

**THE GLUCOCORTICOID RECEPTOR AND FOXO1 SYNERGISTICALLY
ACTIVATE THE SKELETAL MUSCLE ATROPHY ASSOCIATED MURF1 GENE**

**David S. Waddell¹, Leslie M. Baehr¹, Jens van den Brandt⁴, Steven A. Johnsen^{2,3},
Holger M. Reichardt⁴, J. David Furlow^{1,5*}, and Sue C. Bodine^{1,5*}**

¹ Department of Neurobiology, Physiology and Behavior, University of California, Davis 95616-8519

² European Molecular Biology Laboratory, Heidelberg, Germany

³ Department of Molecular Oncology, Göttingen Center for Molecular Biosciences, University of
Göttingen, Göttingen, Germany

⁴ Department of Cellular and Molecular Immunology, University of Göttingen, Göttingen, Germany

Running head: MuRF1 gene regulation by GR and FOXO1

*J. D. Furlow and S. C. Bodine contributed equally to this study.

⁵Contact information:

J. David Furlow, Ph.D., Section of Neurobiology, Physiology & Behavior, University of California,
Davis, One Shields Avenue, Davis, CA 95616. Phone (530)754-8609; Fax: (530) 752-5582, Email:
jdfurlow@ucdavis.edu.

Sue C. Bodine, Ph.D., Section of Neurobiology, Physiology & Behavior, University of California, Davis,
One Shields Avenue, Davis, CA 95616. Phone (530)752-0694; Fax: (530) 752-5582. Email:
scbodine@ucdavis.edu.

The abbreviations used are: GR, glucocorticoid receptor; GR^{dim}, glucocorticoid receptor homodimerization mutant; GRE, glucocorticoid response element; DEX, dexamethasone; FOXO, forkhead transcription factor, class O; FBE, FOXO binding element; LUC, luciferase; SEAP, secreted alkaline phosphatase; ChIP, chromatin immunoprecipitation; IGF-1, insulin-like growth factor-1.

ABSTRACT

The muscle specific ubiquitin E3 ligase MuRF1 has been implicated as a key regulator of muscle atrophy under a variety of conditions, such as during synthetic glucocorticoid treatment. FoxO class transcription factors have been proposed as important regulators of MuRF1 expression, but its regulation by glucocorticoids is not well understood. The MuRF1 promoter contains a near perfect, palindromic glucocorticoid response element (GRE) 200 base pairs upstream of the transcription start site. The GRE is highly conserved in the mouse, rat and human genes, along with a directly adjacent FoxO binding element (FBE). Transient transfection assays in HepG2 cells and C₂C₁₂ myotubes demonstrate that the MuRF1 promoter is responsive to both the dexamethasone-activated glucocorticoid receptor (GR) and FOXO1, while co-expression of GR and FOXO1 leads to a dramatic synergistic increase in reporter gene activity. Mutation of either the GRE or the FBE significantly impairs activation of the MuRF1 promoter. Consistent with these findings, DEX-induced up-regulation of MuRF1 is significantly attenuated in mice expressing a homodimerization deficient glucocorticoid receptor, despite no effect on the degree of muscle loss in these mice versus their wild-type counterparts. Finally, chromatin immunoprecipitation analysis reveals that both GR and FOXO1 bind to the endogenous MuRF1 promoter in C₂C₁₂ myotubes, and IGF-1 inhibition of dexamethasone-induced MuRF1 expression correlates with the loss of FOXO1 binding. These findings present new insights into the role of the GR and FOXO family of transcription factors in the transcriptional regulation of the MuRF1 gene, a direct target of the GR in skeletal muscle.

INTRODUCTION

Skeletal muscle is a dynamic tissue that has the capacity to continuously regulate its size in response to a variety of external cues including mechanical load, neural activity, hormones/growth factors, stress and nutritional status. In addition, skeletal muscle serves as the most significant repository for protein in the body, a source that is tapped to provide a pool of amino acids for tissue repair and gluconeogenesis under conditions of starvation and other metabolic stresses. Muscle loss or “atrophy” occurs as the result of a number of disparate conditions including aging, immobilization, metabolic diseases, cancer, neurodegenerative diseases, and as a serious side effect of therapeutic corticosteroid hormone treatment (15, 27, 32). The recently identified E3 ubiquitin ligase, MuRF1, is proposed to be a key regulator of the atrophy process given that (i) it is expressed predominantly in skeletal muscle (3), (ii) it is upregulated under a variety of atrophy conditions (3, 12, 25), and (iii) deletion of the gene in mice results in significant muscle sparing following denervation (3). While the full physiological functions of MuRF1 are not yet known, it is often assumed that it functions in some manner to regulate protein degradation since it is expressed early in the atrophy process and its peak expression usually occurs during maximum muscle loss. For example, MuRF1 has been shown to play a direct role in myosin heavy chain ubiquitination and degradation during synthetic glucocorticoid treatment (5). However, MuRF1 may have additional important functions in skeletal muscle, such as inhibition of protein synthesis during starvation conditions (22), as well as regulating carbohydrate metabolism (16). Despite considerable interest in MuRF1 as a regulator of skeletal muscle mass and metabolism, there is limited data on the transcriptional regulation of the MuRF1 gene.

Both natural and synthetic glucocorticoid hormones are potent inducers of skeletal muscle atrophy (14). Glucocorticoids exert their physiological actions primarily via a nuclear pathway to directly affect target gene transcription. Natural glucocorticoids such as cortisol and corticosterone, as well as synthetic glucocorticoids such as dexamethasone (DEX) and prednisolone, exert their biological effects predominantly via the glucocorticoid receptor (GR) (56). The GR is a member of the nuclear receptor superfamily, and acts as a ligand dependent transcription factor. Skeletal muscle expresses significant

levels of GR, as do cultured myotubes. In the absence of ligand, the GR is found largely in the cytoplasm in a large complex that includes chaperones such as heat shock protein 90. Upon ligand binding, the GR becomes localized in the cell nucleus and binds to DNA sequences called glucocorticoid response elements (GREs). The consensus GRE sequence is AGAACANNNTGTTCT, where two GRs bind to each six nucleotide half-site of the palindome as a homodimeric complex (2). The three base-pair spacer sequence (NNN) can consist of any nucleotide combination, although that particular sequence length is critical. However, the perfect consensus sequence is rarely found in native glucocorticoid responsive promoters, with two or more nucleotide differences often found in either or both half-sites (45). In addition, GR may positively or negatively influence transcription of target genes in a GRE- and homodimer-independent manner, via interaction with promoter bound STAT5, AP-1, or NFκB transcription factors (reviewed in 50). Development of a mouse strain expressing a dimerization deficient GR (GR^{dim}) has allowed the identification of physiological processes and specific gene expression that are dependent on the classical GRE binding activity of the GR versus an indirect action through other transcription factors (38, 39).

Although the synthetic glucocorticoid DEX induces MuRF1 mRNA accumulation *in vivo* as well as in cultured myotubes (3, 48), it is currently unknown whether the gene is directly regulated by ligand-bound GR *in vivo*. DEX induction could be mediated by the increased expression of other transcription factors and associated coactivators, that in turn bind to and activate the MuRF1 promoter. For example, the class O type forkhead transcription factors, including FOXO1, FOXO3a, and FOXO4, have been recently implicated as key regulators of gene expression during skeletal muscle atrophy (42, 48). FOXO1, FOXO3a and FOXO4 are all expressed in skeletal muscle (17), and FOXO1 and FOXO3a mRNA in particular are up-regulated during fasting and DEX treatment (9, 25). Constitutively active FOXO proteins can activate the endogenous MuRF1 gene (42, 48); however, there is no information on the direct effect of FOXO proteins on the MuRF1 promoter.

Recently, it has been demonstrated that up-regulation of MuRF1 expression following DEX treatment or starvation of C₂C₁₂ cells can be suppressed by IGF-1 (41, 42, 48). The mechanism by which

IGF-1 is able to suppress MuRF1 transcription is believed to be at least in part via the PI3kinase/Akt pathway. Akt phosphorylates members of the FOXO class of forkhead transcription factors (40, 60), and phosphorylated FOXOs are sequestered in the cytoplasm by 14-3-3 proteins, thereby inhibiting transcription of FOXO target genes. In addition, FOXO proteins lacking Akt phosphorylation sites prevent IGF-1 inhibition of DEX induction of MuRF1 (48). Nevertheless, significant questions remain as to whether nuclear FOXO transcription factors alone are sufficient to activate transcription of the MuRF1 gene. In the present study, we provide a detailed analysis of the regulatory elements governing MuRF1 induction following glucocorticoid treatment, with particular attention to the role of the GR and specific FOXO transcription factors.

MATERIALS AND METHODS

Cell culture- HepG2 and C₂C₁₂ cells were cultured in DMEM supplemented with 10% FBS, non-essential amino acids, and antibiotics and grown at 37°C in 5% CO₂. C₂C₁₂ cells were cultured in DMEM, supplemented with 10% FBS, non-essential amino acids, and antibiotics and grown at 37°C in 5% CO₂. C₂C₁₂ myoblasts were differentiated to myotubes by switching confluent cells (usually 24-48 hours post-split) to DMEM supplemented with 2% charcoal dextran treated FBS, non-essential amino acids, and antibiotics and grown for an additional 48-96 hours at 37°C in 5% CO₂. All cell culture reagents except charcoal dextran treated FBS (Hyclone) were from Invitrogen.

Plasmids- The pSG5-GR construct was a kind gift of Dr. Stoney Simons, the pcDNA3-FoxO1 construct was provided by Dr. Masahiko Negishi, pCMV5-cMyc-FoxO3a was provided by Dr. Dominic Accili, and pcDNA3-FoxO4 was provided by Dr. Karen Arden. The pcDNA3.1-GRwt construct contains the full-length mouse GR cDNA and the pcDNA3.1-GR^{dim} construct was derived from pcDNA3.1-GRwt by overlap-PCR introducing the A458T point mutation and a novel BsrGI restriction site similar to the strategy use to create the same point mutation in the GR^{dim} mouse targeting vector described previously (38). The mouse MuRF1 proximal promoter region was obtained by PCR from BAC clones RP23-40E12 (CHORI). BAC DNA was isolated from bacterial cultures using the BACMAX DNA Purification Kit

from Epicentre according to the manufacturer's protocol. Primers were designed to amplify approximately -5000, -2000, -1000 and -500 base pairs from near the transcription start site. The primer sequences used to generate the MuRF1 promoter fragments are: MuRF1-Pro5000-F 5'-*GGA CAG TGC ATC ATG ACC CAG*-3', MuRF1-Pro2000-F 5'-*CCA GAA CTA CAC CAG AAA CTC*-3', MuRF1-Pro1000-F 5'-*GGA GCT GGG AAT ATA GAC TTG*-3', MuRF1-Pro500-F 5'-*CCT TAG AGC TGT TCA GAA TCC AG*-3' and MuRF-Pro-R 5'-*CAC TCG GAT CCT CTT TGT CTT C*-3'. PCR was performed using TaqPlus Long (Stratagene). The resulting PCR products were then sub-cloned into the pGEMT-EZ vector (Promega) and sequenced to confirm that the correct amplicon had been obtained. The promoter fragments were then digested out of the pGEMT-EZ vector with EcoRI, blunted, and cloned into the SmaI site of pGL3-Basic vector (Promega) resulting in fusion with the Firefly luciferase reporter gene. The recombinant plasmids were then subjected to restriction digest analysis and sequenced to confirm correct orientation. MuRF1 promoter fragments, fused to the secreted alkaline phosphatase (SEAP) reporter gene, were constructed by digesting the pGEMT-EZ recombinant plasmids with EcoRI and subcloning the MuRF1 fragments into the EcoRI site of the pSEAP-Basic vector. These constructs were then subjected to restriction digestion analysis and sequenced to confirm correct orientation. Oligonucleotides corresponding to the predicted FOXO binding site (FBE) and the glucocorticoid response element (GRE) in the MuRF1 promoter, as well as a string of 6 Daf Binding Elements (6X-DBE) were designed and ordered from Invitrogen. The oligo sequences are: MuRF1-FBE-F 5'-*CTA GTT CTT GTT TAC GAC C*-3', MuRF1-FBE-R 5'-*CTA GGG TCG TAA ACA AGA A*-3', MuRF1-GRE-F 5'-*CTA GGC TCT GAA CAG TCT GTT CTT GTT*-3', MuRF1-GRE-R 5'-*CTA GAA CAA GAA CAG ACT GTT CAG AGC*-3' 6X-DBE-F 5'-*CTA GAA GTA AAC AAC TAT GTA AAC AAC TAT AAG TAA ACA ACT ATG TAA ACA ACT ATA AGT AAA CAA CTA TGT AAA CAA GAT C*-3' and 6X-DBE-R 5'-*CTA GGA TCT TGT TTA CAT AGT TGT TTA CTT ATA GTT GTT TAC ATA GTT GTT TAC TTA TAG TTG TTT ACA TAG TTG TTT ACT T*-3'. The complementary oligo sequences were annealed by mixing and heating to 95°C and slowly cooling to 25°C over a 35 minute period using a thermocycler. The annealed oligos were then ligated into the SpeI site of the TK-Luc vector (kindly provided by Dr. Ronald Evans). The resulting recombinant

plasmids were then restriction digested to confirm the presence of an insert and sequenced to determine the number and orientation of the concatemered oligos. Site-directed mutagenesis of the FBE and GRE in the pGL3- and pSEAP-MuRF1-Pro500 constructs was performed essentially as described in the Site-Directed Mutagenesis Kit protocol from Strategene, Inc. The primers used to mutate the FBE and GRE are: MuRF1Pro-GRE-Mut-F 5'-*CCT GGC TCT GGT CAG TCT GAC CTT GTT TAC G*-3', MuRF1Pro-GRE-Mut-R 5'-*CGT AAA CAA GGT CAG ACT GAC CAG AGC CAG G*-3', MuRF1Pro-FBE-Mut-F 5'-*CTG TTC TTG GTG ACG ACC CCC*-3' and MuRF1Pro-FBE-Mut-R 5'-*GGG GGT CGT CAC CAA GAA CAG*-3'. The resulting clones were sequenced to confirm the correct mutation had been obtained. The nucleotides mutated are underlined.

