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The promoter activity of human Mfn2 depends on Sp1 in vascular smooth muscle cells.

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Abstract:	Aims: Mitofusin-2 (Mfn2) expression is dysregulated in vascular proliferative disorders and its over-expression attenuates the proliferation of vascular smooth muscle cells (VSMCs) and neointimal lesion development after balloon angioplasty. We sought to gain insight into the mechanisms that control Mfn2 expression in VSMCs. Methods and Results: We cloned and characterized 2 kb of the 5'-flanking region of the human Mfn2 gene. Its TATA-less promoter contains a CpG island. In keeping with this, 5'-RACE, revealed 6 transcriptional start sites (TSSs), of which TSS2 and TSS5 were the most frequently used. The strong CpG island was found non methylated under conditions characterized by large differences in Mfn2 gene expression. The proximal Mfn2 promoter contains 6 putative Sp1 motifs. Sp1 binds to the Mfn2 promoter and its over-expression activates Mfn2 promoter in VSMCs. Inhibition of Sp1 by WP631 reduced Mfn2 expression, and Sp1 silencing reduced transcriptional activity of the Mfn2 promoter. In keeping with this view, Sp1 and Mfn2 mRNA levels were down-regulated in aorta early after an atherogenic diet in Apolipoprotein E-knockout mice or in VSMCs cultured in the presence of low serum. Conclusions: Sp1 is a key factor in maintaining basal Mfn2 transcription in VSMCs.

	Given the anti-proliferative actions of Mfn2, Sp1-induced Mfn2 transcription may represent a mechanism for prevention of VSMCs proliferation and neointimal lesion and development.
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Title: The promoter activity of human *Mfn2* depends on Sp1 in vascular smooth muscle cells.

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Dear Sirs:

I am pleased to enclose the manuscript entitled "The promoter activity of human Mfn2 depends on Sp1 in vascular smooth muscle cells" by Eleonora Sorianello, Francesc X. Soriano, Sergio Fernández-Pascual, Ana Sancho, Deborah Naon, Marian Vila-Caballer, Herminia González-Navarro, José Portugal, Vicente Andrés, Manuel Palacín, and Antonio Zorzano, which we would like to submit to Cardiovascular Research.

In this manuscript we provide evidence on the regulation of Mfn2 gene promoter in vascular smooth muscle cells (VSMCs). Mfn2 is multifunctional protein that plays a relevant role in outer mitochondrial membrane fusion, in the control of endoplasmic reticulum function, and in cell proliferation.   In this manuscript we show by over-expression and silencing studies that Sp1 is a key factor in maintaining basal Mfn2 transcription in VSMCs. Given the anti-proliferative actions of Mfn2, Sp1-induced Mfn2 transcription may represent a mechanism for prevention of VSMCs proliferation and neointimal lesion and development. We think that these findings will be of interest to researchers working in different fields of vascular smooth muscle biology.

We would like to suggest the following experts as potential reviewers of the manuscript:

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Statement of contribution of authors:

ES, FXS and SF-P performed most of studies
AS performed the chromatin precipitation assays
MV-C,HG-N performed in vivo studies
JP provided reagents and participated in the discussion and writing
VA supervised in vivo studies, and participated in the discussion and writing
MP discussed the data
AZ supervised studies and wrote the article

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that the co-authors have read the manuscript and approved its submission to Cardiovascular Research.

One of the figures is in color and the authors declare that they agree to pay for the cost of printing.

Sincerely,

Antonio Zorzano, Ph.D.
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The promoter activity of human *Mfn2* depends on Sp1 in vascular smooth muscle cells.

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Keywords: Vascular smooth muscle cells, mitochondrial dynamics, Mitofusin, Sp1 transcription factor, vascular proliferative disorders.

Introduction.

Vascular proliferative disorders, such as atherosclerosis, restenosis after balloon angioplasty, and vein-graft disease are the most common causes of severe cardiovascular disease. Eventually, arterial wall thickening, plaque rupture, and occlusion lead to the clinical manifestations of cardiovascular disease (eg, myocardial infarction and stroke), the current leading cause of morbimortality in developed countries[1-2]. An important common etiological factor in these inflammatory disorders is the development of neointimal lesions caused by the accumulation of circulating leukocytes, vascular smooth muscle cells (VSMCs), and non-cellular material (eg., circulating lipoproteins, extracellular matrix components). Under normal conditions, VSMCs residing within the arterial tunica media display a so-called 'contractile' phenotype characterized by low proliferative and migratory activity and expression of differentiation markers (eg., contractile proteins). However, unlike terminally differentiated striated muscle cells, VSMCs can revert to a 'synthetic' phenotype featuring suppression of differentiation marker gene expression, abundant synthesis of extracellular matrix components, and high proliferative and migratory activity[3]. These pathological processes are induced by a variety of pathological stimuli, such as atherogenic stimuli, mechanical stress of the vessel wall, and vessel denudation[2]. Thus, VSMCs phenotypic switching, hyperproliferation and migration into the neointimal layer of the arterial wall are essential components of neointimal lesion development.

Multiple signalling pathways and molecular networks appear to control VSMCs growth in response to pathological insults[4-5]. Recently, it has been reported that the mitofusin-2 (*Mfn2*) gene, also named hyperplasia suppressor gene (HSG), encodes a protein whose dysregulation is involved in vascular proliferative disorders[6]. This conclusion is based on a variety of observations, namely: a) *Mfn2* expression is down-regulated in hyper-proliferative VSMCs from spontaneously hypertensive rat arteries, balloon-injured Wistar

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Kyoto rat arteries and apolipoprotein E-knock-out (Apo E-KO) mouse atherosclerotic arteries; and b) adenoviral-mediated over-expression of *Mfn2* suppresses serum-dependent proliferation of cultured VSMCs, and attenuates balloon injury-induced neointimal VSMC growth and vascular occlusive lesion development in rat carotid arteries. Nevertheless, the functional role of *Mfn2* is complex and not limited to the regulation of cell proliferation. *Mfn2* is a mitochondrial outer membrane protein that primarily participates in mitochondrial fusion[7], activates mitochondrial metabolism in mammalian cells[8-9], and protects against apoptosis[10]. Recently, it has been reported that *Mfn2* is enriched at the endoplasmic reticulum-mitochondria interface, and that *Mfn2* in the endoplasmic reticulum is required for efficient mitochondrial Ca^{2+} uptake and normal ER morphology. This gene is essential in rodents, as mice deficient in *Mfn2* die in midgestation, probably because of impaired placental function caused by a marked reduction in the number of polyploid giant cells of the placental trophoblast[11]. Studies in conditional knockout mice indicate that *Mfn2* is required for proper development and maintenance of the cerebellum, and that Purkinje cells require *Mfn2* for their extensive dendritic outgrowth and survival. In humans, *Mfn2* mutations cause Charcot–Marie–Tooth type 2A[12], an autosomal dominant neuropathy. In addition, skeletal muscle *Mfn2* is deficient in human obesity and in type 2 diabetes, which suggests that this gene may be involved in the pathophysiology of these metabolic diseases[8]. On the basis of these observations, the present study was designed to ascertain the mechanisms that regulate *Mfn2* transcription by investigating the promoter region of human *Mfn2*.

Methods.

Cloning of human *Mfn2* promoter. A human *Mfn2* promoter fragment containing positions -1982 to +45 from the transcription start site was prepared by PCR amplification using human genomic DNA templates, and the following primers: 5'-cccgggaggcagaggttgtagtgagttgag-3' and 5'-gaggcgggaggagggaagtggata-3'. The amplified DNA fragment was digested with SacI and SmaI and cloned into the SacI/SmaI sites of pGL3-promoterless vector (Promega).

Databases and computer analysis. Potential transcription factor-binding sites were mapped to the *Mfn2* promoter using AliBaba (http://www.alibaba2.com) and TESS (http://www.cbil.upenn.edu/tess/) software. CpG islands were located using the CpGPlot program (http://www.ebi.ac.uk/emboss/cpgplot/).

Cell culture. Primary rat aorta VSMCs, C3H10T1/2 cells, and the L6E9 rat skeletal muscle cell line were used. Primary rat aorta VSMCs were prepared as previously described[13]. Aorta were collected from adult male Wistar rats euthanized in a CO_2 chamber followed by cervical dislocation. Arteries were dissected free from surrounding tissue and adventitia and cut into small pieces. Aortic tissue was digested with collagenase (2 mg/ml, Worthington). VSMC cells were grown in DMEM-F12 (1:1) supplemented with 10% FBS, 2 mM glutamine, 0.25 µg/ml fungizone, 100 units/ml penicillin and 100 µg/ml streptomycin and 25 mM Hepes. See details in Supplementary Methods.

