

**Development of a bioassay to screen for chemicals mimicking the anti-aging effects of calorie restriction.**

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## **Abstract**

Suppression of the growth hormone/insulin-like growth factor-I pathway in Ames dwarf (DF) mice, and caloric restriction (CR) in normal mice extends lifespan and delays the onset of age-related disorders. In combination, these interventions have an additive effect on lifespan in Ames DF mice. Therefore, common signaling pathways regulated by DF and CR could have additive effects on longevity. In this study, we tried to identify the signaling mechanism and develop a system to assess pro-longevity status in cells and mice. We previously identified genes up-regulated in the liver of DF and CR mice by DNA microarray analysis. Motif analysis of the upstream sequences of those genes revealed four major consensus sequence motifs, which have been named dwarfism and calorie restriction-responsive elements (DFCR-REs). One of the synthesized DFCR-RE sequences bound to hepatocyte nuclear factor-4 $\alpha$  (HNF-4 $\alpha$ ), an important transcription factor involved in liver metabolism. Furthermore, using this sequence information, we developed a highly sensitive bioassay to identify chemicals mimicking the anti-aging effects of CR. When the reporter construct, containing an element upstream of a secreted alkaline phosphatase (SEAP) gene, was co-transfected

with HNF-4 $\alpha$  and its regulator peroxisome proliferator-activated receptor (PPAR)  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ), SEAP activity was increased compared with untransfected controls. Moreover, transient transgenic mice established using this construct showed increased SEAP activity in CR mice compared with *ad libitum*-fed mice. These data suggest that because of its rapidity, ease of use, and specificity, our bioassay will be more useful than the systems currently employed to screen for CR mimetics, which mimic the beneficial effects of CR. Our system will be particularly useful for high-throughput screening of natural and synthetic candidate molecules.

Keywords: calorie restriction, aging, metabolism, biosensing, drug discovery.

Abbreviations: AL: ad libitum, CR: calorie restriction, CRISP: calorie restriction imitating chemicals searching platform, DF: dwarf, DF-CR-RE: dwarfism and calorie restriction-responsive element, HNF-4 $\alpha$ : hepatocyte nuclear factor-4 $\alpha$ , PGC-1 $\alpha$ : peroxisome proliferator-activated receptor (PPAR)  $\gamma$  coactivator-1 $\alpha$ . SEAP: secreted alkaline phosphatase.

## 1. Introduction

Spontaneous mutations and genetic modifications of growth hormone (GH) and suppression of the plasma concentration of insulin-like growth factor-I (IGF-I) produce a dwarf (DF) phenotype and extend the lifespan of rodents (see review [1-3]). Ames DF mice, which are homozygous for a loss of function mutation at the Prop1 locus, which is important for pituitary development, exhibit a 40–70% increase in lifespan compared with normal heterozygous mice [1-3]. Similarly, caloric restriction (CR), in which dietary caloric intake is reduced without causing malnutrition, retards aging and age-related diseases, and increases lifespan in a variety of organisms [1-3]. The lifespan promoting effects of DF and CR could be mediated through the same or related pathways that are more strongly affected by a combination of both interventions, or through distinct molecular pathways that are independently affected by each intervention. Bartke et al [4] suggested that different molecular mechanisms were responsible for the prolonged longevity of Ames DF mice and CR mice. However, they also showed that knockout of the GH receptor gene (GHRKO) in mice suppressed the lifespan extending effect of CR [5]. These results suggest that GHRKO and CR

influence lifespan through the same pathway. We previously observed an additive longevity effect of DF and CR in GH-antisense transgenic rats [6]. That study revealed that CR affects aging predominantly via mechanisms other than suppression of the GH-IGF-I axis in this model. These results suggest that, at least in part, suppression of the GH-IGF-I axis is important for longevity. However, other signaling pathways are also important in the anti-aging effects of CR, and the additive longevity effects on DF and CR could be dependent on strain and species.

To gain insight into the signaling pathways activated by DF in combination with CR, we previously analyzed the hepatic gene expression profiles in normal and Ames DF mice subjected to *ad libitum* (AL) or CR diets using oligonucleotide microarrays [7]. We found that 56 genes were up-regulated by the DF and CR, and that the effects of DF and CR were additive. That study suggested that the additive effects of DF and CR on lifespan are achieved by their additive effects on the expression of genes involved in the regulation of detoxification, oxidative stress, and protein quality control. The study also suggested that DF and CR also might promote longevity through their independent effects on the expression of other genes.

