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Target Specificities of Estrogen Receptor-Related Receptors: Analysis of Binding Sequences and Identification of Rb1-Inducible Coiled-Coil 1 (*Rb1cc1*) as a Target Gene

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Estrogen receptor-related receptors (ERRs) are orphan members of the nuclear receptor superfamily. A single AGGTCA sequence element preceded by three conserved nucleotides has been identified as a specific recognition motif of ERRs. Here we performed systematic analyses of target sequences on all three ERR subtypes, α, β and γ. In electrophoretic gel-mobility shift assay and transcriptional reporter assays, they exhibited similar patterns of recognition specificities, showing extremely broad ranges of target sequences. We searched a mouse promoter database for a gene carrying possible ERR-binding sequences. The Rb-1 inducible coiled-coil 1 (*Rb1cc1*) gene was found to contain two putative ERR binding elements, named response element (RE)-1 and RE-2, in the promoter region. In gene reporter assays, RE-2, but not RE-1, functioned as an effective *cis*-regulatory element for transactivation by ERRα in the presence of a coactivator, peroxisome proliferator-activated receptor γ coactivator-1α. Mutational analyses suggested that RE-2 is recognized by ERRα partly as a monovalent element, but also as a direct repeat motif separated by four spacer nucleotides. *In vivo* binding of ERRα to the *Rb1cc1* promoter region was confirmed by the chromatin immunoprecipitation assay. Thus, *Rb1cc1* is a target gene of ERRα, driven by a novel type of recognition sequence.

Key words: estrogen receptor-related receptor, nuclear hormone receptor, Rb1-inducible coiled-coil 1, response element, target gene, transcriptional regulation.

Abbreviations: ChIP, chromatin immunoprecipitation; DR, direct repeat; EMSA, electrophoretic-gel mobility-shift assay; ER, estrogen receptor; ERR, estrogen receptor-related receptor; IR, inverted repeat; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1 α; Rb1cc1, Rb1-inducible coiled-coil 1; RE, response element.

Nuclear hormone receptors are a large group of transcription factors that are involved in various physiological processes, including metabolism, development and tissue differentiation. Orphan nuclear receptors are members of the nuclear hormone receptor superfamily for which a ligand is not required or has not been identified (*1*, *2*). Estrogen receptor-related receptors (ERRs), a subfamily of orphan nuclear receptors, are close relatives of estrogen receptor (ER) (*3*). ERRs consist of three members, ERRα, β and γ. ERRβ and γ are more closely related to each other than to ERRα (*4*).

Nuclear hormone receptors work by binding to specific DNA elements near the target genes. ERRs bind to an extended monovalent AGGTCA half-site (*5–8*), the estrogen response element, an ER-binding site consisting of an inverted repeat (IR) of AGGTCA half-sites spaced by three nucleotides (IR-3) (*4*, *7*, *9*) and direct repeat (DR) sequences (*10*, *11*). It was found in our laboratory (*12*) that ERRγ binds as a homodimer to a wide spectrum of nucleotide sequences, including a monovalent half-site, IRs and DRs. However, the specificities of ERRα and β were not studied. Possible differences in the binding specificities would be important, when we consider the functions of each ERR subtype. Extensive analyses of the binding sequences for all ERR subtypes, which was one of the aims of the present study, have revealed similar broad specificities, as described in a later section. Coactivation by peroxisome proliferator-activated receptor 1α (PGC-1α), when bound to different sequence elements, is also a subject of this study.

It was previously reported that ERRα is enriched in tissues that rely on mitochondrial oxidative metabolism, such as the heart, brown adipose tissue and slow-twitch skeletal muscle, suggesting its involvement in energy metabolism (*13–15*). Consistent with this, ERRα activates genes related to energy metabolism, including genes of oxidative phosphorylation components (*16–18*), medium chain acyl-CoA dehydrogenase (*5*), pyruvate dehydrogenase kinase 4 (*19*, *20*) and apolipoprotein A-IV (*21*). ERRα has also been shown to be expressed in tissues such as female and male reproductive organs, mature oocytes in the ovary and spermatocytes in the testis (*22*, *23*), where estrogen is also known to play an important role.

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ERRα acts as a regulator of the human aromatase gene in the breast (*24*) and a modulator of estrogen activity on the lactoferrin promoter (*25*, *26*). ERRα seems also to be involved in breast cancer development, pS2, a prognostic marker of human breast cancer, being one of its target genes (*27*). Mouse ERRα is highly expressed in certain adult organs and particularly enriched in the ossification zones of the mouse embryo, suggesting a role in bone remodelling through the regulation of a target gene, osteopontin (*28*, *29*). Moreover, ERRα is expressed during embryonic development and involved in numerous physiological and developmental functions in muscle, as well as the central and peripheral nervous systems (*30*). ERRα expression is increased during the transition to myotubes in C2C12, a myoblast cell line (*30*), implying a role in muscle differentiation.

Based on the broad binding specificity, we sought a candidate target gene of ERRα that carries sequence elements for ERRα binding. Upon searching a mouse gene promoter database, we found that the Rb1-inducible coiled-coil 1 (*Rb1cc1*) gene contains putative ERRα response element (RE) in the upstream region. *Rb1cc1* is abundantly expressed in the mouse embryo throughout development from an early stage (*31*), suggesting a role in the development as well as the maintenance of differentiated tissues. *Rb1cc1* is also expressed at high levels in human embryonic musculoskeletal and cultured osteosarcoma cells (*32*). Moreover, RNAi-mediated knockdown of *Rb1cc1* in C2C12 cells causes a failure of differentiation into myotubes, implying its crucial role in myogenesis (*33*). Based on these observations, the *Rb1cc1* gene seemed to be an attractive candidate for a target gene of ERRα, and in this report, we show that this is indeed the case.

EXPERIMENTAL PROCEDURES

Plasmids—A full-length mouse ERRα cDNA clone (IMAGE 4234647) inserted in the pCMV-SPORT6 vector was obtained from Open Biosystems. The ERRα cDNA was amplified by PCR, using a forward primer AGCagatctA TGTCCAGCCAGGTGGTGG and a reverse primer TATga attcTCAGTCCATCATGGCCTCAAG (small letters representing artificial restriction sites) and inserted into the expression plasmid, pCMX, at the *Eco*RV site, yielding pCMX-ERRα. For the construction of an expression plasmid of HA-tagged ERRα, pHA-ERRα, ERRα cDNA without the start codon was amplified by PCR, using pCMX-ERRα as a template and oligonucleotides AGCagatc tTCCAGCCAGGTGGTGG and TATctcgagTCAGTCCATC ATGGCCTCAAG as primers. The PCR product was digested with *Bgl*II and *Xho*I, and the resulting cDNA fragment was inserted into an expression plasmid, pHA-C1 (*12*), between the *Bgl*II and *Sal*I sites.

For the construction of a VP16-fused ERRα expression plasmid, pCMX-VP16-ERRα, pHA-ERRα was cleaved with *Eco*RI, blunt-ended with Klenow fragment and then digested with *Bam*HI. The resulting cDNA fragment was inserted into a VP16-fusion vector, pCMX-VP16-N, between the *Bam*HI and blunt-ended *Nhe*I sites.

An expression plasmid of myc-tagged ERRα was generated by inserting the *Bam*HI/*Bgl*II fragment of pHA-ERRα into the *Bgl*II site of pCMV5-myc (*34*). For the construction of a recombinant lentivirus expressing myc-ERRα, the myc-ERRα expression plasmid was digested with *Asp*718I and *Nco*I, and blunt-ended with Klenow fragment. The resulting fragment was subcloned into the blunt-ended *Bam*H1 site of CSII-EF-MCS-IRES2-Venus plasmid (a gift from H. Miyoshi), yielding CSII-EF-mycERRα-IRES2-Venus. Other procedures for the viral vector construction were as described previously (*35*).

A full-length mouse ERRβ cDNA clone (RIKEN FANTOM clone D330014B12) inserted in the pFLC1 vector was purchased from Dnaform. The ERRβ cDNA containing the protein-coding region was amplified by PCR using the forward primer ATAggtacCCACCATGTC GTCCGAAGACAGGCAC and the reverse primer TATgct agcTCACACCTTGGCCTCCAGCAT, and inserted into the *Eco*RV site of pCMX, yielding pCMX-ERRβ. The plasmid was digested with *Spe*I and *Bam*HI, and the cDNA fragment generated was inserted between the *Xba*I and *Bam*HI sites of pCMX-VP16. The construction of the ERRγ expression plasmid was as described previously (*12*). In all the fusion constructs the tag moieties and ERR cDNAs were connected in-frame, which was confirmed by nucleotide sequencing. PGC-1α cDNA inserted in the pSVSPORT vector was a gift from B. Spiegelman. To unify the vector backbone, the cDNA was transferred into pCMX in our laboratory. pCMX and pCMX-VP16-N were gifts from R. Evans.

The vector *tk*-Luc (*36*), containing the Herpes simplex thymidine kinase (*tk*) basal promoter upstream of the firefly luciferase gene, was used for the construction of reporter plasmids containing various sequence elements. Double-stranded oligonucleotides listed in Table 1 were inserted into the *Sal*I and *Bam*HI sites of *tk*-Luc.