***In vivo* studies.** Apo E-KO mice (C57BL/6J, Charles River) were fed either control chow or an atherogenic diet for 1 week. Mice were euthanized and aortas were removed, and immediately snap-frozen. Adult male Wistar rats were euthanized and tissues removed and

frozen. Euthanasia of the animals was performed in a CO_2 chamber followed by cervical dislocation. Euthanasia was confirmed by checking for lack of response to limb and tail pinch. All experiments were conducted in accordance with approved institutional operating protocols and the Directive 2010/63/EU of the European Parliament. See details in Supplementary Methods.

Rapid amplification of cDNA ends (RACE). The TSSs of human *Mfn2* in skeletal muscle were identified using the FirstChoice® RACE-Ready cDNA (Ambion) following the manufacturer's protocol. See details in Supplementary Methods.

Construction of luciferase reporter vectors. The pGL3-promoterless vector was generated by excision of the SV40 promoter by digestion with BglIII/HindIII. The -1982/+45 *Mfn2* promoter fragment was cloned into the SacI/SmaI sites of pGL3-promoterless vector (Promega). The subsequent 5' deletion constructs were produced by digestion and religation. See details in Supplementary Methods.

Transfection and luciferase assays. VSMCs, C3H10T1/2 or L6E9 cells were seeded at a density of 25,000 cells per well, 24 hours before transfection. pGL3 reporter constructs (250 ng) and 20 ng of Renilla luciferase were transfected using FuGENE 6. At 40 hours post-transfeccion, luciferase and Renilla activity was measured using the Dual Luciferase Reporter Assay System (Promega). See details in Supplementary Methods.

Site-Directed Mutagenesis. A *QuikChange® Site-Directed Mutagenesis Kit* (Stratagene) was used to introduce mutations into two putative Inr sites (mutI2: 5'-agtcgcggggcagcGgCcgcgtaaggagtaggcg-3'; mutI5: 5'-

acccgggtcgaggCgGCCGctgaggcgactggtgacg-3'; mutations are in capitals), following the manufacturer's protocol.

Bisulfite modification of genomic DNA, PCR and sequencing. Evaluation of CpG methylation status of the samples was performed by bisulfite sequencing. Genomic DNA from different sources was modified with the EzWay DNA Methylation Detection Kit (Koma Biotech) according to manufacturer's protocol. Modified gDNA was used to PCR-amplify a 519 bp region spanning from -489 to +29 of the *Mfn2* gene, and sequenced. See further details in Supplementary Methods.

RNA purification and measurement of mRNA expression. Total RNA was purified using TRIZOL Reagent (GibcoBRL, Invitrogen) and mRNA expression was determined by real time-PCR, as described in Supplementary Methods.

Chromatin immunoprecipitation. Chromatin was isolated from rat L6E9 cells and VSMCs. Cells were cross-linked using a final concentration of 1% formaldehyde for 10 min. The cross-linking reaction was stopped by the addition of 1.25 M glycine. Cross-linked cells were washed, collected, and lysed. Nuclei were then lysed in 50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS and protease inhibitor cocktail (Roche). Isolated chromatin was then sonicated to an average fragment size of 400 to 500 bp. Precleared chromatin was then immunoprecipitated with antibody against Sp1 (sc-59 X, Santa Cruz Biotech) and pre-blocked Protein A beads (Sigma). See further details in Supplementary methods.

Statistical analysis. Results represent the mean±S.E.M. of 3-5 experiments. Statistical differences were evaluated using Student's t-test or ANOVA and further post-hoc t-test.

Results.

The *Mfn2* gene contains a CpG island that is non-methylated under conditions characterized by large differences in *Mfn2* expression.

The human *Mfn2* gene is located in locus 1p36.22 on chromosome 1. *Mfn2* has 19 exons and translation starts in exon 3. *In silico* analysis of the 5'-flanking region of human *Mfn2* identified putative binding sites for several transcription factors, including C/EBP and GATA-1, which may mediate tissue-specific expression of *Mfn2*, and stress-response factors such as NFκB and AP-1 (Figure 1A). Estrogen-related receptor-α (ERRα)-binding element, which is required for PGC-1α and PGC-1β stimulation of *Mfn2* promoter is also shown[14-15]. The region near exon 1 is TATA-less, but it contains a G/C-rich region bearing several potential Sp1-binding motifs (Figure 1A). The mouse (Figure 1B) and rat (Supplementary Figure 1) *Mfn2* promoters showed a high identity to the human promoter and also contained putative binding sites for ERR-α, C/EBP, GATA-1, NFκB, AP-1 and Sp1 transcription factors.

CpG islands are defined as sequences larger than 200 bp with a proportion of C+G greater than 50% and with a CpG observed/CpG expected ratio of 0.6 or higher[16]. Using the CpGPlot program, analysis of the human *Mfn2* promoter region from -600 to +600 (relative to the transcription initiation site) revealed a CpG island that lies between -266 and +362 (Supplementary Figure 2). A CpG island was also detected in the mouse *Mfn2* promoter between -316 and +225, and rat *Mfn2* promoter between -278 and +113 (data not shown). Based on the CpG ratio, GC content and length of the CpG-rich region, the *Mfn2* promoter is defined as a High-CpG promoter (HCP) that represents a strong CpG island[17].

In vertebrates, CpG islands are predominantly non-methylated[18]. Non-methylated CpG islands are organized in a characteristic chromatin structure that predisposes them

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toward promoter activity. As *Mfn2* expression has been shown to be ubiquitous, we

hypothesized that *Mfn2* promoter CpG island should be in a non-methylated state *in vivo*. To

this end, we selected cells or tissues with different expression levels of *Mfn2*. This included

VSMCs or skeletal muscle cells (L6E9) before and after differentiation (Figure 2A and data

not shown), rat INS-1 insulinoma, rat H4IIE hepatoma or rat FAO hepatoma tumoral cell

lines (Figure 2B) or different rat tissues (Figure 2C). Proliferating VSMCs and L6E9

myoblasts showed a low *Mfn2* expression compared to differentiated cells (Figure 2A and

data not shown), and tissues or rat tumour cells showed variable expression levels of *Mfn2*

(Figures 2B and 2C). The bisulfite genomic DNA sequencing technique was used to

determine the DNA methylation status of a fragment of rat *Mfn2* promoter region (-262 to -

52), containing 15 individual CpG sites (Figure 2D). Sequencing data evidenced the absence

of methylation in any CpG site, regardless of the cell or tissue analyzed. As an example we

show a 41 pb gDNA fragment (-107 to −67) of rat *Mfn2* promoter region containing 4 CpG

sites (Figure 2E, first sequence), the same sequence after bisulfite treatment (Figure 2E,

second sequence), and the sequencing result of not differentiated VSMCs (Figure 2E, first

electrospherogram). The methylation control showed protection against bisulfite treatment for

all cytosine residues analyzed (Figure 2E, second electrospherogram). Thus, our results

indicate the absence of *in vivo* methylation in the rat *Mfn2* promoter.

Mfn2 shows multiple transcription start sites.

TATA-less promoters that contain CpG islands usually initiate transcription from multiple

weak start sites, which are often distributed over a region of about 100 bp[19]. 5'-RACE was

performed to determine whether transcription start sites (TSSs) other than those found in the

GeneBank and localized at +1 and +142. We used a commercial 5'-RACE-ready kit with an

adapter linked only to mRNA with CAP (from human skeletal muscle) to avoid the

amplification of partially degraded mRNA, and nested primers located in exon 2. Several

bands were detected after 5'-RACE (Supplementary Figure 3A), which were cloned in the T-

A cloning vector pGEM-T. The sequence of 22 randomly selected clones revealed two

predominant TSSs, one in a narrow window between -7 and -4 (8 clones; TSS2) and another

at position +58 (8 clones; TSS5) (Supplementary Figure 3B). Additional TSSs were detected

at -47 (2 clones; TSS1), +37 (1 clone; TSS3), +52 (2 clones; TSS4) and +124 (1 clone; TSS6)

(Supplementary Figure 3B). Three of these start sites showed homology with Inr motifs.

Transcription in Inr core promoters can initiate in a single site or in a cluster of multiple sites

in the vicinity of Inr, and not necessarily at A_{+1} position, which seems to be the case of

TSS2[20]. It is likely that the TSS localized at +142 according to GeneBank corresponds to an

incomplete sequence. To examine the role of the most prevalent TSS of human *Mfn2*, we

cloned the *Mfn2* fragment -229/+348 into a luciferase reporter plasmid and performed 5'

deletions. In transiently transfected 10T1/2 cells, the construct -229/+348-Luc exhibited 200-

fold greater luciferase activity than the promoter-less construct, and 5' deletions up to +84

produced a progressive loss in promoter activity (~70-fold greater activity than the promoter-

less construct) (Figure 3A). Construct +123/+348-Luc did not show significant differences in

luciferase activity compared to the promoterless vector (Figure 3A). Although the +123/+348

Mfn2 fragment maintained the TSS6, its loss of promoter activity may be attributable to the

lack of 5' sequences required for the positioning of basal transcription machinery. Given that

TSS2 and TSS5 are the two most represented TSSs found by RACE analysis, we next

examined their relevance for *Mfn2* promoter activity. To this end, the related Inr elements in

TSS2 and TSS5 in -229/+348-luc were subjected to mutagenesis by substituting the A_{+1} and

the base at position +3 by G and C, respectively, which greatly reduces Inr activity both *in*

vitro and *in vivo*[21]. As commonly found in other CpG island-containing promoters(18), single

TSS2 or TSS5 mutations did not modify the activity of the -229/+348 promoter in transient transfection assays, but double mutants showed a 50% reduction in this activity (Figure 3B).