Cell culture reporter gene and Northern blot assays- 125-150 x 10³ HepG2 cells/well were plated into 12-well plates and cultured for 24 hours or until an approximate confluency of 30-40% was reached. Using FuGene 6 (Roche), 1 µg of total DNA per well was transiently transfected (including 0.250 µg/well of the indicated reporter construct, 0.070 µg/well of SV40-*Renilla* luciferase (Promega), 0.250 µg/well GR expression vector (i.e. pSG5-GR, pcDNA3.1-GRwt, or pcDNA3.1- GR^{dim}), 0.125 µg/well FOXO expression vector, i.e. pcDNA3-FoxO1, pCMV5-cMyc-FoxO3a, or pcDNA3-FOXO4, and pBluescript as filler DNA) for 12-16 hours. Cells were then treated for 18-24 hours with either vehicle, 1 µM Dexamethasone (Sigma, from a 1 mM stock in ethanol), 20 ng/ml IGF-1 (Sigma) or both as indicated. Dual-Luciferase Reporter assays were performed in HepG2 cells essentially as described previously (7). Briefly, transfected cells were lysed with 1X Passive Lysis Buffer (Promega), scraped, transferred to microfuge tubes, centrifuged at high speed for 5 minutes to clear cellular debris, and then 25 µl of supernatant from each tube (corresponding to each well) was transferred to a 96-well plate. Working Firefly buffer (25 mM glycylglycine pH 8.0, 5 mM K₂HPO₄, 4 mM EGTA, 15 mM MgSO₄, 4 mM ATP, 1.25 mM DTT, 0.1 mM CoA, 80 µM D-Luciferin) and working *Renilla* buffer (1.1M NaCl, 2.2 mM Na₂EDTA, 0.22 M K₂HPO₄, pH 5.1, 0.5 mg/ml BSA, 1.5 mM NaN₃, and 1.5 µM coelenterazine) were

both prepared fresh prior to each assay. D-Luciferin was purchased from ICN. 5 mg Luciferin was dissolved in 18 ml of 25 mM Glycylglycine (pH 8.0), aliquoted, and stored at -80°C . Coelenterazine, (Promega), was dissolved in EtOH to a final concentration of 3 mM. All other chemicals were purchased from Sigma. Following treatments, cells were lysed and supernatants analyzed for luciferase activity using a Beckman Coulter LD 400 luminometer programmed to dispense 100 μl of working Firefly Buffer with a 1 sec delay and a 10 sec integration, followed by injection of 100 μl of working *Renilla* Buffer with a 2 sec delay and a 10 sec integration. Firefly luciferase activities were normalized to *Renilla* Luciferase activity to correct for variations in transfection efficiency.

For C_2C_{12} transfection experiments, 125-150 $\times 10^3$ cells/well were plated into 12-well plates and cultured as described above for 24 hours. Using FuGene 6 (Roche), 1 μg of total DNA per well was transiently transfected (including 0.250 μg /well of the indicated reporter construct, 0.125 μg /well of a TK- β -galactosidase construct, and pBluescript as filler DNA) for 12-16 hours. Cells were then switched to DMEM containing 2% charcoal/dextran treated FBS. 24 hours following the switch, the cells were treated with vehicle, either 1 μM Dexamethasone (Sigma, from a 1 mM stock in ethanol), 20 ng/ml IGF-1 (Sigma) or both as indicated. Medium from each well of the transfected, differentiated C_2C_{12} myotubes was sampled immediately prior to ligand treatment and then every 24 hours following ligand treatment for 3 days. Fresh ligand was added to the differentiated C_2C_{12} myotubes every 24 hours. 10-15 μl of each collected medium was then processed and analyzed using the Great EscAPe SEAP Detection Kit (BD Biosciences) following the instructions of the manufacturer. At the end of the experiment, the C_2C_{12} myotubes were lysed and β -galactosidase activities were determined and used to correct for variations in secreted alkaline phosphatase (SEAP) activities resulting from variations in transfection efficiencies.

For C_2C_{12} Northern blot experiments, cells were differentiated and treated as above, and total RNA was isolated using using RNeasy columns (Qiagen) according to the manufacturer's instructions. Northern analysis was conducted using random-hexamer labeled MuRF1 and rpL8 cDNAs essentially as described previously (8).

Animal studies. The generation of GR^{dim} mice was described previously (38). The mice were backcrossed to Balb/c mice for more than 20 generations. Homozygous mutants and wild-type controls were obtained by intercrossing heterozygous GR^{dim} mice. Animals were housed individually in ventilated cages under SPF conditions with *ad libitum* access to food and water and a normal dark-light cycle. For the 6 and 24 hours dexamethasone treatments: 10 mg/kg water soluble Dexamethasone (Sigma) was given by i.p. injection, and 50 mg/l Dex subsequently provided in the drinking water. For the three and eight day Dex treatments, 50 mg/l water soluble Dexamethasone (Sigma) was provided in the drinking water and replenished every second day. At each time point, animals were weighed, euthanized, and spleens as well as tibialis anterior and gastrocnemius muscles dissected and weighed. Muscles were frozen in liquid nitrogen until further processing. Splenocytes were isolated by passing the freshly isolated spleen through a 40 µm Nylon mesh, washed in PBS and subsequently treated with OptiLyse (Beckman Coulter) to remove the erythrocytes. Total splenocyte numbers were determined by counting the cells under the microscope using a *Neubauer* chamber. Total RNA from treated wild-type and GR^{dim} gastrocnemius muscle was isolated and analyzed by Northern hybridization using MuRF1, FOXO1, and FOXO3a, and rpl32 cDNA probes as described above.

Chromatin immunoprecipitation (ChIP). Chromatin immunoprecipitations were performed as modified from Metivier *et al.* (30) and Nelson *et al.* (35). Chemicals were obtained from Sigma unless stated otherwise. C₂C₁₂ cells were grown to confluence in 100mm tissue culture plates in DMEM supplemented with 10% FBS. Cells were then shifted to 2% FBS and allowed to differentiate into myotubes for four days. One plate per time point for the 1 µM Dexamethasone time course treatment or four sets of plates in quadruplicate were treated with 1 µM dexamethasone, 20 ng/ml R3-IGF-1, or a combination of 1 µM dexamethasone and 20 ng/ml R3-IGF-1 for 15 or 30 minutes. Untreated cells were used as a control. At the indicated times, medium was removed and cells were incubated with 1% formaldehyde in PBS for ten minutes at 37°C. Formaldehyde cross-linking was quenched by adding glycine to a final concentration of 125 mM and incubating for an additional ten minutes at room temperature. Cells were washed twice in

PBS, scraped in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and collected by centrifugation for five minutes at 3000 x g. Cell pellets were resuspended in 400 μ l 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 10 mM β -glycerophosphate, 1 mM sodium orthovanadate, and protease inhibitor cocktail (PIC; Roche). Cells were sonicated twice for 30 seconds each on setting “3” and “pulsed” using a Branson microtip sonicator. The resulting lysates were then centrifuged at full speed in an Eppendorf microfuge for ten minutes. Supernatants were diluted to 10 ml with ChIP dilution solution (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, PIC, 10 mM β -glycerophosphate, and 1 mM sodium vanadate). The indicated antibody (anti-HA tag IgG HA-probe (Y-11); anti-FOXO1 IgG (H-128); or anti-glucocorticoid receptor (M-20) were obtained from Santa Cruz Biotechnology, Inc.) was added to the diluted chromatin at a concentration of 1 μ g/ml and samples were incubated overnight at 4 °C with tumbling. 50 μ l Protein A (50% slurry) pre-absorbed with sheared salmon sperm DNA was added and incubated with tumbling at 4 °C for two hours. Immunoprecipitations were transferred to BioRad minicolumns (pre-rinsed with 500 μ l ChIP dilution solution) with an additional 500 μ l dilution solution to rinse the tubes and ensure complete transfer of beads to the minicolumns. Columns were then washed twice each with 1 ml TSEI (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl), TSEII (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl), and TSEIII (1 mM EDTA, 10 mM Tris-HCl, pH 8.0, 1% NP-40, 1% sodium deoxycholate, 0.25 M LiCl) followed by three washes with Wash Buffer IV (10 mM Tris (pH 8.0), 1 mM EDTA). Washed beads were transferred to new tubes with 400 μ l Wash Buffer IV, adding an additional 500 μ l Wash Buffer IV to the columns to ensure that all beads were transferred. The beads were then pelleted by centrifugation at 3000 x g for 2 minutes. After removing the supernatant by careful aspiration, 100 μ l of 10% Chelex-100 (BioRad) was added to the beads, followed by vortexing and incubation at 95 °C for 10 minutes to reverse the cross-linking. 2 μ l of 20U/ml Proteinase K solution (Invitrogen) were added, followed by vortexing and incubated at 55 °C for 30 minutes, followed by heat inactivation at 95 °C for 10 minutes. After centrifugation at full speed in an Eppendorf microfuge for two minutes, the supernatant was transferred to a new tube and combined with a second extraction of the

Chelex beads with 100 μ l of water. Input (non-immunoprecipitated chromatin) was prepared by adding 100 μ l of 10% Chelex directly to 100 μ l of the diluted chromatin extract and processed as above. Quantitative PCR of input and immunoprecipitated chromatin samples was performed using 2 μ l of DNA as template and Perkin-Elmer 2X SYBR Green master mix on an ABI 7700. Cycling conditions were one cycle at 95 °C for 10 minutes, 40 cycles at 95 °C for 10 seconds, 60 °C for 60 seconds. Primers flanking the putative mouse MuRF1 GRE/FBE region were Forward 5'-TATCTGGCTCTCCCCTGAAC-3' and Reverse 5'-CCTCAAAGATTTGGCCCTCT-3'. Values for each time point and hormone treatment were normalized to input values. For agarose gel analysis of PCR products, reactions were stopped at 30 cycles, samples from each treatment group pooled, and run on a 2% agarose gel containing ethidium bromide.

RESULTS

The MuRF1 promoter contains a conserved near perfect palindromic glucocorticoid response element. Since MuRF1 is known to be induced by synthetic glucocorticoids, as well as in several catabolic conditions associated with elevated endogenous glucocorticoids, we sought to identify the key glucocorticoid responsive elements in the gene. We began by isolating BAC clones encompassing the entire mouse MuRF1 transcription unit, and amplifying 5000 base pairs upstream of the transcription start site of the gene. Human, rat and mouse promoter sequences were aligned to detect conserved sequences that may be functionally relevant for MuRF1 expression. The MuRF1 (Figure 1A) promoter shows a high degree of homology across species for about 400 base pairs upstream of the transcription start site, and there are additional, sporadic pockets of homology far upstream of the proximal promoter, 3' of the transcription unit and within some introns (not shown). We searched for potential transcription factor binding sites by scanning the promoters for published and well-established consensus sequences. Previous reports identified and characterized consensus Class O forkhead (FOXO) binding sites in the promoter of the muscle atrophy related MAFbx gene (42) and we found multiple potential AT rich FOXO binding elements (FBEs) in the MuRF1 promoter as well, including one overlapping the consensus TATA

box (Figure 1A). Importantly, the MuRF1 promoter has a near perfect consensus GRE approximately 200 base pairs upstream of the transcription start site, supporting our hypothesis that this gene could be a direct target of the ligand activated glucocorticoid receptor (GR). The potential GREs from the human, mouse, and rat MuRF1 genes differ only by a single nucleotide in the 3 bp spacer region whose exact sequence is not critical for GR binding (Figure 1B). Interestingly, a consensus FBE was found directly adjacent to the putative MuRF1 GRE, raising the possibility that FOXO and the GR function together to regulate the MuRF1 promoter. In addition to FOXO and GR binding sites, we detected a cluster of potential NF κ B response elements in the MuRF1 promoter, consistent with the up-regulation of this promoter by pro-inflammatory cytokines and upon activation of NF κ B (4). Several C/EBP sites were also detected in the MuRF1 promoter that are of interest since C/EBP β and δ are up-regulated by DEX in skeletal muscle cells (57, 58). Finally, numerous conserved, consensus muscle specific E-box sites were found in the promoter as expected given its highly selective cardiac and skeletal muscle expression pattern.

The MuRF1 promoter is a direct target of activated glucocorticoid receptors. Since the mere presence of consensus transcription factor binding sites does not guarantee their functional importance, MuRF1 promoter fragments, or the predicted GRE and FBE in a minimal heterologous promoter, were placed in front of the firefly luciferase reporter and transiently transfected into HepG2 cells, along with expression vectors for the murine GR and/or FOXO family members. For these initial studies, HepG2 cells were chosen for their ease of transfection and their previous use in transfection studies of gluconeogenic gene promoters that are also regulated by both GR and FOXO family members (23, 24, 52). Consistent with the presence of the consensus GRE within 500 base pairs of the transcription start site, DEX was able to induce all MuRF1 promoter constructs containing between 500 and 5000 base pairs (Figure 2A). Indeed, the isolated MuRF1 GRE is sufficient to support potent DEX induced transcription, either alone (14 fold) or as a multimer (19-45 fold) (Figure 2B).

FOXO transcription factors differentially activate the MuRF1 promoter. Next, we tested whether the native MuRF1 promoter would respond to co-transfected FOXO1, FOXO3a, or FOXO4 expression vectors, since these proteins are candidates for intermediate, DEX inducible regulatory factors. As shown in Figure 3A, FOXO factors only marginally induce the proximal MuRF1 promoter (2-3 fold). As with the GRE, we tested whether the isolated FOXO binding site (FBE) would respond to co-transfected FOXO1, FOXO3a, or FOXO4 expression vectors; in this context, only FOXO3a and FOXO4 induced transcription of the reporter containing a single FBE (Figure 3B). However, FOXO1 is able to activate multimerized DAF binding element (DBE) or the MuRF1 FBE suggesting that this FOXO family member is most active as a multimeric complex rather than a monomer (Figure 3C).