Luciferase reporter plasmids containing the upstream region of the *Rb1cc1* gene were as described previously (*32*). The sequences of oligonucleotides encompassing RE-1 and RE-2, the putative ERR-binding sites in the promoter region of the mouse *Rb1cc1* gene (see RESULTS), and their mutant sequences are shown in Table 1. These oligonucleotides were used for the construction of reporter plasmids and as the competitors in EMSA. Site-directed mutagenesis of RE-1 and RE-2 in the *Rb1cc1* promoter was performed by the PCR-based overlap extension method as described previously (*37*), using primers containing mutations in the RE-1 and RE-2 sequences, respectively. For replacement of RE-1 by the consensus IR-3 in the *Rb1cc1* upstream region, forward primer 5′-GCCAGAAGGTCACACTGACCTTGGGGCCGT-3′ and reverse primer 5′-ACGGCCCCAAGGTCAGTGTGACCTT CTGGC-3′ were used.

Cell Culture and DNA Transfection—C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10% fetal bovine serum at 37°C under 5% CO_2. The cells were induced to differentiate with DMEM containing 2% horse serum (*20*). HeLa cells were cultured in F-12 medium containing 10% fetal bovine serum under 5% CO_2 at 37°C. For DNA transfection studies, HeLa cells were used throughout. Transfection was carried out by a calcium phosphate method (*38*). For luciferase assay, 2×10^4 cells were seeded in each well of 24-well plates and cultured

Table 1. **Oligonucleotides used for this study.**

Oligonucleotide	Sequence
M1 top	GATC**TCAAGGTCA**GACGTGCTGCACACTCGA
bottom	TCGAGTGTGCAGCACGTC**TGACCTTGA**
M2 top	GATC**CAGAGGTCA**GACGTGCTGCACACTCGA
bottom	TCGAGTGTGCAGCACGTC**TGACCTCTG**
DR-0 top	GATCCAG**AGGTCAAGGTCA**CTGCACAC
bottom	TCGAGTGTGCAG**TGACCTTGACCT**CTG
DR-1 top	GATCCAG**AGGTCAcAGGTCA**CTGCACA
bottom	TCGATGTGCAG**TGACCTgTGACCT**CTG
DR-2 top	GATCCAG**AGGTCAcaAGGTCA**CTGCAC
bottom	TCGAGTGCAG**TGACCTtgTGACCT**CTG
DR-3 top	GATCCAG**AGGTCAcacAGGTCA**CTGCA
bottom	TCGATGCAG**TGACCTgtgTGACCT**CTG
DR-4 top	GATCCAG**AGGTCAcacaAGGTCA**CTGC
bottom	TCGAGCAG**TGACCTtgtgTGACCT**CTG
DR-5 top	GATCCAG**AGGTCAcacacAGGTCA**CTG
bottom	TCGACAG**TGACCTgtgtgTGACCT**CTG
IR-0 top	GATCCAG**AGGTCATGACCT**CTGCACAC
bottom	TCGAGTGTGCAG**AGGTCATGACCT**CTG
IR-1 top	GATCCAG**AGGTCAcTGACCT**CTGCACA
bottom	TCGATGTGCAG**AGGTCAgTGACCT**CTG
IR-2 top	GATCCAG**AGGTCAcaTGACCT**CTGCAC
bottom	TCGAGTGCAG**AGGTCAtgTGACCT**CTG
IR-3 top	GATCCAG**AGGTCAcacTGACCT**CTGCA
bottom	TCGATGCAG**AGGTCAgtgTGACCT**CTG
IR-4 top	GATCCAG**AGGTCAcacaTGACCT**CTGC
bottom	TCGAGCAG**AGGTCAtgtgTGACCT**CTG
IR-5 top	GATCCAG**AGGTCAcacacTGACCT**CTG
bottom	TCGACAG**AGGTCAgtgtgTGACCT**CTG
Wt RE-1 top	GCCAGA**AGGTCAcgcTCACCT**TGG
Bottom	GATCCCA**AGGTGAgcgTGACCT**TCT
Mut RE-1 top	GATCAGA**CCATCAcgcTCACCT**TGG
Bottom	GATCCCA**AGGTGAgcgTGATGG**TCT
Wt RE-2 top	GATCCCA**GGGTCAgggaAGGACA**GACA
Bottom	GATCTGTC**TGTCCTtcccTGACCC**TGG
Mut RE-2 (mut1) top	GATCCCA**CCCTCAgggaAGGACA**GACA
Bottom	GATCTGTC**TGTCCTtcccTGAGGG**TGG
Mut RE-2 (mut2) top	GATCCCA**GGGTCAgggaTCCACA**GACA
bottom	GATCTGTC**TGTGGAtcccTGACCC**TGG
Mut RE-2 (mut1/2) top	GATCCCA**CCCTCAgggaTCCACA**GACA
bottom	GATCTGTC**TGTGGAtcccTGAGGG**TGG

Sequences are written in 5′ to 3′ directions. Nucleotides of the elements are indicated with bold letters, spacer nucleotides with small letters, and mutated bases with underlines.

overnight. The next day, the cells in each well were incubated with DNA/calcium phosphate precipitates containing plasmid mixtures of 0.8 μg of reporter plasmids, 0.1 μg of one of the expression plasmids of ERRs, 0.4 μg of an expression plasmid of PGC-1α when necessary and 0.2 μg of pCMVβ as an internal control. The total amount of DNA was adjusted to 1.5 μg with an empty vector, pCMX. After 4 h, the precipitates were removed, and cells were cultured for a further 24 h.

Luciferase Assay—Cells were lysed with a cell lysis buffer (*39*), and luciferase activities measured using a PicaGene reagent kit (Toyo Ink) in a Lumat LB9501 luminometer (Berthold) or a Lucy2 microplate luminometer (Anthos). β-Galactosidase activity derived from the pCMVβ plasmid was measured as described previously (*38*), and luciferase activity was normalized for transfection efficiency based on the β-galactosidase activity. Assays were carried out in triplicate, and the averages are shown, together with standard deviations.

In Vitro Transcription/Translation and Electrophoretic-Gel Mobility Shift Assay (EMSA)—cDNAs of ERR subtypes were subcloned in the pEU vector (*12*). The resulting plasmids were used as the templates for *in vitro* transcription/translation employing a TNT T7quick-coupled transcription/translation system (Promega), according to the manufacturer's protocol. EMSA was performed as described previously (*38*), using the *in vitro*-synthesized ERR proteins.

Preparation and Infection of Recombinant Lentivirus—293FT cells were cultured according to the protocol of the supplier (Promega). Lentivirus encoding myc-ERRα was produced by transfection of 293FT cells in 60-mm dishes with a plasmid mixture of 7 μg of CSII-EF-mycERRα-IRES2-Venus, 5 μg of pCAG-HIVgp and 4 μg of pCMV-VSV-G-RSV-Rev. These helper vectors for lentivirus production (*40*) were kindly provided by H. Miyoshi and A. Miyawaki. The supernatant containing the recombinant lentivirus was collected 48 h after transfection, passed through a 0.45-μm filter, and used for infection. C2C12 cells were infected with the recombinant virus for 6 h, washed once with PBS, and used for experiments. Infection efficiencies were checked using an IX-70 fluorescence microscope by monitoring the fluorescence of Venus, a GFP derivative, whose gene was inserted into the lentiviral vector.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed as described previously (*41*) for chromatin from differentiated C2C12 cells stably expressing myc-tagged ERRα. Binding of myc-ERRα was assessed employing anti-myc monoclonal antibody. Quantitative PCR was performed using the following primer pairs: proximal, 5′-ATCTGTGCTGGTGCTCACT CG-3′ (corresponding to positions −292 to −272 relative to the cap site of the *Rb1cc1* gene) and 5′-TCTGCGCAG ATCCGGAAGTCG-3′ (positions −59 to −79); and distal, 5′-TGTATCAGCAGTTAAGAGCACTCACTTC-3′ (positions 3812 to 3839) and 5′-ATAGGGTGTTTCCAGAAAG GGGAGGAAC (positions 4049 to 4022). The region amplified with the proximal primers encompassed both RE-1 and RE-2, whereas that with the distal primers was within intron 1 of the *Rb1cc1* gene.