CR might be effective for human longevity, but it is difficult to continue CR in our daily lives because of the ensuing perpetual state of hunger. In addition, we do not know the degree or length of restriction required to elicit meaningful effects in humans. Thus, research in this field is focusing on the development of molecules that mimic the beneficial effects of CR without reducing food intake [8, 9]. The candidate molecules include synthetic chemical compounds and plant-derived functional chemicals (phytochemicals) [10], and some of molecules are currently being tested in clinical trials as anti-diabetic drugs. These studies hold great promise to improve the quality of life of elderly people. Thus, the development of an efficient system to screen CR mimetics would undoubtedly accelerate and expand research in this field.

The secreted alkaline phosphatase (SEAP) reporter system has been widely used to investigate the activity of known or putative enhancer/promoter elements [11]. To construct a reporter vector, we need to identify the regulatory sequence elements of the target genes. Approaches for regulatory motif discovery have been described that extract upstream regulatory sequences from microarray-derived yeast expression data [12]. In the present study, we used a similar method to identify the regulatory elements

of putative pro-longevity genes up-regulated in both DF and CR. Up-regulation of these co-regulated genes seem likely to activate the pathways that increase lifespan. Here, we describe the motifs identified using this approach, and the development of an efficient bioassay to screen candidate CR mimetics.

## **2. Materials and Methods**

### *2.1. Motif isolation*

We previously identified 56 genes that were additively up-regulated in the liver of DF and CR mice [7]. The 5' untranslated regions of these genes (3000 bp from the open reading frame) were isolated using EZ Retrieve [13] (<http://siriusb.umdnj.edu:18080/EZRetrieve/index.jsp>). The sequences of 43 of these genes were isolated and consensus motifs of these sequences were analyzed using MEME [14] (<http://meme.sdsc.edu/meme/cgi-bin/meme.cgi>). The results for the four most highly conserved motifs (except for putative repetitive sequence) are shown as a sequence logo generated using WebLogo (<http://weblogo.berkeley.edu/>) [14].

### *2.2. Cell culture*

CHO cells and 293 cells were purchased from the American Type Culture Collection. The cells were maintained in Dulbecco's modified minimum essential medium (D-MEM; Wako) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (Nacalai Tesque). Incubation and



isolation of protein samples were performed as previously described [15, 16].

### 2.3. Gel shift assay

The gel shift assay was performed essentially as previously described [17, 18].

Probe DNAs best describing the isolated motifs were synthesized. Double-stranded

DNA probes were end-labeled with [ $\bullet$ - $^{32}$ P]ATP and T4 polynucleotide kinase

(Amersham Biosciences). The sequences of the probes are as follows: DFCR-RE1

(5'-AGACCAGGCTGGCCT-3'), DFCR-RE2

(5'-AGCACTTGGGAGGCAGAGGCAGGGGGAG-3'), DFCR-RE3

(5'-CTGCCTCTGCCTCCC-3'), and DFCR-RE4

(5'-AGTGAGTTCCAGGCCAGCCAG-3'). Five micrograms of nuclear extract (CHO

cell or mouse liver) was incubated with 1  $\mu$ g of poly(dI-dC) (Roche Diagnostics),

followed by the addition of the end-labeled oligonucleotide (8 fmol). Samples were then

incubated at room temperature for 30 min in binding buffer (20 mM Hepes pH 7.9, 50

mM KCl, 5% glycerol, 1 mM EDTA, 10 mM DTT). The HNF-4 $\alpha$  binding assay was

performed as follows. HNF-4 $\alpha$  protein was produced *in vitro* using a TNT Quick

Coupled Transcription/Translation System (Promega). Double-stranded DNA probes (DFCR-RE2) were biotin-labeled using a LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology). *In vitro* transcribed/translated HNF-4 $\alpha$  (4  $\mu$ L) was incubated with 1  $\mu$ g of poly(dI-dC), followed by the addition of biotin-labeled oligonucleotide (40 fmol), and incubated at room temperature for 30 min in binding buffer (10 mM Tris pH 7.5, 60 mM KCl, 4 mM MgCl, 12% glycerol, 1 mM EDTA, 2 mM DTT, 20 mM PMSF). Anti-Flag monoclonal antibody M2 (Sigma-Aldrich, 5  $\mu$ g) was added for supershift, or 4 pM (100-fold excess) of unlabeled competitor DNA was added to confirm binding specificity. The reaction mixtures were loaded onto 5% nondenaturing polyacrylamide gels containing 1 $\times$  TBE (90 mM Tris-HCl pH 8.0, 89 mM boric acid, 2 mM EDTA). After electrophoresis for 2 h at 100 V at room temperature, the radioactive gels were dried and analyzed with an image analyzer [19, 20]. For chemiluminescence detection of the biotinylated probes, the DNA/protein complex was transferred to a nylon membrane (Hybond N+, Amersham Biosciences) and analyzed by an image analyzer [19, 20].