Identification of the core promoter of human *Mfn2*.

Next, we aimed to identify the functionally relevant *cis*-regulatory elements in the human *Mfn2* promoter. Several fragments spanning from -1982 to +45 and -54 to +45 (all containing TSS1, TSS2 and TSS3) were cloned in a luciferase reporter vector and the resulting constructs were transiently transfected in rat VSMCs or in murine 10T1/2 cells, which show endogenous expression of *Mfn2*[8-9]. The -1982/+45-Luc construct showed 25% stronger promoter activity than the viral SV40 promoter when transfected in 10T1/2 cells (data not shown). In VSMCs and 10T1/2 cells, 5' deletion from -1332 to -532 caused a marked increase in promoter activity, suggesting that repressor factors may negatively regulate *Mfn2* expression in these cells by binding to sequences within -1332/-532 (Figure 4A). Furthermore, deletion of the region between -229 to -54 markedly reduced promoter activity in VSMCs and 10T1/2 cells (Figure 4A). Elimination of this region, while maintaining upstream and downstream sequences, also caused an 80% reduction in the promoter activity in 10T1/2 cells (Figure 4B). Altogether, our data indicate that the region -229/-54 is critical for maintaining high transcriptional activity of the human *Mfn2* promoter in VSMCs and fibroblasts.

Regulatory role of Sp1.

The -229/-54 region of the *Mfn2* promoter is a GC-rich region with multiple consensus binding sites for Sp1 (Figure 1). It has been suggested that in promoters contained within CpG islands, Sp1 would direct the basal transcriptional machinery to form a pre-initiation complex within a loosely defined window(18). To examine whether *Mfn2* promoter

activity is affected by the Sp1, we performed transient transfection experiments with the -1984/+45-pGL3 construct and expression vectors encoding Sp1. In 10T1/2 cells, Sp1 induced a 3-fold increase in luciferase activity (Figure 5A). In VSMCs, Sp1 also enhanced the promoter activity (4.5-fold) (Figure 5A).

In order to assess Sp1 binding to *Mfn2* promoter, chromatin immunoprecipitation (ChIP) assays were performed. Endogenous chromatin of primary rat VSMCs or L6E9 muscle cell cultures was immunoprecipitated with or without anti-Sp1 antibodies. The precipitated genomic DNA was then analyzed by PCR using the primers encompassing the Sp1-binding site in the *Mfn2* promoter. Chromatin fragments containing the Sp1 DNA binding site from the *Mfn2* promoter region were specifically immunoprecipitated by anti-Sp1 antibody (Figure 5B) showing specific amplification band for both VSMCs and L6E9 cells. No chromatin fragments were immunoprecipitated in the absence of antibody (Figure 5B). Binding of Sp1 to the promoter region of *Mfn2* was specific, as no chromatin fragments in the coding region of *Mfn2* (exon 2) or in the intergenic region ∼10 kb upstream of the *Mfn2* transcription start site were immunoprecipitated with the anti-Sp1 antibody (Figure 5B). Thus, endogenous Sp1 binds to *Mfn2* promoter in primary VSMCs and L6E9 cells.

Considering that Sp1 binds to *Mfn2* promoter and increases *Mfn2* promoter activity in cell cultures, we wanted to assess if this effect/phenotype was also present in a more physiological *in vivo* model. Consistent with previous studies showing reduced *Mfn2* expression in arteries from Apolipoprotein E-null mice (ApoE-KO) subjected to a high-fat diet (6), we found a marked reduction in Sp1 mRNA levels in aorta from Apo E-KO mice fed an atherogenic diet for 1 week (50% decrease versus control diet) (Figure 5C, left panel). Under these conditions, *Mfn2* mRNA levels also underwent a significant reduction (60% decrease) (Figure 5C, right panel). The effects of an atherogenic diet were transient and no effects on Sp1 or *Mfn2* gene expression were detected in aorta after more prolonged treatment

(2 months) (data not shown). These data suggest that downregulation of Sp1 at the onset of atherosclerosis contributes to reduced *Mfn2* transcription in aortic tissue. A similar co-regulation of Sp1 and Mfn2 was detected upon culturing VSMCs in normal (10% FBS) or low serum (0.2% during 48h) conditions. Total RNA was obtained and real-time PCR assays were performed. Sp1 gene expression was lower in VSMCs cultured in 0.2% FBS than in the normal serum condition (Figure 5D, left panel). Accordingly, *Mfn2* mRNA levels were also downregulated in this situation (Figure 5D, right panel).

Searching for the *in vivo* effects of Sp1 depletion on *Mfn2* protein expression, a shRNA approach was used. In order to knock down Sp1 expression, we infected VSMCs and L6E9 cultures with lentivirus encoding specific Sp1 shRNA (shSp1) or scramble control sequences (C). Western blot analysis revealed an almost total depletion of Sp1 protein in shSp1 transduced VSMCs and an 85% downregulation in shSp1 transduced L6E9 cells (Figure 6A and B).

In order to evaluate the impact of Sp1 loss-of-function on *Mfn2* promoter activity control and Sp1 depleted cells were transfected with the -532/+45-pGL3 or -229/-54-pGL3 and promoter activity was tested. As expected, the luciferase assay showed a significant decrease in promoter activity in Sp1 knock-down cells compared to controls (Figure 6C), The construct containing the -1984/+45 promoter region showed the same result (data not shown). Thus, these data confirms the essential role of Sp1 in *Mfn2* promoter activation.

Sp1 consensus binding sites are target for specific inhibitors. Thus, next we examined the role of Sp1 in the control of *Mfn2* gene expression, by using WP631, a specific inhibitor of Sp1[22-23]. For this purpose, L6E9 cells transfected with -532/+45-pGL3 or -229/-54-pGL3 constructs were incubated in the presence of 300 nM WP631 during 24h. As shown in Figure 6D, WP631 caused a 90% inhibition in luciferase activity for both constructs. In order to further assay WP631 action, we incubated primary rat VSMCs with the compound and

analyzed *Mfn2* transcript expression. Incubation of VSMCs with different nanomolar

concentrations of WP631 for 24 hours caused a significant reduction of *Mfn2* mRNA levels

(Figure 6E). In addition, incubation with WP631 markedly reduced *Mfn2* mRNA levels in

MCF-7/VP cells (data not shown), in agreement with a direct effect of WP631 on Sp1-

regulated *Mfn2* transcription.

Discussion.

In the present study, we have both identified and characterized the promoter regions of human, mouse and rat *Mfn2* genes. These promoters contain a region of approximately 500 bp which satisfies the criteria for the presence of a strong CpG island(15, 16). Under *in vivo* conditions no evidence for methylation has been found, which is in agreement with data indicating that strong CpG islands-containing promoter are not methylated[17, 24]. We have also shown that the human *Mfn2* promoter is TATA-less and that the -229/+123 region, which contains 6 putative binding sites for the transcription factor Sp1, is essential for normal transcriptional activity. In keeping with the above, human *Mfn2* shows 6 transcriptional start sites. The functional relevance of Sp1 transcription factor in the control of *Mfn2* expression is suggested by the following findings: a) Sp1 over-expression enhances the transcriptional activity of the *Mfn2* promoter, b) Sp1 ablation down-regulates *Mfn2* promoter activity in VSMCs and L6E9 cells; c) the stimulatory effect of Sp1 on *Mfn2* promoter activity is abolished pharmacologically by using the specific Sp1 inhibitor WP631, d) Sp1 binds *in vivo* to the *Mfn2* promoter; and e) Apo E-KO mice subjected to an atherogenic diet undergo a concomitant reduction in *Mfn2* and Sp1 mRNA levels in aorta. Altogether, our data demonstrate that the human *Mfn2* promoter belongs to the class of CpG dispersed promoters[25], in which methylation is prevented under *in vivo* conditions and in which transcription is driven and regulated by Sp1.

