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Transcriptional regulation of FoxO3 gene by glucocorticoids in murine myotubes

Taiyi Kuo,^{1,2} Patty H. Liu,² Tzu-Chieh Chen,² Rebecca A. Lee,^{1,2} Jenny New,² Danyun Zhang,² Cassandra Lei,² Andy Chau,² Yicheng Tang,² Edna Cheung,² and  Jen-Chywan Wang^{1,2}

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Kuo T, Liu PH, Chen TC, Lee RA, New J, Zhang D, Lei C, Chau A, Tang Y, Cheung E, Wang JC. Transcriptional regulation of FoxO3 gene by glucocorticoids in murine myotubes. *Am J Physiol Endocrinol Metab* 310: E572–E585, 2016. First published January 12, 2016; doi:10.1152/ajpendo.00214.2015.—Glucocorticoids and FoxO3 exert similar metabolic effects in skeletal muscle. FoxO3 gene expression was increased by dexamethasone (Dex), a synthetic glucocorticoid, both in vitro and in vivo. In C2C12 myotubes the increased expression is due to, at least in part, the elevated rate of FoxO3 gene transcription. In the mouse FoxO3 gene, we identified three glucocorticoid receptor (GR) binding regions (GBRs): one being upstream of the transcription start site, –17kbGBR; and two in introns, +45kbGBR and +71kbGBR. Together, these three GBRs contain four 15-bp glucocorticoid response elements (GREs). Micrococcal nuclease (MNase) assay revealed that Dex treatment increased the sensitivity to MNase in the GRE of +45kbGBR and +71kbGBR upon 30- and 60-min Dex treatment, respectively. Conversely, Dex treatment did not affect the chromatin structure near the –17kbGBR, in which the GRE is located in the linker region. Dex treatment also increased histone H3 and/or H4 acetylation in genomic regions near all three GBRs. Moreover, using chromatin conformation capture (3C) assay, we showed that Dex treatment increased the interaction between the –17kbGBR and two genomic regions: one located around +500 bp and the other around +73 kb. Finally, the transcriptional coregulator p300 was recruited to all three GBRs upon Dex treatment. The reduction of p300 expression decreased FoxO3 gene expression and Dex-stimulated interaction between distinct genomic regions of FoxO3 gene identified by 3C. Overall, our results demonstrate that glucocorticoids activated FoxO3 gene transcription through multiple GREs by chromatin structural change and DNA looping.

glucocorticoids; glucocorticoid receptor; FoxO3; myotube; chromatin structure; gene transcription

GLUCOCORTICIDS PLAY A CRITICAL role in the regulation of skeletal muscle physiology. Glucocorticoids inhibit glucose utilization and protein synthesis while promoting protein degradation in skeletal muscle. Inhibiting glucose utilization preserves plasma glucose, the primary energy source for brain. Promotion of protein degradation and repression of protein synthesis produce free amino acids, which can be used as the substrates for hepatic gluconeogenesis. These effects are important metabolic adaptations for the survival of mammals during stress conditions, such as fasting and starvation. However, chronic or excess glucocorticoid treatment can cause severe metabolic disorders, such as muscle atrophy and insulin resistance in skeletal muscle (21, 32, 35, 39, 40).

Glucocorticoids exert their biological functions mainly through binding to an intracellular receptor, the glucocorticoid receptor (GR). Upon binding to glucocorticoids, GR is recruited to genomic glucocorticoid response elements (GRE) to regulate the transcription of its target genes. These primary target genes then trigger glucocorticoid-regulated physiological responses. Our previous chromatin immunoprecipitation sequencing (ChIPseq) experiments identified four GR binding regions (GBR) in or near the mouse FoxO3 gene (22). The induction of FoxO3 mRNA and protein levels by glucocorticoids has been shown previously both in vitro and in vivo (15, 29, 34, 50, 52). The FoxO3 gene encodes a transcription factor that plays a vital role in skeletal muscle protein and glucose metabolism (9, 33). First, the transcription factor inhibits glucose oxidation by activating the transcription of pyruvate dehydrogenase kinase 4 (PDK4) (23). Interestingly, glucocorticoids also induce PDK4 gene transcription (23). In the human PDK4 gene promoter, the binding of both FoxO3 and FoxO1, another member of the FoxO transcription factor family, is necessary for the maximum level of glucocorticoid-induced PDK4 transcription (23). Second, like glucocorticoids, the FoxO3 transcription factor stimulates the transcription of genes that activate protein degradation, such as MuRF1 and atrogin-1, and genes that suppress protein synthesis, such as Eif4ebp1 (37, 47). MuRF1 and atrogin-1 are muscle-specific ubiquitin ligases that have been shown to mediate muscle atrophy caused by various conditions. Constitutively active FoxO3 has been shown to be sufficient to induce muscle atrophy, whereas dominant negative FoxO3 has been shown to prevent the muscle atrophy caused by either disuse or glucocorticoids (37, 47). Furthermore, the dominant negative form of FoxO3 suppresses glucocorticoid-induced atrogin-1 gene expression (37, 47). Recently, microRNA that decreases FoxO3 gene expression has been shown to decrease glucocorticoid-induced expression of atrogin-1 gene in C2C12 myotubes (15). Overall, these results strongly suggest that glucocorticoid-regulated glucose and protein metabolism requires the participation of FoxO3.

Because of the important role of FoxO3 in glucocorticoid action, we systematically examined the mechanisms of glucocorticoid-regulated FoxO3 gene expression in this report. With nuclear run-on assay, we examined whether the FoxO3 gene was transcriptionally regulated by glucocorticoids. We characterized the four GBRs in the FoxO3 gene identified by ChIPseq and further identified 15-bp GREs that mediate the glucocorticoid response within these GBRs. The acetylation status of histone H3 and H4 surrounding these GBRs was checked. Moreover, we mapped the position of nucleosomes wrapped by these GBRs and studied the effect of glucocorticoid treatment

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on their chromatin structures. Finally, we used chromatin conformation capture (3C) assay to test the potential interactions between GBRs and the genomic region near the transcription start site (TSS).

EXPERIMENTAL PROCEDURES

Cell culture. Mouse C2C12 cells were purchased from the Cell and Tissue Culture Facility at the University of California Berkeley. They were maintained in Dulbecco's modified Eagle's medium (DMEM; Mediatech) containing 10% fetal bovine serum (FBS; Tissue Culture Biologicals) and incubated at 37°C with 5% CO₂. Upon reaching 95–100% confluence, C2C12 myoblasts were differentiated into myotubes with 2% horse serum (J. R. Scientific) in DMEM. The C2C12 cells were maintained in 2% horse serum-containing DMEM, changed every 2 days, until fully differentiated into myotubes, in ~4–6 days. For cell culture experiments, C2C12 myotubes were treated with various concentrations of Dex (Sigma), RU486 (Mifepristone; Sigma), or an equal volume (0.05% vol/vol of media) of vehicle control ethanol (EtOH) or DMSO.

Animals. Male 8-wk-old C57BL/6 mice were purchased from Charles River. Mice were injected with 5 mg/kg Dex (Sigma) or PBS for 1 or 4 days. After the treatment, gastrocnemius muscles were isolated from mice for gene expression analysis. For *in vivo* ChIP, male 8-wk-old C57BL/6 mice were injected with PBS or Dex at 11 AM for 2 or 3 h. Gastrocnemius muscles were then isolated for ChIP. Gastrocnemius muscle of 14-wk-old transgenic mice overexpressing corticotropin-releasing hormone (CRH) (46) and wild-type (WT) C57BL/6 mice were provided by the laboratory of Charles Harris (Washington University, St. Louis, MO). In these CRH-Tg transgenic mice, the CRH gene was driven by mouse metallothioneine-1 promoter. Thus the CRH gene was overexpressed in almost all tissues in these transgenic mice. The levels of corticosterone were ~10 times higher than normal C57BL/6 mice (46). The Office of Laboratory Animal Care at the University of California Berkeley (Approval No. R306-0111) approved all animal experiments conducted in this study.

Nuclear run-on. C2C12 myotubes were untreated or treated with 1 μM Dex for 30 min and 1, 2, or 4 h. Cells were then washed once with PBS, and 3 ml of lysis buffer (10 mM Tris-HCl pH 7.4, 3 mM MgCl₂, 10 mM NaCl, 150 mM sucrose, and 0.5% NP40) were added to each plate, followed by incubation at 4°C for 10–15 min. Cell lysate was collected and spun at 170 g at 4°C for 5 min to pellet the nuclei. Nuclei were washed once with lysis buffer without NP40 and resuspended in freezing buffer (50 mM Tris-HCl at pH 8.3, 40% glycerol, 5 mM MgCl₂, and 0.1 mM EDTA). The total number of nuclei from each of the untreated and Dex-treated samples was counted, and 1 × 10⁶ nuclei were used for *in vitro* transcription. Two aliquots from each sample were used: one sample was incubated in 100 μl of 2× *in vitro* transcription buffer [200 mM KCl, 20 mM Tris-HCl pH 8.0, 5 mM MgCl₂, and 4 mM dithiothreitol (DTT); 4 mM each of ATP, GTP and CTP; 200 mM sucrose; and 20% glycerol] with 8 μl biotin-UTP (Roche, or equal amount from Epicentre), and the other sample was incubated in 100 μl 2× *in vitro* transcription buffer with 8 μl UTP (negative control) for 60 min at 30°C. Then, 6 μl of 250 mM CaCl₂ and 6 μl of RNase-free DNase (10 U/ml; Roche) were added to stop the reactions. Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel).

Dyna beads M-280 (Invitrogen) were washed twice in *solution A* (0.1 mM NaOH and 0.5 M NaCl) for 5 min, once in *solution B* (0.1 M NaCl) for 5 min, and then resuspended in binding/wash buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, and 2 M NaCl) with 1 μl (40 units) RNasin per 100 μl of beads. Then, 50 μl of beads were added to total RNA isolated, incubated at 42°C for 30 min, followed by vigorous shaking on a shaker at room temperature for 3 h. The beads were precipitated with magnets and centrifugation, and the supernatant was discarded. The beads were then washed once for 15 min with 500 μl 15% formamide with 2× saline-sodium citrate (SSC) buffer,

twice for 5 min with 1 ml 2× SSC buffer, and then resuspended in 30 μl RNase- and DNase-free water. Finally, 10 μl of beads were used for each reverse transcription (RT) reaction before real-time PCR (qPCR). These primers were used in qPCR: mFOXO3_runon_F, ACTCCCGTCTTTTCCTCTCC; mFOXO3_runon_R, GGAAGT-GATCTTGGCAGGTC; mRPL19_cDNA_F, ATGGAGCACATC-CACAAGC; and mRPL19_cDNA_R, TCCTTGGTCTTAGACC-TGCG.