#### *2.4. Plasmid*

Expression vectors for HNF-4 $\alpha$  and PGC-1 $\alpha$  were kind gifts from Dr. Shimano (Tsukuba) and Dr. Spiegelman (Harvard), respectively. The pcDNA3 vector (Invitrogen) was used as an empty vector control. Five copies of the DFPCR-RE2 sequence were subcloned into the *NheI*/*BglIII* site of pSEAP2-Control (Clontech), and pDFPCR-RE2-SEAP was constructed. The secreted luciferase gene containing pMetLuc-Control vector (Clontech) was used to evaluate transfection and delivery efficiency.

#### *2.5. Animals*

Weaned 6-week-old male ICR mice (Slc:ICR) were purchased from Japan SLC, and housed in our specific-pathogen-free facility under temperature- and light-controlled conditions (21–24°C, 12-h light/dark cycle). Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University, and were approved by the Institutional Animal Care and Use Committee. Mice were fed *AL* with a CR-LPF diet (Oriental Yeast). From

12 weeks of age, mice were divided into two groups: one group was fed AL, and the other group was subjected to CR (70% calorie intake). CR was performed as previously described [21].

### 2.6. *In vitro* reporter assay

Subconfluent 293 cells plated on six-well plates were used for the *in vitro* reporter assay. Transfection was performed using FuGENE HD Transfection Reagent (Roche Diagnostics). pDFCR-RE2-SEAP and pMetLuc-Control plasmid were included in each transfection experiment with or without HNF-4 $\alpha$  and PGC-1 $\alpha$  expression vectors. The total transfected DNA content was adjusted to 3  $\mu$ g by the addition of pcDNA3 vector. Before and 2 days after transfection, the medium was collected and SEAP and luciferase activities were measured as described below.

### 2.7. *In vivo* reporter assay

Twenty-eight-week-old ICR mice were used for the *in vivo* reporter assay. Using a TransIT-EE Hydrodynamic Delivery Solution (Mirus Bio), 10  $\mu$ g of

pDFCR-RE2-SEAP and 5  $\mu\text{g}$  of pMetLuc-Control plasmid were introduced to the mouse liver. Before and 2 days after injection, blood samples were collected and SEAP and luciferase activities were measured as described below.

### 2.8. SEAP and luciferase activity assay

SEAP and luciferase activities in the conditioned media and sera were evaluated by a chemiluminescent method using the Ready-To-Glow Dual Secreted Reporter Assay kit (Clontech). In brief, for the SEAP assay, 25  $\mu\text{L}$  of cell culture medium from transfected cells or serum (undiluted or diluted 10-fold with PBS, respectively) was mixed with 75  $\mu\text{L}$  of 1 $\times$  dilution buffer and incubated for 30 min at 65°C to inactivate endogenous alkaline phosphatase. After incubation, the samples were mixed with 100  $\mu\text{L}$  of SEAP substrate solution, and left at room temperature for 30 min. SEAP activity was then assessed using a luminometer (AccuFLEXLumi 400, Aloka). For the luciferase assay, 50  $\mu\text{L}$  of cell culture medium from transfected cells or serum (undiluted or 10-fold diluted with PBS, respectively) was mixed with 5  $\mu\text{L}$  of 1 $\times$  substrate/reaction buffer and incubated for 10 min at room temperature. Luciferase

activity was then assessed using a luminometer. All assays were performed in duplicate.

### 2.9. Statistical analysis

All experiments were repeated at least three times. Data are expressed as means  $\pm$  standard deviation (SD). Statistical analysis of the *in vivo* reporter assay was performed using Student's *t* test to compare each group. A *p* value of  $< 0.05$  was considered to indicate a statistically significant difference.