The *Mfn2* promoter represents a model to understand the elements responsible for the core promoter function of promoters located in CpG islands. This aspect is particularly relevant since the specific mechanisms that control the activity of promoters located in CpG islands are not well understood[19] in spite of the fact that almost 60% of the promoter regions of human genes are located within these islands[26]. Using 5'-RACE, we found that

transcription of *Mfn2* in human skeletal muscle can be initiated in at least 6 TSS, which show homology with Inr elements, a common feature of promoters in CpG islands[19]. The two main transcriptional start points identified by sequencing of 5'-RACE products, TSS2 and TSS5, were mutated to examine their functional importance. Individually, mutation did not change the strength of the promoter although the double mutant reduced promoter activity by 50%. These results indicate that the human *Mfn2* gene constitutes a redundant system capable of maintaining high promoter activity even after mutation of the most frequently used individual TSSs. In addition, our data demonstrate the presence of 5' heterogeneity of the *Mfn2* transcripts of human origin, which may have an impact on the biological activities of the products of this gene.

DNA methylation involves the addition of methyl groups to cytosine located 5' to guanine in CpG nucleotide pairs, and methyl-CpG is now recognized as a gene-silencing signal[27-28]. In healthy adult tissue, strong CpG islands are usually unmethylated. Thus, *de novo* methylation depends on the local CpG density, making it more likely for weak CpG islands[17]. In agreement with these observations, we found no methylation of the *Mfn2* promoter in cultured cells from tumor origin, adult rat tissues or rat VSMCs or skeletal muscle cells before and after differentiation. However, methylation of human *Mfn2* is undertaken *in vitro* by HpaII or SssI methylases (data not shown), which suggests that under *in vivo* conditions different factors may cause steric hindrance and prevent methylation. The fact that we did not found any tissue or cell type in which *Mfn2* expression was completely absent points to the essentiality of *Mfn2* function in cells. Thus, the demethylated CpG island status and the concomitant open chromatin structure of the promoter seem to be necessary to guarantee at least a minimal *Mfn2* promoter activity/*Mfn2* expression. Whether other pathological conditions cause methylation of the *Mfn2* promoter remains to be elucidated.

We have identified *cis*-regulatory elements and transcription factors involved in the regulation of human *Mfn2* promoter activity. In the cell types tested (rat VSMCs, and murine 10T1/2 fibroblasts), deletion of the -1332/-532 region enhanced promoter activity, thereby providing evidence for the operation of repressor factors. In addition, deletion of the -229/-54 region suppressed promoter activity, thereby providing evidence that this is a critical region of the core *Mfn2* promoter. Consistent with the observation that the presence of several putative Sp1-binding sites is a common feature of CpG islands[19], computer-assisted sequence analysis of the human *Mfn2* promoter revealed 6 potential consensus sequences for the Sp1 family of transcription factors in the -229/-54 region. Importantly, Sp1 overexpression increased *Mfn2* promoter transcription in fibroblasts and VSMCs, and this activation was abolished by *in vitro* promoter methylation. In addition, Sp1 binding to *Mfn2* promoter was confirmed for VSMCs and L6E9 cells, suggesting that its function as a transcriptional activator on *Mfn2* is not restricted to a specific cell type.

Further *in vivo* evidence demonstrated that in aortas from Apo-E –KO mice subjected to a high-fat diet, Sp1 as well as *Mfn2* expression were decreased. The effects of an atherogenic diet were transient and no effects on Sp1 or *Mfn2* gene expression were detected in aorta after more prolonged treatment (2 months). The transient repression of *Mfn2* detected early after the implementation of an atherogenic diet is similar to the transient repression of *Mfn2* detected in carotid arteries after a mechanical injury induced by angioplasty[6]. In keeping with the observations in aorta, VSMCs showed a parallel reduction in Sp1 and *Mfn2* mRNA expression levels when grown in low serum conditions. In addition, Sp1 inhibition caused *Mfn2* gene repression in VSMCs. Therefore, our studies provide solid evidence that Sp1 binds to *Mfn2* promoter and regulates *Mfn2* expression in several *in vivo* systems.

In conclusion, Sp1 binds to the *Mfn2* promoter in different cells types, which includes VSMCs and skeletal muscle cells, and transactivates the basal machinery of *Mfn2*. Sp1 has been reported to inhibit VSMCs proliferation by induction of p27 and by repression of p21 expression and cyclin D1-Cdk4-p21 complex formation[29-30]. In this study we provide evidence for a stimulatory role of Sp1 on *Mfn2* expression in VSMCs. Based on the antiproliferative action of *Mfn2* in VSMCs, our data reveal a new mechanism by which Sp1 may prevent the development of vascular proliferative disease.

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Figure Legends.

Figure 1. Nucleotide sequence of the 5' region of human (panel A) and mouse (panel B) *Mfn2*.

+1 indicates the transcription start site reported in NCBI for human *Mfn2* (NM 014874.2) and the homologous site for mouse *Mfn2* (NP 573464). The first exon is shaded. Putative transcription-regulatory motifs are in bold and underlined. Functional estrogen-related receptor-α (ERRα)-binding element is shown inside a rectangle.

Figure 2. Lack of methylation of the 5´-flanking region of human *Mfn2*.

A) Left: Western blot showing the expression of SMα-actin and the constitutive protein ERK2 in differentiated (D) and not differentiated (ND) VSMCs. Right: *Mfn2* and Acidic Ribosomal Protein (ARP) mRNA levels from differentiated and not differentiated VSMCs were measured by real time-PCR. (Mean ± S.E.M., * $p<0.01$)

B) and C) *Mfn2* and Acidic Ribosomal Protein (ARP) mRNA levels from INS-1, H4IIE and FAO cell lines, and from lung, testis, kidney and heart from adult rat, and aorta from young and old rat were measured by real time-PCR. (Mean ± SEM)

D) Above: Bisulfite modified gDNA from differentiated (D) and not differentiated (ND) VSMCs, L6E9 myoblasts (Mb) and myotubes (Mt), and methylation control (Met) were used as template for bisulfite sequencing analysis. Second round PCR generated a single 312 bp product. N, negative control. *, DNA-ladder. Below: 312 bp-PCR product and its position in the *Mfn2* promoter (-315 to -4). The grey fragment of 211 pb (-262 to -52), corresponds to the sequencing result.

E) Representative direct sequencing results showing 4 out of the 15 CpG sites studied. Original gDNA (-107 to -67) with the CpG sites indicated in bold, bisulfite modified gDNA, sequencing result of bisulfite modified unmethylated gDNA (in this case not differentiated

VSMCs) and methylation control were shown. ↓: SssI methylated cytosine from the CpG motives.

Figure 3. Functional analysis of the proximal human *Mfn2* promoter.

A) The region -229/+348 and deletions at 5' of human *Mfn2* were cloned in the promoterless pGL3 reporter vector and cotransfected with Renilla reporter plasmid in 10T1/2 cells. Transcriptional activity was normalized for the level of Renilla activity and expressed as fold activity of pGL3 promoterless vector.

B) The two main transcription start sites (TSS2 and TSS5) were mutated in the construction -229/+348-Luc as indicated (crossed) and cotransfected with Renilla reporter plasmid in 10T1/2 cells. Transcriptional activity was normalized for the level of Renilla activity and expressed as fold activity of -229/+348-Luc wild type. (Mean ± S.E.M. of 3 experiments performed in triplicate;*, $p < 0.001$).

Figure 4. Functional analysis of human *Mfn2* promoter.

A) -1982/+45-Luc and 5' deletions, as indicated on the left panel, were cotransfected with Renilla reporter plasmid in 10T1/2 (□), and VSMCs (■). Cells were harvested 40 hours after transfection and luciferase activity was determined. Transcriptonal activity was normalized by Renilla and expressed as fold activity of pGL3 promoterless vector.

B) The region -229/-54 was deleted in the construction -532/+45-Luc by restriction digestion. Each construct was cotransfected with Renilla reporter plasmid in 10T1/2 cells. Transcriptional activity was normalized by Renilla and expressed as fold activity of -532/+45-Luc wild type vector. (Mean ± S.E.M. of 3 experiments performed in triplicate).

Figure 5. *Mfn2* promoter is regulated by Sp1 transcription factor.

A) The -1982/+45-Luc vector was cotransfected with GFP and Sp1 expression vectors in 10T1/2 cells (□) or was cotransfected with Renilla in VSMCs (■). Transcriptional activity was normalized and expressed as fold activation of -1982/+45-Luc (basal). (Mean ± S.E.M. of 3 experiments performed in triplicate;*, $p < 0.01$).

B) Chromatin immunoprecipitation of endogenous Sp1 in the *Mfn2* promoter. *In vivo* cross-linked chromatin from rat VSMCs or L6E9 cells was immunoprecipitated with anti-Sp1 or without antibody, followed by PCR amplification using specific primers for the *Mfn2* promoter, the exon 2 and an intergenic region ~10 kb upstream of the *Mfn2* transcription start site.