RESULTS

Broad Recognition Sequence Specificities of ERR Subtypes—We previously found that ERRγ has a broad range of binding sequence specificity (*12*). ERRα and ERRβ were also reported to recognize several sequence elements with different arrangements of the half-sites (*5–8*, *11*, *23*). Accordingly, we extended the systematic analysis of recognition sequence specificities to all ERR subtypes. For this purpose, different oligonucleotides were designed to contain the AGGTCA motif as a monovalent element as well as DR and IR elements with different spacings (Table 1). These oligonucleotides

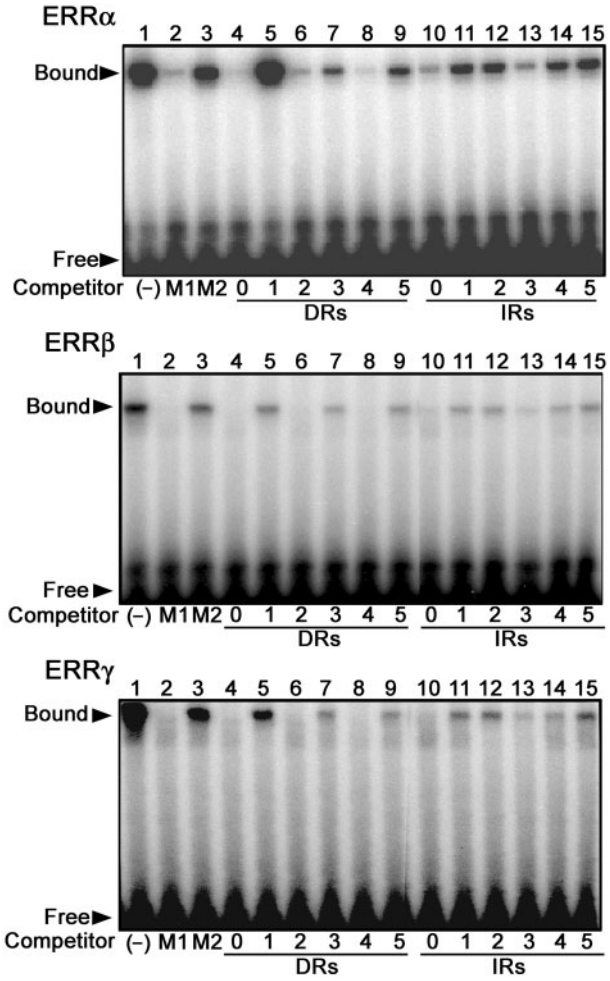

Fig. 1. **Broad binding specificities of ERR subtypes.** Competition efficiencies of different oligonucleotides were examined by EMSA with the binding of ERRα, β or γ to an IR3 probe. The sequences of competitor oligonucleotides are listed in Table 1. The ^{32}P-labelled IR3 oligonucleotide was used as a probe. ERR proteins were synthesized in an *in vitro* reticulocyte lysate system. The probe was incubated with ERR proteins, and a 100-fold molar excess of competitors was added to the binding reaction (lanes 2–15). The positions of protein-bound and free forms of the probe are indicated by arrowheads.

were used as competitors against an IR3 probe in EMSA to examine the binding sequence specificities of ERRs (Fig. 1). M1, a monovalent half-site carrying a TCA trinucleotide extension just upstream, was a highly effective competitor for ERRs (lane 2), supporting the importance of this sequence in the regulation of many genes by ERRs. M2, in which the preceding three nucleotides were mutated, competed only weakly (lane 3). DR0, DR2, DR4, IR0 and IR3 strongly competed for all ERR subtypes (lanes 4, 6, 8 10 and 13), whereas DR3 and DR5 competed less efficiently (lanes 7 and 9), and IR1, IR2, IR4 and IR5 were weak competitors (lanes 11, 12, 14 and 15), except for the case of IR4 for ERRγ. DR1 was a poor competitor, especially for ERRα (lane 5). Thus, all ERR subtypes showed largely similar patterns of broad binding sequence specificities, though the relative affinities varied depending on the sequences.

We next tested the abilities of these nucleotide sequences to support transactivation by ERRs. To this end, these oligonucleotides were cloned in front of the *tk* promoter linked to a luciferase reporter gene, and the resulting reporter plasmids were transfected into HeLa cells together with expression plasmids of ERRs. The constitutive activities of ERRs alone were too low to compare accurately the relative transcriptional activities among the different constructs (data not shown). Accordingly, ERRs were fused to the activation domain of VP16, a virus-derived transactivating protein. These fusion proteins were expected to activate gene transcription independent of natural transactivating functions, directly reflecting the binding efficiencies to each element within the cells. We performed gene reporter assays for the VP16-fusions with reporters containing the same sequence elements that were used in the binding experiments. Significant luciferase expression was observed with many constructs to different extents (Fig. 2). Compared to other elements, IR3 exhibited the highest transcriptional activation with all VP16-fused versions of ERR subtypes. We also found that M1, DR0, DR3 and DR4 caused efficient transactivation. Transactivation was not efficient with M2, DR1, DR5, IR1 or IR2, and that with IR0 was intermediate. DR2 was effective for ERRα, but not ERRβ and ERRγ. These results were mostly consistent with the binding results, though exceptions were also observed. That is, most notably, significant transcriptional activation was observed with IR4 and IR5 for ERRα and ERRγ, but not for ERRβ.

It was previously reported that ERRs exhibited significant transactivation when the coactivator PGC-1α was coexpressed (*14*). This result led to a notion that the transactivating functions of ERRs are regulated by cofactor availability, instead of low molecular weight ligands (*15*, *42*). Accordingly, we examined transactivation by ERRs in the presence of PGC-1α, using the same luciferase reporter plasmids as used earlier. We found significant transactivation by ERRs and PGC-1α through many target sites (Fig. 3). The results were similar to those with the VP16-fused ERRs, though IRs exhibited relatively higher activities as compared with the activities with VP16 fusions. It was also notable that even DR1, which was almost ineffective for transactivation by VP16 fusions, conferred significant transactivation by ERRs assisted by PGC-1α. Thus, when the binding sequences were less favourable, PGC-1α apparently coactivated ERRs more effectively. Taken together, these findings indicate that ERRs cause transactivation through a broad range of sequence elements, but the extent of transactivation is variable depending on the sequence, which is consistent with the results of binding assays.

ERRα Activates Transcription from the Rb1cc1 Gene Promoter—To identify a novel target gene of ERRα, we performed a computational search of preferable ERRα-binding sites in a database containing mouse promoter sequences, DBTSS, (http://dbtss.bioinf.med.uni-goettingen.de/). This database collects the sequences around the transcriptional start sites of human and mouse genes, and users can search for any sequence

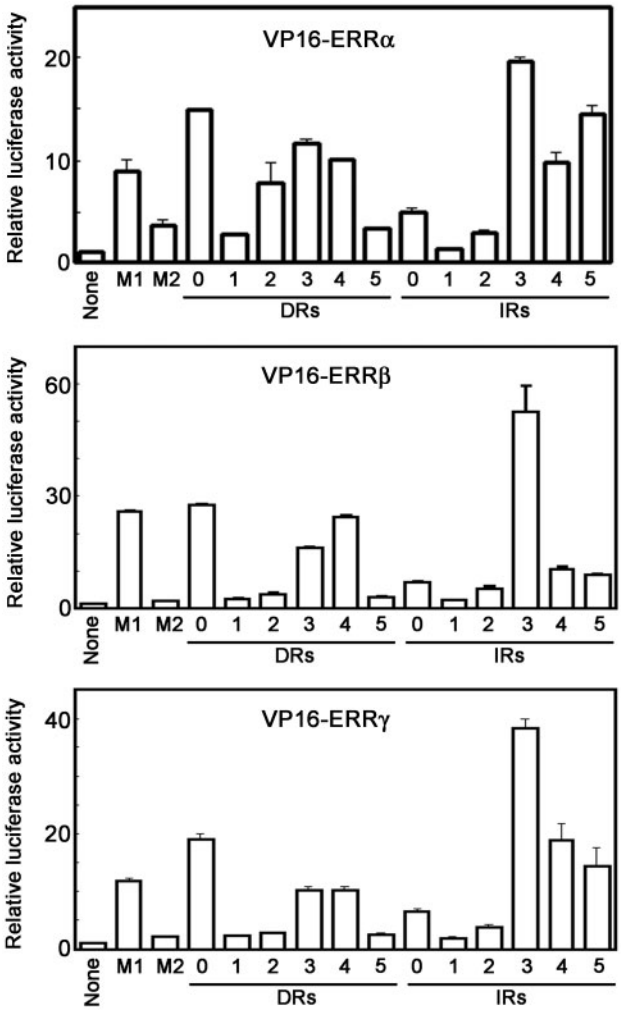

Fig. 2. **Transactivation by VP16-ERRs through different target sites.** Luciferase reporter plasmids containing the *tk* promoter and one of the oligonucleotides listed in Table 1 were used. HeLa cells were transfected in 24-well plates with 0.8 μg of one of the reporter plasmids and 0.1 μg of the expression plasmid of VP16-fused ERRα, β or γ. A β-galactosidase expression vector, pCMVβ (0.2 μg), was used as an internal control. Cells were harvested 24 h after transfection and assayed for the activities of luciferase and β-galactosidase. Other conditions were as described in EXPERIMENTAL PROCEDURES. Relative luciferase activities are given, taking the value with tk-Luc (marked "None") as 1. Means of triplicate samples together with standard deviations are shown.