The MuRF1 promoter is synergistically activated by FOXO1 and GR. Remarkably, however, FOXO1 showed strong synergistic activation with the GR on the 500 base pair MuRF1 promoter (Figure 4A). This potent synergy was consistently observed (20-40 fold) over multiple experiments, and on all promoter fragments tested up to 5000 bp from the transcription start site. FOXO3a and FOXO4 did not synergize with the GR; indeed, expression of FOXO3a somewhat inhibits GR activation of the MuRF1 promoter. The GR-FOXO1 synergy is also reflected in dose-response experiments, where this combination supports strong activation of the MuRF1 promoter with as little as 1 nM DEX in the culture media (Figure 4B). Both the GRE and the adjacent FBE play an important role in the observed GR-FOXO synergy. DEX induction of the 500 base pair MuRF1 promoter is nearly abolished when the GRE is mutated and is completely abolished when the GRE is intact but the adjacent FOXO site is mutated (Figures 4C and D).

DEX induction of MuRF1 expression is inhibited in GR^{dim} mice that express a dimerization deficient GR. The functional significance of the near perfect GRE in the MuRF1 promoter was tested *in vivo* through the use of the GR^{dim} mutant mouse strain that expresses a GR with a point mutation in the DNA binding domain that prevents binding of the GR to classical, palindromic GREs (38). This mutant GR still retains the ability to be tethered to target genes via protein-protein interactions such as the NFκB or AP-1 transcription factor complexes. Both wild-type and GR^{dim} mice were treated with water soluble

DEX either by injection (6 and 24 hour time points) or in the drinking water (24 hour, 3 day, and 8 day time points). The experiment was terminated at 8 days of treatment due to the severe weight loss observed in the wild-type mice, which was not observed in the GR^{dim} mice ($p < 0.001$). As shown in Figure 5A, despite the inhibited overall body weight decline in GR^{dim} versus wild-type mice in response to DEX, the degree of atrophy induced in the tibialis anterior and gastrocnemius muscles was not statistically different in the two strains. By contrast, DEX induced splenocyte apoptosis was strongly inhibited in GR^{dim} mice ($p < 0.001$).

We next examined MuRF1 expression as well as FOXO1 and FOXO3a expression in GR^{dim} versus GR^{+/+} mice (Figure 5B). MuRF1 expression is clearly up-regulated by six hours after DEX injection and continues to increase up to 3 days with continuous DEX treatment in wild-type mice. MuRF1 induction is strongly inhibited in GR^{dim} mice which is particularly apparent at the earliest time points. After eight days of DEX treatment, however, MuRF1 expression declines in wild-type mice until it is essentially equal to its induced expression in GR^{dim} animals. In addition, FOXO1 and FOXO3a are also rapidly induced by DEX in wild-type mice, in agreement with a previous study (9), and expression is reduced in GR^{dim} mice at all time points tested. We then tested the ability of the homodimerization mutant GR expressed in GR^{dim} mice to upregulate the proximal MuRF1 promoter, again using transient transfection assays (Figure 5C). The homodimerization mutant GR lost the ability to induce the MuRF1 promoter on its own, as expected, and showed a strongly reduced, but not completely abolished, ability to augment FOXO1 induction of the promoter. These transfection results may at least partially explain the residual DEX induced expression of MuRF1 in GR^{dim} mice.

The glucocorticoid receptor and FOXO1 bind to the endogenous MuRF1 promoter in C₂C₁₂ myotubes. Given the strong dependence on GR homodimerization *in vivo* for full MuRF1 gene induction, we next sought to determine the role of the GRE and FOXO1 sites in cultured skeletal myotubes. As we observed in HepG2 cells, the 500 base pair MuRF1 promoter is also DEX inducible in differentiated C₂C₁₂ mouse skeletal myotubes (Figure 6A). DEX induction of the promoter is nearly abolished when the GRE is mutated and the adjacent FOXO site is intact, and completely abolished when the GRE is intact

but the adjacent FOXO site is mutated (Figure 6B). Furthermore, addition of 20 ng/ml IGF-1 strongly inhibits DEX induction of the endogenous MuRF1 gene, as has been previously reported (41, 48) (Figure 6C), and IGF-1 likewise inhibits DEX induction of the MuRF1 promoter (Figure 6D). Therefore, the 500 base pair proximal promoter of MuRF1 contains the critical elements necessary to respond positively to glucocorticoids (via synergy with a forkhead site) and negatively to IGF-1 in a manner identical to the endogenous gene in differentiated myotubes.

In order to determine whether FOXO1 and GR can directly occupy the MuRF1 promoter *in vivo*, we established a modified chromatin immunoprecipitation (ChIP) protocol suitable for use with differentiated C₂C₁₂ myotubes. C₂C₁₂ myoblasts were plated on 100 mm dishes and cultured until confluent, then differentiated for four days in reduced serum media. Myotubes were then treated with vehicle, 1 μ M DEX, 20 ng/ml IGF-1, or 1 μ M DEX plus 20 ng/ml IGF-1 for the indicated times. Sonicated chromatin from fixed cells were analyzed with control IgG, an anti-FOXO1 antibody, or two anti-GR antibodies. Both GR and FOXO1 are present on the endogenous MuRF1 promoter in untreated C₂C₁₂ myotubes (Figure 7A), and binding of each factor is enhanced by DEX treatment as soon as 15 minutes after ligand addition (Figure 7B and 7C), especially the GR. GR binding to the MuRF1 promoter is unaffected by IGF-1 (Figure 7B) even though the DEX induction of the endogenous gene or the transfected MuRF1 promoter is strongly inhibited. However, IGF-1 addition rapidly induces the loss of FOXO1 from the MuRF1 promoter in the presence or absence of DEX (Figure 7C). We also attempted to detect FOXO3a binding to the MuRF1 promoter by chromatin immunoprecipitation as well; only one of two commercially available antibodies we tested (Upstate versus Santa Cruz) gave a weak positive signal and the binding pattern was highly similar to FOXO1 (not shown).

DISCUSSION

While it is known that MuRF1 expression increases under numerous atrophy-inducing conditions (11) the mechanism of transcriptional regulation of this gene is poorly understood. The present study

provides new insights into how the glucocorticoid receptor and the FOXO family of transcription factors regulate the transcription of the MuRF1 gene. Specifically, we demonstrate here that (i) the MuRF1 proximal promoter is directly activated by glucocorticoids via a conserved GRE that strongly depends on GR homodimerization for full activation, at least in the earliest time points after DEX treatment, (ii) FOXO1 and GR synergistically and specifically induce the MuRF1 proximal promoter, and (iii) IGF1 inhibition of DEX-induced MuRF1 expression correlates with the loss of FOXO1 binding to the endogenous MuRF1 promoter. Importantly, these data also reveal that not all FOXO family members equally activate the FOXO binding motif in the MuRF1 promoter.

The presence of an essentially perfect palindromic GRE in the MuRF1 proximal promoter likely explains the strong induction of the gene by exogenous synthetic glucocorticoids (3, 48), and how the gene is induced under a variety of catabolic conditions associated with increased endogenous corticosteroid levels (25, 53). Each half-site of the MuRF1 GRE is perfectly conserved between the human, rat, and mouse genes with the only difference found in the spacer region that, nevertheless, still retains the three base pair length necessary for GR homodimer binding (43). It is surprisingly rare to find such a perfect GRE in an endogenous glucocorticoid target gene promoter, although the specific sequences of the divergent half-sites found in most direct GR target genes are strongly conserved across species (45, 46). Most GREs, such as the pair found in the well studied phosphoenolpyruvate carboxykinase (PEPCK) gene in the liver (18) or the first described GREs in the mouse mammary tumor virus long terminal repeat (37), have multiple base pair changes compared to the perfect palindrome consensus GRE that is present in the MuRF1 promoter. Previous studies on the estrogen regulated family of vitellogenin genes in the frog liver demonstrate that a single perfect estrogen response element supports essentially the same fold induction by estradiol as a pair of imperfect palindromic elements (29). The physiological relevance of having such a well-conserved, consensus GRE in the MuRF1 promoter is an interesting and important question.

Analysis of MuRF1 induction by DEX in GR^{dim} mice revealed a critical role for GR homodimerization, which is most evident at the earliest time points examined in these experiments.

Interestingly, despite the clear overall reduction in MuRF1 expression, as well as FOXO1 and FOXO3a, the degree of muscle atrophy in GR^{dim} mice after eight days of DEX treatment was exactly the same in the mutant as in wild-type mice. Consequently, the residual level of MuRF1 expression in GR^{dim} mice may well be enough to support full DEX induced atrophy. The rapid rise and gradual decline of MuRF1 expression we observed in DEX treated Balb/c mice is in agreement with experiments in rats dosed with the synthetic glucocorticoid prednisolone (1). Concerning the mechanism of residual MuRF1 induction in GR^{dim} mice, it is conspicuous that co-transfection of FOXO1 with the homodimerization mutant GR supports at least modest induction of the MuRF1 promoter in transient transfection assays, whereas the induction without FOXO1 is completely lost (Figure 5). This is in line with the previous observation that the GR^{dim} receptor by its own fails to bind to a palindromic GRE. Nevertheless, it is conceivable that FOXO1, when present together, still tethers the homodimerization mutant GR to the composite GRE-FBE motif, since protein-protein-interactions with other transcription factors are not compromised by the GR^{dim} mutation. Regardless, it will be important to identify DEX responsive genes that are less affected than MuRF1 and FOXOs in GR^{dim} mice that may ultimately be more important for the loss of muscle mass in response to DEX. For example, activation of the N-end rule ubiquitin pathway was recently implicated in MyoD degradation in response to DEX in C₂C₁₂ cells (49). In addition, transgenic overexpression of MuRF1 was not sufficient to induce atrophy but led to altered levels of enzymes involved in carbohydrate metabolism (16). Thus, consideration of these and other studies, and our present results with GR^{dim} mice, may force a re-evaluation of the role of MuRF1 (and possibly even FOXO1 and FOXO3a) in the reduction of muscle mass in catabolic states, although the protein certainly may still play a significant role in structural protein degradation in denervation induced atrophy. Further analysis of the GR^{dim} model may allow for a genetic dissection of the induction of atrophy versus metabolic effects of glucocorticoids in this important GR target tissue.

Several recent papers have implicated FOXO family transcription factors as being important for the up-regulation of gene expression during skeletal muscle atrophy, including after DEX treatment or nutrient starvation in C₂C₁₂ cells. For example, Stitt *et al.* (48) reported that activated FOXO1 is

necessary but not sufficient to activate the MuRF1 gene in cultured myotubes; however, the effects of FOXO3a or FOXO4 were not reported. Interestingly, selective transgenic over-expression of FOXO1 in skeletal muscle produced mice with significantly smaller muscles than wild type litter mates, without apparent upregulation of MuRF1 (19). The lack of MuRF1 induction in these instances is not surprising given our present findings. Assuming that FOXO1 was the only transcription factor over-expressed in these transgenic mice, and that circulating corticosteroid levels are normal, then MuRF1 would not necessarily be upregulated. The reduction in muscle mass observed in these mice may be due to the activation of other atrophy-inducing pathways. For example, over-expression of FOXO1 leads to an upregulation of 4E-BP1 and inhibition of mTOR signaling resulting in a decrease in protein synthesis (47).

There is important precedence for the collaborative role of FOXO1 on GR target genes from studies of inversely regulated insulin and glucocorticoid responsive genes in the liver. The IGF binding protein-1 (10) and glucose-6 phosphatase promoters contain GREs with directly adjacent and even overlapping FOXO binding sites, and the phosphoenolpyruvate carboxykinase (PEPCK) (13) and pyruvate dehydrogenase kinase-4 (PDK4) (24) genes have FOXO sites in relative close proximity to their GREs. In each case, integrity of the FOXO site is important for maximal glucocorticoid responsiveness. In addition, like MuRF1, the ability of FOXO1 and FOXO3a to have differential effects on a single promoter has recently been demonstrated by Onuma *et al.* for the glucose-6-phosphatase promoter (36). It is intriguing that key genes involved in gluconeogenesis have clustered functional GREs and FOXO binding sites in critical glucocorticoid and insulin sensitive enhancers reminiscent of our findings with the muscle specific MuRF1 gene promoter. It is tempting to speculate that if indeed one role of MuRF1, as an E3 ubiquitin ligase, is to target specific skeletal muscle proteins for degradation, then a gene involved in providing a source of amino acid substrates for the liver to use to make glucose *de novo* might logically be under similar regulatory control as important gluconeogenic enzymes. The common regulatory strategy of MuRF1 expression with liver gluconeogenic enzymes also supports the notion of a potential prominent role for MuRF1 in skeletal muscle glucose metabolism.

The role of FOXO1 as a key component of a composite glucocorticoid responsive unit in MuRF1 gene regulation is further emphasized by our chromatin immunoprecipitation data. IGF-1 not only virtually eliminates DEX up-regulation of the 500 base pair MuRF1 promoter and the endogenous MuRF1 gene (see Figure 7) but also rapidly clears the MuRF1 promoter of FOXO1 binding without affecting GR binding. On the PEPCK enhancer in rat liver H4IIE cells, insulin treatment causes a rapid loss of FOXO binding in control and DEX treated cells, with significant reduction already apparent by as little as 3 minutes after insulin addition (13). In this case, GR binding was reduced to approximately 40% compared to the DEX only induced level after 30 minutes of treatment, which we did not observe upon IGF-1 addition to C₂C₁₂ cells. Nevertheless, the most impressive change in promoter occupancy examined in both our experiments and the PEPCK gene experiments was decreased FOXO1 binding. In addition to inhibition of FOXO1 (and, to a lesser extent GR) binding to the PEPCK promoter in response to insulin treatment, dramatic change in histone post-translational modifications were observed that paralleled decreased transcription (13). Whether these changes occur on the MuRF-1 promoter in response to DEX and/or IGF-1 treatment remains an interesting question, as is whether removal of FOXO1 is the key event triggering the reversal of transcriptional activation and associated chromatin modifications induced by DEX.