### 3. Results and Discussion

Fig. 1 shows the isolated motifs of the putative pro-longevity genes. We generated dsDNAs best describing these motifs (Supplement Table S1) and performed gel shift assays. As shown in Fig. 2, the nuclear proteins bound to these sequences, particularly DFCE-RE2 and 4 (marked by an asterisk). Supplementary Table S2 lists the gene containing DFCE-RE2 motifs in their up-stream region. As reported previously [7], most of these genes are related to the regulation of metabolism, detoxification, oxidative stress, and protein quality control. Some of these genes have been shown to be under the control of PGC-1 $\alpha$ , an important transcriptional regulator in liver [22]. The major target transcriptional factors of PGC-1 $\alpha$  are HNF-4 $\alpha$ , FoxO1 and Nrf2, and we have recently shown the importance of HNF-4 $\alpha$  [23] and FoxO1 [21] in the effects of CR. Importantly, HNF-4 $\alpha$ , FoxO1 could interact and regulate the transcription of HNF-4 $\alpha$  target genes [18]. Furthermore, we found that DFCE-RE2 showed some homology to a HNF-4 $\alpha$  binding motif identified in an earlier study [17]. Therefore, we next assessed whether HNF-4 $\alpha$  could bind to the DFCE-RE2 sequence. As shown in Fig. 3, the *in vitro* translated Flag-tagged HNF-4 $\alpha$  protein could bind to DFCE-RE2. This binding was

reduced by the addition of anti-Flag antibody, probably due to steric blocking of the DNA binding region of HNF-4 $\alpha$  by the antibody. Moreover, the addition of unlabeled competitor DNA diminished the shifted band. These results suggest the DNA/protein interaction is specific, although mutational analysis of DF $\alpha$ CR-RE2 will be needed to further validate this specificity and to identify the sequences crucial for this binding.

We constructed a reporter assay system using DF $\alpha$ CR-RE2 linked upstream of the SEAP gene. As shown in Fig. 4A, SEAP activity was dose-dependently increased by HNF-4 $\alpha$ /PGC-1 $\alpha$  transfection in 293 cells. These results support the notion that DF $\alpha$ CR-RE2 binds to and is activated by HNF-4 $\alpha$ /PGC-1 $\alpha$  *in vitro*. Because DF $\alpha$ CR-RE2 is a relatively long sequence for an enhancer element, other factors might also play important roles in its transcriptional transactivation. This appears likely, since our previous results in CR rats [23] demonstrated that CR increases the interaction between HNF-4 $\alpha$  and PGC-1 $\alpha$  in the liver, and increases the expression of target genes, such as phosphoenolpyruvate carboxykinase. The mRNA for this gene is also increased in the liver of CR and DF mice (Supplementary Table S2). The SEAP reporter assay can be used for *in vivo* studies because secreted SEAP can be detected in mouse serum.



Therefore, we investigated whether this reporter construct is activated in the liver of CR mice. As shown in Fig. 4B, SEAP activity was significantly greater in CR mice than in AL mice. Although we still need to conduct mutational studies of DFCE-RE, these results indicate that DFCE-RE2 is activated by CR in the liver. The construction of combined DFCE-RE reporters, such as a reporter with DFCE-RE2 linked in cis with DFCE-RE4, might increase their responsiveness to CR mimetics. These motifs may act in concert to transactivate their target genes.

Kurachi et al [24, 25] identified an age-related stability element (ASE) that regulates age-related gene expression. They found an ASE, (C/G)AGGAAG, containing an Ets transcription factor consensus motif (GGAA), that was bound specifically by Ets1. Interestingly, DFCE-RE2 contains two sequences similar to this ASE, namely GAGGCAG, in its middle region. Mutational studies demonstrated that this sequence (GAGGCAG) does not bind to any proteins present in nuclear extracts, including Ets1 [25]. Further studies are needed to determine whether there is a functional relationship between the ASE and DFCE-RE2. Because loss of the ASE causes an age-related decline in gene expression, DFCE-RE2 might regulate stable gene expression for the

entire lifespan of an animal. Therefore, DF and CR might have similar functions. They may stabilize (suppress down-regulation) or up-regulate target genes containing the DF-CR-RE2. Such regulation would avoid the age-related reductions in gene expression which appear to result in age-related functional decline.