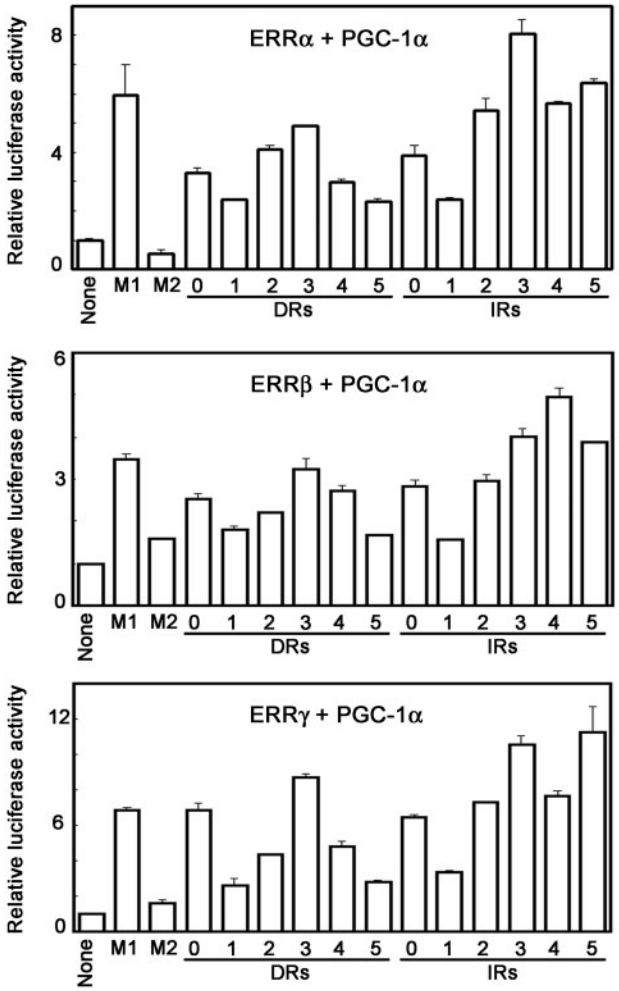

Fig. 3. **Transactivation by ERRs and PGC-1α through different target sites.** HeLa cells were transfected in 24-well plates with 0.8 μg of one of the luciferase reporter plasmids, 0.1 μg of the expression plasmid of ERRα, β or γ, as well as 0.4 μg of the expression plasmid of PGC-1α. Cells were collected 24 h after transfection, and luciferase activity was measured as described in EXPERIMENTAL PROCEDURES. The results are expressed as in Fig. 2. Representative data of two independent experiments are shown.

motifs within the −1000/+200 regions relative to the start sites of the genes. We searched for an IR3-like sequence, AGGTNANNNTNACCT, in the mouse database, hitting 17 genes in total (Table 2). Information was available about the protein products, for about a half of these genes. Among them, we were particularly interested in *Rb1cc1*, which is expressed in skeletal muscle cells and has been suggested to be involved in muscle differentiation (*32*, *33*). Because ERRα was reported to be involved in cellular energy metabolism in muscle (*5*, *16*, *17*) and tissue differentiation (*28*), we expected that the *Rb1cc1* gene would be a target of ERRα. The *Rb1cc1* gene carries sequences similar to the consensus IR3 and DR4 elements in the promoter region (Fig. 4A). These sequences, named RE-1 (AGGTCAcgcTCACCT) and RE-2 (AGGTCAgggaAGGACA), were located at positions −206 to −192 and −155 to −140, respectively, relative to the cap site. Notably, the nucleotide sequences of RE-1 and RE-2 are perfectly conserved between human and mouse, except for the spacer regions. As shown before, IR3 and DR4 were the most favourable recognition sequences of ERRα, and both motifs, particularly IR3, conferred strong transactivation by ERRα.

To examine whether RE-1 and/or RE-2 are involved in the regulation of *Rb1cc1* transcription, we performed a transfection experiment using a luciferase reporter plasmid containing a 1.7-kb promoter region of *Rb1cc1*, in the presence or absence of expression vectors of ERRα and PGC-1α. Luciferase expression was significantly

Table 2. **Mouse genes carrying IR3-like sequences in the promoter regions, hit in the DBTSS search.**

NM ID	Product	Chromosome
023144	Non-POU-domain-containing, octamer binding	X
009531	Xeroderma pigmentosum, complementation group C	6
009357	Testis expressed gene 261	6
028982	RIKEN cDNA 8430419L09	6
026309	RIKEN cDNA 1010001J12	6
133997	Expressed sequence AI255964	10
009227	Small nuclear ribonucleoprotein E	1
029453	RIKEN cDNA 4930511H01	1
008976	Protein tyrosine phosphatase, non-receptor type	1
009826	Rb1-inducible coiled-coil 1	1
153387	Gamma tubulin ring complex protein	2
011130	Polymerase (DNA directed), beta	8
008583	Multiple endocrine neoplasia 1	19
146102	Expressed sequence AU041783	19
134064	Ring finger protein 44	13
025989	RIKEN cDNA 2310037I18	7
021493	Hypothetical protein, MNCb-1301	11

enhanced with both ERRα and PGC-1α (Fig. 4B). Their effects were seemingly additive, rather than synergistic, which will be discussed later. We also examined the transcriptional activation of the *Rb1cc1* gene promoter by VP16-fused ERRα. The reporter expression was increased with an increasing amount of VP16-fused ERRα expression vector (Fig. 4C), whereas no transactivation was observed with simple VP16 (data not shown). Thus, the *Rb1cc1* gene promoter responds to transactivation by ERRα. We observed similar transactivation of *Rb1cc1* promoter by ERRβ and γ (data not shown).

We next studied whether ERRα activates the *Rb1cc1* gene promoter through binding to RE-1 and/or RE-2. For this purpose, we first carried out a competitive EMSA. We compared the binding affinities of RE-1 and RE-2, based on the relative efficiencies as competitors against the consensus IR3 probe for binding with ERRα. The consensus IR3 itself and wild-type RE-1 efficiently competed with the probe (Fig. 5B; lanes 3 and 4). Wild-type RE-2 also competed, though relatively weakly as compared with RE-1 (lane 6). To confirm the specificities of these sequences, a set of oligonucleotides carrying base substitutions were designed (Fig. 5A). Mut RE-1 largely lost the ability to compete for binding (Fig. 5B, lane 5). Mut RE-2 (mut1/2) and Mut RE-2 (mut1), in which both half-sites and only the first half-site were mutated, respectively, were totally unable to compete (lanes 7 and 8). On the other hand, Mut RE-2 (mut2), which was mutated only in the second half-site, retained weaker but considerable affinity to ERRα (lane 9). The results of competitive EMSA were confirmed with different molar excesses of competitors (data not shown). These data indicate that RE-1 and RE-2 of the *Rb1cc1* gene promoter specifically bind with ERRα, the affinity of RE-1 being higher than that of RE-2.

We then investigated whether these putative REs, RE-1 and RE-2, indeed act as regulatory sites for gene transcription, by transfection assays employing reporter constructs carrying mutations in these elements. The reporter containing a mutation in the first half-site of RE-1 (Mut RE-1) did not exhibit a significant reduction of ERRα-dependent luciferase expression, as compared with the wild-type reporter (Fig. 5C, left panel). ERRα-dependent transactivation was not affected, even when both half-sites of RE-1 were simultaneously mutated (Mut RE-1 <1/2>; Fig. 5C, right panel). Notably, however, PGC-1α by itself exhibited significant transactivation, and the mutations of RE-1 decreased it. This was probably due to the presence of an endogenous transcription factor that is also coactivated by PGC-1α, which recognizes wild-type, but not mutated, RE-1. We next investigated the function of RE-2. A set of base substitutions that were the same as those used in the binding experiment (Fig. 5A) were introduced into the first and second half-sites of RE-2, in a reporter construct containing the *Rb1cc1* gene upstream region up to position −289 (Fig. 5D). This truncated version of reporter showed transactivation by ERRα and PGC-1α at a level comparable with that of the longer version containing the 1.7-kb upstream region (data not shown). The base substitutions in RE-2 reduced the transcriptional activity as compared with that of the wild-type. Simultaneous mutations of both half-sites of RE-2 (mut1/2) almost completely abolished the transactivation. The mutant of the first half-site was affected for transactivation function more severely than that of the second half-site, indicating that recognition of RE-2 by ERRα depends to a larger extent on the first half-site than the second one. These results indicate the importance of RE-2 in the transcriptional activation of the *Rb1cc1* gene promoter by ERRα.