The exact nature of the synergy between the GR and FOXO1 is clearly an important area for further investigation, as it is also not well understood for liver gluconeogenic genes. FOXO1 has been demonstrated to physically interact in solution with several steroid hormone receptors, such as the estrogen, progesterone, and androgen receptors (21, 26, 44, 59), but not to date with the glucocorticoid receptor. The functional significance of this interaction (either repressing or enhancing receptor activity) varies greatly depending on the cell-type and target gene promoter context. For example, FOXO1 synergistically activates the IGFBP1 promoter with ligand activated progesterone receptors in endometrial adenocarcinoma cells but not in endometrial fibroblasts (20). FOXO1, FOXO3a, and FOXO4 all contain an LXXLL motif that can interact with a ligand-induced pocket on the hormone binding domain of steroid receptors (59). Since FOXO1 binding to the MuRF1 promoter is (at least modestly)

enhanced by DEX treatment of C₂C₁₂ cells along with the strong recruitment of GR (Figure 7), a situation also observed on the PEPCK and glucose-6 phosphatase genes (13, 51), these proteins may be mutually bound and stabilized on the DEX activated MuRF1 promoter. Synergy may also be achieved via co-recruitment of transcriptional coactivators such as histone acetyltransferases like CBP/p300 that have been shown to be independently recruited by the liganded GR or FOXO1 in other contexts (28, 34). Irrespective of the precise nature of the GR-FOXO1 synergy, our results reveal that each factor is required to create an optimal glucocorticoid inducible MuRF1 promoter.

There are several important implications of our findings on MuRF1 gene regulation. First, the activated glucocorticoid receptor may be an important player in the control of muscle atrophy, but the presence of nuclear FOXO1 is critical to achieve full induction of the MuRF1 promoter and, quite possibly, multiple glucocorticoid regulated genes in skeletal muscle. Furthermore, the glucocorticoid concentration needed to induce MuRF1 is significantly reduced in the presence of FOXO1. A logical prediction based on these results is that conditions with both elevated corticosteroids and active FOXO1 would lead to the greatest degree of muscle wasting and altered metabolism. In fact, diabetes mellitus presents such a condition (53). In several models of diabetes, Akt is less active, potentially leading to decreased inhibition of FOXO activity and/or increased nuclear localization (55). Elevated nuclear FOXO1 would be available to interact with the GR on the MuRF1 promoter to further induce the gene above the level induced by corticosteroids alone. Prolonged glucocorticoid exposure also leads to insulin resistance (54), which might in turn lead to higher MuRF1 induction, accelerated muscle atrophy and metabolic changes as part of a vicious cycle. In contrast, the need for both nuclear FOXO1 and activated GR to fully activate MuRF1 may be protective and prevent unnecessary muscle breakdown. For example, following intense exhaustive exercise, circulating cortisol levels increase (6, 31), yet elevated cortisol is generally not associated with an increase in the breakdown of contractile proteins, nor is loss of muscle mass a consequence of endurance training (33). Thus, the need for additional factors to fully activate MuRF1 gene expression could protect the muscle from breakdown under certain physiological conditions.

In summary, our results demonstrate a potent synergy between the GR and FOXO1 in transcriptional control of an important gene linked to skeletal muscle atrophy and metabolic control. Further understanding the molecular details of the opposing effects of corticosteroids and insulin/IGF-1 on gene expression in skeletal muscle, the breadth of the GR-FOXO1 co-operation on muscle gene expression in general, and the very nature of this robust synergy should have important ramifications for preventing inappropriate induction of genes involved in muscle atrophy and altered metabolism in catabolic disease states.

REFERENCES

1. **Almon RR, DuBois DC, Yao Z, Hoffman EP, Ghimbovschi S, and Jusko WJ.** Microarray analysis of the temporal response of skeletal muscle to methylprednisolone: comparative analysis of two dosing regimens. *Physiological genomics* 30: 282-299, 2007.
2. **Beato M, Chalepakis G, Schauer M, and Slater EP.** DNA regulatory elements for steroid hormones. *J Steroid Biochem* 32: 737-747, 1989.
3. **Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, and Glass DJ.** Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 294: 1704-1708, 2001.
4. **Cai D, Frantz JD, Tawa NE, Jr., Melendez PA, Oh BC, Lidov HG, Hasselgren PO, Frontera WR, Lee J, Glass DJ, and Shoelson SE.** IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. *Cell* 119: 285-298, 2004.
5. **Clarke BA, Drujan D, Willis MS, Murphy LO, Corpina RA, Burova E, Rakhilin SV, Stitt TN, Patterson C, Latres E, and Glass DJ.** The E3 Ligase MuRF1 degrades myosin heavy chain protein in dexamethasone-treated skeletal muscle. *Cell Metab* 6: 376-385, 2007.
6. **Duclos M, Corcuff JB, Arsac L, Moreau-Gaudry F, Rashedi M, Roger P, Tabarin A, and Manier G.** Corticotroph axis sensitivity after exercise in endurance-trained athletes. *Clinical endocrinology* 48: 493-501, 1998.
7. **Dyer BW, Ferrer FA, Klinedinst DK, and Rodriguez R.** A noncommercial dual luciferase enzyme assay system for reporter gene analysis. *Anal Biochem* 282: 158-161, 2000.
8. **Furlow JD, Berry DL, Wang Z, and Brown DD.** A set of novel tadpole specific genes expressed only in the epidermis are down-regulated by thyroid hormone during *Xenopus laevis* metamorphosis. *Dev Biol* 182: 284-298, 1997.
9. **Furuyama T, Kitayama K, Yamashita H, and Mori N.** Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation. *Biochem J* 375: 365-371, 2003.
10. **Gan L, Pan H, and Unterman TG.** Insulin response sequence-dependent and -independent mechanisms mediate effects of insulin on glucocorticoid-stimulated insulin-like growth factor binding protein-1 promoter activity. *Endocrinology* 146: 4274-4280, 2005.
11. **Glass DJ.** Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* 37: 1974-1984, 2005.

12. **Gomes MD, Lecker SH, Jagoe RT, Navon A, and Goldberg AL.** Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proceedings of the National Academy of Sciences of the United States of America* 98: 14440-14445, 2001.
13. **Hall RK, Wang XL, George L, Koch SR, and Granner DK.** Insulin represses phosphoenolpyruvate carboxykinase gene transcription by causing the rapid disruption of an active transcription complex: a potential epigenetic effect. *Mol Endocrinol* 21: 550-563, 2007.
14. **Hasselgren PO.** Glucocorticoids and muscle catabolism. *Curr Opin Clin Nutr Metab Care* 2: 201-205, 1999.
15. **Hasselgren PO and Fischer JE.** Muscle cachexia: current concepts of intracellular mechanisms and molecular regulation. *Ann Surg* 233: 9-17, 2001.
16. **Hirner S, Krohne C, Schuster A, Hoffmann S, Witt S, Erber R, Sticht C, Gasch A, Labeit S, and Labeit D.** MuRF1-dependent Regulation of Systemic Carbohydrate Metabolism as Revealed from Transgenic Mouse Studies. *J Mol Biol*, 2008.
17. **Hribal ML, Nakae J, Kitamura T, Shutter JR, and Accili D.** Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *J Cell Biol* 162: 535-541, 2003.
18. **Imai E, Stromstedt PE, Quinn PG, Carlstedt-Duke J, Gustafsson JA, and Granner DK.** Characterization of a complex glucocorticoid response unit in the phosphoenolpyruvate carboxykinase gene. *Mol Cell Biol* 10: 4712-4719, 1990.
19. **Kamei Y, Miura S, Suzuki M, Kai Y, Mizukami J, Taniguchi T, Mochida K, Hata T, Matsuda J, Aburatani H, Nishino I, and Ezaki O.** Skeletal muscle FOXO1 (FKHR) transgenic mice have less skeletal muscle mass, down-regulated Type I (slow twitch/red muscle) fiber genes, and impaired glycemic control. *J Biol Chem* 279: 41114-41123, 2004.
20. **Kim J, Jia L, Stallcup MR, and Coetzee GA.** The role of protein kinase A pathway and cAMP responsive element-binding protein in androgen receptor-mediated transcription at the prostate-specific antigen locus. *J Mol Endocrinol* 34: 107-118, 2005.
21. **Kim JJ, Buzio OL, Li S, and Lu Z.** Role of FOXO1A in the regulation of insulin-like growth factor-binding protein-1 in human endometrial cells: interaction with progesterone receptor. *Biol Reprod* 73: 833-839, 2005.
22. **Koyama S, Hata S, Witt CC, Ono Y, Lerche S, Ojima K, Chiba T, Doi N, Kitamura F, Tanaka K, Abe K, Witt SH, Rybin V, Gasch A, Franz T, Labeit S, and Sorimachi H.** Muscle RING-finger protein-1 (MuRF1) as a connector of muscle energy metabolism and protein synthesis. *J Mol Biol* 376: 1224-1236, 2008.
23. **Krones-Herzig A, Mesaros A, Metzger D, Ziegler A, Lemke U, Bruning JC, and Herzig S.** Signal-dependent control of gluconeogenic key enzyme genes through coactivator-associated arginine methyltransferase 1. *J Biol Chem* 281: 3025-3029, 2006.
24. **Kwon HS, Huang B, Unterman TG, and Harris RA.** Protein kinase B-alpha inhibits human pyruvate dehydrogenase kinase-4 gene induction by dexamethasone through inactivation of FOXO transcription factors. *Diabetes* 53: 899-910, 2004.
25. **Lecker SH, Jagoe RT, Gilbert A, Gomes M, Baracos V, Bailey J, Price SR, Mitch WE, and Goldberg AL.** Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *Faseb J* 18: 39-51, 2004.
26. **Li P, Lee H, Guo S, Unterman TG, Jenster G, and Bai W.** AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR. *Mol Cell Biol* 23: 104-118, 2003.
27. **Lynch GS, Schertzer JD, and Ryall JG.** Therapeutic approaches for muscle wasting disorders. *Pharmacology & therapeutics* 113: 461-487, 2007.
28. **Ma H, Hong H, Huang SM, Irvine RA, Webb P, Kushner PJ, Coetzee GA, and Stallcup MR.** Multiple signal input and output domains of the 160-kilodalton nuclear receptor coactivator proteins. *Mol Cell Biol* 19: 6164-6173, 1999.

29. **Martinez E and Wahli W.** Cooperative binding of estrogen receptor to imperfect estrogen-responsive DNA elements correlates with their synergistic hormone-dependent enhancer activity. *Embo J* 8: 3781-3791, 1989.
30. **Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, and Gannon F.** Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* 115: 751-763, 2003.
31. **Minetto MA, Lanfranco F, Baldi M, Termine A, Kuipers H, Ghigo E, and Rainoldi A.** Corticotroph axis sensitivity after exercise: comparison between elite athletes and sedentary subjects. *Journal of endocrinological investigation* 30: 215-223, 2007.
32. **Mitch WE and Goldberg AL.** Mechanisms of muscle wasting. The role of the ubiquitin-proteasome pathway. *N Engl J Med* 335: 1897-1905, 1996.
33. **Nader GA.** Concurrent strength and endurance training: from molecules to man. *Med Sci Sports Exerc* 38: 1965-1970, 2006.
34. **Nasrin N, Ogg S, Cahill CM, Biggs W, Nui S, Dore J, Calvo D, Shi Y, Ruvkun G, and Alexander-Bridges MC.** DAF-16 recruits the CREB-binding protein coactivator complex to the insulin-like growth factor binding protein 1 promoter in HepG2 cells. *Proceedings of the National Academy of Sciences of the United States of America* 97: 10412-10417, 2000.
35. **Nelson JD, Denisenko O, Sova P, and Bomsztyk K.** Fast chromatin immunoprecipitation assay. *Nucleic Acids Res* 34: e2, 2006.
36. **Onuma H, Vander Kooi BT, Boustead JN, Oeser JK, and O'Brien RM.** Correlation between FOXO1a (FKHR) and FOXO3a (FKHRL1) binding and the inhibition of basal glucose-6-phosphatase catalytic subunit gene transcription by insulin. *Mol Endocrinol* 20: 2831-2847, 2006.
37. **Payvar F, DeFranco D, Firestone GL, Edgar B, Wrangle O, Okret S, Gustafsson JA, and Yamamoto KR.** Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. *Cell* 35: 381-392, 1983.
38. **Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P, and Schutz G.** DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* 93: 531-541, 1998.
39. **Reichardt HM, Tuckermann JP, Gottlicher M, Vujic M, Weih F, Angel P, Herrlich P, and Schutz G.** Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. *Embo J* 20: 7168-7173, 2001.
40. **Rena G, Guo S, Cichy SC, Unterman TG, and Cohen P.** Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. *J Biol Chem* 274: 17179-17183, 1999.
41. **Sacheck JM, Ohtsuka A, McLary SC, and Goldberg AL.** IGF-1 stimulates muscle growth by suppressing protein breakdown and expression of atrophy-related ubiquitin-ligases, atrogin-1 and MuRF1. *Am J Physiol Endocrinol Metab*, 2004.
42. **Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, and Goldberg AL.** Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* 117: 399-412, 2004.
43. **Schoneveld OJ, Gaemers IC, and Lamers WH.** Mechanisms of glucocorticoid signalling. *Biochim Biophys Acta* 1680: 114-128, 2004.
44. **Schuur ER, Loktev AV, Sharma M, Sun Z, Roth RA, and Weigel RJ.** Ligand-dependent interaction of estrogen receptor- α with members of the forkhead transcription factor family. *J Biol Chem* 276: 33554-33560, 2001.
45. **So AY, Chaivorapol C, Bolton EC, Li H, and Yamamoto KR.** Determinants of cell- and gene-specific transcriptional regulation by the glucocorticoid receptor. *PLoS Genet* 3: e94, 2007.
46. **So AY, Cooper SB, Feldman BJ, Manuchehri M, and Yamamoto KR.** Conservation analysis predicts in vivo occupancy of glucocorticoid receptor-binding sequences at

glucocorticoid-induced genes. *Proceedings of the National Academy of Sciences of the United States of America* 105: 5745-5749, 2008.

47. **Southgate RJ, Neill B, Prelovsek O, El-Osta A, Kamei Y, Miura S, Ezaki O, McLoughlin TJ, Zhang W, Unterman TG, and Febbraio MA.** FOXO1 regulates the expression of 4E-BP1 and inhibits mTOR signaling in mammalian skeletal muscle. *J Biol Chem* 282: 21176-21186, 2007.