C) Apo E-KO mice were fed during 1 week with control chow (□) or atherogenic diet (■). Sp1 and *Mfn2* mRNA from aorta were measured by real time-PCR and expressed relative to Cyp mRNA levels. (Mean ± S.E.M. 4 pools of each diet, *, $p<0.05$).

D) Sp1 and *Mfn2* mRNA expression from rat VSMCs cultured in 10% or 0.2% FBS during 48h were determined by real-time PCR. (Mean ± S.E.M. of 3 experiments performed in triplicate;*, $p < 0.05$).

Figure 6. *Mfn2* promoter activity is abolished in response to Sp1 deficiency.

A) Western blot of total extracts obtained from rat VSMCs and L6E9 cells transduced with lentiviral vectors encoding Sp1 shRNA (shSp1) or scramble control sequences (C). Sp1 and Tubulin proteins were detected using specific antibodies. n=3-5.

B) Relative protein expression of Sp1 from rat VSMCs and L6E9 cultures transduced with lentiviral vectors encoding Sp1 shRNA (shSp1) or scramble control sequences (C) (Mean ± S.E.M. of 3-5 experiments, *, $p<0.05$).

C) The -532/+45-pGL3 or -229/-54-pGL3 vectors were cotransfected with Renilla expression vector in L6E9 cells transduced with lentiviral vectors encoding Sp1 shRNA (shSp1) or control plasmid (C). Transcriptional activity was expressed as fold change of control. (Mean ± S.E.M. of 3 experiments performed in triplicate;*, p< 0.01).

D) The -532/+45-pGL3 or -229/-54-pGL3 vectors were cotransfected with Renilla expression vector in L6E9 cells. Four hours after transfection, cells were incubated with 300 nM WP631 (WP631) or PBS (Basal) during 24h. Transcriptional activity was expressed as fold change of control. (Mean ± S.E.M. of 3 experiments performed in triplicate; *, p<0.01).

E) VSMCs were treated with various concentrations of WP631 for 24 hours. Thereafter, total RNA was extracted, and *Mfn2* and Cyclophilin A mRNA levels were analyzed by real time-PCR. (Mean ± S.E.M. of 5 experiments, *, p<0.05).

Figures

A Human Mfn2

```
-950   ttctcctgcctcttcctcccgagtgcactatcatgcctggctaattttttg
                                       Myogenin
-900   tattttttagtacagacggggtttcaccatgttggccaggctggtcttgaa
-850   ctcccgatgcacccgcctcgccctcctaaaatgctggaattacaggcttg
-800   agccaccgcgcccggtcgatatttttttttcctaaaaattgagatagttt
                         NFAT              GATA-1
-750   aacagcctttttttgtcctaagtcttatagcgcatctccgtttgcactggc
-700   tcaggactcgagagccacacgtggctagtgcctactgttctggcgcgctc
-650   aggcttggagccttctctgcggaggccaagtcctgcaagggatctgtttg
                                                    AP-1
-600   ctgcggtttcctagtagtgtctgccgcatcactggggcccggtgatctgg
-550   gtaaccatgtgagcttgggtaacctctcctctccctctcctgggactgct
                        C/EBP            NFκB
-500   gtggacgttaaatacagcggtggatgttagagacgcagttcagtcactgg
                                                AP-1
-450   cattgattcgccgttgttgctgctcttctttcctcaaaggcgactgaagg
                          C/EBP          ERRa
-400   gcagcaggcccatgctcgcttctgtctgcccgatgagtcacttcacccta
-350   ggcctggccctctagagaacactcctctaagattacagaatgcaaatctg
                                        C/EBP
-300   gattccagagctggcagaggcccccagggtcaggctctaggacaaccggcg
-250   aaaaagcgggaacctctactccacgcccttggttttttcgccccttgacag
                           Sp1
-200   cttgaggctccgccccgcttttgccccgcccccccagctccggccgacgcc
             Sp1          Sp1
-150   caccggaactacagcccccatgatgcagtgggagtccgagcctctgcgtc
                                       Sp1
-100   gtccgcttgggacgcgccggcggaggagtggcgcgcggaggagtggcgcg
                              Sp1            Sp1
-50    ctgagacgccgctcgaagcgccgagtcgcggggcagcagaggcgtaagga
+1     gtaggcggggcgagccggctgggctcagggtccaccagctcacccgggtc
+51    gagggcaatctgaggcgactggtgacgcgcttatccacttccctcctcc
+101   cgcctccccccggggtggcgctcgctggtgacgtagtgagtgtgatggcc
+151   gccgcgaggccgggaaggtgaaggtgagagggcgagcaagccggggtggc
+201   ggggactggcccggcccggcccggcgagtcctgagcagctcggcgtgggc
+251   cgacgggattctggggccacgggggtctgggatgcagtcaagcggggtcc
```