It was unexpected that RE-1, an IR-3-like element that efficiently bound ERRα *in vitro*, did not seem to be effective for transactivation of the *Rb1cc1* gene by ERRα. To address this issue, we studied whether RE-1 and RE-2 confer transactivation by ERRα in a different promoter context. We cloned RE-1 and RE-2 in front of the *tk* promoter and performed transfection experiments as before. Significant transactivation by ERRα through RE-1 and RE-2 was observed, similar to those through consensus IR3 and DR4 elements (Fig. 6A). When RE-1 or RE-2 was mutated in the same construct, the reporter

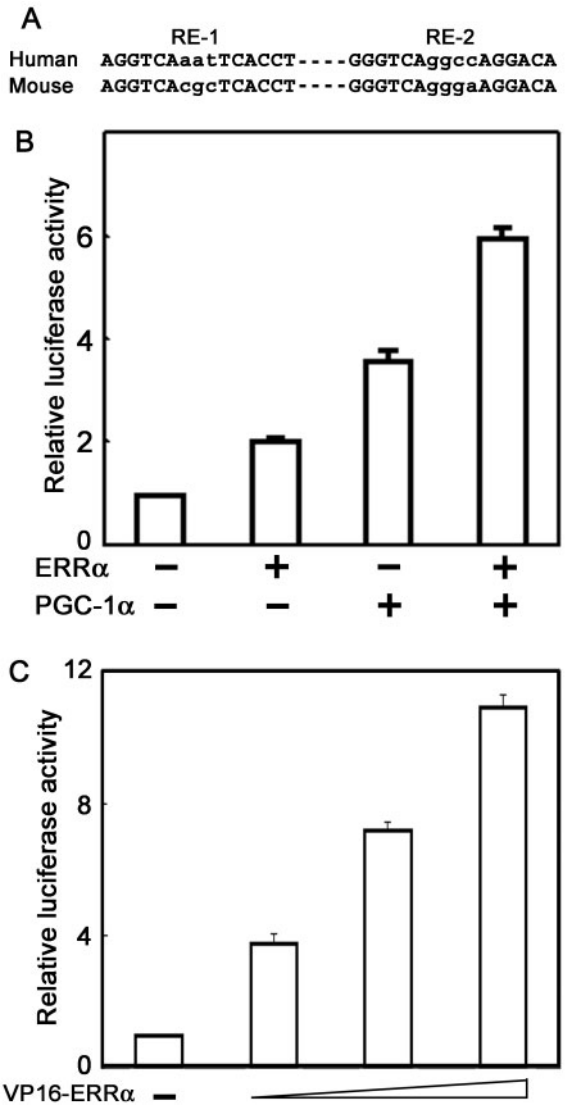

Fig. 4. ***Rb1cc1* is a possible target gene of ERRα.** (A) Alignment of RE-1 and RE-2 elements of human and mouse *Rb1cc1* genes. In the human gene, RE-1 and RE-2 are located at nucleotide positions −227 to −213 and −169 to −154, respectively, whereas in the mouse gene, they are located at −207 to −193 and −156 to −141, respectively, relative to the putative cap sites assigned based on the longest EST clones. (B) Activation of *Rb1cc1* promoter by ERRα and PGC-1α. HeLa cells were transfected in 24-well plates with the reporter construct (0.8 μg) containing the −1689 to +27 region of the mouse *Rb1cc1* gene, in the presence or absence of expression vectors of ERRα (0.1 μg) and PGC-1α (0.4 μg). The cells were harvested 24 h after transfection and assayed for luciferase activity. Results are given as in Fig. 2, except that the value without any expression vectors was taken as 1. (C) Activation of *Rb1cc1* promoter by VP16-ERRα. HeLa cells were transfected with the same reporter construct as used in (B), as well as increasing amounts of pCMX-VP16-ERRα (0.1, 0.2 and 0.4 μg). Other experimental conditions were as described in EXPERIMENTAL PROCEDURES.

expression was diminished, thus confirming the functional specificities of both elements.

These results indicate that both RE-1 and RE-2 effectively cooperate with a viral promoter, suggesting that their combinations with different promoters can affect the transcriptional activity. Accordingly, we studied the functional difference between consensus IR3 and RE-1 when placed in the *Rb1cc1* gene promoter. For this purpose, RE-1 was replaced by consensus IR3 in the construct containing the −289/+27 region of the *Rb1cc1* gene. When the wild-type RE-2 was retained, the reporter containing either RE-1 or consensus IR3 behaved similarly with respect to activation by ERRα and PGC-1α (Fig. 6B). Interestingly, even when RE-2 was mutated, a reporter construct in which RE-1 was replaced by consensus IR3 was activated by ERRα and PGC-1α, in sharp contrast to the case of the combination of natural RE-1 and mutated RE-2. Thus, in the context of the *Rb1cc1* promoter, the consensus IR3, but not RE-1, is an effective *cis*-element for ERRα function. Hence, RE-2 solely contributes to transactivation of this gene by ERRα.

Finally, we investigated the *in vivo* binding of ERRα to the *Rb1cc1* gene promoter by the ChIP assay. We employed C2C12 cells, a model myoblastic cell line in which endogenous expression of *Rb1cc1* gene was reported (*33*). However, endogenous expression of ERRα was very low in C2C12, and moreover, a commercially available monoclonal antibody against ERRα did not effectively immunoprecipitate chromatin (data not shown). Accordingly, we overexpressed myc-tagged ERRα using a lentivirus vector containing the EF-1α promoter. Efficient expression of myc-tagged ERRα was confirmed by western blotting using an anti-myc antibody (Fig. 7A). Chromatin from C2C12 cells expressing myc-ERRα was immunoprecipitated with control IgG and an anti-myc antibody. A significant signal for myc-ERRα binding was detected in the *Rb1cc1* gene promoter region, but not in a distal region (Fig. 7B). Hence, it is concluded that ERRα binds with the *Rb1cc1* gene promoter *in vivo*. We could not discriminate, however, which binding site, RE-1, RE-2 or both, ERRα bound to *in vivo*, because these sites are only 50 bp apart, which is below the limit of resolution of ChIP analysis.

We found that expression of the endogenous *Rb1cc1* gene was increased by about 20% in the myc-ERRα-overexpressing cells as compared with that in control cells (data not shown). As judged by RT-PCR, total ERRα mRNA, derived from exogenous myc-ERRα plus endogenous ERRα genes, was about 3.5-fold more abundant in the overexpressing cells than the level of endogenous ERRα mRNA in the control cells (data not shown). On the other hand, we failed to overexpress PGC-1α with the lentiviral vector despite many trials. Thus, probably due to the limiting level of PGC-1α, the endogenous *Rb1cc1* gene did not respond remarkably to the several fold increase in ERRα.

DISCUSSION

The present results of competitive EMSA have revealed that the three subtypes of ERR bind to a broad range of nuclear receptor-binding motifs with variable affinities. The patterns of the relative effectiveness of individual binding sites were similar among all the ERR subtypes, and largely consistent with our previous study on