DRESSA (dioxin-responsive-element-based sensing via secreted alkaline phosphatase) has been used to detect toxic materials in assays similar to the SEAP assay described here [11]. The advantages of these assays have been widely reported. For these reasons, we adapted this strategy to identify molecules offering potential health benefits. Because DF-CR-REs were isolated from long-lived mice, the transcriptional regulators of this sequence could be beneficial for longevity. We found that our DF-CR-RE2 reporter construct was up-regulated in CR mice. Hence, compounds that activate this reporter may be candidate CR mimetics.

Using the approach described here, we can analyze the effects of candidate molecules by adding them to the culture medium *in vitro* or mixing them with mouse food for *in vivo* studies. Our approach could be used to identify activators and well as inhibitors of the negative regulator of this element. Therefore, our approach offers

advantages compared with the single molecule targeted methods. Sirtuin activators such as resveratrol and other small molecules were thought to be promising candidates for CR mimetics [10]. However, recent studies have revealed the limitations of the sirtuin activator screening system [26, 27]. One limitation is that the screening system was established as an *in vitro*, cell-free system that targeted a single protein, SIRT1.

Furthermore, the studies used a problematic fluorescently labeled substrate which produce non-specific SIRT1 activation. Our strategy offers distinct advantages because it can be performed *in vitro* or *in vivo*, and is not limited in terms of possible candidate molecules because a target protein is not specified in this assay. The present results suggest that mice transgenic for DF<sub>CR</sub>-RE-SEAP reporter genes will provide an even more convenient vehicle for *in vivo* screening of CR mimetics.

#### 4. Conclusions

Here, we have described a rapid and highly sensitive bioassay that is expected to help screen potential anti-aging agents (CRISP: calorie restriction imitating chemicals searching platform), such as drugs, including traditional Chinese medicines, and phytochemicals *in vitro* and *in vivo*. Molecules identified by this assay could ultimately be used to treat metabolic diseases such as diabetes, cancer and neurodegenerative diseases because of the properties of CR mimetics [8, 9]. Because of its ease of use, specificity, and time- and cost-effectiveness, the approach described here is expected to be more useful than other currently available screening systems and would be particularly suited to high-throughput screening of CR mimetics.

## **Acknowledgments**

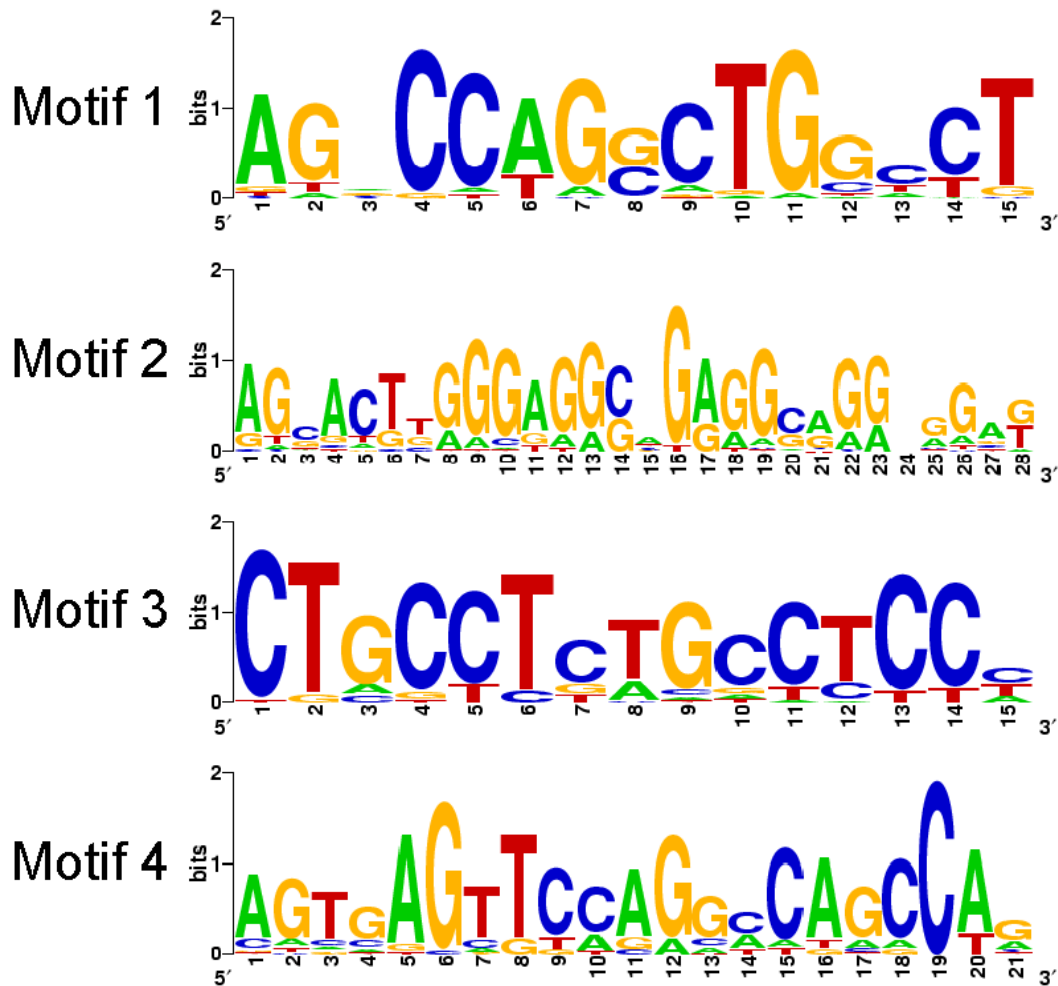
We are grateful to the staff at the Biomedical Research Center at Center for Frontier Life Sciences, Nagasaki University, for technical assistance and animal care.