48. **Stitt TN, Drujan D, Clarke BA, Panaro FJ, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, and Glass DJ.** The IGF-1/PI3K/Akt Pathway Prevents Expression of Muscle Atrophy-Induced Ubiquitin Ligases by Inhibiting FOXO Transcription Factors. *Mol Cell* 14: 1-14, 2004.

49. **Sun L, Trausch-Azar JS, Muglia LJ, and Schwartz AL.** Glucocorticoids differentially regulate degradation of MyoD and Id1 by N-terminal ubiquitination to promote muscle protein catabolism. *Proceedings of the National Academy of Sciences of the United States of America* 105: 3339-3344, 2008.

50. **Tuckermann JP, Kleiman A, McPherson KG, and Reichardt HM.** Molecular mechanisms of glucocorticoids in the control of inflammation and lymphocyte apoptosis. *Crit Rev Clin Lab Sci* 42: 71-104, 2005.

51. **Vander Kooi BT, Onuma H, Oeser JK, Svitek CA, Allen SR, Vander Kooi CW, Chazin WJ, and O'Brien RM.** The glucose-6-phosphatase catalytic subunit gene promoter contains both positive and negative glucocorticoid response elements. *Mol Endocrinol* 19: 3001-3022, 2005.

52. **Vander Kooi BT, Streeper RS, Svitek CA, Oeser JK, Powell DR, and O'Brien RM.** The three insulin response sequences in the glucose-6-phosphatase catalytic subunit gene promoter are functionally distinct. *J Biol Chem* 278: 11782-11793, 2003.

53. **Wang X, Hu Z, Hu J, Du J, and Mitch WE.** Insulin resistance accelerates muscle protein degradation: Activation of the ubiquitin-proteasome pathway by defects in muscle cell signaling. *Endocrinology* 147: 4160-4168, 2006.

54. **Weinstein SP, Paquin T, Pritsker A, and Haber RS.** Glucocorticoid-induced insulin resistance: dexamethasone inhibits the activation of glucose transport in rat skeletal muscle by both insulin- and non-insulin-related stimuli. *Diabetes* 44: 441-445, 1995.

55. **Whiteman EL, Cho H, and Birnbaum MJ.** Role of Akt/protein kinase B in metabolism. *Trends Endocrinol Metab* 13: 444-451, 2002.

56. **Wright AP, Zilliacus J, McEwan IJ, Dahlman-Wright K, Almlöf T, Carlstedt-Duke J, and Gustafsson JA.** Structure and function of the glucocorticoid receptor. *J Steroid Biochem Mol Biol* 47: 11-19, 1993.

57. **Yang H, Mammen J, Wei W, Menconi M, Evenson A, Fareed M, Petkova V, and Hasselgren PO.** Expression and activity of C/EBPbeta and delta are upregulated by dexamethasone in skeletal muscle. *J Cell Physiol* 204: 219-226, 2005.

58. **Yang H, Menconi MJ, Wei W, Petkova V, and Hasselgren PO.** Dexamethasone upregulates the expression of the nuclear cofactor p300 and its interaction with C/EBPbeta in cultured myotubes. *J Cell Biochem* 94: 1058-1067, 2005.

59. **Zhao HH, Herrera RE, Coronado-Heinsohn E, Yang MC, Ludes-Meyers JH, Seybold-Tilson KJ, Nawaz Z, Yee D, Barr FG, Diab SG, Brown PH, Fuqua SA, and Osborne CK.** Forkhead homologue in rhabdomyosarcoma functions as a bifunctional nuclear receptor-interacting protein with both coactivator and corepressor functions. *J Biol Chem* 276: 27907-27912, 2001.

60. **Zhao X, Gan L, Pan H, Kan D, Majeski M, Adam SA, and Unterman TG.** Multiple elements regulate nuclear/cytoplasmic shuttling of FOXO1: characterization of phosphorylation- and 14-3-3-dependent and -independent mechanisms. *Biochem J* 378: 839-849, 2004.

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FIGURE LEGENDS

Fig. 1. Schematic of the MuRF1 promoter and sequence alignment of the proximal regulatory regions. *A.* Promoter sequences from mouse, rat, and human MuRF1 (5000 base pairs upstream of the transcription start site (+1) through the first exon) were downloaded from the Ensembl database (www.ensembl.org) and aligned using the ClustalW algorithm. Approximate positions of potential transcription factor binding sites are indicated in the schematics of the MuRF1 promoter on top and/or boxed in the alignment below: The O class, or FOXO, Forkhead binding site (G/A)TAAA(T/C)AA (black ovals); Glucocorticoid response element (GRE) (A/T)GAACANNNTGTTT(A/T) (hatched rectangle); C/EBP TT(G/T)NGNAA (white diamonds); NF- κ B consensus binding sequence GGG(G/A)N(C/T)(C/T)(C/T)CC (gray hexagons) and muscle specific E box CANGTG (MyoD, etc.). (N) represents any nucleotide. The sequence alignments for approximately 400 base pairs upstream and 26 base pairs downstream of the rat, mouse, and human MuRF1 promoters are shown below the corresponding promoter schematics. Identical sequences for the indicated regions are highlighted in black. Arrow indicates transcription start site. *B.* Comparison of a consensus GRE, and perfect palindrome GRE, and putative GREs from the mouse, rat and human MuRF1 promoters. Arrows indicate half-sites.

Fig. 2. Dexamethasone-activated GR induces the MuRF1 proximal promoter. *A.* HepG2 cells were transfected with luciferase reporter constructs (LUC) containing varying lengths of the MuRF1 (pGL3-

MuRF1) promoter, a SV40-*Renilla* luciferase reporter construct, and a mouse GR expression vector (pSG5-GR). Cells were treated with or without 1 μ M dexamethasone (DEX) for 24 hours. Luciferase activities in cell extracts were normalized to *Renilla* luciferase activity to control for transfection efficiency. Numbers on the Y-axis indicate distance from the transcription start site fused to the reporter, pGL3-Basic, a promoterless LUC vector. *B*. The isolated MuRF1 GRE supports DEX-mediated transcriptional activation of a heterologous promoter. The DEX regulation of the thymidine kinase (TK) promoter was tested with 0 (Control), 1 (1XGRE), 3 (3XGRE), and 4 (4XGRE) inserted GREs. The arrows below the indicated GRE constructs labeled on the Y axis depict the orientation of each individual GRE oligonucleotide. In A and B, fold induction was obtained by dividing values from DEX-treated samples by the mean of values from matched untreated samples. Each condition was done in triplicate, and error bars reflect SD.

Fig. 3. FOXO transcription factor induction of the MuRF1 promoter. *A*. HepG2 cells were transfected with 500 base pair MuRF1-Promoter –LUC constructs, SV40-*Renilla* luciferase reporter construct, and expression vectors for murine FoxO1, FoxO3A, or FoxO4 (pcDNA3-FoxO1, pcDNA3-FoxO3A, or pcDNA3-FoxO4) or pcDNA3 alone (dash). Luciferase activities in cell extracts were normalized to *Renilla* luciferase activity to control for variations in transfection efficiency. Each point was done in triplicate; error bars reflect SD. *B*. The isolated MuRF1 FBE supports FOXO3a and FOXO4, but not FOXO1, mediated transcriptional activation of a heterologous promoter. FOXO regulation of the thymidine kinase (TK) promoter was tested by inserting a single FBE and cotransfecting HepG2 cells with the control pcDNA3 vector alone (dash) or each FOXO expression vector as in A. *C*. Concatamerized FOXO binding elements from the MuRF1 promoter (FBE-4X) and Daf-16 binding elements (DBE-6X) support transcriptional activation of the TK promoter by FoxO1. White bars indicate normalized luciferase values from the cells transfected with the TK-Luc-4XFBE and TK-Luc-6XDBE in the absence of exogenous FoxO1 expression, while black bars indicate normalized luciferase values from

the TK-Luc-4XFBE and TK-Luc-6XDBE transfected cells in the presence of exogenous FoxO1 expression. Data was processed as in A and B.

Fig. 4. Dexamethasone-activated GR Synergizes with FoxO1 to potently induce the MuRF1 promoter. *A.* HepG2 cells were transfected with the pGL3-MuRF1-Promoter (-500) reporter construct, SV40-*Renilla* luciferase reporter construct, pSG5-GR, and/or pcDNA3-FoxO1, pcDNA3-FoxO3A, or pcDNA3-FoxO4. Cells were treated with or without 1 μ M DEX for 24 hours. Firefly Luciferase activities in cell extracts were normalized to *Renilla* luciferase activity to control for variations in transfection efficiency. Fold induction was obtained by dividing values from DEX-treated samples by the mean of values from matched untreated samples. Each point was done in triplicate, and errors reflect SD. *B.* HepG2 cells were transfected with pGL3-MuRF1-Promoter (-500), SV40-*Renilla*, and pBluescript as filler DNA (Control) and either GR (GR) or GR and FoxO1 (GR + FoxO1) expression vectors. 24 hours post-transfection, cells were treated with the indicated dexamethasone concentration and incubated overnight. Each point was done in triplicate. *C* and *D.* Mutation of either the GRE or the FBE is sufficient to abolish dexamethasone-induced MuRF1 promoter activity. HepG2 cells were transfected with either the wild-type MuRF1 promoter construct (-500), or MuRF1 promoter constructs that have either the GRE mutated (GRE-Mut) or the FBE mutated (FBE-Mut) as shown in *C* in combination with expression vectors for FoxO1 and/or GR as indicated. The cells were then treated and assayed for luciferase activity as in *A.*

Fig. 5. Dexamethasone induced MuRF1 expression is inhibited in mutant GR^{dim} mice versus in wild-type GR^{+/+} mice. *A.* GR^{+/+} and GR^{dim} mice were treated with DEX in their drinking water for eight days, weighed (top left), and sacrificed for determination of splenocyte number (top right) as well as gastrocnemius (bottom left) and tibialis anterior (bottom right) weights. White bars, vehicle treated; black bars, DEX treated. GR^{+/+}, n=8; GR^{dim}, n=7. *B.* Northern analysis of MuRF1, FOXO1, and FOXO3a expression in gastrocnemius muscle from DEX treated GR^{+/+} and GR^{dim} mice. Top panels, Control, 6 hour, and 24 hour treated mice; Bottom panels, Control, 3 day and 8 day treated mice. rpL32

expression is shown as a loading control below each set of Northern blots. Errors bars reflect SEM. C. Effect of wild-type GR and the homodimerization mutant GR on DEX induction of the MuRF1 promoter in a transient transfection assay. HepG2 cells were transfected with the pGL3-MuRF1-Promoter (-500) reporter construct, SV40-*Renilla* luciferase reporter construct (Control), pcDNA3.1-GRwt (GR) or pcDNA3.1-GR^{dim} (GR^{dim}) and/or pcDNA3-FoxO1 (FOXO1). Cells were treated with or without 1 μ M DEX for 24 hours. Firefly Luciferase activities in cell extracts were normalized to *Renilla* luciferase activity to control for variations in transfection efficiency. Fold induction was obtained by dividing values from DEX-treated samples by the mean of values from matched untreated samples. Each point was done in triplicate, and errors reflect SD.

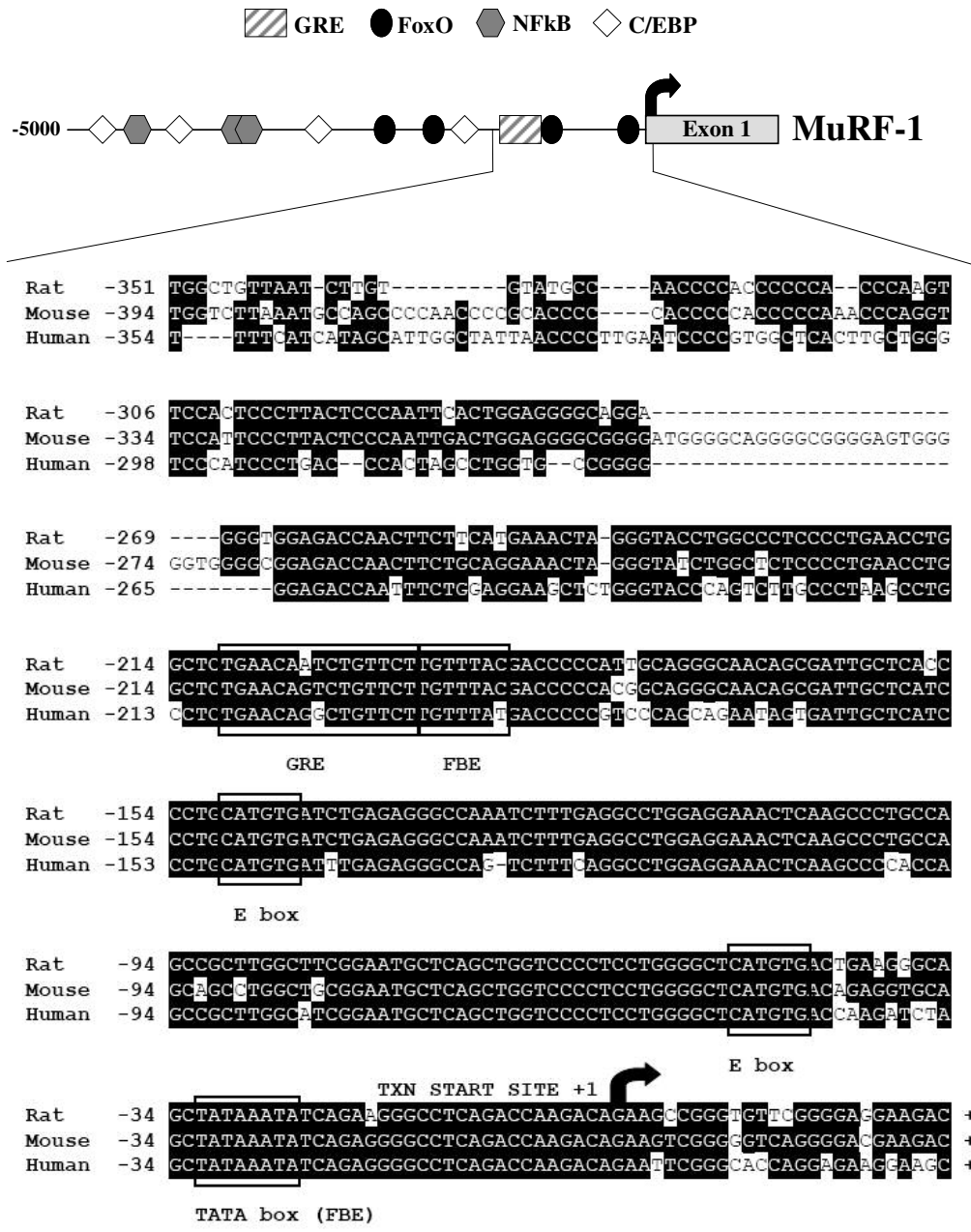
Fig. 6. Dexamethasone induces the MuRF1 promoter in differentiated C₂C₁₂ myotubes. A. C₂C₁₂ myoblasts were transfected with a reporter construct containing 500bp of the MuRF1 promoter fused to the Secreted Alkaline Phosphatase (SEAP) gene. The myoblasts were then differentiated by switching to low serum media, followed by treatment with 1 μ M Dex over a period of three days. The media was sampled every 24 hours to measure for SEAP activity. Conditions were done in triplicate and SEAP numbers were normalized with β -galactosidase to correct for variations in transfection efficiency. Each point was done in triplicate, and errors reflect SD. B. Mutation of either the GRE or the FBE is sufficient to abolish dexamethasone-induced MuRF1 promoter activity in C₂C₁₂ cells. C₂C₁₂ myoblasts were transfected with either the wild-type MuRF1 promoter construct (-500), or MuRF1 promoter constructs that have either the GRE mutated (GRE-Mut) or the FBE mutated (FBE-Mut) SEAP constructs, differentiated, and treated with 1 μ M Dex over a period of three days. The media was sampled every 24 hours to measure for SEAP activity as in A. Each time point was done in triplicate and normalized with β -galactosidase to correct for variations in transfection efficiency. C. Northern blot analysis of C₂C₁₂ cells differentiated for 48 hours and then treated with DEX (10 μ M), IGF-1 (20ng/ml), or DEX (10 μ M) +IGF-1 (20 ng/ml) for 24, 48, and 72 hours. Ligand was refreshed every 24 hours. D. IGF-1 potently inhibits DEX-induced activation of the MuRF1 promoter. C₂C₁₂ myotubes were treated for 24 hours with either