RNA isolation and quantitative PCR. Total RNA was isolated from mouse gastrocnemius muscles using TRI Reagent RT (Molecular Research Center). To synthesize randomly primed cDNA, 0.5 μg of total RNA, 4 μl of 2.5 mM dNTP, and 2 μl of 15 μM random primers (New England Biolabs) were mixed at a volume of 16 μl and incubated at 70°C for 10 min. Then, a 4-μl cocktail containing 25 units of Moloney murine leukemia virus (M-MuLV) Reverse Transcriptase (New England Biolabs), 10 units of RNasin (Promega), and 2 μl of 10 × reaction buffer (New England Biolabs) was added, and samples were incubated at 42°C for 1 h and then at 95°C for 5 min. The cDNA was diluted and used to perform real-time quantitative PCR (qPCR) using the EVA QPCR SuperMix Kit (Biochain), following manufacturer's protocol. qPCR was performed in either a 7900HT, 7500HT, or StepOne PCR System (Applied Biosystems) and analyzed with the ΔΔ-Ct method, as supplied by the manufacturer (Applied Biosystems). Rpl19 gene expression was used for internal normalization. Primer sequences for qPCR were as follows: Rpl19_cDNA_F, ATGGAGCACATCCACAAGC, Rpl19_cDNA_R, TCCTTGGTCTTAGACCCTGCG; FoxO3_cDNA_forward, TTCAA-CAGTACCGTGTGGAC; and FoxO3_cDNA_reverse, AGTGT-GACACGGAAGAGAAGGT.

Western blotting. RIPA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 5% glycerol, 0.1% sodium deoxycholate, 0.1% SDS, and 1% Triton X-100), supplemented with protease inhibitors, was added to cell pellet. The mixture was gently rocked at 4°C for 1 h. The supernatant was then collected as protein sample. NuPAGE Novex Bis-Tris mini gels (Invitrogen) were used, following the manufacturer's protocol, and proteins were transferred to nitrocellulose membranes (Amersham) using semidry transfer (Bio-Rad) overnight. The next day, membranes were blocked for 4 h at room temperature with 10% (wt/vol) nonfat milk in TBS (50 mM Tris-base and 200 mM NaCl pH 7.5). Membranes were then incubated in 5% milk in TBS with appropriate primary antibody with gentle rocking overnight at 4°C. The following day, membranes were washed with TBS plus 0.5% Tween-20 at pH 7.5 (TBST), and then incubated in 5% milk in TBS containing appropriate secondary antibody for at least 2 h at room temperature. The membranes were then washed with TBST, and proteins were detected by chemiluminescence (Western Lighting Plus-ECL; Perkin Elmer). For additional protein detection on the same membrane, membranes were soaked in TBS overnight at 4°C, and stripped for 30 min in PBS with 7 μl/ml β-mercaptoethanol, followed by 30 min in PBS only, and 4 h in 10% milk in TBS before reprobing with other primary antibodies. The following antibodies were used: FoxO3 (07-702; Millipore), p300 (sc-584; Santa Cruz Biotechnology), β-actin (C4) mouse monoclonal IgG1 (sc-47778; Santa Cruz Biotechnology), GAPDH (ab9483; abcam), anti-rabbit IgG₁-HRP (Cell Signaling), and anti-goat IgG-HRP (sc-2768; Santa Cruz Biotechnology). Blots were scanned and analyzed with ImageJ software (<http://rsbweb.nih.gov/ij/>). β-Actin or GAPDH was used as an internal control.

Plasmids, transfection, and luciferase reporter assay. pGL4.10-E4TATA reporter plasmid was generated by insertion of a 50-bp minimal E4 TATA promoter sequence (25) into the Bgl II to Hind III sites of vector pGL4.10 to drive luciferase expression (1). Each chosen GBR fragment, extending 100–150 bp upstream and downstream of the GBR, was amplified from genomic C2C12 DNA (primer sequences are available in Supplemental Material S1; Supplemental Material for this article is available online at the Journal website), using the Expand Long Template PCR System (Roche Applied

Science) and cloned into the pGL4.10-E4TATA vector with Kpn I/Xho I sites. We also replaced E4 TATA promoter region with the promoter region of FoxO3 gene (−70 to +25 bp relative to its transcription start site, TSS). The QuikChange Lightning mutagenesis kit (Stratagene) was used to make site-directed mutations per the manufacturer's instructions. Lipofectamine 2000 (Invitrogen) was used to transfect C2C12 myoblast according to the technical manual. Twenty-four hours posttransfection, cells were treated with either 1 μ M Dex or control EtOH in differentiation media for 16–20 h. Cells were then harvested and their luciferase activities were measured with the Dual-Luciferase Reporter Assay kit (Promega) according to procedures in the technical manual.

Chromatin immunoprecipitation. Fully differentiated C2C12 myotubes were treated with 1 μ M Dex or control EtOH for 1 h and cross linked in 2% formaldehyde for 3 min at 37°C. The reactions were quenched with 0.125 M glycine. The cells were then washed with 1 \times PBS, scraped, and lysed in cell lysis buffer (50 mM HEPES-KOH at pH 7.4, 1 mM EDTA, 150 mM NaCl, 10% glycerol, and 0.5% Triton X-100), supplemented with protease inhibitor cocktails (Calbiochem). The cell lysate was incubated for 1 h at 4°C, and the crude nuclear extract was collected by centrifugation at 600 *g* for 5 min at 4°C. The nuclei were resuspended in 1 ml of ice-cold RIPA buffer (10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 150 mM NaCl, 5% glycerol, 1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% SDS, supplemented with protease inhibitor). The chromatin was fragmented with a Branson Sonifier 250 sonicator (13 min total, 20-s pulse at 35% power followed by 40-s pause). To remove insoluble components, we centrifuged the samples at 13,000 rpm for 15 min at 4°C and recovered the supernatant. Fifty microliters of lysates were retained and prepared as input. After proteinase K treatment and reverse cross linking, DNA fragments were purified using QIAquick PCR purification kit (Qiagen) as described at the end of this paragraph. For GR ChIP, 1 μ g of rabbit polyclonal anti-GR antibody (N499, provided by Keith R. Yamamoto laboratory, University of California, San Francisco) was added to the supernatant to immunoprecipitate GR-bound chromatin at 4°C overnight. For histone modification ChIP, the following antibodies were used: anti-histone H3 (ab1791; abcam), anti-acetyl histone H3 (ab47915; abcam), anti-histone H4 (05–858; Millipore), anti-acetyl histone H4 (06–866; Millipore), CBP (sc-7300x; Santa Cruz Biotechnology), and p300 (sc-584x; Santa Cruz Biotechnology). Normal rabbit IgG antibody (sc-2027; Santa Cruz Biotechnology) was used as negative control for all ChIP. The next day, 100 μ l of 50% protein A/G (GR ChIP; Santa Cruz Biotechnology) or protein A (histone modification ChIP; Upstate) bead slurry, containing 100 μ g/ml salmon sperm DNA, were added into each immunoprecipitation and nutated at 4°C for 2 h. The beads were then washed twice with RIPA buffer, three times with RIPA buffer containing 500 mM NaCl, twice with LiCl buffer (20 mM Tris at pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, and 0.5% sodiumdeoxycholate), and one time with RIPA buffer, all supplemented with protease inhibitor. After the remaining wash buffer was removed, 75 μ l of proteinase K solution (TE pH 8.0, 0.7% SDS, and 200 μ g/ml proteinase K) was added to each immunoprecipitation reaction, followed by incubation at 55°C for 3 h and 65°C overnight to reverse formaldehyde cross linking. ChIP DNA fragments were purified with QIAquick PCR purification kit (Qiagen), eluting in 60 μ l of Qiagen Elution Buffer. Primers used for ChIP are in Supplemental Material S1.

For each ChIP experiment, the percentage of input was calculated by taking the ratio of ChIP DNA fragment to input DNA fragment. Since input DNA fragments were isolated from 50 μ l whereas ChIP DNA fragments were purified from 950 μ l lysate, the 19 \times difference was taken into consideration for data calculation.

In vivo ChIP. The in vivo ChIP protocol was modified from two previous reports (41, 42). Mice were injected intraperitoneally with Dex (5 mg/kg body wt, in 200 μ l solution) or an equal volume of PBS. Gastrocnemius muscles were excised and snap frozen in isopentane

cooled by liquid nitrogen and then crushed into a fine powder using a mortar and pestle. Samples were suspended in a 1% formaldehyde solution in PBS and incubated at 37°C for 10 min and incubated for 5 min in 125 mM of glycine. Samples were then re-suspended in 50 mM Tris pH 8, 1% SDS, 10 mM EDTA, 1 mM DTT, and protease inhibitors and then incubated at 10°C for 10 min and sonicated at 60% output for a total of 50 s. Cleared lysates were then diluted threefold in a dilution buffer of 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8, 167 mM NaCl, and protease inhibitor. One-hundred microliters of 50% protein A/G beads with 5 μ g of IgG antibody were added and incubated for 1 h at 4°C. Supernatants were collected and samples were incubated with antibodies overnight at 4°C. Beads were washed once with a low-salt washing buffer (150 mM NaCl, 20 mM Tris pH 8.1, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), a high-salt washing buffer (500 mM NaCl, 20 mM Tris pH 8.1, 0.1% SDS, 1% Triton X-100, and 2 mM EDTA), a LiCl washing buffer (250 mM LiCl, 1% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, and 10 mM Tris pH 8.1), and twice with Tris-EDTA buffer. Protein-chromatin complexes were eluted with 10 mM DTT, 1% SDS, and 100 mM NaHCO₃ for 1 h, and the supernatant was then incubated overnight at 65°C with 200 mM NaCl. Proteins were digested with Proteinase K for 3 h at 55°C and DNA fragments were isolated using QIAquick DNA purification kit (Qiagen, Valencia, CA). Real-time PCR were used to monitor the recruitment of GR to various GBRs.