B Mouse Mfn2

```
-950   cttggtagcagccatttatacctactgagcctttttttgtgaccgccacta
-900   ttagctctactttttagaagtaagaagaaacggggtggggggggcgattta
                                         Sp1
-850   attaagataatgtatttcatttagcccagtttatccaaagctataacaac
       C/EBP   GATA-1                          GATA-1
-800   acatcaatataaaattactaataaggtagtttgcatttctttttatatta
                                                GATA-1
-750   aacattggggcatttctcagtttgtagcagctgcatttcagggtctcagt
-700   agctacaagtggctggtgggtgttactacagagcccaggcttggagccct
               AP-1                   NFκB
-650   cccagcttggggtccaatgcagtatcccagttcttggaacaaaatgaggg
-600   gccagtgaatggcatgtgaggttggataatcatgtcctgaatgtcctggg
                                  C/EBP
-550   actgagtgaaaattaaaaactgcatgttagatagccagtgcaatgactgt
          AP-1                                    AP-1
-500   cacctagtagctgcctgatgcgttgtggctgctgtttagttgcctcaaag
                             C/EBP
-450   gcaactgaaggacaattacttcaccctaacggtctgtttgttccctttga
       ERRa       RORα
-400   agacactcggattatgaaatgcgagtctggatttcaaaactgacaggtgt
-350   tatacagggtcaaattctggagcagcaacaacaaaaaaggcgcaacaaca
-300   aaaaaggcgcacgcaaacctctaatcggtacccttgtcgtgtcctgcccg
-250   ctccccttccccgccgtccacccctctccctccccaaccccgtcccgtt
       Sp1                     Sp1           Sp1
-200   ctcccacccctccaacccctgcttgacagctcgaggctccgccctacccc
                                            Sp1
-150   accctgacggacgctgatccaaactacaactcccatgacgcgctgggagc
       Sp1
-100   cccacccctacagcttacgtcatctgcacgagacacgggacggttaccagg
       Sp1
-50    cacgcggggggctccttttggagcgcggagttcctagacactgtggtatag
                Sp1
+1     gagtggtaaggttgaggtgacagcgttcagaggccatcggctcgtccggg
+51    ctgcagcggggcgagtgacgagcgcagcccccctcccctcccgcttctctc
+101   ggtgtggcgccggctagtgacgtgttgggtgtgatggccgccgcgaggcc
+151   gggaaggtgaaggtgagagggcgagcgcgccggggtggtggaaccggcag
+201   gcccgggccgggagagtcctgagccacccggcttaggacgctgggcctct
+251   ggggcctctgggcctcgggctcaggctcgggtcacacgggcgtttcccct
```
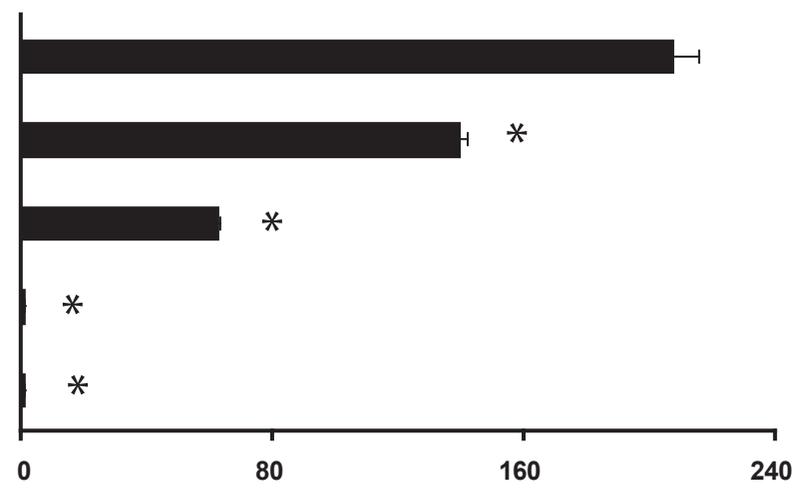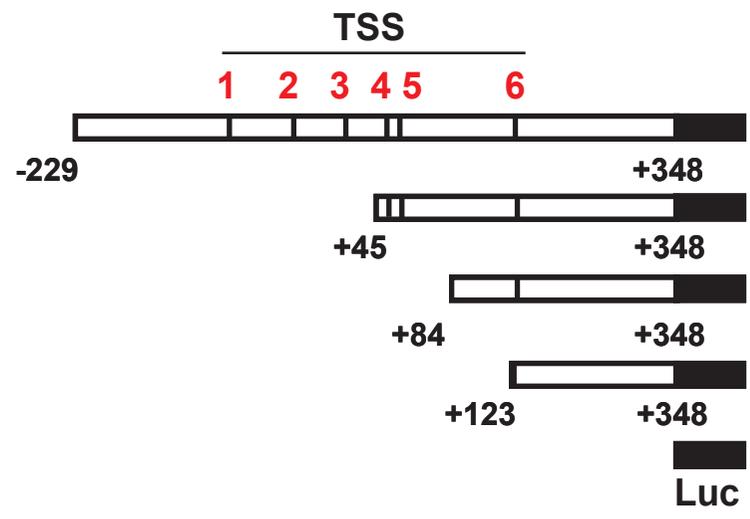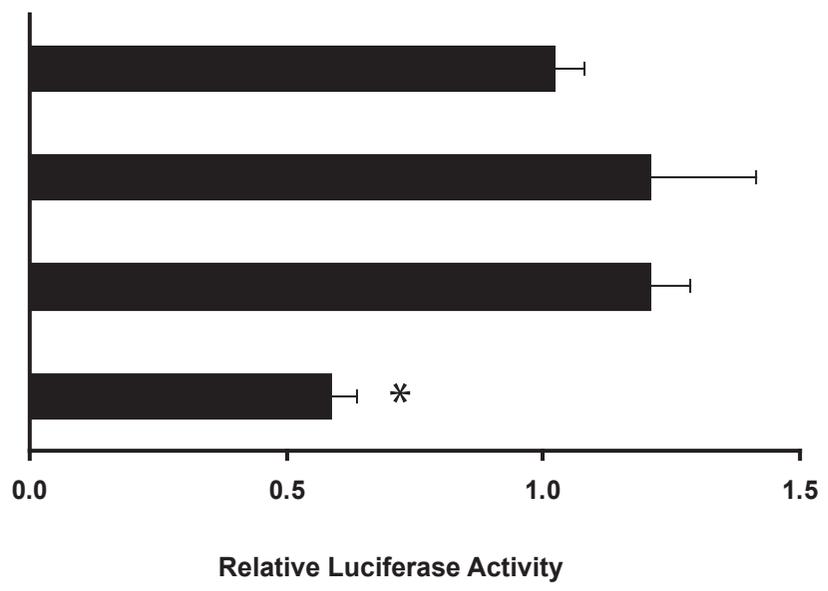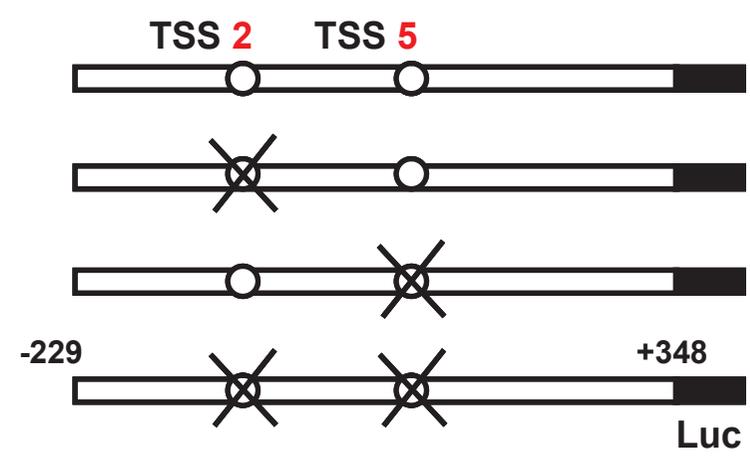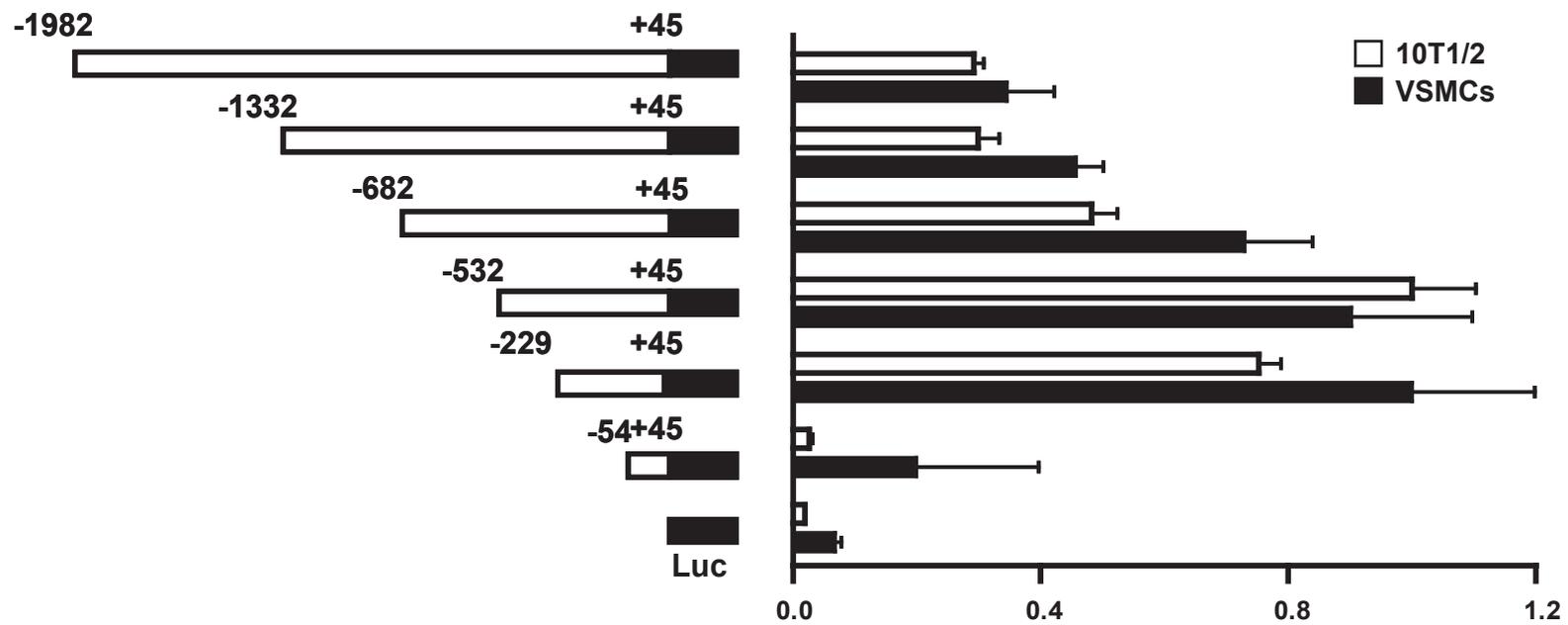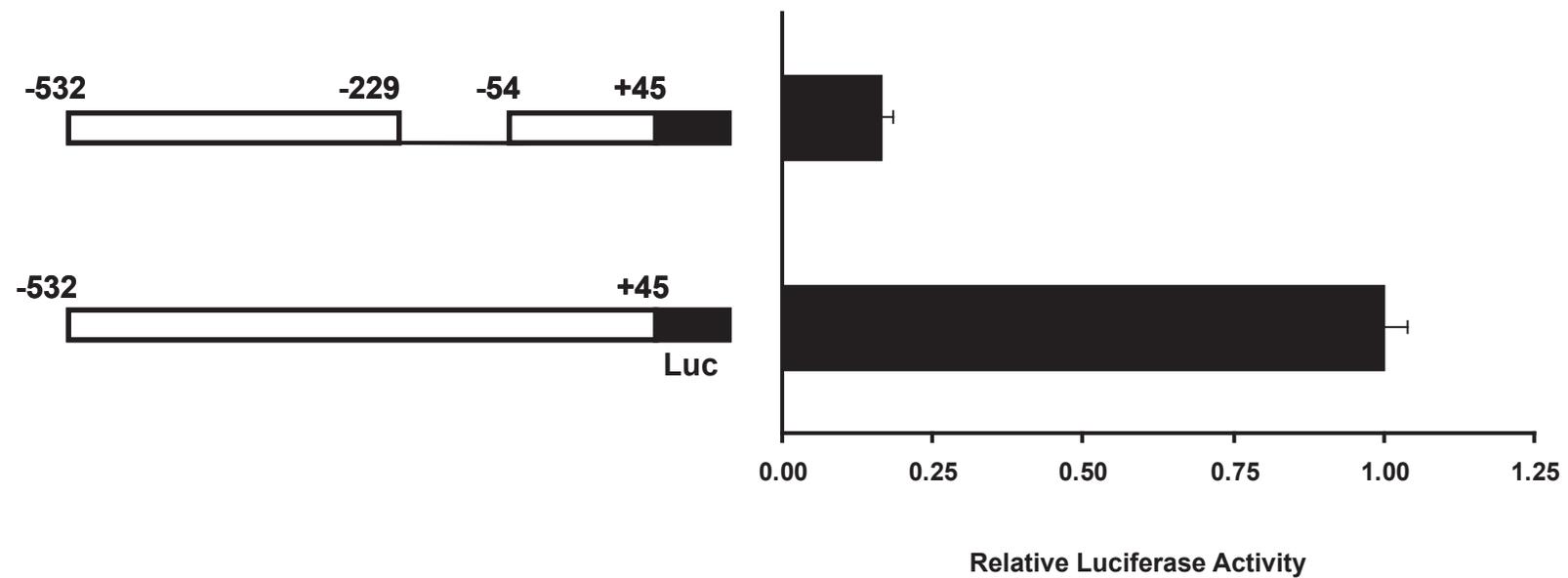
FIGURE 1