1 μ M DEX, 20ng/ml IGF-1, or 1 μ M DEX and 20ng/ml IGF-1 followed by sampling of the media for SEAP activity as described in A. Fold induction was obtained by dividing values from DEX-treated samples by the mean of values from matched untreated samples. Each point was done in triplicate, and errors reflect SD.

Fig. 7. GR and FoxO1 associate directly with the MuRF1 promoter in C₂C₁₂ myotubes. *A.* C₂C₁₂ myotubes treated with 1 μ M DEX for the indicated times (one 10 cm plate per time point), and cross-linked chromatin was immunoprecipitated with normal rabbit IgG (dashed line, circles), anti-FOXO1 antibody (solid line, triangles) and an anti-GR antibody (solid line, squares). Quantitative PCR was used to determine the amount of MuRF1 promoter immunoprecipitated using primers flanking the predicted GRE and FOXO sites in the mouse MuRF1 promoter. Values were normalized as a percentage of input DNA used at each time point. *B* and *C.* C₂C₁₂ myotubes were treated with vehicle (white bar), 1 μ M DEX, 20ng/ml R3-IGF-1, or 1 μ M DEX + 20ng/ml R3-IGF-1 for 15 minutes (black bars) or 30 minutes (gray bars) before harvest. Four plates for each treatment were processed in parallel, as in *A*; values (fold enrichment) are expressed as the mean MuRF1 promoter copies immunoprecipitated with either the anti-GR antibody (*B*) or anti-FOXO1 antibody (*C*) divided by that immunoprecipitated by a non-specific antibody (anti-HA). Error bars reflect SEM.

Figure 1

A.



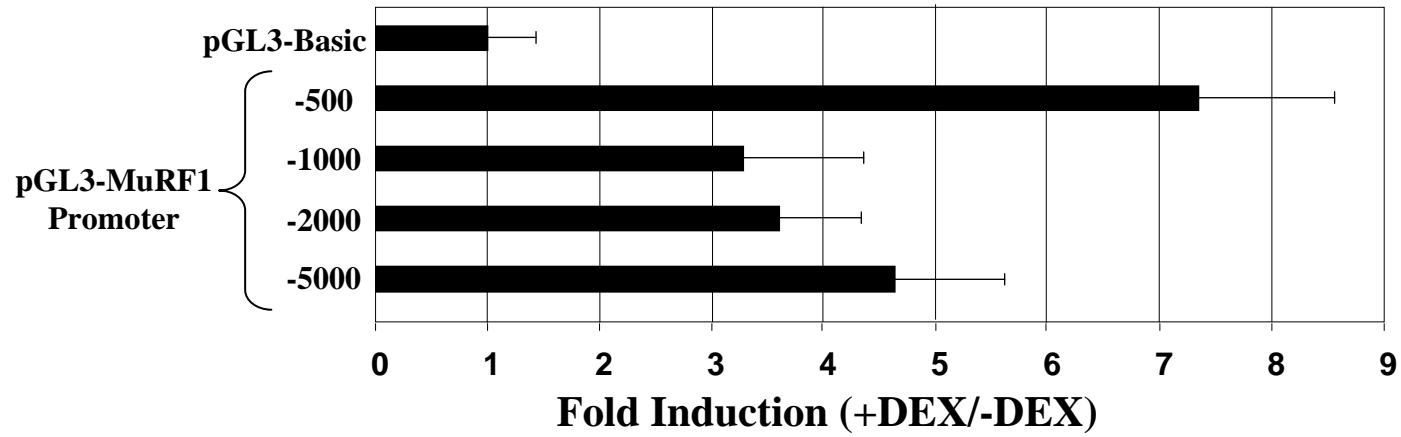
B.

Consensus GRE → ←
 Palindromic GRE
 mMURF1 GRE
 rMURF1 GRE
 hMURF1 GRE

GGTACAnnnTGTTCT
 AGAACAnnnTGTTCT
 TGAACAgtcTGTTCT
 TGAACAatcTGTTCT
 TGAACAaggcTGTTCT

Figure 2

A.



B.

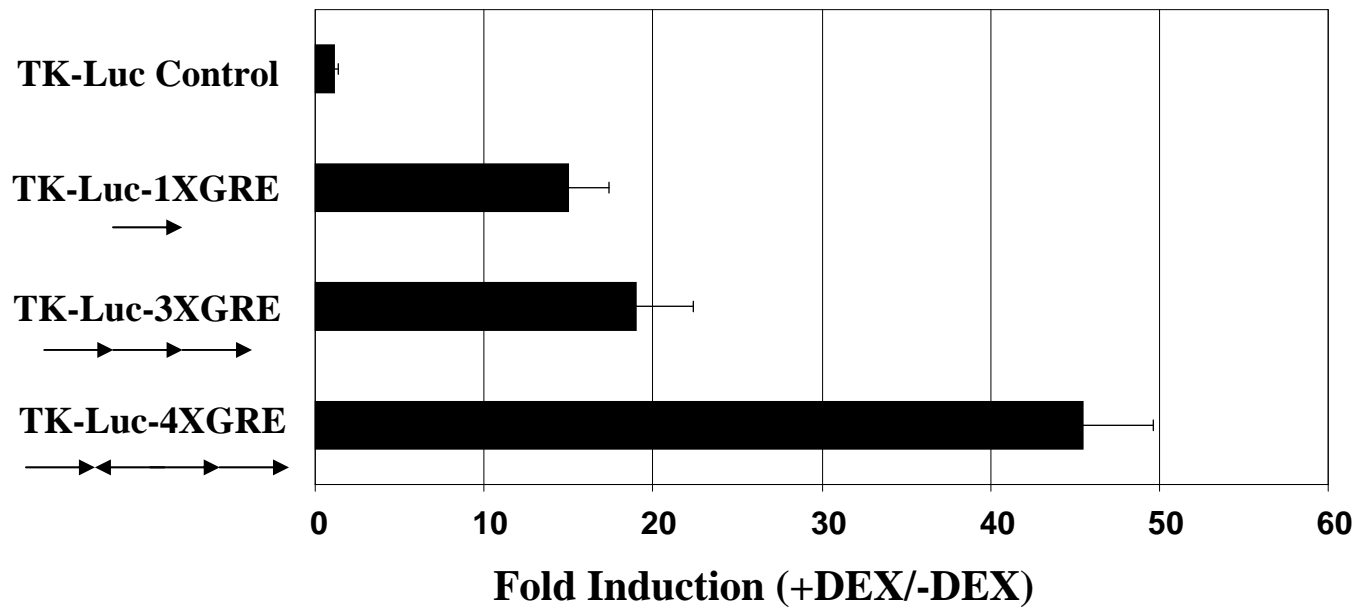
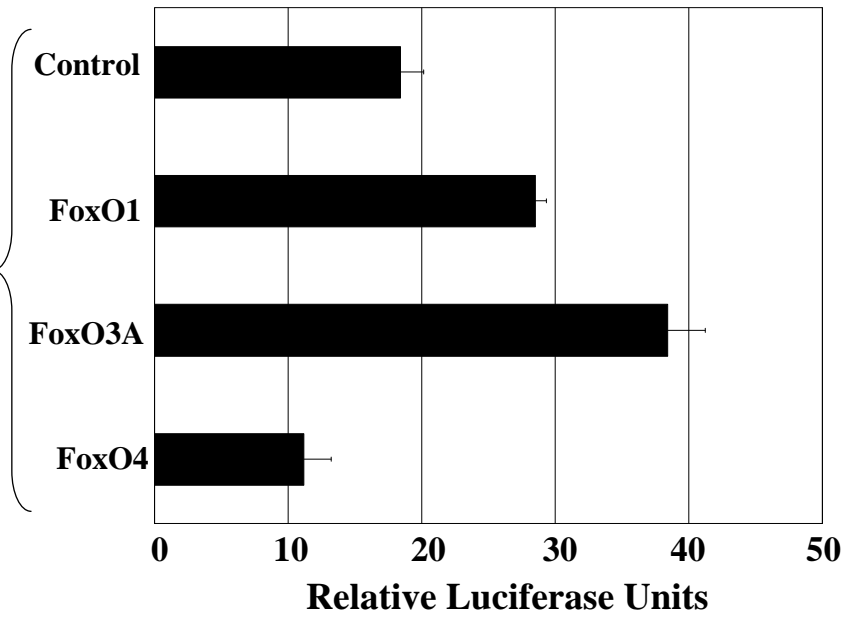


Figure 3

A.

pGL3-MuRF1
-500 Promoter



B.