MNase assay. The protocol for MNase assay was previously described (43). Briefly, C2C12 myotubes were treated with 1 μ M Dex or an equal volume (0.05% vol/vol of media) of vehicle control ethanol (EtOH) for 30 or 60 min. Cells were cross linked with 1% formaldehyde for 3 min at 37°C, and the reaction was quenched by the addition of glycine to a final concentration of 0.125 M. Cells were then washed once with PBS and scraped in ice-cold MNase NP-40 lysis buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP-40, 0.15 mM spermine, and 0.5 mM spermidine). After being shaken for 3–5 h at 4°C in MNase lysis buffer, nuclei were collected by centrifugation and washed in ice-cold MNase digestion buffer without CaCl₂ (10 mM Tris at pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, and 0.5 mM spermidine). Samples were then resuspended in ice-cold MNase digestion buffer with CaCl₂ (10 mM Tris at pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, and 1 mM CaCl₂). Nuclei were treated with 1 unit of MNase (nuclease micrococcal from *Staphylococcus aureus*; N5386-200UN; Sigma-Aldrich) for 60–90 min at 25°C. Reactions were stopped by the addition of 80 μ l MNase digestion buffer with CaCl₂, 20 μ l MNase stop buffer (100 mM EDTA and 10 mM EGTA), 75 μ g proteinase K, and 20 μ l 10% SDS, and then cells were incubated at 65°C overnight. Samples were run on 1.5% agarose gel, and single nucleosome-wrapped DNA (~150 bp) was purified with Qiagen gel extraction kit. The concentration of the samples was measured and diluted to 0.3 ng/ μ l for use in qPCR. The qPCR primers were designed to span ~500-bp regions, covering the identified GREs in each of the GBRs. When there were gaps between primers, the gaps were no more than 20 bp long. Primers used for MNase assay are available in Supplemental Material S1. Notably, a titration curve using genomic DNA was performed for each primer in individual MNase experiment to calculate for the absolute DNA amount.

Lentiviral infection. Mouse C2C12 myoblasts were infected with p300 shRNA lentiviral particle (sc-29432v; Santa Cruz Biotechnology) or control shRNA lentiviral particle (sc-108080; Santa Cruz Biotechnology) and selected with 5 μ g/ml puromycin for several days. Sh-p300 and sh-scr C2C12 myoblasts were then differentiated into myotubes. Three days after differentiation, 1 μ M Dex or EtOH was added to sh-p300 or sh-scr myotubes for 6 h, followed by Western blotting or gene expression assay.

Chromatin conformation capture. Two 15-cm plates of C2C12 myotubes were used: one treated with 1 μ M Dex, the other with control EtOH for 1 h. After treatment, cells were fixed in 2%

formaldehyde and incubated at 37°C for 3 min. Glycine was added to a final concentration of 0.125 M at room temperature for 5 min to quench the cross linking. Cells were then washed with ice-cold PBS and resuspended in 5 ml of ice-cold PBS. The cells were transferred through a 40- μ m nylon cell strainer (BD Falcon 352340) and centrifuged at 320 g (100 rpm) at 4°C for 7 min. The pellet was then resuspended in 20 ml ice-cold lysis buffer (10 mM Tris·HCl at pH 8.0, 10 mM NaCl, 0.2% NP-40, and complete protease inhibitor) and rotated at 4°C for at least 1 h. Samples were centrifuged at 15,000 to 18,000 rpm at room temperature for 5 min and resuspended in 2 ml of 1.1 \times Bgl II restriction enzyme buffer, 60 μ l of 10% SDS (final concentration 0.3%), and 200 μ l of 20% Triton X-100 (final concentration 1.8%) and shaken at 37°C for 1 h. Then, 1,600 units of Bgl II restriction enzyme were added to each sample and incubated at 37°C overnight. The next day, 320 μ l of 10% SDS (final concentration 1.3%) were added and incubated at 65°C for 30 min, followed by the addition of 1.5 ml of 20% Triton X-100 (final concentration 1%), 2.8 ml of 10 \times T4 DNA ligase buffer (NEB), and up to 28 ml of deionized water, followed by incubation at 37°C for 1 h. Then, 1 μ l of T4 DNA ligase (400 units) was added to each sample, and samples were kept at 4°C overnight. The next day, 60 μ l of proteinase K (20 mg/ml; Fermentas) were added, and samples were incubated at 65°C overnight. The next morning, 120 μ l of RNase A (10 mg/ml; Fermentas) were added, followed by a 45-min incubation at 37°C. Four rounds of phenol/chloroform extraction were used to clear SDS, and the aqueous phase was recovered. Then, DNA was precipitated with 0.1 vol of 3 M sodium acetate at pH 4.8 and 2 vol of 100% ethanol in -80°C overnight, followed by centrifugation at 4,000 rpm at 4°C for 60 min. The DNA pellets were washed with 70% ethanol and spun at 4,000 rpm at 4°C for 10 min. The DNA pellets were dissolved in 500 μ l of TE buffer, pH 8.0. Forward and reverse primers for qPCR are designed to flank the junction of Bgl II restriction enzyme site and were paired (see Fig. 6). Primers for 3C are listed in Supplemental Material S1.

Statistical analysis. Error bars are means \pm SE for each group. Student's *t*-test was used for all statistical analyses.

RESULTS

Glucocorticoids increase the expression of FoxO3 gene in vitro and in vivo. C2C12 myotubes were treated with vehicle control EtOH or Dex (1 μ M), a synthetic glucocorticoid, for 2, 6, 24, or 48 h. Except for the 2-h treatment, Dex markedly increased FoxO3 gene expression at all time points (Fig. 1A). We then used the shortest yet effective treatment time (6 h) for the subsequent dose-response analysis. As shown in Fig. 1B, while treatment of 10 and 100 nM of Dex significantly stimulated the FoxO3 gene expression, 1 μ M of Dex provided the strongest induction. By treating C2C12 myotubes with a combination of Dex and RU486, a GR antagonist, we found that RU486 cotreatment dose dependently reduced Dex-induced FoxO3 gene expression (Fig. 1C). These results demonstrated that Dex response on FoxO3 gene expression in C2C12 myotubes is GR dependent.

To study the glucocorticoid effect on FoxO3 gene expression in vivo, WT mice were injected with control PBS or Dex (5 mg·kg $^{-1}$ ·day body wt $^{-1}$) for 4 days. FoxO3 gene expression was 2.5-fold higher in the gastrocnemius muscle from Dex-injected WT mice than that from PBS-treated ones (Fig. 1D). Next, we utilized transgenic mice overexpressing CRH (4, 14) to examine long-term glucocorticoid effects on FoxO3 gene expression. These transgenic mice have chronically elevated levels of CRH, which stimulates the secretion of adrenocorticotropin hormone (ACTH). ACTH further increases the secretion of corticosterone to circulation. We found that FoxO3

gene expression was \sim 1.8-fold higher in gastrocnemius muscle from CRH-Tg mice than that from WT ones (Fig. 1E). Overall, these experiments demonstrated that the expression of FoxO3 was induced by glucocorticoids both in vitro and in vivo.

To confirm that Dex-upregulated FoxO3 mRNA levels were correlated with FoxO3 protein levels, C2C12 myotubes were treated with Dex or control EtOH for 24 h. Immunoblotting revealed that Dex treatment resulted in 1.5-fold increase of FoxO3 protein levels (Fig. 1F).

Next, we performed nuclear run-on assay to test the induction of FoxO3 gene in transcriptional level by glucocorticoids. Since transcriptional activation of the FoxO3 gene by Dex should occur before the observation of its mRNA increase, we chose time points earlier than 6 h for the nuclear run-on assay. Figure 1G shows that FoxO3 transcription was significantly induced by 0.5, 1, 2, and 4 h of the treatment. These results indicate that the increased FoxO3 gene expression was due to, at least in part, the induction of its transcription, as early as 0.5 h after Dex treatment.

Insulin antagonizes the effects of glucocorticoids on protein metabolism, as it activates protein synthesis and reduces protein degradation (21, 40). To investigate whether insulin represses glucocorticoid-induced FoxO3 gene expression, C2C12 myotubes were treated with control EtOH, Dex, insulin, or a combination of Dex and insulin for 6 h. As shown in Fig. 1H, insulin reduces the basal expression of FoxO3 gene. The absolute level of FoxO3 induction by Dex is also decreased by insulin; however, the fold change of Dex-induced FoxO3 gene expression in the presence of insulin is similar to that in the absence of insulin (3.2- vs. 3.2-fold, represented as hash tags in Fig. 1H). These results suggest that insulin reduces the expression of basal expression of FoxO3 gene rather than suppressing Dex-stimulated FoxO3 gene transcription.

Identification of GBRs in the genomic region of FoxO3 gene. Previously, our ChIPseq identified four potential GBRs in or near FoxO3 genomic region (22). These include the genomic region between $-17,455$ to $-17,126$ (relative to the TSS, referred to as the -17kbGBR), between $+45,231$ and $+45,317$ (called the $+45\text{kbGBR}$), between $+71,380$ and $+71,565$ (called the $+71\text{kbGBR}$), and between $+98,640$ and $+98,777$ (called the $+93\text{kbGBR}$). The $+45\text{kbGBR}$ and the $+71\text{kbGBR}$ were located in introns, whereas the $+93\text{kbGBR}$ was located in the 3'-untranslated region. Using conventional ChIP we found that GR was recruited to the -17kbGBR , the $+45\text{kbGBR}$, and the $+71\text{kbGBR}$, but not the $+93\text{kbGBR}$, upon 1-h treatment of Dex on C2C12 myotubes (Fig. 2A). We individually inserted each GBR upstream of the TATA box in a luciferase reporter, pGL4.10-E4TATA, and performed reporter assay. For C2C12 myoblasts transfected with reporters containing the -17kbGBR , the $+45\text{kbGBR}$ or the $+71\text{kbGBR}$, Dex-treated cells gave a significantly higher luciferase activity than EtOH-treated ones (Fig. 2, C, E, and G). These results indicate that the -17kbGBR , the $+45\text{kbGBR}$, and the $+71\text{kbGBR}$ contain functional GREs that confer glucocorticoid responses.

We also replaced the promoter region of pGL-17kbWT, pGL+45kbWT, and pGL+71kbWT with -70 to $+25$ (relative to TSS) region of FoxO3 gene. With FoxO3 promoter, all three GBRs again conferred Dex response, although the levels of response were lower (Fig. 2H).