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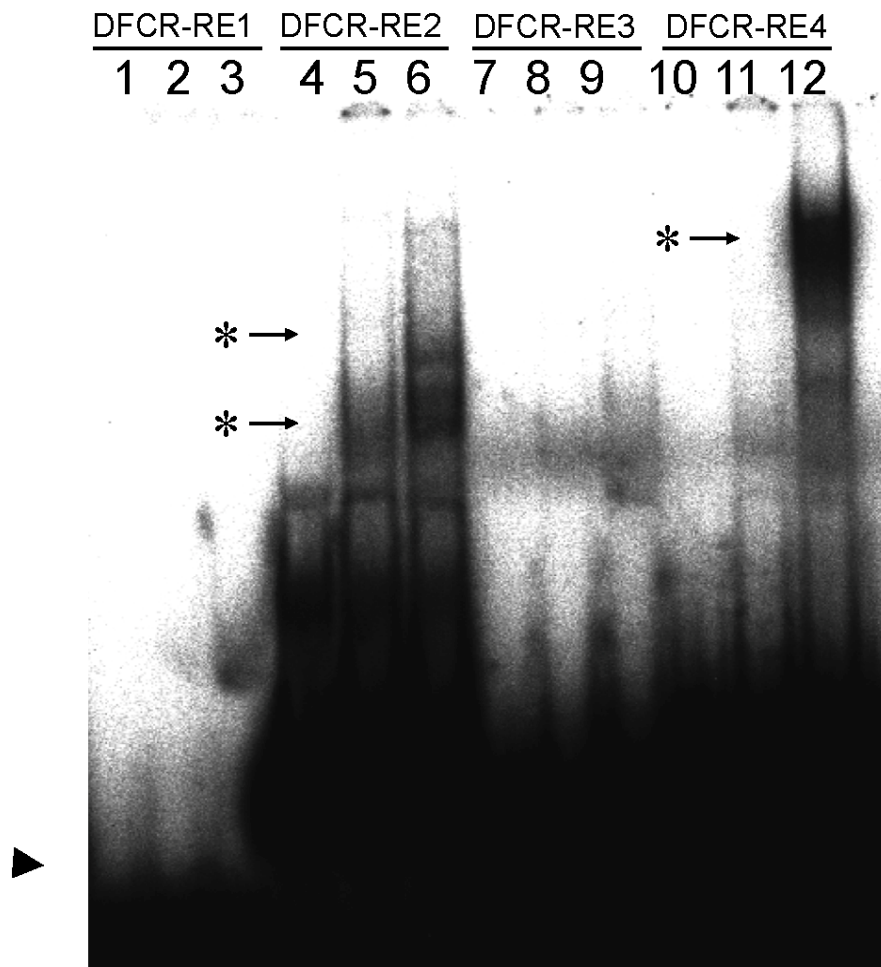
## **Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:xxx