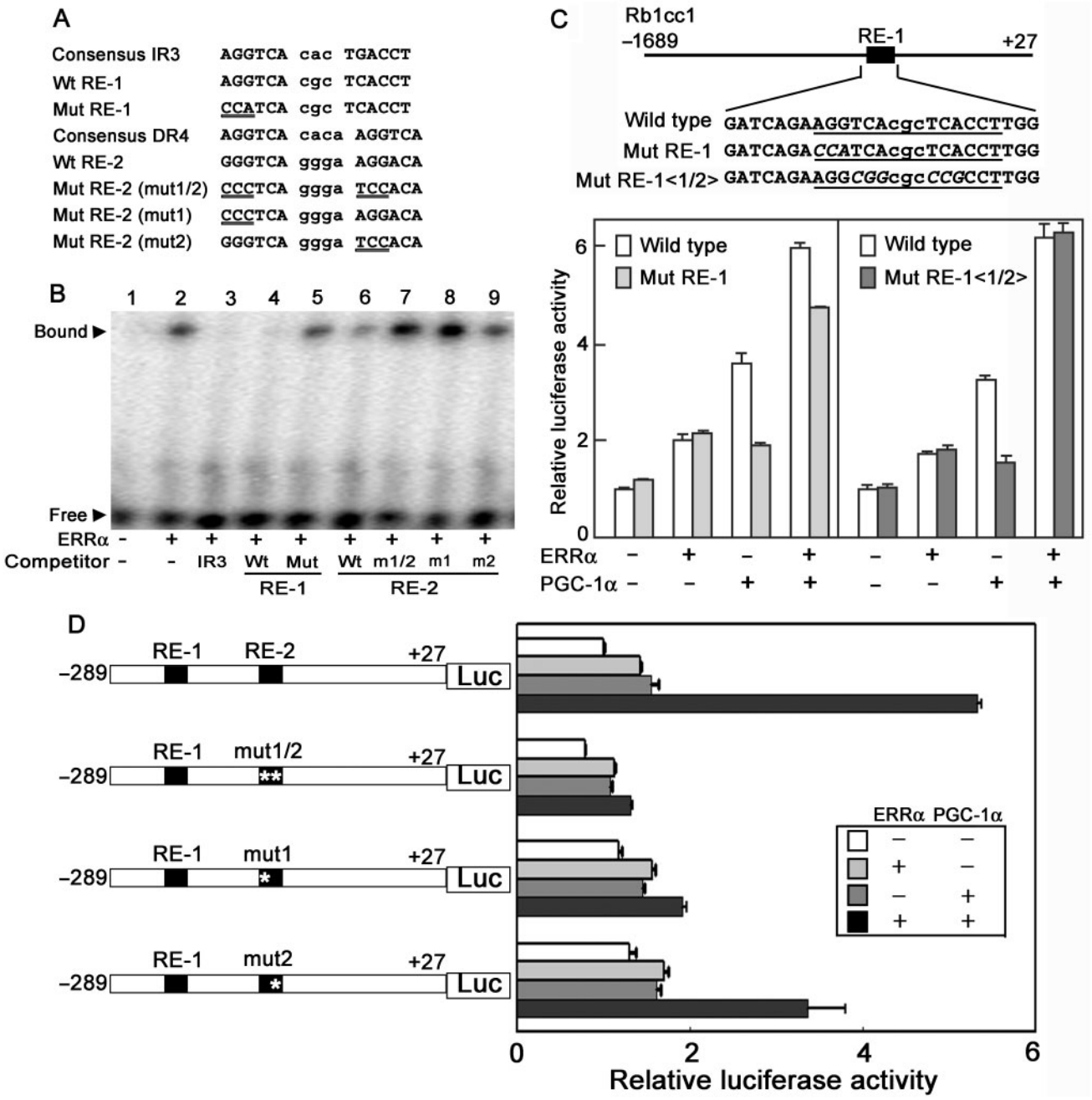

Fig. 5. **ERRα activates the *Rb1cc1* promoter through a *cis*-acting element within the proximal region.** (A) List of wild-type and mutated sequence elements employed in the experiments of Figs. 5 and 6. Mutated bases are underlined and spacer nucleotides are shown with small letters. (B) Binding of ERRα to RE-1 and RE-2 *in vitro*. EMSA was performed using a ^{32}P-labelled double-stranded oligonucleotide containing consensus IR3 (the same as that used in Fig. 1), as a probe. The probe was incubated without ERRα (lane 1), with ERRα (lane 2) or with ERRα in the presence of competitors (lanes 3–9). The sequences of the competitors are given in Table 1. m1/2, m1 and m2 denote Mut RE-2 (mut1/2), Mut RE-2 (mut1) and Mut RE-2 (mut2), respectively. Arrowheads indicate the protein-bound and free probes. (C) Effect of mutation in RE-1 on activation of *Rb1cc1* promoter by ERRα and PGC-1α. Upper panel, the wild-type and mutated RE-1 sequences in the *Rb1cc1* reporter constructs. The IR3-like sequence of wild-type RE-1 and the corresponding mutant sequences are underlined. Mutated bases are italicized, whereas the spacer nucleotides are written in small letters. Lower panel, effects of mutations in RE-1 on transactivation of *Rb1cc1* promoter by ERRα and PGC-1α. Experimental conditions were as in Fig. 4B. (D) Effects of mutations in RE-2 on transactivation of *Rb1cc1* promoter by ERRα and PGC-1α. Left, schematic diagram of luciferase (LUC) reporter gene constructs. Asterisks denote mutations in RE-2. Right, the results of the reporter assay. HeLa cells were transfected with the wild-type and mutant constructs with or without ERRα and PGC-1α. Other experimental conditions were as in Fig. 4B.

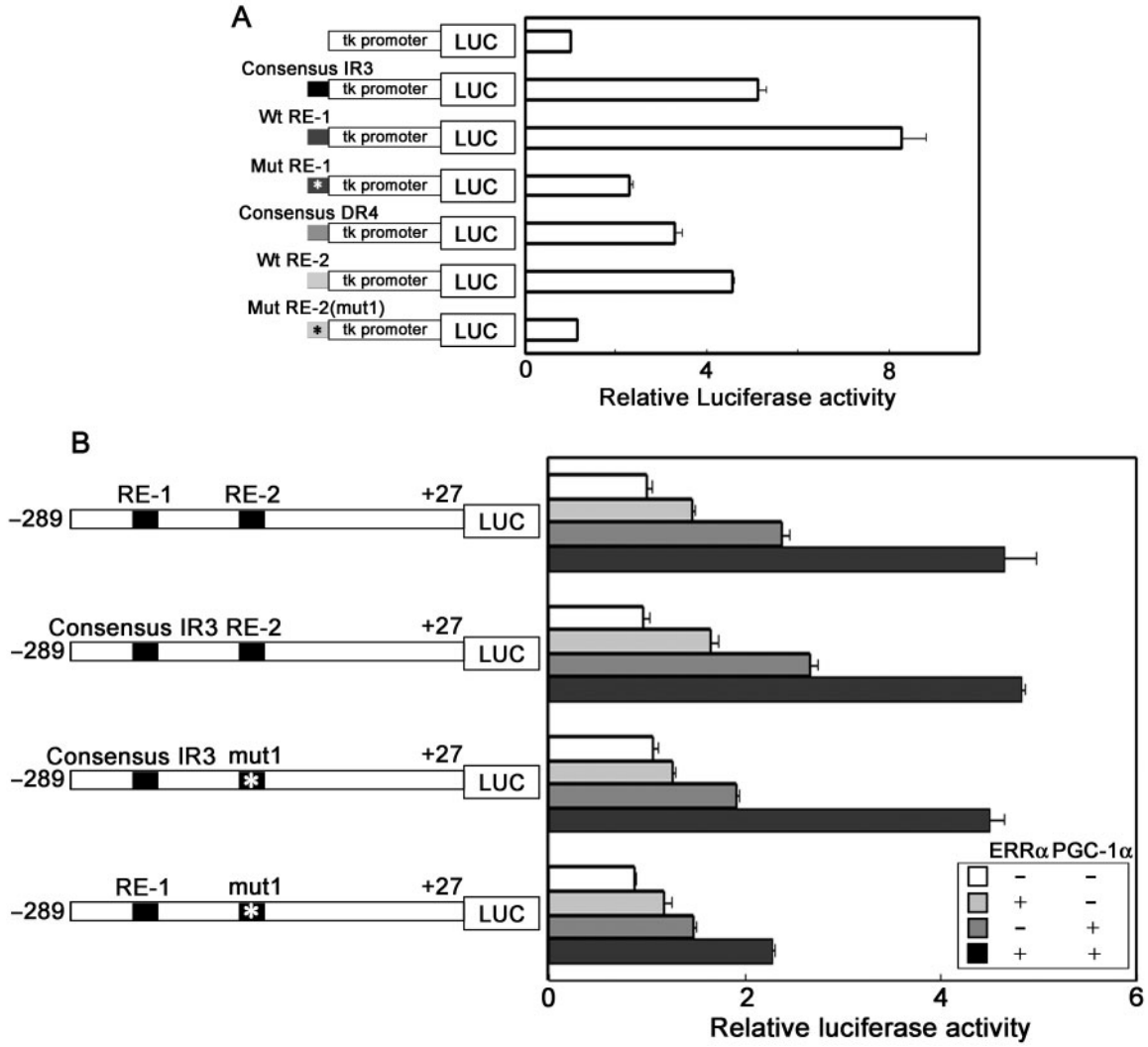

Fig. 6. **Functions of RE-1 and RE-2 in different promoter contexts.** (A) Results of transfection assays employing the *tk* promoter. Sequence elements as indicated were placed just upstream of the Herpes simplex *tk* gene promoter linked to the luciferase gene. Transfection experiment was performed in the presence of both ERRα and PGC-1α. Other experimental conditions were as in Fig. 4B. (B) Functional difference between consensus IR3 and RE-1 in the context of *Rb1cc1* gene promoter. Left, diagram of reporter constructs. RE-1 was replaced by consensus IR3, and RE-2 was mutated as indicated, in the *Rb1cc1* upstream region. Right, results of transfection assay. Experimental conditions were as in Fig. 5D.

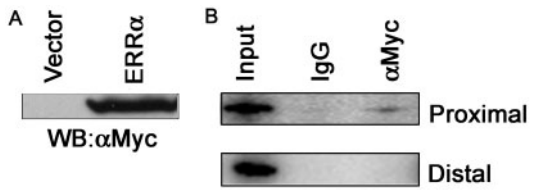

Fig. 7. **ERRα binds to the *Rb1cc1* gene promoter region *in vivo*.** (A) Successful expression of myc-ERRα in C2C12 cells. Equal amounts of lysates of cells infected with empty lentivirus and lentivirus encoding myc-ERRα were analysed by western blotting with an anti-myc monoclonal antibody. (B) ChIP assay for binding of ERRα to the *Rb1cc1* promoter region. Chromatin was immunoprecipitated from the myc-ERRα-expressing C2C12 cells with control IgG or a monoclonal anti-myc antibody. Recovery of chromatin region proximal or distal to the *Rb1cc1* promoter was examined by PCR. Other experimental conditions were as described in EXPERIMENTAL PROCEDURES.

ERRγ (*12*). Transactivation by VP16-fused ERRs was conferred by many of the binding motifs tested, though highly variably. The results were largely consistent with the EMSA results. Thus, motifs with higher affinities to ERRs exhibited higher transactivation, whereas poor binding motifs had almost negligible effects on transactivation. The major inconsistencies were higher transactivation with IR4 and IR5 for ERRα and ERRγ in spite of lower binding affinities, and poor transactivation through IR2 by ERRβ and ERRγ despite efficient binding in EMSA. Supposing that transactivation by VP16-fused ERRs directly reflects the efficiencies of binding to the binding sites, these inconsistencies may reflect different affinities of the binding sites to ERRs under the conditions of EMSA and transfection studies. In the former, experiments are performed *in vitro* using oligonucleotides as probes, whereas in the latter,

ERRs must bind to the elements contained in supercoiled plasmids in a nuclear milieu.