A

SMα-actin

ERK2

D   ND   kDa

- 37

- 50

- 37

Mfn2 / ARP

0.02

0.015

0.01

0.005

0

*

D   ND

B

Mfn2 / ARP

0.03

0.025

0.02

0.015

0.01

0.005

0

INS-1   H4IIE   FAO

C

Mfn2 / ARP

0.7

0.6

0.5

0.4

0.3

0.2

0.1

0

Lung   Testis   Kidney   Heart   Young aorta   Old aorta

D

VSMCs   L6E9

Met   N   *   D   ND   Mb   Mt

312 bp

-315 →   ← -4

-262   -52

E

-107   -67

Genomic DNA   CAT**CG**GTTCATC**CG**GGCTTCAGCAGGGCTAGTGA**CG**AG**CG**C

Bisulfite modified DNA   TAT**TG**GTTTATT**TG**GGTTTTAGTAGGGTTAGTGA**TG**AG**TG**T

ND VSMCs

TATTGGTTTATTTGGGTTTTAGTAGGGTTAGTGATGAGTGT

Methylation control

↓   ↓   ↓   ↓

TATCGGTTTATTCGGGTTTTAGTAGGGTTAGTGACGAGCGT

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

FIGURE 6

Supplementary Data

Supplementary Methods

Cell culture. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 ºC. C3H10T1/2 cells were grown in DMEM supplemented with 10% FBS and 100 units/ml penicillin and 100 µg/ml streptomycin. Primary rat aorta VSMCs were prepared as previously described[1] and were grown in DMEM-F12 (1:1) supplemented with 10% FBS, 2 mM glutamine, 0.25 µg/ml fungizone, 100 units/ml penicillin and 100 µg/ml streptomycin and 25 mM Hepes. Preconfluent VSMCs were induced to differentiate by incubating them in medium containing 0.5% FBS during 48h. The L6E9 rat skeletal muscle cell line was cultured in DMEM containing 10% FBS, 100 units/ml penicillin G and 100 µg/ml streptomycin and 25 mM Hepes (pH 7.4) (Growth Medium). Pre-confluent myoblasts (80-90%) were induced to differentiate by lowering FBS to a final concentration of 2% (vol/vol) (Differentiation Medium). Cells were completely differentiated after 4 days in this medium. INS-1 rat insulinoma cell line was cultured in RPMI 1640 supplemented with 10% FBS, 10 mM Hepes (pH 7.4), 1 mM sodium piruvate, 50 µM beta-mercaptoethanol, 2 mM glutamine, 100 units/ml penicillin G and 100 µg/ml streptomycin. H4IIE rat hepatoma cell line was cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin G and 100 µg/ml streptomycin. FAO rat hepatoma cell line was cultured in F-12 Coon´s Modification (Sigma) supplemented with 10% FBS, 2 mM glutamine, 100 units/ml penicillin G and 100 µg/ml streptomycin.

***In vivo* studies.** Four-month-old Apo E-KO mice (C57BL/6J, Charles River) were fed either control chow or an atherogenic (ATG) diet for 1 week (10.8% total fat, 0.75% cholesterol, S8492-E010, Ssniff, Germany). After sacrifice, the aorta was collected (pooled from 1 male and one female), immediately snap-frozen and stored at -80ºC. For some other purposes, tissues from adult male Wistar rats (Harlan Laboratories, Spain) were obtained, immediately snap-frozen and stored at -80ºC. Ascending aorta and aortic arch from young (2 months) and old (24 months) male rats were collected and frozen immediately in liquid nitrogen. Aortic tissues were pooled (each pool contained the ascending aorta and the aortic arch from the same rat). All experiments were conducted in accordance with approved institutional operating protocols and the Directive 2010/63/EU of the European Parliament.

Rapid amplification of cDNA ends (RACE). The TSSs of human *Mfn2* in skeletal muscle were identified using the FirstChoice® RACE-Ready cDNA (Ambion) following the manufacturer's protocol. The 5' end of human *Mfn2* cDNA was amplified using a standard *nested* PCR protocol[2]. To avoid the amplification of partially degraded mRNA an adapter linked only to mRNA with CAP (from human skeletal muscle) was used. Primers were designed to match sequences in exon 2: 5'-agattctgagttctcttctgtccta-3' and nested 5'-ttgattgactgtgttgactccaccag-3'. The resulting PCR products were purified, cloned in pGEM-T easy (Promega) and sequenced.

Construction of luciferase reporter vectors. The pGL3-promoterless vector was generated by excision of the SV40 promoter by digestion with BglIII/HindIII. The -1982/+45 *Mfn2* promoter fragment was cloned into the SacI/SmaI sites of pGL3-promoterless vector (Promega). The subsequent 5' deletion constructs were produced by double digestion and religation. Forward primer 5'-cagggtcaggctctaggaca-3' and reverse primer 5'-gcttgactgcatcccagac-3' were used to generate the human -276/+275 *Mfn2* promoter region. The amplified DNA fragment was digested with SacI/NcoI and cloned into SacI/NcoI sites of pGL3-promoterless vector (Promega). The subsequent 5' deletion constructs were produced by double digestion and religation. The construct -532/+45Δ-229/-54-pGL3 was produced by double digestion BssHII/BstXI of the construct -532/+45-pGL3 and subsequent religation.

Transfection and luciferase assays. VSMCs, C3H10T1/2 or L6E9 cells were seeded into 24-well plates at a density of 25,000 cells per well, 24 hours before transfection. pGL3 reporter constructs (250 ng) and 20 ng of Renilla luciferase (used as an internal control for transfection efficiency) were transfected using FuGENE 6 (Roche), following the manufacturer's protocol. At 40 hours post-transfeccion, luciferase and Renilla activity was measured using the Dual Luciferase Reporter Assay System (Promega), following the manufacturer's protocol. For Sp1 transfections, 250 ng of the expression plasmid[3] and 50 ng of GFP in C3H10T1/2 cells or 40 ng of Renilla luciferase in VSMCs, together with 250 ng of pGL3 reporter construct, were transfected. GFP was measured by flow cytometry. Expression of reporter genes was normalized to GFP measured by flow cytometry or Renilla activity.

RNA purification and measurement of mRNA expression. RNA was extracted with TRIZOL Reagent (GibcoBRL, Invitrogen) according to manufacturer's instructions. The

Invitrogen's SuperScrip™ II Reverse Transcriptase was used to obtain cDNA from RNA. One or two µg of RNA was used for retro-transcription. Real time-PCR was performed with specific primers and using SYBR-Green PCR Master Mix (Applied Biosystems) according to manufacturer's instructions. In all cases, a single PCR product was detected. Results were expressed as number of mRNA copies relative to Cyp gene expression. For the Apo E-KO mice experiment, fold change values were calculated as the ratio of the ΔCt sample averages. Relative quantification (RQ) is defined as $2^{-\Delta\Delta Ct}$. For each gene, results in ATG diet are normalized versus control diet (RQ=1). PRIMERS: rat Cyp (rCyp-f ctttgacttgcgggcattttac ; rCyp-r aagaacttcagtgagagcagagattacagg), rat ARP (rARP-f gagccagcgaagccacact ; rARP-r gatcagcccgaaggagaagg), rat *Mfn2* (rMfn2-f gacctgcaccgagaagagaca ; rMfn2-r ctgcaggggggtagggagtaac), rat Sp1 (rSp1-f cggcaaagtatatggcaaaacct ; rSp1-r agtaaagcgcttcccacagtatga), mouse Cyp (mCyp-f caaatgctggaccaaacacaaac ; mCyp-r tgccatccagccattcagtc), mouse *Mfn2* (mMfn2-f gtaagggctcggagaaggtatgtg ; mMfn2-r tggcaagaagggaggcaagtc), mouse Sp1 (mSp1-f catgccaggcctccagacc ; mSp1-r caccagatccatgaagaccaagtt).