## Figure legends



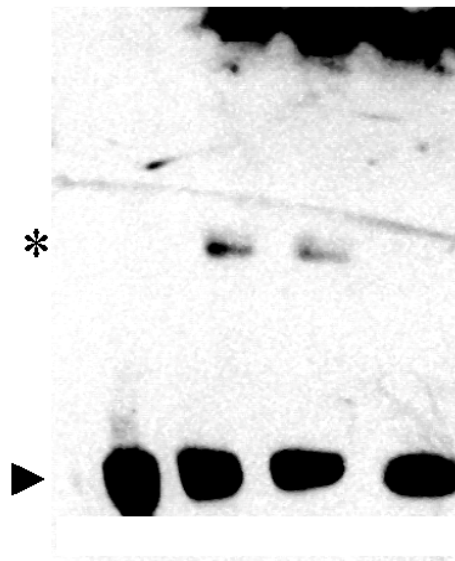
**Fig. 1.** Motifs identified by analyzing the combined effects of dwarfism and calorie restriction. The four most significant motifs are shown. The identified motif matrices are represented as a sequence logo.



**Fig. 2.** Identification of dwarfism and calorie restriction response element

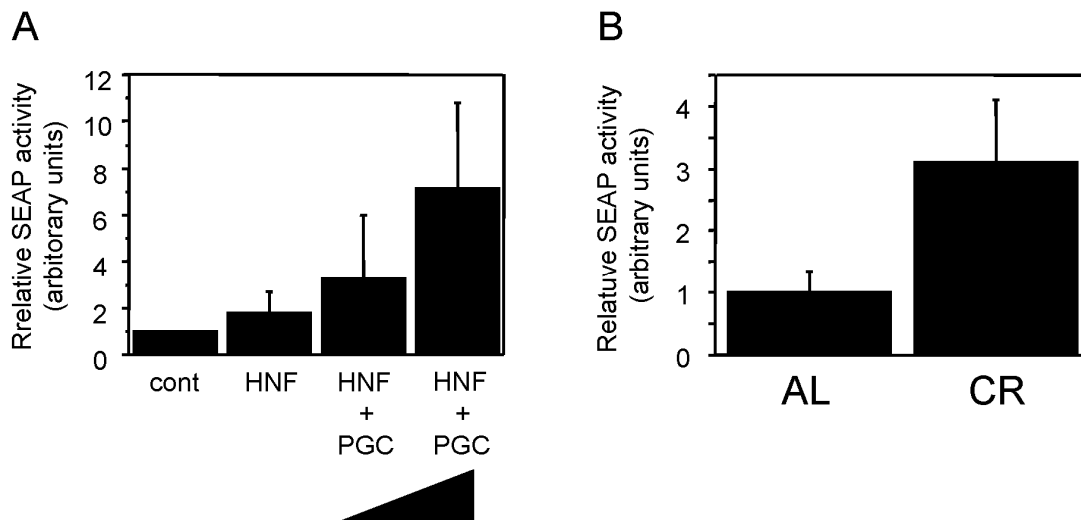
(DFCR-RE)-binding nuclear proteins. A gel shift assay was performed using nuclear extracts from mouse liver (lanes 3, 6, 9 and 12) and CHO cells (lanes 2, 5, 8 and 11), or in the absence of protein (lanes 1, 4, 7 and 10). Lane 1–3 contain the  $^{32}\text{P}$ -labeled DFCR-RE1 probe, lanes 4–6 contain the  $^{32}\text{P}$ -labeled DFCR-RE2 probe, lanes 7–9 contain the  $^{32}\text{P}$ -labeled DFCR-RE3 probe, and lanes 10–12 contain the  $^{32}\text{P}$ -labeled DFCR-RE4 probe. Asterisk: major shifted bands; arrowhead: free probe.

antibody	-	-	+	-
competitor	-	-	-	+
protein	-	+	+	+
	1	2	3	4



**Fig. 3.** The dwarfism and calorie restriction-response element 2 (DFCR-RE2) is a binding site for HNF-4 $\alpha$ . A gel shift assay was performed to detect specific interactions between the DFCR-RE2 sequence and HNF-4 $\alpha$ . A biotin-labeled DFCR-RE2 probe was incubated with (lane 2–4) or without (lane 1) the *in vitro* translation product of HNF-4 $\alpha$ . Anti-Flag antibody (5  $\mu$ g, lane 3) or a 100-fold molar excess of unlabeled competitor DNAs (lane 4) were added to confirm specific binding. Asterisk: shifted band corresponding to the HNF-4 $\alpha$ -DNA complex; arrowhead: free probe.