On the other hand, in the presence of PGC-1α, ERRs activated transcription through versatile sequence motifs. The coactivating effect of PGC-1α seemed more prominent for motifs with lower affinities, and hence the variability in the reporter expression through different sequence motifs was smaller than that with VP16-ERRs (compare Figs. 2 and 3). PGC-1α coactivates nuclear receptors through binding to the hormone-binding domains, without direct interaction with DNA. To our knowledge, there has been no suggestion that PGC-1α may affect the DNA-binding specificities of nuclear receptors, and hence it is less likely that PGC-1α improves the affinities of ERRs to less favourable binding sites. Rather, the net transactivating functions of ERRs, which depend on both the efficiencies of DNA-binding and PGC-1α-assisted transactivation, are possibly saturable. Hence, the coactivating effect of PGC-1α might be apparently smaller, when the binding affinity is high enough. As pointed out by other investigators (*43*), another interesting, but yet to be examined, possibility is that PGC-1α adopts different conformations depending on binding sequences, which may affect the coactivator functions.

Thus, although the mechanism remains unclear, ERRs would activate transcription through an even broader range of binding sites than expected by *in vitro* binding studies, when assisted by PGC-1α. Given that PGC-1α is a close functional partner of ERRs, many different configurations of nuclear receptor binding motifs may confer transactivation of physiological target genes by ERRs. To date, all the natural ERRα-target genes so far reported have REs composed of an extended monovalent half-site. As discussed subsequently for the *Rb1cc1* gene, even slight deviations from the idealized IR or DR binding sequences may seriously affect transactivation, when they are placed in the natural gene promoter context. Thus, naturally occurring ERR-target genes that are driven by IR or DR-type of RE might be less abundant than expected from the simple probability of occurrence. Nevertheless, the present results suggest that it would be worth seeking for such ERR-target genes in the genomes.

We identified *Rb1cc1* as a new target gene of ERRα. This gene was first noted upon searching a promoter database, DBTSS. *Rb1cc1* was one of the genes carrying IR3-like sequences in the promoters, being hit at RE-1. Because *Rb1cc1* has been suggested to be involved in muscle differentiation (*31–33*) and ERRα is enriched in the muscle (*28*), we hypothesized that this gene is activated by ERRα through RE-1. We indeed found that the *Rb1cc1* promoter was transactivated by ERRα and PGC-1α, and ERRα bound to the promoter region *in vivo*, strongly supporting the notion that this gene is a bona fide target of ERRα. Unexpectedly, however, studies showed that a DR4-like element, RE-2, rather than RE-1, mediated the action of ERRα. Based on the result of mutational analysis, the first half-site of RE-2 was more important than the second one for ERRα binding. The first half-site sequence is ccaGGGTCA (the preceding three nucleotides being denoted with small letters), while the second one is ggaAGGACA, the former being closer than the latter to the consensus extended half-site, tcaAGGTCA. Hence, RE-2 would be recognized in part as a monovalent binding motif with the first half-site, and also as a DR4 motif. Although the extended monovalent half-site motif has been implicated in transactivation by ERRs (*8*, *18*), our present results indicate that bivalent sites would also work as functional binding sites of ERRs.

To our surprise, RE-1 did not confer transactivation by ERRα in the *Rb1cc1* promoter, though it was effective when combined with the viral *tk* promoter. In contrast, the consensus IR3 motif was fully active for supporting ERRα function, when placed in the *Rb1cc1* promoter, even if the RE-2 sequence was ablated. The sequence of RE-1, AGGTCAcgcTCACCT, deviates from the consensus IR3, AGGTCAnnnTGACCT, at a single position in the second half-site. This deviation would possibly make RE-1 slightly less efficient for ERRα binding under the conditions of the transfection assay, although the difference was hardly discriminated by EMSA. Thus, RE-1 cannot support transactivation by ERRα in the *Rb1cc1* promoter context, even though it is functional when combined with a strong viral promoter. On the other hand, the reporter gene expression driven by the *Rb1cc1* promoter was enhanced by PGC-1α even in the absence of ERRα (Fig. 4B), and this enhancement was decreased by the mutations of RE-1 (Fig. 5C). This is probably due to the presence of an endogenous transcriptional factor that is also coactivated by PGC-1α, which binds to RE-1 and hence transactivates the *Rb1cc1* promoter. This would explain why the effects of ERRα and PGC-1α on *Rb1cc1* expression are additive, rather than synergistic (Fig. 4B). Thus, although RE-1 is not effective for transactivation by ERRα, it seems to be functional for the regulation by other transcriptional factors. An attractive candidate for such factor would be ER, considering the notion that a mutation in *Rb1cc1* is associated with human breast cancer (*44*). In the present study, however, it is not clear whether ER indeed mediates the endogenous activity of transactivation through RE-1, because HeLa cells have been reported to lack ER expression (*45*).

We found that all ERR subtypes transactivated the *Rb1cc1* promoter. ERRα has been implicated in energy metabolism in tissues highly dependent on mitochondrial oxidative functions, including cardiac and skeletal muscles. ERRβ expression is restricted to embryos (*9*), whereas ERRγ is expressed in many tissues, with a distribution overlapping that of ERRα (*46*). On the other hand, *Rb1cc1* has been suggested to be involved in muscle differentiation (*31–33*). Thus, the present findings raise the possibility that ERRα, and possibly ERRγ, also participate in promoting the muscle functions at the stage of differentiation.

Rb1cc1 was not found in the lists of target genes in the genome-wide search for *in vivo* binding sites of ERRα/γ on the chromatin (*47*), or in the search for down-regulated genes in ERRα-knockout mice (*16*, *48*). A possible reason why *Rb1cc1* was missed in these analyses would be that these studies focused on the cardiac muscle or adipose tissue, rather than the skeletal muscle. Further studies would be required for elucidating the physiological significance of *Rb1cc1* regulation by ERRα.