TK-Luc-1X FBE

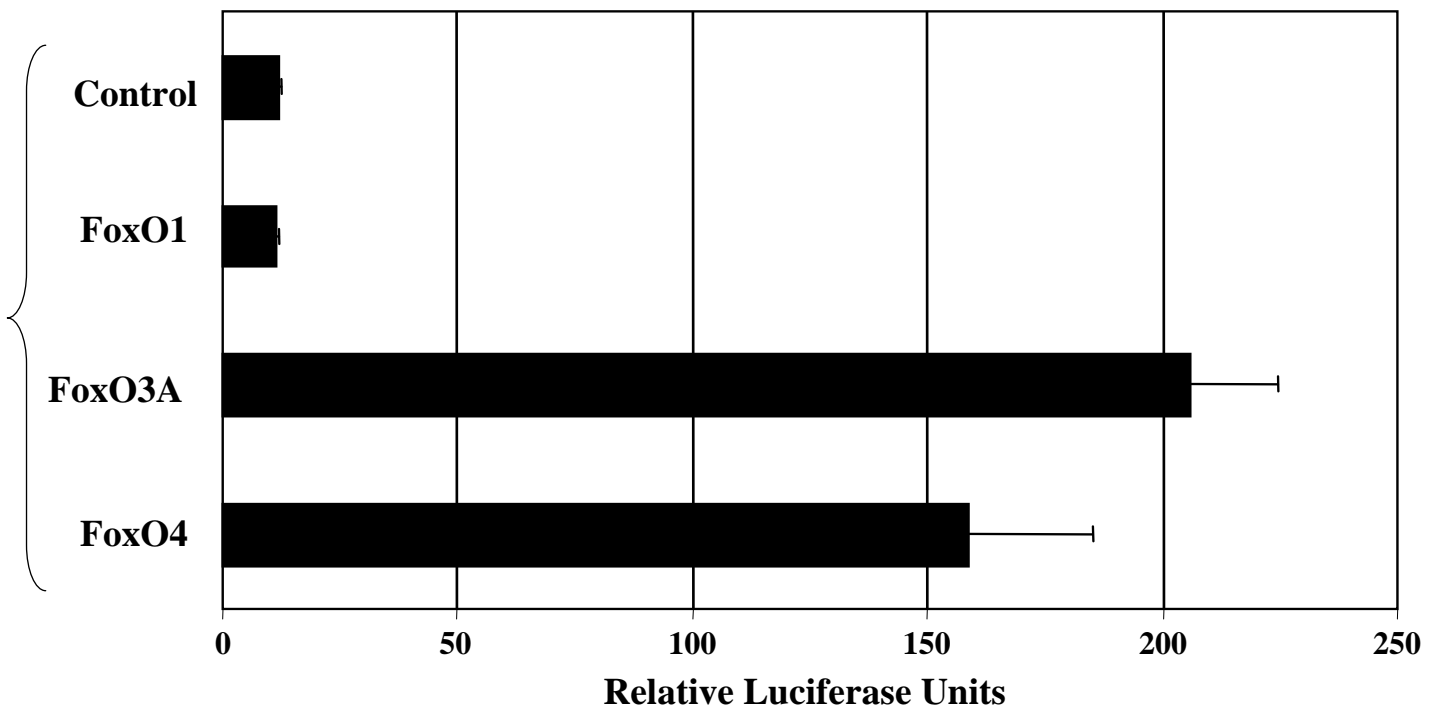


Figure 3

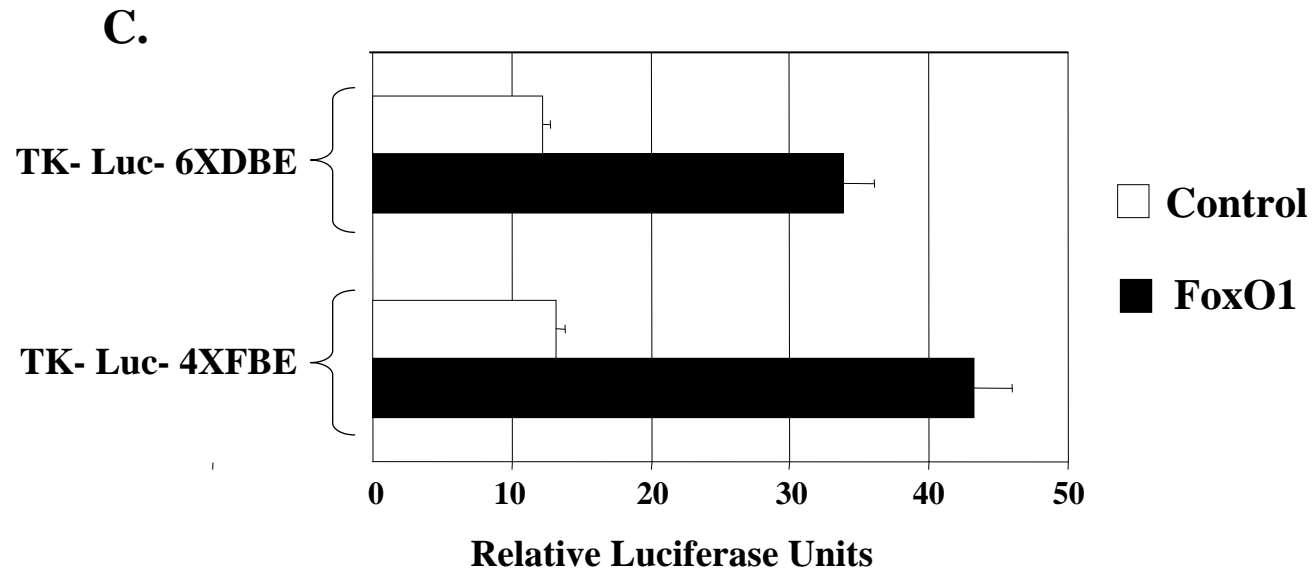


Figure 4

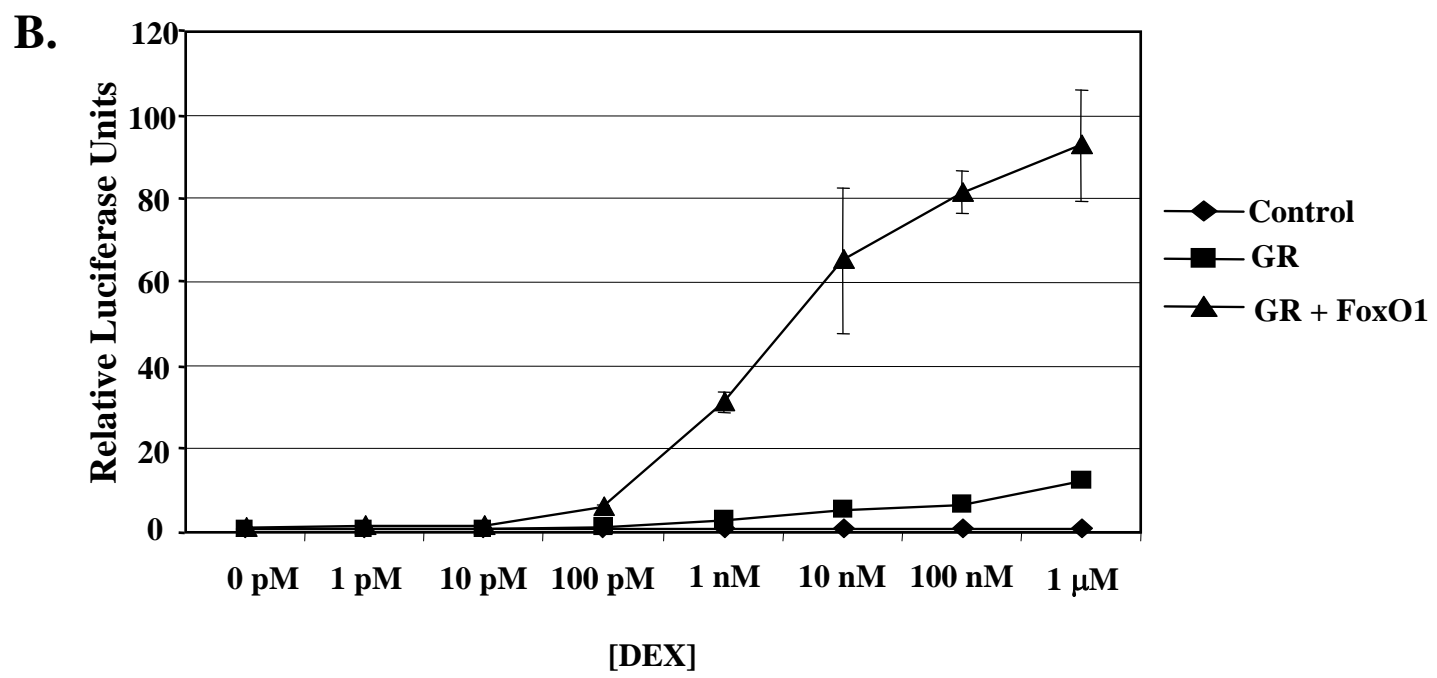
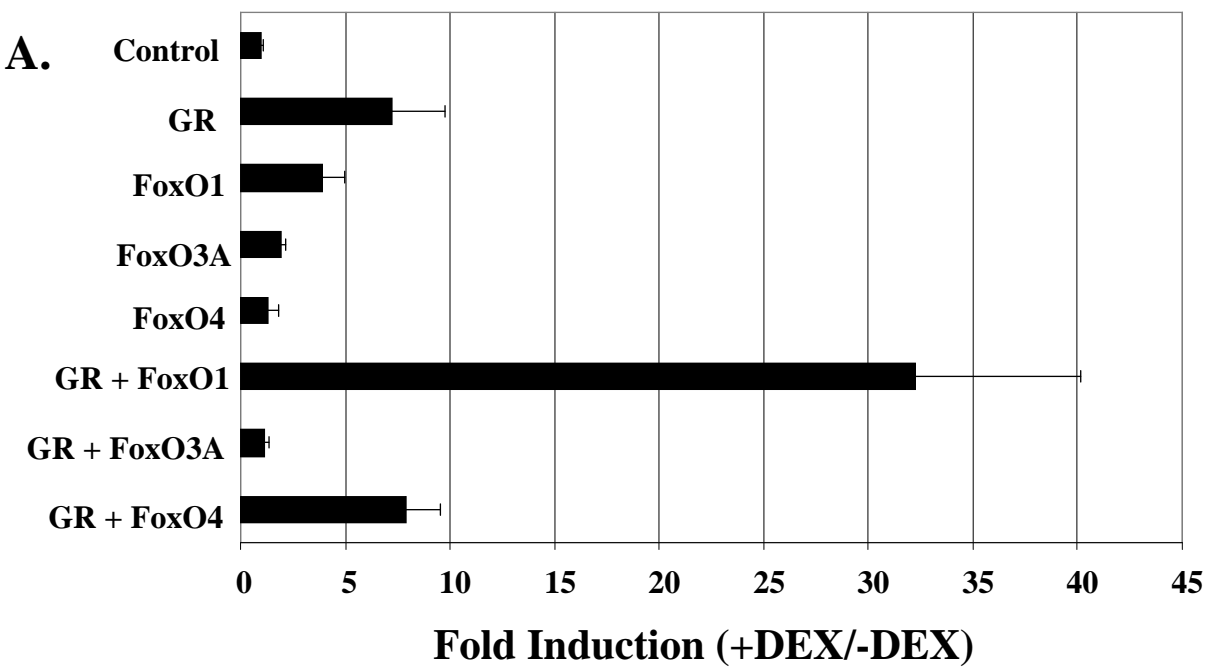


Figure 4



D.

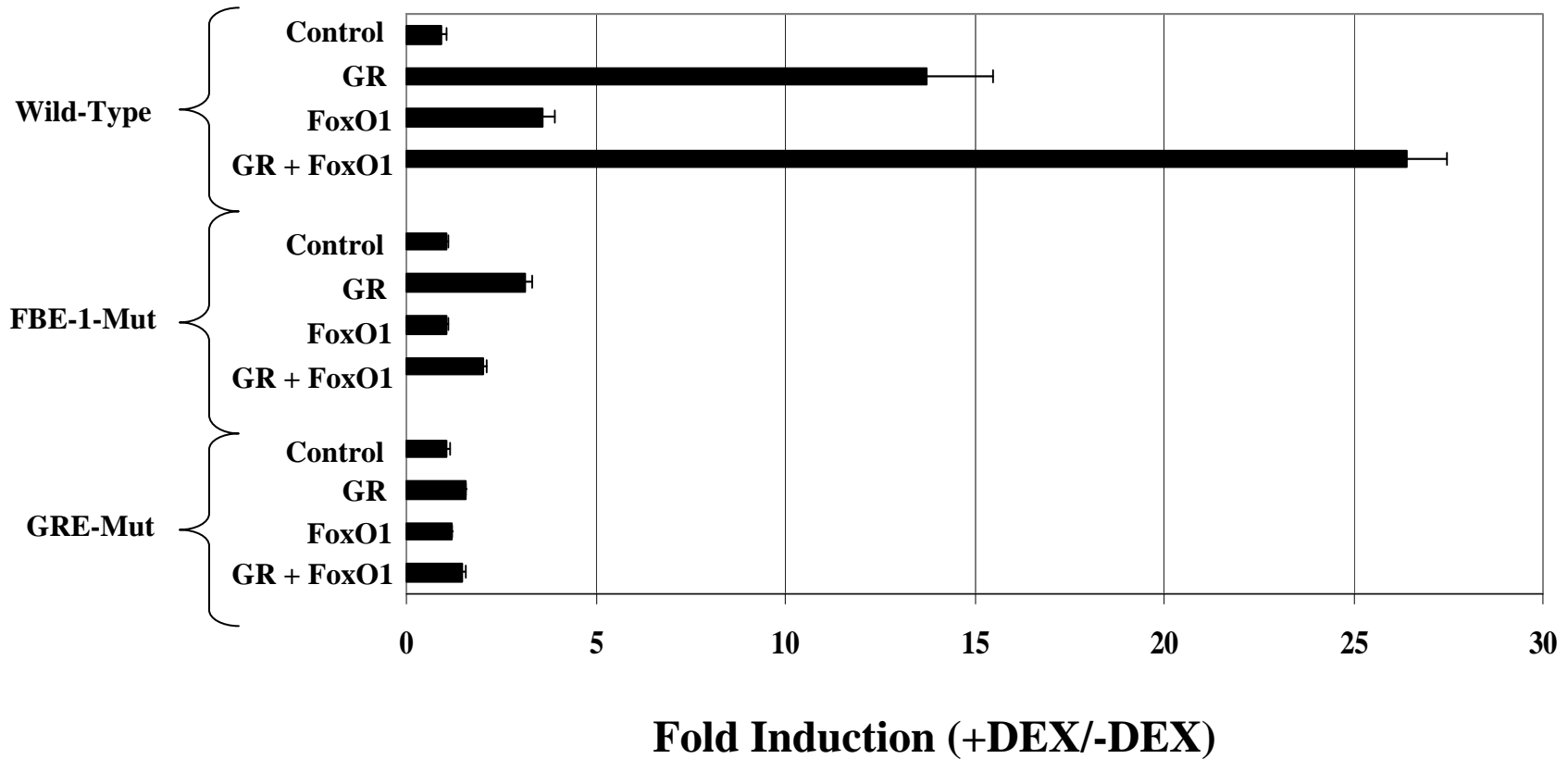


Figure 5A.

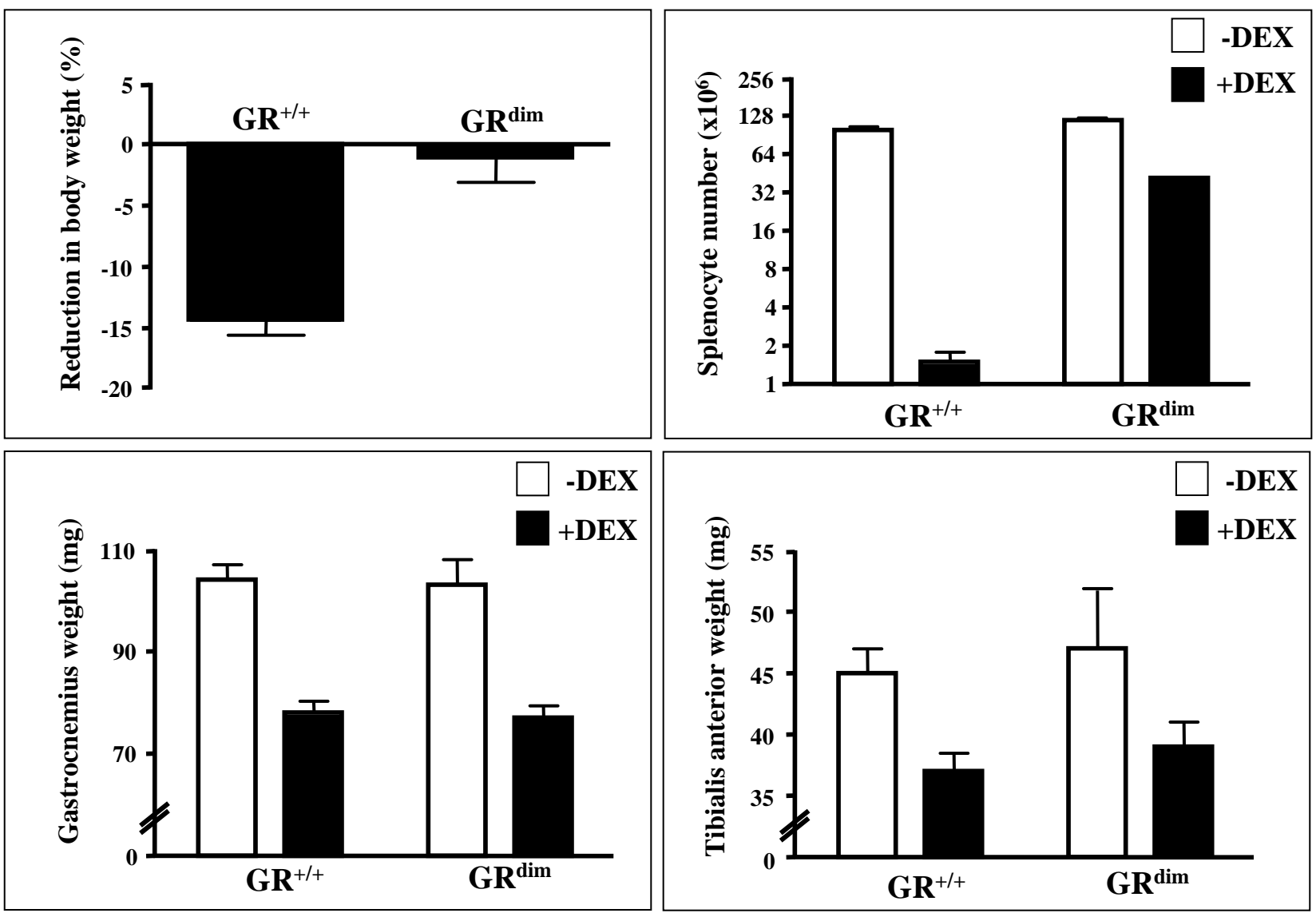


Figure 5B.