**Fig. 4.** (A) The dwarfism and calorie restriction-response element 2 (DFCR-RE2) is activated by HNF-4 $\alpha$ /PGC-1 $\alpha$  *in vitro*. 293 cells were transfected with a secreted alkaline phosphatase (SEAP) reporter, pDFCR-RE2-SEAP (500 ng) and pMetLuc-Control plasmid (500 ng) in the presence of HNF-4 $\alpha$  (500 ng) alone or HNF-4 $\alpha$  with PGC-1 $\alpha$  (500 ng or 1  $\mu$ g each) or empty vectors (adjusted total DNA, 3  $\mu$ g). Values are normalized to the internal control, secreted luciferase activity. Results are presented as relative SEAP activity (means  $\pm$  SD). (B) Activation of DFCR-RE2 by CR *in vivo*. Transient transgenic reporter mice (35–55 g body weight; five male mice per group) fed AL or CR. At 28 weeks of age, the mice were injected with 10  $\mu$ g of pDFCR-RE2-SEAP and 5  $\mu$ g of pMetLuc-Control vector by the hydrodynamic method. Values are normalized to the internal control secreted luciferase activity. Results are

presented as relative SEAP activity (means  $\pm$  SD). Statistically significant differences were observed between the AL and CR groups ( $p < 0.05$ ).

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and resveratrol are not direct activators of SIRT1. *J Biol Chem* 285 (2010)

8340-51.

**Supplementary Table S1.** DFCR-RE probe sequences best describing the motifs

shown in Fig. 1.

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Motif 1	AGACCAGGCTGGCCT
Motif 2	AGCACTTGGGAGGCAGAGGCAGGGGGAG
DFCR	
Motif 3	CTGCCTCTGCCTCCC
Motif 4	AGTGAGTTCCAGGCCAGCCAG

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**Supplementary Table S2.** Genes containing the DFCR-RE2 sequence

<b>Accession number</b>	<b>Gene</b>	<b>Function</b>	<b>Fold-change</b>
J03952	<i>Gstm1</i>	Phase II protein	1.4
AW047116	<i>Rpl37</i>	Ribosomal protein	1.4
AI854771		EST	1.4
U73039	<i>Nbr1</i>	Unknown	1.4
AB017616	<i>Rragc</i>	GTP binding protein	1.4
AJ245569	<i>Dennd5a</i>	Unknown	1.4
Y13087	<i>Casp6</i>	Apoptosis	1.4
AI852608		EST	1.5
Z54179	<i>Gtl3</i>	Unknown	1.5
AI854482		EST	1.5
M32032	<i>Selenbp1</i>	Selenium metabolism	1.5
AF093140	<i>Nxf1</i>	Nuclear RNA export factor	1.5
AI846123	<i>Grsf1</i>	RNA binding factor	1.5
U96116	<i>Hadh2</i>	Lipid metabolism	1.5
U89491	<i>Ephx1</i>	Phase II protein	1.5
AW125626	<i>Cnn3</i>	Cytoskeleton	1.5
AI838709	<i>Spnr</i>	RNA binding protein	1.5

X74938	<i>Foxa3</i>	Gluconeogenesis	1.6
U85414	<i>Gclc</i>	Anti-oxidant	1.6
AF009605	<i>Pck1</i>	Gluconeogenesis	1.6
M27168	<i>Cyp2d9</i>	Phase I protein	1.6
AF080580	<i>Clk-1</i>	Respiratory chain	1.6
Z37107	<i>Ephx2</i>	Phase II protein	1.6
AI846938	<i>Herpud1</i>	Protein degradation	1.6
Z50159	<i>Elf1</i>	Translation initiation factor	1.6
U90535	<i>Fmo5</i>	Phase I protein	1.7
AI843802	<i>Lpin2</i>	Lipid metabolism	1.7
L21221	<i>Pcsk4</i>	Serine protease	1.7
Z22216	<i>Apoc2</i>	Lipid metabolism	1.8
J03953	<i>Gstm3</i>	Phase II protein	2.0
AW124049		EST	2.3
X53451	<i>Gstp2</i>	Phase II protein	2.3
X81580	<i>Igfbp2</i>	Growth suppression	3.0
AB016424	<i>Rbm3</i>	RNA binding protein	3.5
AI853773	<i>Fbxo21</i>	Protein degradation	3.7

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GenBank accession number, gene name, function, and fold up-regulation versus control

are shown based on results of our previous study [7]. EST: expression sequence tag.