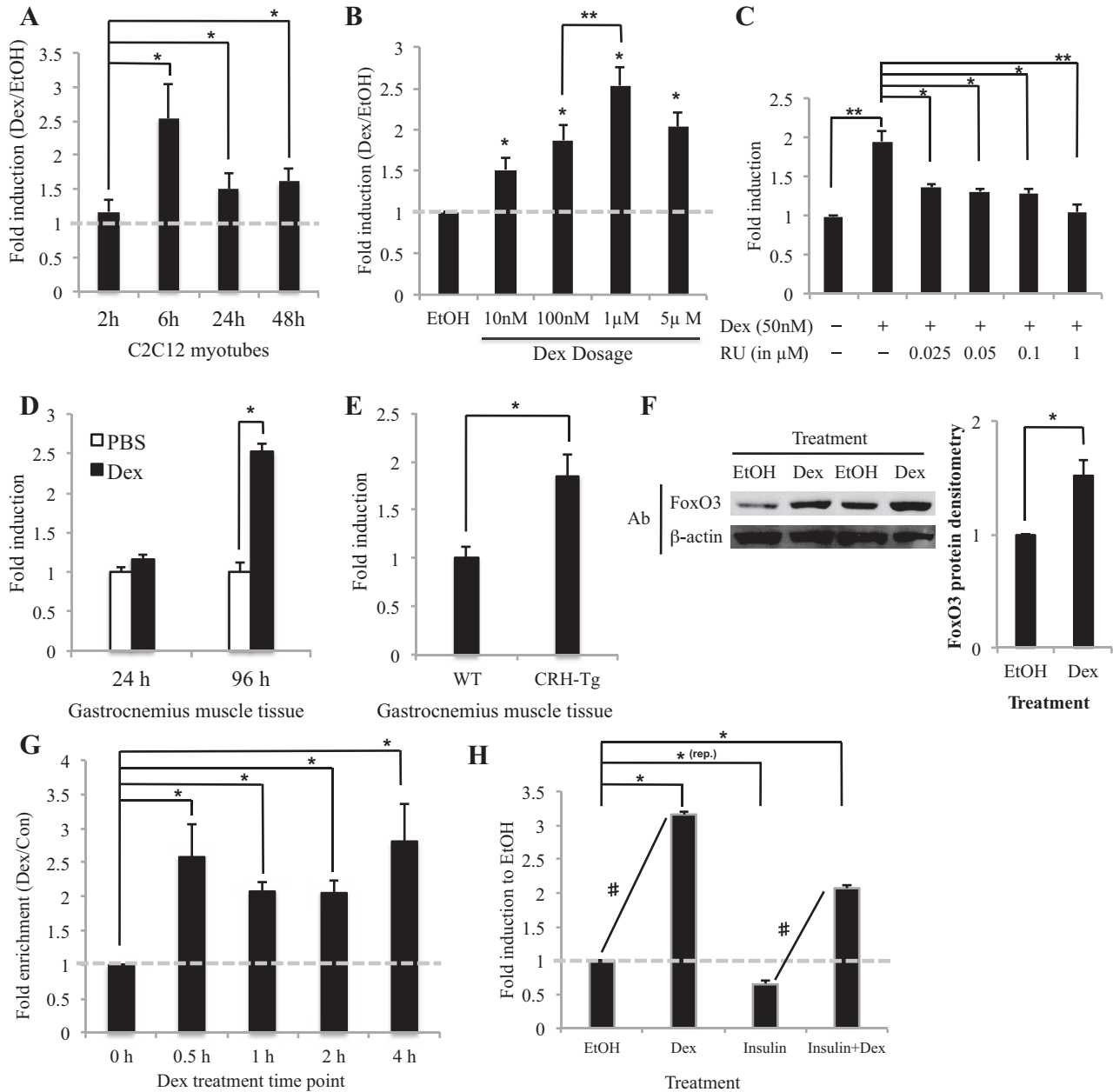


Fig. 1. Induction of FoxO3 gene and protein expression by glucocorticoids in vitro and in vivo. **A**: C2C12 myotubes were treated with 1 μ M dexamethasone (Dex) or control ethanol (EtOH) for 2, 6, 24, or 48 h; $n = 6$. **B**: C2C12 myotubes were treated with 10 nM, 100 nM, 1 μ M, and 5 μ M Dex or control EtOH for 6 h; $n = 6$. **C**: C2C12 myotubes were treated with 50 nM Dex and with DMSO (vehicle control) or 25 nM, 50 nM, 100 nM, and 1 μ M of RU486 for 6 h; $n = 5$. **D**: male C57BL/6 mice were injected with 5 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ of Dex or control PBS for 1 day (24 h) or 4 days (96 h). Their gastrocnemius muscles were isolated; $n = 10$. **E**: gastrocnemius muscles were collected from wild-type and corticotropin-releasing hormone (CRH)-Tg mice; $n = 12$. In **A**, **B**, **C**, and **D**, primers specific to the FoxO3 and the Rpl19 (internal control) genes were used in quantitative (q)PCR for gene expressions analysis. Fold induction was calculated by normalizing to Rpl19 and taking the ratio of Dex over EtOH. **F**: C2C12 myotubes were treated with 1 μ M Dex or control EtOH for 24 h, and FoxO3 protein levels were measured; $n = 3$. **G**: nuclear run-on assay for monitoring FoxO3 in in vitro transcription. C2C12 myotubes were untreated or treated with 1 μ M Dex for 0, 0.5, 1, 2, or 4 h. Primers specific to FoxO3 (across exon 1 and intron 1) and Rpl19 (internal control) were used in qPCR; $n = 4$. **H**: FoxO3 gene expression after cotreatment of Dex and insulin; $n = 3$. C2C12 myotubes were treated with control EtOH, Dex, insulin (100 nM), or a combination of Dex and insulin for 6 h. Fold induction is calculated by comparing treatment to EtOH. For all, error bars indicate means \pm SE. * $P < 0.05$. #3.2-fold induction. *(rep)Statistical significant repression compared with EtOH. **Statistically significant difference in fold induction between 100 nM and 1 μ M Dex treatment in **B**, and among PBS, 50 nM Dex, and 1 μ M RU486 treatment in **C**.

Next, we searched for sequences resembling the consensus GRE identified from our ChIPseq (51), RGXACAnnT-GTXCY, in the -17kbGBR , the $+45\text{kbGBR}$, and the $+71\text{kbGBR}$. Based on the consensus sequences, nucleotide positions 2, 4, 5, 6, 10, 11, 12, and 14 have to be a specific nucleotide.

We looked for sequences that contain at least six of these eight nucleotides. We mutated position 11 of this consensus GRE from a G to a C residue, or position 5, from C to G (Fig. 2, **B**, **D**, and **F**). These residues have been previously shown to make direct contact with the GR (28). In the -17kbGBR , two

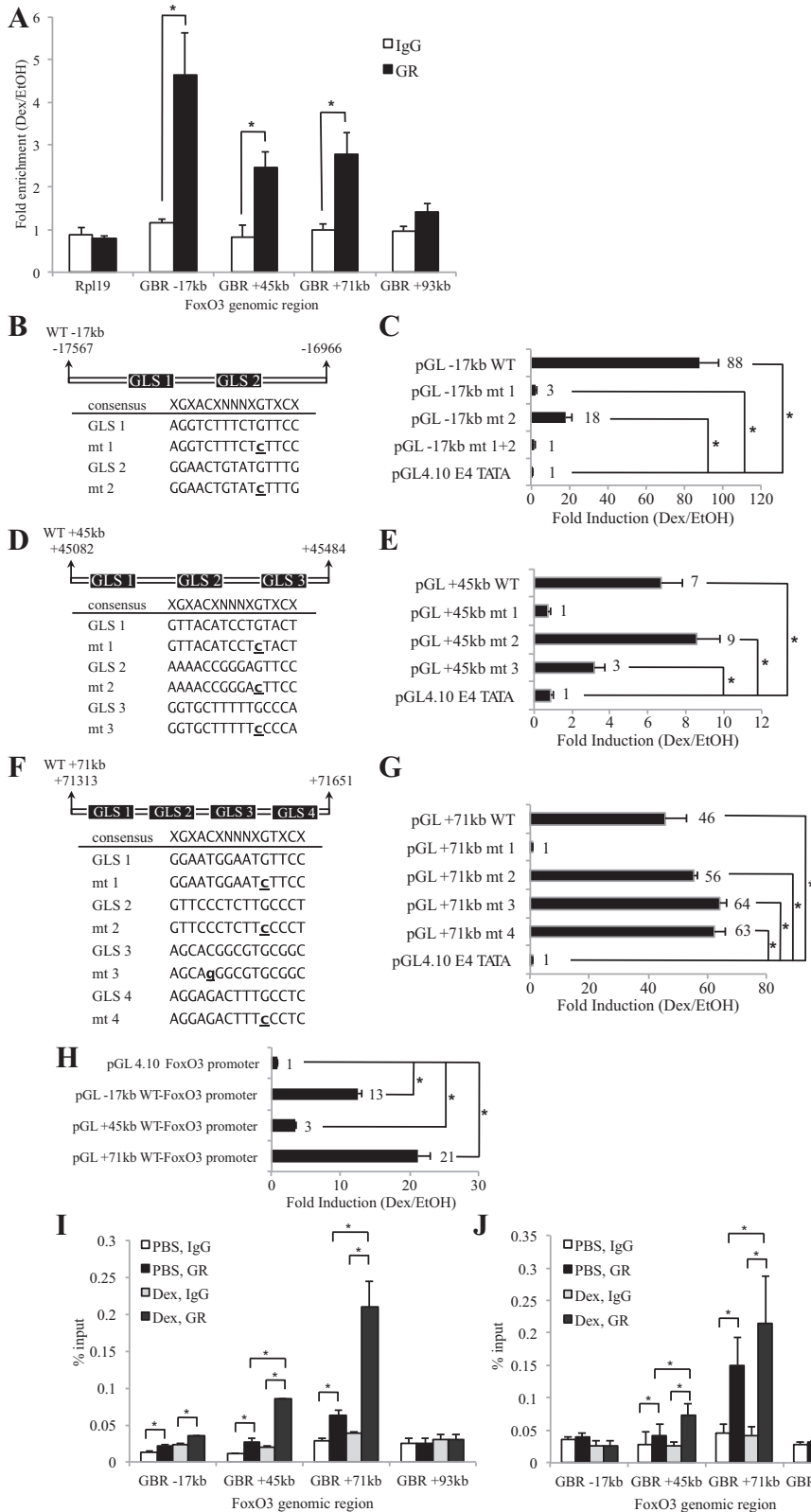


Fig. 2. The identification of glucocorticoid response elements (GREs) in FoxO3 glucocorticoid receptor (GR) binding regions (GBRs). *A*: chromatin immunoprecipitation (ChIP) to confirm GR recruitment to GBRs identified from ChIP (ChIPseq). C2C12 myotubes were treated with 1 μ M Dex or control EtOH for 1 h. Primers flanking the -17kbGBR, the +45kbGBR, the +71kbGBR, the +93kbGBR, and Rpl19 (negative control) were used in qPCR. Error bars represent the means \pm SE of the fold enrichment from 3 independent experiments. * P < 0.05. *B*: -17kbGBR genomic region. *C*: reporter assay for plasmid containing -17kbGBR. *D*: +45kb-GBR genomic region. *E*: reporter assay for plasmid containing +45kbGBR. *F*: +71kbGBR genomic region. *G*: reporter assay for plasmid containing +71kbGBR. For *B*, *D*, and *F*, the location of each GBR, the sequence of consensus GRE, the sequence of GRE-like sequences (GLSs), and each mutated nucleotide are shown. For *C*, *E*, and *G*, reporter plasmids described in *B*, *D*, and *F* were cotransfected with a human GR expression vector and a Renilla internal control plasmid in C2C12 myoblasts, and their luciferase activities are shown. The *t*-test is calculated by comparing each reporter plasmid to pGL4.10 E4TATA vector. *H*: FoxO3 neutral promoter region -70 to +25 bp, relative to its transcription start site, was cloned into pGL4.10 reporter plasmid to give pGL4.10 FoxO3 promoter reporter; $n \geq 5$. Fold induction was calculated by taking the ratio of luciferase activity in Dex-treated samples over EtOH-treated ones. Error bars represent means \pm SE. * P < 0.001. *I* and *J*: ChIP to confirm GR recruitment to GBRs in gastrocnemius muscle after 2 h (*I*) and 3 h (*J*) PBS or Dex treatment. Error bars represent the means \pm SE of the fold enrichment from at least 3 independent experiments. * P < 0.05.