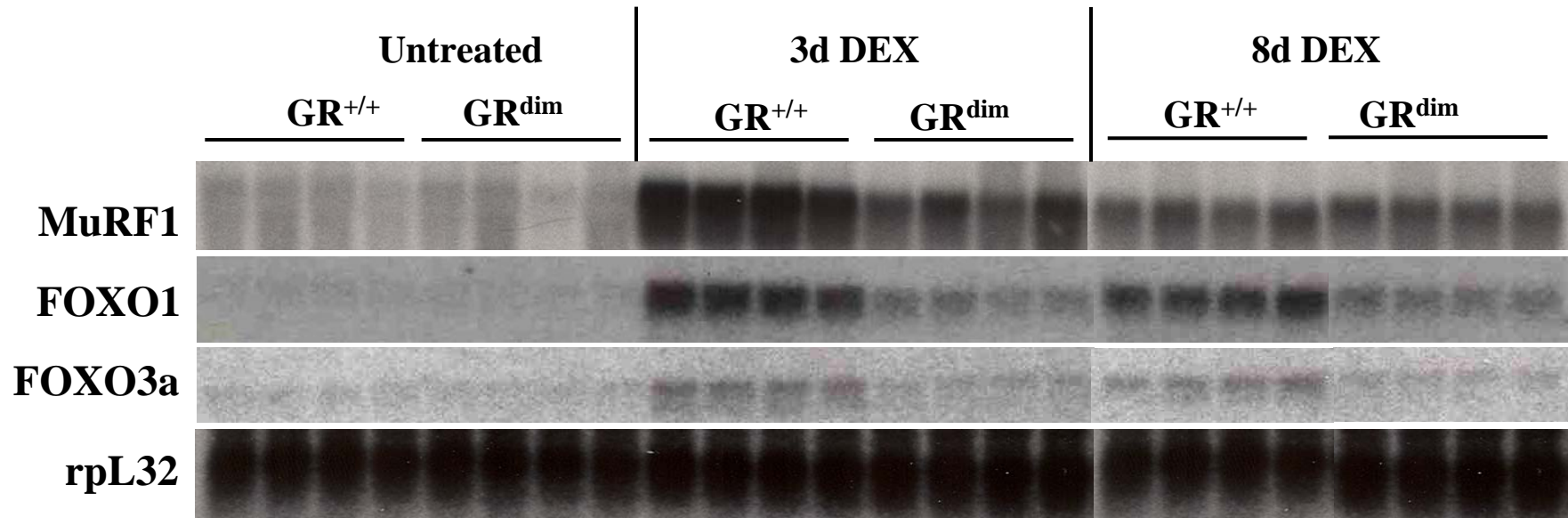
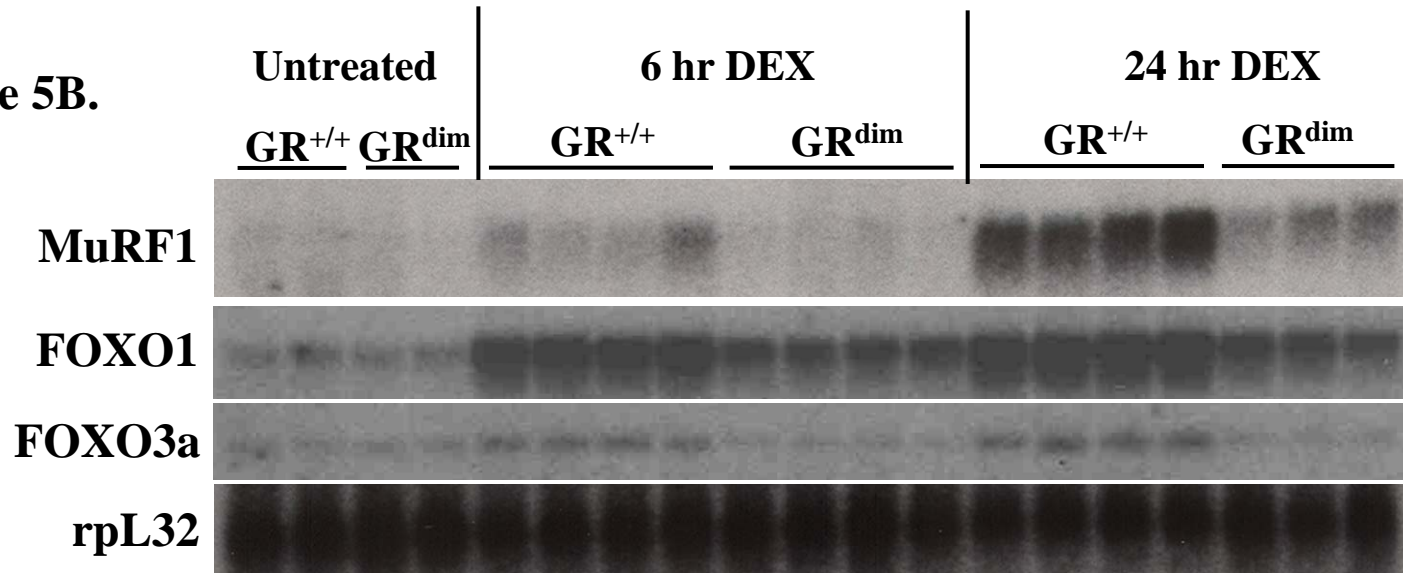


Figure 5C.

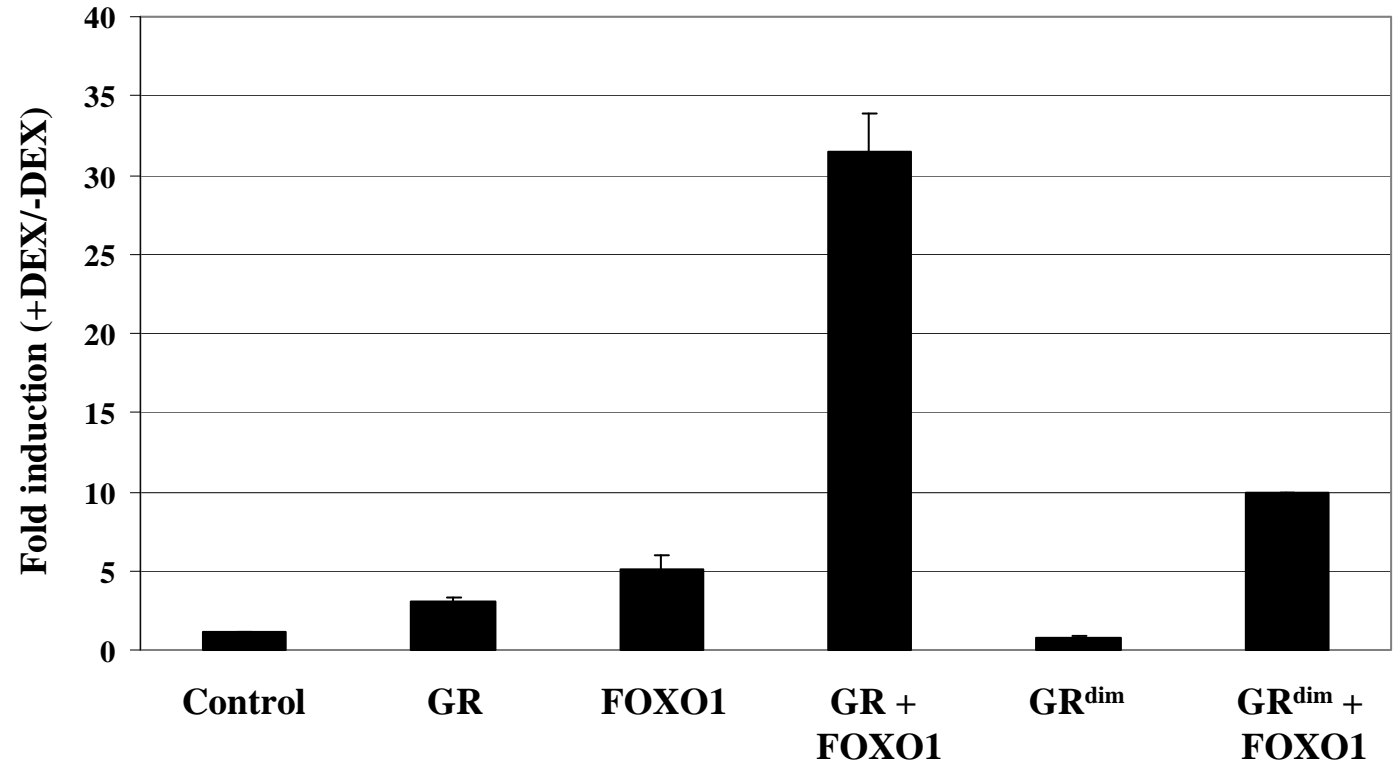


Figure 6

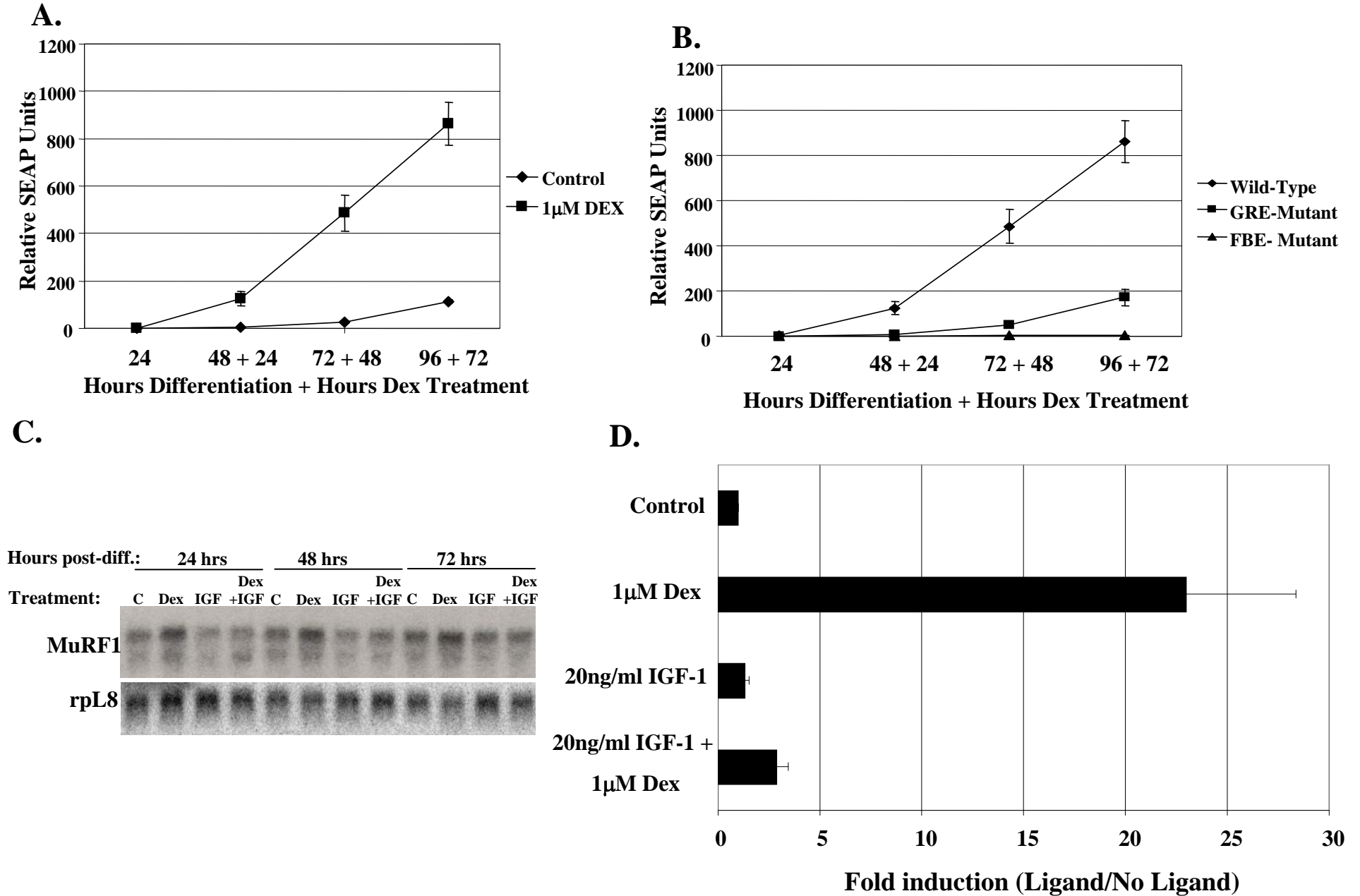


Figure 7

