complex, as well as ERα through a different domain of the gelsolin-like C-terminal region of FLII, and both interactions are critical for the coactivator function of FLII [21]. In addition, transient transfection assays with SW13 cells clearly showed that FLII requires the SWI/SNF complex to activate ER-driven transcription, suggesting a role of FLII in remodeling of chromatin structures [21]. In this study, we showed that specific silencing of FLII by siRNA dramatically decreased the E2-induced chromatin accessibility at the enhancer and promoter regions of *TFF1* (Fig. 3A) and other ERα target genes (Fig. 3B) that were observed in siNS-transfected cells. Given that FLII recruits the SWI/SNF complex to ERα target genes, our results indicate that FLII is a key protein that regulates the expression of ERα target genes by facilitating E2-induced chromatin accessibility to EREs. Finally, we demonstrated that silencing of endogenous FLII by siRNA in MCF-7 cells cultured in normal media resulted in significant inhibition of proliferation (Fig. 4), confirming that hormone-dependent growth of MCF-7 cells requires FLII.

Although FLII depletion affected chromatin accessibility of multiple ERα target genes, it appears that the coactivator function of FLII follows a gene-specific pattern during ERα-mediated transcription [23,33]. Previously, it has been shown that genes *TFF1*, *GREB1*, *CCND1*, and *MYC* require FLII for their optimal transcription; however, the expression of PGR is not affected by FLII depletion [23]. At this time, the precise number of ER target genes occupied by and requiring FLII for their chromatin regulation remains unknown. Genome-wide analysis may help to identify the global function of FLII in nuclear receptor-mediated transcription.

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