GRE-like sequences (GLSs) were found (Fig. 2B). Mutation of GLS1 resulted in more than a 95% decrease in response to Dex, whereas mutation of GLS2 caused about 80% reduction of Dex response. Double mutation of GLS1 and 2 completely

abolished its response to Dex (Fig. 2C). These results suggested that both GLS1 and 2 are required to confer glucocorticoid response for -17kbGBR, while GLS1 plays a more prominent role. In this regard, the sequence of GLS1, but not

the sequence of GLS2, is highly conserved in human and rat FoxO3 gene (Table 1).

For the +45kbGBR, three GLSs were located (Fig. 2D). Mutation of GLS2 had no effect on Dex response, whereas mutation of GLS3 gave a 57% decrease in response to Dex (Fig. 2E). Furthermore, mutation of GLS1 completely eliminated its response to Dex (Fig. 2E). These results indicated that GLS1 plays a primary role and GLS3 plays an accessory role in mediating glucocorticoid response. Notably, the GLS1 sequence is also highly conserved in human and rat FoxO3 genes (Table 1).

For the +71kbGBR, four GLSs were found (Fig. 2F). Mutation of GLS2, 3, or 4 had no effect on the Dex response (Fig. 2G). However, mutation in GLS1 completely removed its response to Dex (Fig. 2G). Therefore, GLS1 alone conferred a complete glucocorticoid response in the +71kbGBR, and its sequence is also conserved in human and rat FoxO3 genes (Table 1).

To test whether three GBRs identified from C2C12 myotubes are occupied by GR in vivo, mice were injected with PBS or Dex at 11 AM. Two or three hours postinjection their gastrocnemius muscles were isolated for ChIP. We found that GR occupied +45kbGBR and +71kbGBR in gastrocnemius muscle after 2- and 3-h PBS treatment (Fig. 2, I and J, respectively). This suggests that endogenous corticosterone was enough to induce GR recruitment to these two GBRs. Treating mice with Dex for 2 and 3 h further enhanced GR recruitment on these two GBRs in gastrocnemius muscle (Fig. 2, I and J). On the contrary, GR was recruited to -17kbGBR after 2 h PBS treatment (Fig. 2, I and J), but the recruitment was not enhanced by Dex. Overall, these results confirmed that GR is recruited to the three GBRs in vivo.

Glucocorticoids increase the level of acetylated histones in FoxO3 genomic region surrounding GBRs. Histone hyperacetylation is highly associated with transcription activation. We monitored the levels of acetylated histone H3 and H4 (AcH3 and AcH4, respectively) as well as total H3 and H4 in FoxO3 genomic regions containing GRE and regions located upstream and downstream from each GRE. For the -17kbGBR, the +45kbGBR, and the +71kbGBR, there is no significant change in the total H3 and H4 levels between control EtOH and Dex treatment (Fig. 3, A–C). All three genomic regions present significant levels of acetylated H3 and H4 before Dex treatment (Fig. 3, D–F). These observations sug-

gest that those are likely regulatory regions that play a role in controlling basal FoxO3 gene expression. Dex treatment markedly induced hyperacetylation of H3 or H4 in genomic regions surrounding all three GREs (Fig. 3, D–F), although the status of histone acetylation was not affected on the GRE region of each GBR. The ratio of acetylated histones vs. total histones was calculated to more precisely reflect histone hyperacetylation status. Dex treatment increased AcH4/H4 levels upstream of the +45kbGBR (Fig. 3H), as well as downstream of all three GBRs (Fig. 3, G–I). Dex treatment also elevated AcH3/H3 level downstream of the -17kbGBR (Fig. 3, G). Overall, these results indicate that glucocorticoids increase the acetylation status of histones surrounding, but not within, each GRE.

Glucocorticoid treatment differentially induce chromatin structural changes in GBRs. Treatment of glucocorticoids has been shown to disrupt nucleosome assembly or change the position of nucleosomes in the genome. We used MNase to map the position of nucleosomes surrounding the three FoxO3 GBRs.

For the -17kbGBR, three nucleosomes were detected: -17,550 to -17,400, -17,400 to -17,250, and -17,200 to -17,050. Dex treatment did not affect the position of these three nucleosomes, as their sensitivity to MNase was similar between EtOH-treated and Dex-treated cells. Interestingly, the major GRE (-17,231 to -17,217) in the -17kbGBR is located in a linker region between nucleosome 2 and 3 (Fig. 4, A and B).

For the +45kbGBR, two nucleosomes were observed: +44,850 to +45,050 and +45,250 to +45,450. These nucleosomes appeared to cover more than 146 bp of DNA, probably due to the lack of overlapping primer sets in certain GC-rich regions. Nonetheless, the effect of glucocorticoids on these nucleosomes is apparent. Dex treatment for 30 min markedly increased the sensitivity to MNase of both nucleosomes (Fig. 4C). This increase of sensitivity, however, was not seen in cells treated with Dex for 60 min (Fig. 4D). Thus the density of chromatin structure of these two nucleosomes in the +45kbGBR region was reduced upon 30-min Dex treatment, and it was transient.

For the +71kbGBR region, three nucleosomes were detected: +71,300 to +71,420, +71,420 to +71,560, and +71,560 to +71,700. Dex treatment for 30 min did not significantly affect the position or sensitivity to MNase of these nucleosomes (Fig. 4E). However, 60-min treatment signifi-

Table 1. Conservation of GRE in FoxO3 GBR

Type	Location	Sequence	n
Mouse -17KbGBR GLS1 (-17,231 to -17,217)	(chr 10, 42013789–42013775)	AGGTCTTTCTGTTCC	[6/8]
Human (-16,519 to -16,533)	(chr 6, 108971185–108971199)	AGGTCTTTCTGTTCC	[6/8]
Rat (-12,290 to -12,276)	(chr 20, 46275109–46275095)	AGGGCATTCACTTCC	[6/8]
Mouse +45kbGBR GLS1 (+45,266 to +45,252)	(chr 10, 41951297–41951283)	GTTACATCCTGGACT	[6/8]
Human (+61,314 to +61,328)	(chr 6, 109049034–109049048)	GGCACATTCGTACT	[8/8]
Rat (+44,026 to +44,012)	(chr 20, 46218771–46218757)	GGTACGTCCTGTACT	[7/8]
Mouse +71kbGBR GLS1 (+71,492 to +71,478)	(chr 10, 41925071–41925057)	GGAATGGAATGTTCC	[6/8]
Human (+94,403 to +94,417)	(chr 6, 109082123–109082137)	GGAATGGAATGTTCC	[6/8]
Rat (+70,364 to +70,350)	(chr 20, 46192453–46192439)	GGAATGGAATGTTCC	[6/8]

Genomic location of mouse glucocorticoid response element (GRE) and its conserved counterparts in human and rat genome are shown. Based on the consensus GRE sequence identified from chromatin immunoprecipitation sequencing (ChIPseq), RGXACANNNTGTXY, we looked for sequences that have at least 6 identical nucleotides of 8 underlined nucleotides. The number of identical nucleotide (underlined) is shown in brackets. Mouse sequences are based on mm9 assembly, human sequences are based on hg18 assembly, and rat sequences are based on Baylor 3.4/rn4 assembly. GBR, glucocorticoid receptor binding region.