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REFERENCES

1. Gronemeyer, H. and Laudet, V. (1995) Transcription factors 3: nuclear receptors. *Protein Profile* **2**, 1173–1308
2. Mangelsdorf, D.J, Thummel, C., Beato, M., Herrlich, P., Schüz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M. (1995) The nuclear receptor superfamily: the second decade. *Cell* **83**, 835–839
3. Giguè, V., Yang, N., Segui, P., and Evans, R.M. (1988) Identification of a new class of steroid hormone receptors. *Nature* **331**, 91–94
4. Hong, H., Yang, L., and Stallcup, M.R. (1999) Hormone-independent transcriptional activation and coactivator binding by novel orphan nuclear receptor ERR3. *J. Biol. Chem.* **274**, 22618–22626
5. Sladek, R., Bader, J.A., and Giguère, V. (1997) The orphan nuclear receptor estrogen-related receptor α is a transcriptional regulator of the human medium-chain acyl coenzyme A dehydrogenase gene. *Mol. Cell. Biol.* **17**, 5400–5409
6. Johnston, S.D., Liu, X., Zuo, F., Eisenbraun, T.L., Wiley, S.R., Kraus, R.J., and Mertz, J.E. (1997) Estrogen-related receptor alpha 1 functionally binds as a monomer to extended half-site sequences including ones contained within estrogen-response elements. *Mol. Endocrinol.* **11**, 342–352
7. Vanacker, J.M., Pettersson, K., Gustafsson, J.A., and Laudet, V. (1999) Transcriptional targets shared by estrogen receptor- related receptors (ERRs) and estrogen receptor (ER) α, but not by ERβ. *EMBO J.* **18**, 4270–4279
8. Barry, J.B., Laganiere, J., and Giguère, V. (2006) A single nucleotide in an estrogen related receptor α site can dictate mode of binding and PGC-1α activation of target promoters. *Mol. Endocrinol.* **20**, 302–310
9. Pettersson, K., Svensson, K., Mattsson, R., Carlsson, B., Ohlsson, R., and Berkenstam, A. (1996) Expression of a novel member of estrogen response element-binding nuclear receptor is restricted to the early stages of chorion formation during mouse embryogenesis. *Mech. Dev.* **54**, 211–223
10. Hentschke, M., Susens, U., and Borgmeyer, U. (2002) Domains of ERRγ that mediate homodimerization and interaction with factors stimulating DNA binding. *Eur. J. Biochem.* **269**, 4086–4097
11. Xie, W., Hong, H., Yang, N.N., Lin, R.J., Simon, C.M., Stallcup, M.R., and Evans, R.M. (1999) Constitutive activation of transcription and binding of coactivator by estrogen-related receptors 1 and 2. *Mol. Endocrinol.* **13**, 2151–2162
12. Razzaque, M.A., Masuda, N., Maeda, Y., Endo, Y., Tsukamoto, T., and Osumi, T. (2004) Estrogen receptor-related receptor γ has an exceptionally broad specificity of DNA sequence recognition. *Gene* **340**, 275–282
13. Vega, R.B. and Kelly, D.P (1997) A role for estrogen-related receptor α in the control of mitochondrial fatty acid β-oxidation during brown adipocyte differentiation. *J. Biol. Chem.* **272**, 31693–31699
14. Huss, J.M., Kopp, R.P., and Kelly, D.P. (2002) Peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-α and -γ. Identification of novel leucine-rich interaction motif within PGC-1α. *J. Biol. Chem.* **277**, 40265–40274
15. Schreiber, S.N., Knutti, D., Brogli, K., Uhlmann, T., and Kralli, A. (2003) The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor α (ERRα). *J. Biol. Chem.* **278**, 9013–9018
16. Luo, J., Sladek, R., Carrier, J., Bader, J.A., Richard, D., and Giguère, V. (2003) Reduced fat mass in mice lacking orphan nuclear receptor estrogen-related receptor α. *Mol. Cell. Biol.* **23**, 7947–7956
17. Huss, J.M., Torra, I.P., Staels, B., Giguère, V., and Kelly, D.P. (2004) Estrogen-related receptor α directs peroxisome proliferator-activated receptor α signaling in the transcriptional control of energy metabolism in cardiac and skeletal muscle. *Mol. Cell. Biol.* **24**, 9079–9091
18. Schreiber, S.N., Emter, R., Hock, M.B., Knutti, D., Cardenas, J., Podvinec, M., Oakeley, E.J., and Kralli, A. (2004) The estrogen-related receptor α (ERRα) functions in PPARγ coactivator 1α (PGC-1α)-induced mitochondrial biogenesis. *Proc. Natl. Acad. Sci. USA* **101**, 6472–6477
19. Wende, A.R., Huss, J.M., Schaeffer, P.J., Giguère, V., and Kelly, D.P. (2005) PGC-1α Coactivates PDK4 gene expression via the orphan nuclear receptor ERRα: a mechanism for transcriptional control of muscle glucose metabolism. *Mol. Cell. Biol.* **25**, 10684–10694
20. Araki, M. and Motojima, K. (2006) Identification of ERRα as a specific partner of PGC-1α for the activation of PDK4 gene expression in muscle. *FEBS J.* **273**, 1669–1680
21. Carrier, J.C., Deblois, G., Champigny, C., Levy, E., and Giguère, V. (2004) Estrogen-related receptor α (ERRα) is a transcriptional regulator of apolipoprotein A-IV and controls lipid handling in the intestine. *J. Biol. Chem.* **279**, 52052–52058
22. Vanacker, J.M., Bonnelye, E., Delmarre, C., and Laudet, V. (1998) Activation of the thyroid hormone receptor α gene promoter by the orphan nuclear receptor ERRα. *Oncogene* **17**, 2429–2435
23. Horard, B., Rayet, B., Triqueneaux, G., Laudet, V., Delaunay, F., and Vanacker, J.M. (2004) Expression of the orphan nuclear receptor ERRα is under circadian regulation in estrogen-responsive tissues. *J. Mol. Endocrinol.* **33**, 87–97
24. Yang, C., Yu, B., Zhou, D., and Chen, S. (2002) Regulation of aromatase promoter activity in human breast tissue by nuclear receptors. *Oncogene* **21**, 2854–2863
25. Yang, N., Shigeta, H., Shi, H., and Teng, C.T. (1996) Estrogen-related receptor, hERR1, modulates estrogen receptor-mediated response of human lactoferrin gene promoter. *J. Biol. Chem.* **271**, 5795–5804
26. Zhang, Z. and Teng, C.T. (2000) Estrogen receptor-related receptor α1 interacts with coactivator and constitutively activates the estrogen response elements of the human lactoferrin gene. *J. Biol. Chem.* **275**, 20837–20846
27. Lu, D., Kiriyama, Y., Lee, K.Y., and Giguère, V. (2001) Transcriptional regulation of the estrogen-inducible pS2 breast cancer marker gene by the ERR family of orphan nuclear receptors. *Cancer Res.* **61**, 6755–6761
28. Bonnelye, E., Vanacker, J.M., Dittmar, T., Begue, A., Desbiens, X., Denhardt, D.T., Aubin, J.E., Laudet, V., and Fournier, B. (1997) The ERR-1 orphan receptor is a transcriptional activator expressed during bone development. *Mol. Endocrinol.* **11**, 905–916
29. Bonnelye, E. and Aubin, J.E. (2005) Estrogen receptor-related receptor α: a mediator of estrogen response in bone. *J. Clin. Endocrinol. Metab.* **90**, 3115–3121
30. Bonnelye, E., Vanacker, J.M., Spruyt, N., Alric, S., Fournier, B., Desbiens, X., and Laudet, V. (1997) Expression of the estrogen-related receptor 1 (ERR-1) orphan receptor during mouse development. *Mech. Dev.* **65**, 71–85

31. Bamba, N., Chano, T., Taga, T., Ohta, S., Takeuchi, Y., and Okabe, H. (2004) Expression and regulation of RB1CC1 in developing murine and human tissues. *Int. J. Mol. Med.* **14**, 583–587
32. Chano, T., Saeki, Y., Serra, M., Matsumoto, K., and Okabe, H. (2002) Preferential expression of RB1-inducible coiled-coil 1 in terminal differentiated musculoskeletal cells. *Am. J. Pathol.* **161**, 359–364
33. Watanabe, R., Chano, T., Inoue, H., Isono, T., Koiwai, O., and Okabe, H. (2005) Rb1cc1 is critical for myoblast differentiation through Rb1 regulation. *Virchows Arch.* **447**, 643–648
34. Yamaguchi, T., Matsushita, S., Motojima, K., Hirose, F., and Osumi, T. (2006) MLDP, a novel PAT family protein localized to lipid droplets and enriched in the heart, is regulated by peroxisome proliferator-activated receptor α. *J. Biol. Chem.* **281**, 14232–14240
35. Fumoto, T., Yamaguchi, T., Hirose, F., and Osumi, T. (2007) Orphan nuclear receptor Nur77 accelerates the initial phase of adipocyte differentiation in 3T3-L1 cells by promoting mitotic clonal expansion. *J. Biochem.* **141**, 181–192
36. Nagai, K., Yamaguchi, T., Takami, T., Kawasumi, A., Aizawa, M., Masuda, N., Shimizu, M., Tominaga, S., Ito, T., Tsukamoto, T., and Osumi, T. (2004) SKIP modifies gene expression by affecting both transcription and splicing. *Biochem. Biophys. Res. Commun.* **316**, 512–517
37. Akter, M.H., Razzaque, M.A., Yang, L., Fumoto, T., Motojima, K., Yamaguchi, T., Hirose, F., and Osumi, T. (2006) Identification of a gene sharing a promoter and peroxisome proliferator-response elements with acyl-CoA oxidase gene. *PPAR Res*, doi:10.1155/PPAR/2006/71916
38. Osada, S., Tsukamoto, T., Takiguchi, M., Mori, M., and Osumi, T. (1997) Identification of an extended half-site motif required for the function of peroxisome proliferator-activated receptor α. *Genes Cells* **2**, 315–327
39. Shimizu, M., Takeshita, A., Tsukamoto, T., Gonzalez, F.J., and Osumi, T. (2004) Tissue-selective, bidirectional regulation of PEX11α and perilipin genes through a common peroxisome proliferator response element. *Mol. Cell. Biol.* **24**, 1313–1323
40. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F.H., and Verma, I.M. (1998) Development of a self-inactivating lentivirus vector. *J. Virol.* **72**, 8150–8157
41. Yamashita, D., Sano, Y., Adachi, Y., Okamoto, Y., Osada, H., Takahashi, T., Yamaguchi, T., Osumi, T., and Hirose, F. (2007) hDREF regulates cell proliferation and expression of ribosomal protein genes. *Mol. Cell. Biol.* **27**, 2003–2013
42. Kallen, J., Schlaeppi, J.M., Bitsch, F., Filipuzzi, I., Schilb, A., Riou, V., Graham, A., Strauss, A., Geiser, M., and Fournier, B. (2004) Evidence for ligand-independent transcriptional activation of the human estrogen-related receptor α (ERRα): crystal structure of ERRα ligand binding domain in complex with peroxisome proliferator-activated receptor coactivator-1α. *J. Biol. Chem.* **279**, 49330–49337
43. Knutti, D. and Kralli, A. (2001) PGC-1, a versatile coactivator. *Trends Endocrinol. Metab.* **12**, 360–365
44. Chano, T., Kontani, K., Teramoto, K., Okabe, H., and Ikegawa, S. (2002) Truncating mutations of RB1CC1 in human breast cancer. *Nat. Genet.* **31**, 285–288
45. Touitou, I., Mathieu, M., and Rochefort, H. (1990) Stable transfection of the estrogen receptor cDNA into HeLa cells induces estrogen responsiveness of endogenous cathepsin D gene but not of cell growth. *Biochem. Biophys. Res. Commun.* **169**, 109–115
46. Liu, D., Zhang, Z., and Teng, C.T. (2005) Estrogen-related receptor-γ and peroxisome proliferator-activated receptor-γ coactivator-1α regulate estrogen-related receptor-α gene expression via a conserved multi-hormone response element. *J. Mol. Endocrinol.* **34**, 473–487
47. Dufour, C.R., Wilson, B.J., Huss, J.M., Kelly, D.P., Alaynick, W.A., Downes, M., Evans, R.M., Blanchette, M., and Giguère, V. (2007) Genome-wide orchestration of cardiac functions by the orphan nuclear receptors ERRα and γ. *Cell Metab.* **5**, 345–356
48. Huss, J.M., Imahashi, K., Dufour, C.R., Weinheimer, C.J., Courtois, M., Kovacs, A., Giguère, V., Murphy, E., and Kelly, D.P. (2007) The nuclear receptor ERRα is required for the bioenergetic and functional adaptation to cardiac pressure overload. *Cell Metab.* **5**, 25–37