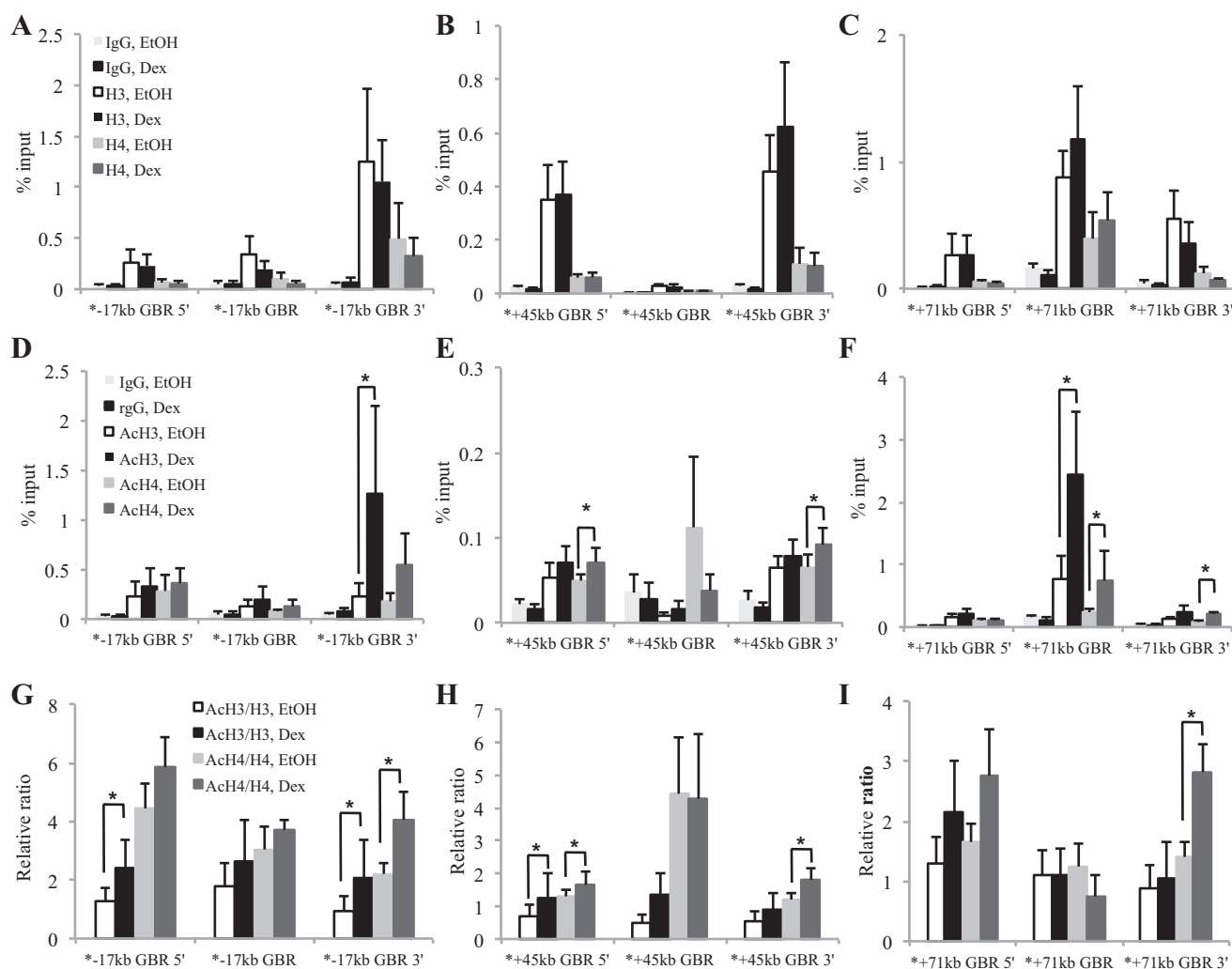


Fig. 3. Assessment of histone H3 and H4 acetylation near GREs of the FoxO3 gene. C2C12 myotubes were treated with 1 μ M Dex or control EtOH for 30 min. ChIP was performed with antibodies recognizing total histone H3 and H4 (H3 and H4, respectively) in A–C and with antibodies recognizing acetylated H3 and H4 (AcH3 and AcH4, respectively) in D–F. The ratio of AcH3 or AcH4 over H3 or H4 is presented in G–I. For A, D, and G, the –17kbGBR primer marks FoxO3 genomic region of –17,308 to –17,184; the –17kbGBR 5' primer, –17,678 to –17,570; and the –17kbGBR 3' primer, –16,909 to –16,786. For B, E, and H, the +45kbGBR primer defines FoxO3 genomic region of +45,219 to +45,302; the +45kbGBR 5' primer, +44,853 to +44,929; and the +45kbGBR 3' primer, +45,631 to +45,683. For C, F, and I, the +71kbGBR primer identifies FoxO3 genomic region of +71,443 to +71,555; the +71kbGBR 5' primer, +71,290 to +71,381; and the +71kbGBR 3' primer, +72,129 to +72,215. Error bars are means \pm SE; $n \geq 6$ and $*P < 0.05$.

cantly increased the sensitivity to MNase of the second nucleosome. In fact, this nucleosome is unrecognizable in the 60-min Dex-treated sample (Fig. 4F). This result strongly suggests that chromatin structure was loosened up in the region of +71,420 to +71,560. Interestingly, this region harbors the highly conserved +71kb GLS1 sequence.

Overall, these results show that glucocorticoids differentially modulate the chromatin structure of the three GBRs in the FoxO3 genomic region, suggesting that distinct mechanisms are adapted by these GBRs to participate in GR-activated FoxO3 gene transcription.

Dex-induced FoxO3 gene transcription requires p300. One key hallmark for active GREs is the recruitment of transcriptional coactivators by GR. We examined the recruitment of transcriptional coregulators, p300 and CBP, to FoxO3 GBRs upon 30-min Dex treatment. We found that p300, but not CBP, was recruited to all three GBRs upon Dex treatment (Fig. 5A). Reducing p300 in C2C12 myotubes with shRNA lentiviruses

(Fig. 5B) markedly decreased the induction of FoxO3 gene and protein expression by Dex (Fig. 5, C and D). These results indicated that p300 directly participates in GR-activated FoxO3 gene transcription.

The potential interactions between genomic regions near FoxO3 GBRs and 578 bp downstream of TSS. All three FoxO3 GBRs are located far away from the TSS. Therefore, to stimulate FoxO3 transcription, they may need to interact with the genomic region near the TSS, where the basal transcription machinery is located. We performed 3C to examine the potential interaction between GBRs and TSS genomic regions. C2C12 myotubes were treated with control EtOH or Dex for 30 or 60 min, cross linked, followed by nuclei isolation. The samples were digested with restriction enzyme Bgl II and then diluted for intracellular religation. With primers pairing between genomic regions near each GBR and TSS, qPCR was used to assess the change in relative cross-linking frequencies between Dex- and EtOH-treated samples. We tested more than

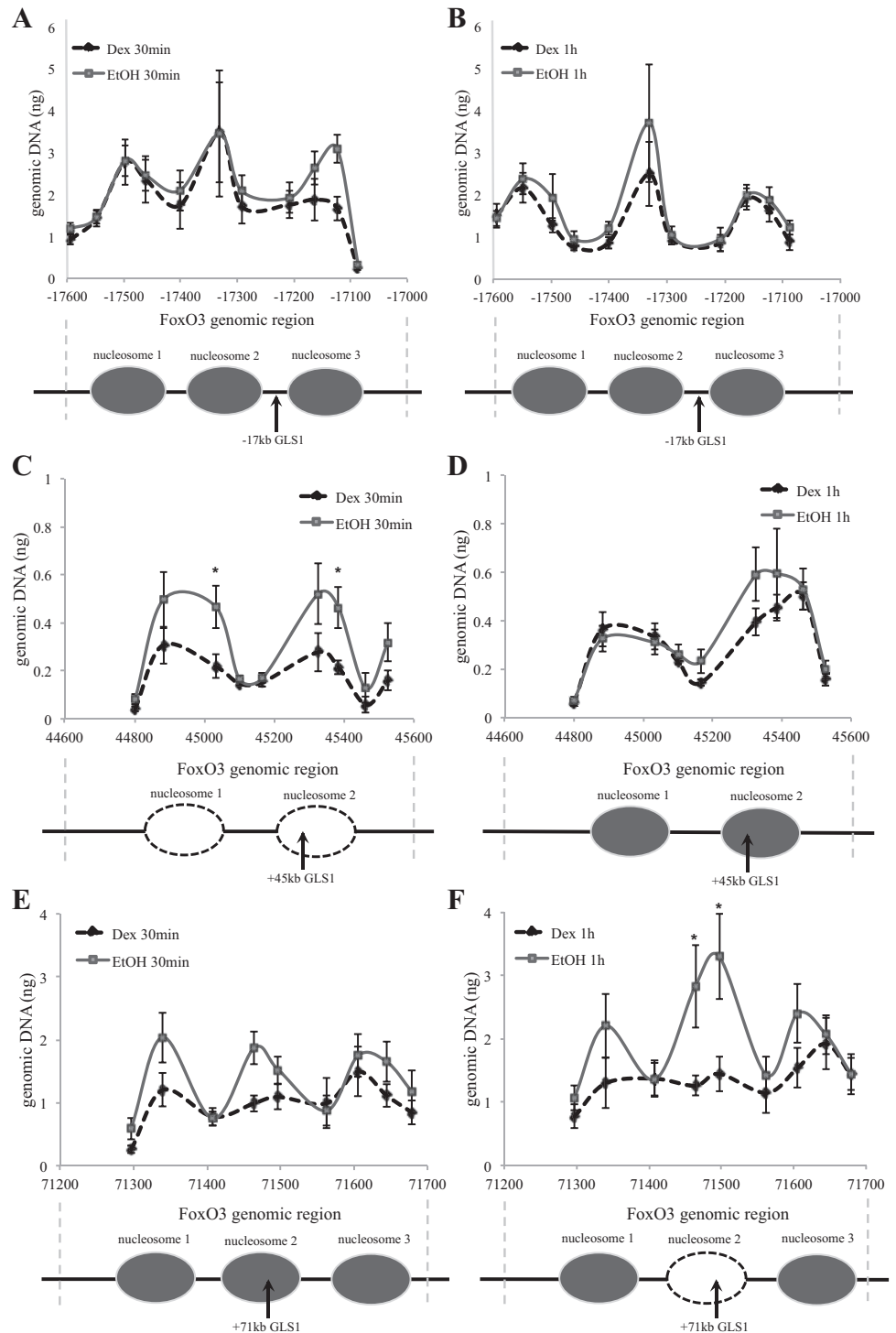


Fig. 4. Nucleosome mapping of the FoxO3 GBRs. C2C12 myotubes were treated 1 μ M Dex or control EtOH. Nucleosome positions were analyzed with MNase digestion, followed by qPCR with primers spanning FoxO3 GBRs. For *A* and *B*, nucleosome mappings between -17,600 and -17,100 of the FoxO3 gene are shown for 30-min and 1-h treatment, respectively. Eleven primers were used to span this region. For *C* and *D*, nucleosome mappings between +44,800 and +45,600 of the FoxO3 gene are shown for 30-min and 1-h treatment, respectively. Nine primers were used to span this region. For *E* and *F*, nucleosome mappings between +71,300 and +71,700 of the FoxO3 gene are shown for 30 min and 1 h, respectively. Nine primers were used to span this region. For Fig. 4, the positions of nucleosomes are drawn. Error bars represent means \pm SE of the genomic DNA amount from at least 5 independent experiments. * $P < 0.05$, for the indicated primer comparing Dex to EtOH-treated samples.

a 100 primer pairs, where most of them did not detect any PCR products. Nevertheless, we found 14 primer pairs that detected significant levels of PCR products. Figure 6A showed the location of these primers (the nucleotide number represent the midpoint of each primer sequence) in the FoxO3 genomic region. Levels of these 14 ligated products were not affected by 30-min Dex treatment (data not shown). However, upon 60-min Dex treatment, the levels of two ligated products were significantly increased (Fig. 6, *B* and *C*): between primer pair

+73,506 and -17,648 (2.3-fold induction, primer 7) and primer pair -17,648 and +578 (2.3-fold induction, primer 14). This finding suggests that Dex treatment enhanced DNA looping in a time-dependent manner, and resulted in increased interaction between the 3' end of the +71kbGBR and the -17kbGBR, and between the -17kbGBR and the genomic region ~578 bp downstream of TSS.

To test whether p300 is involved in Dex-induced DNA looping, we performed 3C in EtOH- or Dex-treated C2C12

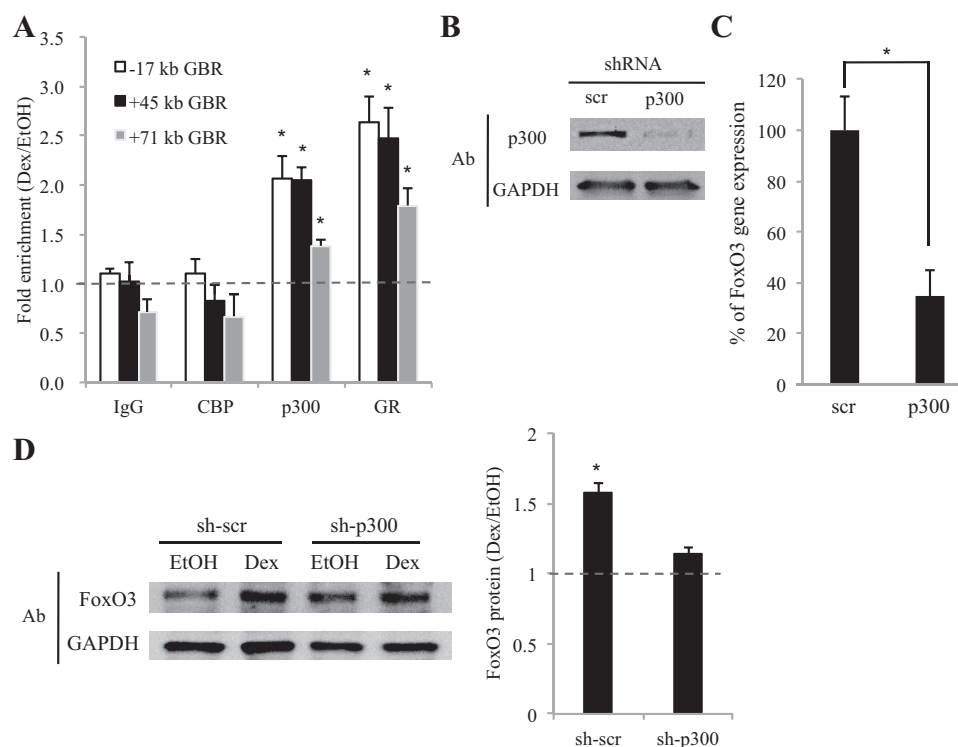


Fig. 5. Acetyltransferase p300 is recruited to all 3 FoxO3 GBRs and is involved in Dex induced FoxO3 transcription. **A:** C2C12 myotubes were treated with 1 μ M Dex or EtOH for 30 min. ChIP was performed with antibodies recognizing IgG (control), CBP, p300, or GR, followed by qPCR with primers spanning FoxO3 GBRs. Fold enrichment was calculated by taking the ratio Dex-treated samples over EtOH-treated ones. Dashed line indicates EtOH-treated basal level of 1; $n = 3$. **B:** Western blotting shows that p300 is knocked down in C2C12 myotubes treated with shRNA lentiviral particle against p300 compared with that against control scramble (scr). **C:** FoxO3 gene expression in Dex or EtOH-treated myotubes infected with shRNA lentiviral particle against p300 or control scr. Fold induction represents the ratio of Dex-treated samples to EtOH-treated ones. Error bar represents means \pm SE; $n = 3$. $*P < 0.05$. **D:** FoxO3 protein expression in Dex or EtOH-treated myotubes infected with shRNA lentiviral particle against p300 or control scr. One representative result was shown from 2 independent experiments. The intensity of bands were measured using ImageJ. The bar graph shows the average of 2 independent experiments. Error bar represents means \pm SE. $*P < 0.05$.

myotubes that express scramble shRNA (control) or p300 shRNA. Consistent with the results above, Dex treatment resulted in 1.8-fold induction between primer pair +73,506 and -17,648, and 1.6-fold induction between primer pair -17,648 and +578 in C2C12 myotubes that express scramble shRNA (Fig. 6D). In contrast, both inductions were abolished with the presence of p300 shRNA (Fig. 6D). These results indicate that p300 is required for Dex-induced interaction between these genomic elements in FoxO3 gene.

DISCUSSION

Glucocorticoids and FoxO3 play similar roles in the regulation of protein and glucose metabolism in skeletal muscle. The induction of FoxO3 gene expression by glucocorticoids could have important physiological and/or pathological implications. Here, we extensively studied the mechanism of glucocorticoid-activated FoxO3 gene expression. We showed that glucocorticoids activated the transcription of the FoxO3 gene in C2C12 myotubes. We identified three GBRs in or near the FoxO3 gene and the functional GREs within the GBRs. Interestingly, these three GBRs are far away from FoxO3 TSS. Several lines of evidence suggest that they all play a role in glucocorticoid-activated FoxO3 gene transcription.

First, all three GBRs confer glucocorticoid response when individually inserted into a reporter plasmid. Among these GBRs, four functional GREs were identified from nine GRE-like sequences. Intriguingly, the sequences of three out of the four GREs (one from each GBR) are highly conserved in human and rat FoxO3 gene (Table 1), suggesting that these GREs in human and rat may also play a role in glucocorticoid-regulated FoxO3 gene transcription. Alternatively, these GREs are conserved due to selective pressure in evolution. Previous studies have shown that certain glucocorticoid-regulated genes,

such as tyrosine aminotransferase and dual specificity phosphatase 1, contain multiple GREs (7, 48). Moreover, ChIP sequencing results revealed many glucocorticoid-regulated genes contain multiple GR binding regions in their genome (8, 22, 36, 51), which suggests that regulation of gene transcription by multiple GREs is likely a common mechanism.

We also showed that GR was recruited to these GBRs in vivo. GR was recruited to +45kb and +71kbGBR in gastrocnemius muscle of PBS-treated mice, which indicates a basal level of GR occupancy at these GBRs. Notably, Dex further enhanced GR recruitment to both +45kb and +71kbGBR. In contrast, GR occupancy on -17kbGBR was not enriched with Dex treatment, which suggests that further GR recruitment to -17kbGBR likely required other signals or transcription factors.

Second, we observed changes in chromatin structure surrounding two GBRs: +45kbGBR and +71kbGBR. An increase in sensitivity to MNase was observed in the nucleosome that contains the GRE in the +45kbGBR upon 30-min glucocorticoid treatment. However, there is no significant change in chromatin structure of the +71kbGBR at the same time point. Instead, the chromatin structure was disrupted upon 60-min glucocorticoid treatment. Furthermore, upon 30-min glucocorticoid treatment, while histone H4 was hyperacetylated both immediately upstream and downstream of the +45kbGBR and downstream of -17kbGBR and +71kbGBR, H3 was hyperacetylated upstream and downstream of -17kbGBR and upstream of +45kbGBR.

The chromatin structure surrounding the -17kbGBR was not affected at either time point. Interestingly, its GRE is located within the linker region between two nucleosomes. Thus, unlike the GREs in the +45kbGBR and the +71kbGBR, which are located within nucleosome, the GRE in the -17kb-

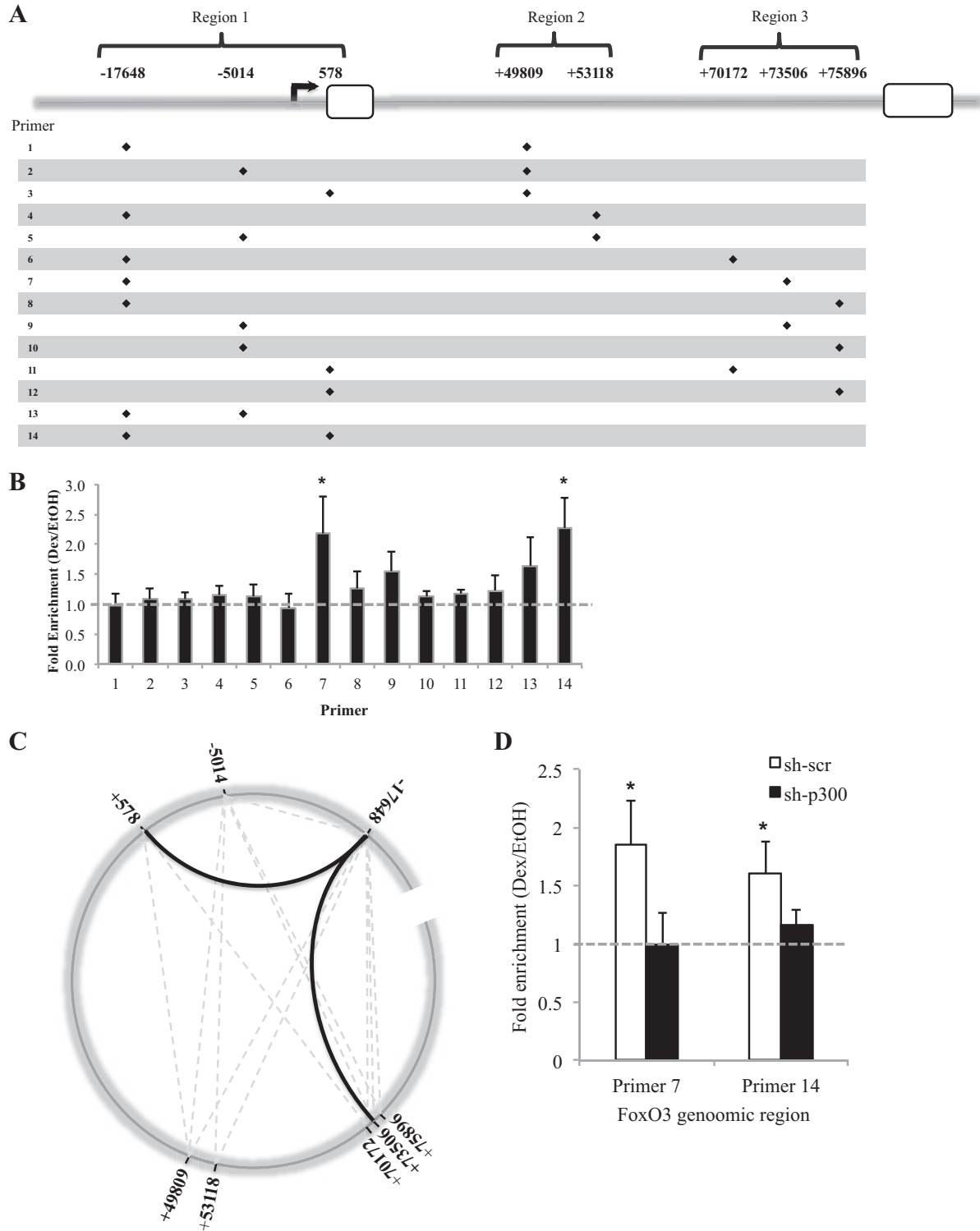


Fig. 6. Chromatin conformation capture (3C) identifies potential physical interaction between genomic regions near GBRs and transcription start site (TSS) of FoxO3 gene. C2C12 myotubes were treated with 1 μ M Dex or control EtOH for 1 h. DNA was digested with Bgl II and re-ligated with DNA ligase intermolecularly, and qPCR was used to detect the amount of ligated products obtained from individual primer set pairing region 1 (-17,648, -5,014, -578), region 2 (+49,809, +53,118), and region 3 (+70,172, +73,506, +75,896). The nucleotide position represents mid-point of each primer. **A**: schematic diagram of Bgl II sites in regions 1, 2, and 3 of the FoxO3 gene. White boxes represent the two exons. **B**: PCR amplification results of primer set 1–14. The data represent means \pm SE of fold enrichment (Dex- to EtOH-treated samples) from 7 independent experiments. A bacterial artificial clone (BAC, RP24-177H14) harboring the entire FoxO3 genomic region was used as control for random chromatin interaction. The data shown here all have higher amount of ligated products in Dex- and EtOH-treated samples compared with PCR amplification from BAC. Ligated products from primer set 7 and 14 are significantly enriched after Dex treatment, pairing +73,506 to -17,648 and -17,648 to +578, respectively. * $P < 0.05$. **C**: Circos plot interprets the results from **B** and indicates relative crosslinking frequencies observed between positions in regions 1, 2, and 3. **D**: 3C performed with C2C12 myotubes expressing scramble shRNA or p300 shRNA. Fold enrichment was calculated by taking the ratio of Dex- to EtOH-treated cells from 2 independent experiments. * $P < 0.05$.

GBR is already exposed for GR binding. It could explain the absence of chromatin structural change in the -17kbGBR . In the mean time, increasing histone acetylation surrounding GBRs would loosen the chromatin structure near GREs to allow other transcription factors to associate with their respective binding sites to further assist transcriptional activation.

It is intriguing that these three GBRs respond to glucocorticoid treatment differently to modify their chromatin structure. Previous studies have shown that the nucleotide sequence of a GRE plays a central role in modulating GR function (24, 45), as distinct GRE sequences were shown to induce different conformational change of GR (30). This is likely affecting the ability of GR to associate with transcriptional coregulators and other DNA-binding transcription factors (27, 45). We therefore hypothesize that distinct sets of transcriptional coregulators may be recruited to FoxO3 GBRs in a time-dependent fashion. So far, p300 was the only transcriptional coregulator that we found to be recruited to all three GBRs upon Dex treatment for 30 min. Dex-induced FoxO3 gene transcription appears to require p300, as p300 reduction markedly decreased the ability of Dex to stimulate FoxO3 gene expression. It is possible that p300, a histone acetyltransferase, participates in the hyperacetylation of genomic regions at and/or surrounding GREs shown in Fig. 3. To understand the differential effects of Dex on the chromatin structure change, one would need to identify transcription coregulators that are differentially recruited to GBRs, possibly at distinct time points of Dex treatment, in future studies.

Third, in 3C experiments, we found that 60-min Dex treatment increases the interaction between genomic region downstream of $+71\text{kbGBR}$ and the -17kbGBR and between the -17kbGBR and the genomic region around $+578\text{ bp}$. Based on these results, we contemplated a model for FoxO3 genomic configuration upon GR activation (Fig. 7). In this model, the -17kbGBR is drawn to the $+578\text{-bp}$ genomic region upon glucocorticoid treatment to be closer to the TSS. If we consider these interactions in a three-dimensional geometry, the $+71\text{kbGBR}$ could also be close to the TSS through its communication with the -17kbGBR (Fig. 7). Likely, there is no direct interaction between genomic regions around $+73\text{kb}$ and $+578\text{ bp}$, as we did not detect any ligated PCR product with respective primers. Nonetheless, such spatial arrangements could still play a role in transcription regulation (12, 31). There is no

available restriction site that allows us to study genomic regions closer to TSS than $+578$ region. Nevertheless, we speculate that $+578$ region may contain regulatory elements required for maximal glucocorticoid response in FoxO3 gene transcription.

It is likely that certain levels of DNA looping occur before the Dex treatment, as we had positive qPCR results from 14 primer pairs in EtOH-treated C2C12 myotubes. These results are not surprising, as DNA looping may be required to provide basal transcription of FoxO3. Nuclear run-on first showed Dex-induced FoxO3 gene transcription at 30-min time point, whereas DNA looping was augmented after 1-h Dex treatment in 3C analysis. This suggests that the basal level of DNA looping is sufficient to support the initiation of FoxO3 transcription by Dex. Enhanced DNA looping induced by Dex at the 1-h time point may be required to sustain prolonged FoxO3 gene transcription. Alternatively, we cannot exclude the possibility that DNA looping is increased outside of tested genomic regions by 30 min of Dex treatment. We did not observe an increased interaction between the $+45\text{kbGBR}$ and genomic regions tested. However, it does not exclude the possibility that the $+45\text{kbGBR}$ is drawn to the TSS upon glucocorticoid treatment at an earlier or later time point or the possibility that the $+45\text{kbGBR}$ may interact with genomic regions outside the ones tested.

The induction of DNA looping by glucocorticoids has been shown in other cases (11, 13, 17). However, the mechanism of glucocorticoid-induced DNA looping is not clear. One DNA binding protein that can induce DNA looping is CTCF (10, 26, 38, 44). Cohesin has been shown to act with CTCF to induce DNA looping (5, 6). Based on CTCF ChIP sequencing results from the University of California, Santa Cruz genome browser, there are multiple CTCF binding sites in the FoxO3 genomic region. It is unclear whether these CTCF binding sites play a role in basal and Dex-induced DNA looping in FoxO3 genomic region. In addition to CTCF, other transcription cofactors may participate in DNA looping event in a glucocorticoid-dependent manner. It has been shown that Mediators form a complex with cohesin to participate in the induction of DNA looping (18). SWI/SNF chromatin remodeling complex has also been shown to induce chromatin looping (19). It is well established that GR can recruit Mediator (2, 3) and SWI/SNF complexes (16, 49). So far we were not able to detect the recruitment of

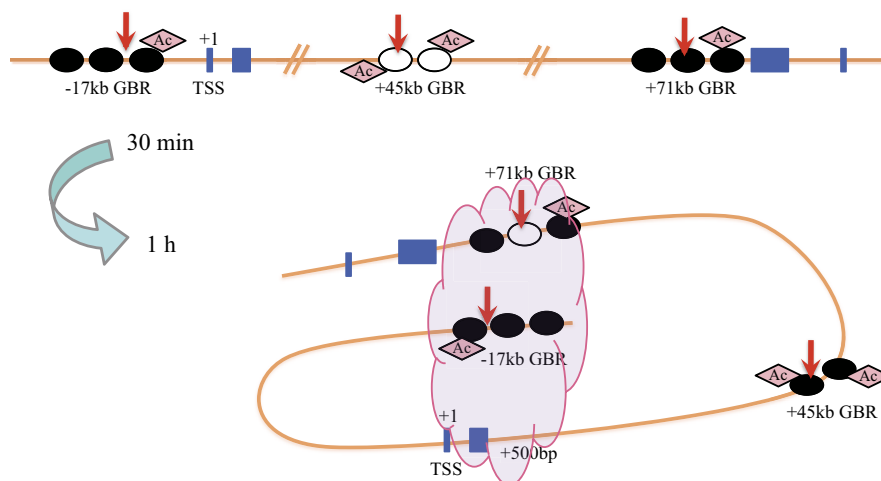


Fig. 7. Schematic model of glucocorticoid-induced FoxO3 chromatin structural change. The chromatin structure of the FoxO3 gene upon 30-min and 1-h Dex treatment. The arrow points to the location of each conserved GRE. The black ovals indicate packed nucleosomes, while the white ovals represent nucleosomes that are loosened or disrupted. Ac indicates hyperacetylated histones. Before Dex treatment there are basal levels of DNA looping in FoxO3 gene. Dex treatment for 1 h, however, induces DNA looping, which in turn increases the interaction between $+71\text{kbGBR}$ and -17kbGBR and between genomic regions near -17kbGBR and TSS.

components in Mediator and SWI/SNF to FoxO3 GBRs. However, p300 was recruited to all three GBRs. Recent studies suggest that histone hyperacetylation induced by p300 contributes to DNA looping between locus control region and globin gene promoter (20). Indeed, when we reduced p300 expression in C2C12 myotubes, the ability of glucocorticoids to induce DNA looping within FoxO3 gene was markedly reduced. It is unclear how p300 exerts this effect; nonetheless, these experiments established a role of p300 in this process.

Since the three GREs showed distinct regulatory characteristics, it is likely that they serve different functions in the activation of FoxO3 gene transcription. We speculate that +45kbGBR is involved in the initial step of transcriptional activation, as chromatin structure surrounding +45kbGBR was loosened upon 30-min Dex treatment (Fig. 7). This initial step may include the recruitment of proteins to initiate DNA looping, as we observed the interaction between +71kbGBR and -17kbGBR upon 1-h Dex treatment, at which time point the chromatin structure surrounding +45kbGBR has returned to the basal state. At this time point, chromatin structure surrounding +71kbGBR has loosened up, which may in turn recruit additional GR and/or other transcription factors and interact with -17kbGBR to activate transcription (Fig. 7). To test the role of each GRE, it would be necessary to delete or mutate them individually and endogenously to examine the effects on transcriptional activation process.

In summary, in this report we have shown that glucocorticoids employ a novel mechanism in regulating FoxO3 gene transcription, where three GBRs, with a total of four GREs, are involved in the transcriptional activation process. The interaction between two GBRs and genomic region ~578 bp downstream of TSS likely plays a role in Dex-induced FoxO3 gene transcription. Further study of these transcriptional regulations not only is important for understanding glucocorticoid action in skeletal muscle but also provides a valuable model for elucidating the complex mechanisms of GR-regulated gene transcription.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.K., P.H.L., T.-C.C., and J.-C.W. conception and design of research; T.K., P.H.L., T.-C.C., R.A.L., J.N., D.Z., C.L., A.C., Y.T., and E.C. performed experiments; T.K., P.H.L., T.-C.C., R.A.L., J.N., D.Z., C.L., A.C., Y.T., E.C., and J.-C.W. analyzed data; T.K., P.H.L., T.-C.C., R.A.L., and J.-C.W. interpreted results of experiments; T.K., T.-C.C., R.A.L., and J.-C.W. prepared figures; T.K. and J.-C.W. drafted manuscript; T.K. and J.-C.W. edited and revised manuscript; T.K., P.H.L., T.-C.C., R.A.L., J.N., D.Z., C.L., A.C., Y.T., E.C., and J.-C.W. approved final version of manuscript.

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