Bisulfite modification of genomic DNA, PCR and sequencing. Genomic DNA (gDNA) purification was performed using the traditional phenol/chlorophorm/isoamyl alcohol method. gDNA isolated from not differentiated and differentiated rat VSMCs and L6E9 cell line as well as from tumoral cell lines and rat tissues were modified with the EzWay DNA Methylation Detection Kit (Koma Biotech) according to manufacturer's protocol. Briefly, 1 µg gDNA was treated with sodium bisulfite to modify unmethylated cytosine residues to uracil, while 5-methyl cytosines remain unaltered. Modified gDNA was purified using the Qiaquik PCR Purification Kit (Qiagen). A 519 bp region spanning from -489 to +29 of the *Mfn2* gene was hot start PCR amplified using forward degenerated 5'-aygtaagtttggattt-3' and reverse 5'-cctctcaccttcacct-3' primers that recognized bisulfite modified DNA. PCR conditions were: 10 min at 94°C, (30 s at 94°C, 35 s at 52°C, and 40 s at 72°C) for 40 cycles, and 5 min at 72°C. Then, a 1/10 dilution of the first round PCR products was subjected to a nested second round hot start PCR amplification using forward degenerated 5'-tgatagttygaggtttygt-3' and reverse 5'-accatcacacccaac-3' primers, yielding a product of 312 pb (-315 to –4). Second round PCR conditions were: 10 min at 94°C, (30 s at 94°C, 35 s at 56°C, and 40 s at 72°C) for 40 cycles, and 5 min at 72°C. Not bisulfite treated genomic DNA and bisulfite-treated *in vitro* SssI methylated rat DNA were used as controls. Water without addition of DNA served as negative control. The last PCR products (312 bp) were run on 2% agarose gels and stained

with ethidium bromide. 1 Kb Plus-DNA-ladder (Gibco) was used. The bands were gel purified using the illustra GFX PCR DNA and Gel Band Purification Kit (Amersham), and sequenced with the same second round PCR primers using the ABI BigDye V3.1 sequencing kit (Applied Biosystems) and an automated DNA sequencer (3730XL Applied Biosystems). Therefore, we amplified a bisulfite treated DNA fragment containing 22 CpG dinucleotides, and were able to analyze 15 individual CpG sites.

Chromatin immunoprecipitation. Chromatin was isolated from rat L6E9 cells and VSMCs grown in high (10% FBS) or low serum (0.2% FBS during 48h) conditions. Briefly, cells were cross-linked using a final concentration of 1% formaldehyde for 10 min. The cross-linking reaction was stopped by the addition of 1.25 M glycine. Cross-linked cells were washed in 1x PBS with 0.5 mM PMSF and collected. Cells were resuspended in cell lysis buffer (10 mM EDTA, 0.5 mM EGTA, 10 mM Hepes at pH 6.5, 0.25% TX-100 and protease inhibitor cocktail). Nuclei were then lysed in 50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS and protease inhibitor cocktail (Roche). Isolated chromatin was then sonicated to an average fragment size of 400 to 500 bp. Precleared chromatin was then immunoprecipitated with antibody against Sp1 (sc-59 X, Santa Cruz Biotech) and pre-blocked Protein A beads (Sigma). Immunoprecipitation with no antibody was performed as a control for nonspecific interaction of DNA. Samples were washed in buffer 1 (0.1% SDS, 1% TX-100, 2 mM EDTA, 20 mM Tris-HCl at pH 8.1 and 150 mM NaCl), followed by another wash using buffer 2 (buffer 1 with 500 mM NaCl). Following this, samples were washed with buffer 3 (10 mM Tris-HCl at pH 8, 0.25 M LiCl, 1% NP-40, 1% deoxycholate and 1 mM EDTA) and then 1x TE (pH 8). Precipitated chromatin was then subjected to reverse cross-link overnight and purified by phenol/chloroform extraction. Input and immunoprecipitated DNA were subjected to PCR analysis with primers flanking the Sp1 site on the *Mfn2* promoter. The coding region 2 and an intergenic region ∼10 kb upstream of the *Mfn2* transcription start site were also amplified as controls for nonspecific binding of genomic DNA. The following primers were used: for the *Mfn2* promoter (forward: 5'-GCCTCTGAACACCGTCACTT-3' and reverse: 5'-CGCACCCTTATCGTTTTTGT-3'), for the *Mfn2* exon 2 (forward: 5'-CACCATCAGTAGCCAATCTGGA-3' and reverse: 5'-TTCACAGGTTGGGCATCATG-3') and for the intergenic region (forward: 5'-GCCTTGGGATGATCCTCCTT-3' and reverse 5'-CCCTAACTAAAGCAAACAGGCATG-3').

Western blot. Cells were collected in ice-cold PBS 1X and homogenized using a dounce homogenizer in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM sodium ortovanadate, 50 mM NaF, 20 mM sodium pyrophosphate and protease inhibitors cocktail tablet, Roche). Fifteen micrograms proteins from each sample were resolved in 7.5% acrylamide gels for SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, USA). The following antibodies were used: Sp1 (1:1000, Santa Cruz), and tubulin (1:8000, Sigma). Peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins (1:10000) were used as secondary antibodies.

shRNA. For Sp1 expression silencing, 5 different mouse shRNA sequences (MISSION shRNA clones, Sigma-Aldrich) included in shRNA lentiviral plasmids (pLKO.1-puro) were used. Clone ID, target sequence: TRCN0000071603, CCGGGCAGCAGTAATACCACCCTAACTCGAGTTAGGGTGGTATTACTGCTGCTTT TTG; TRCN0000071604, CCGGCCCAACTTACAGAACCAGCAACTCGAGTTGCTGGTTCTGTAAGTTGGGTTT TTG; TRCN0000071605, CCGGGCAGGATGGTTCTGGTCAAATCTCGAGATTTGACCAGAACCATCCTGCTTT TTG; TRCN0000071606 CCGGCCTTCACAACTCAAGCTATTTCTCGAGAAATAGCTTGAGTTGTGAAGGTTTT TG; and TRCN0000071607 CCGGCCAATGCCAATAGTTATTCAACTCGAGTTGAATAACTATTGGCATTGGTTTT TG.
shRNA lentiviral non-target control plasmid (MISSION® Non-Target shRNA Control Transduction Particles, Sigma-Aldrich) was used as a control.

Lentivirus packing and infection. Lentiviruses encoding scrambled and Sp1 shRNA were produced by triple transient transfection of HEK293T [4]. Briefly, the lentivirus expression plasmid (pLKO.1-puro-shSp1 or control vector), together with pCMV-dR8,74 (helper packaging construct) and pMD2.G (vector encoding for envelope protein) plasmids kindly provided by Dr. D. Trono from the Ecole Polytechnique Federale de Lausanne (Switzerland), were transiently co-transfected into HEK293T cells in the presence of 0.05 mg/ml PEI pH7 (Linear MW 25.000, Polyscience, Inc.) and 150 mM NaCl. Lentivirus was harvested at 48 and 72 h posttransfection, centrifuged to get rid of cell debris, and then filtered through 0.45 cellulose acetate filters followed by ultracentrifugation.

For lentivirus infection, rVSMC and L6E9 cells were plated at 10.000 cells/ml in 6 well plates, infected with 1:10 of total obtained Sp1-shRNA lentivirus or control lentivirus and incubated at 33ºC. Twenty four hours after infection, virus containing media was removed, standard culture media was added and incubation continued at 37ºC. Puromycin selection (6 ug/ul for rVSMC and 3 ug/ul for L6E9) was performed four days after infection and resistant cells were amplified for 1-2 additional passages. At 70–80% confluence cells were harvested and lysed.

Legends to Supplementary Figures

Supplementary Figure 1. Nucleotide sequence of the 5' region of rat *Mfn2*.

+1 represents the transcription start site reported in NCBI (NM 130894 for rat sequence) and the first exon is shaded. Putative transcription-regulatory motifs are in bold and underlined. Functional estrogen-related receptor-α (ERRα)-binding element is shown inside a rectangle.

Supplementary Figure 2. Human *Mfn2* 5'-flanking region is located in a CpG island.

Position and characteristics of the CpG island associated with the *Mfn2* promoter region, showing observed versus expected CpG, (G+C) content and the location of the CpG island.

Supplementary Figure 3. Determination of human *Mfn2* transcription start sites in human skeletal muscle.

A) 5'-RACE analysis of human *Mfn2* mRNA from skeletal muscle. The PCR products (second round of amplification) were resolved on agarose gel, at least three bands were visualized by ethidium bromide staining (arrows); C is the negative control. The PCR product was cloned and 22 clones were sequenced.
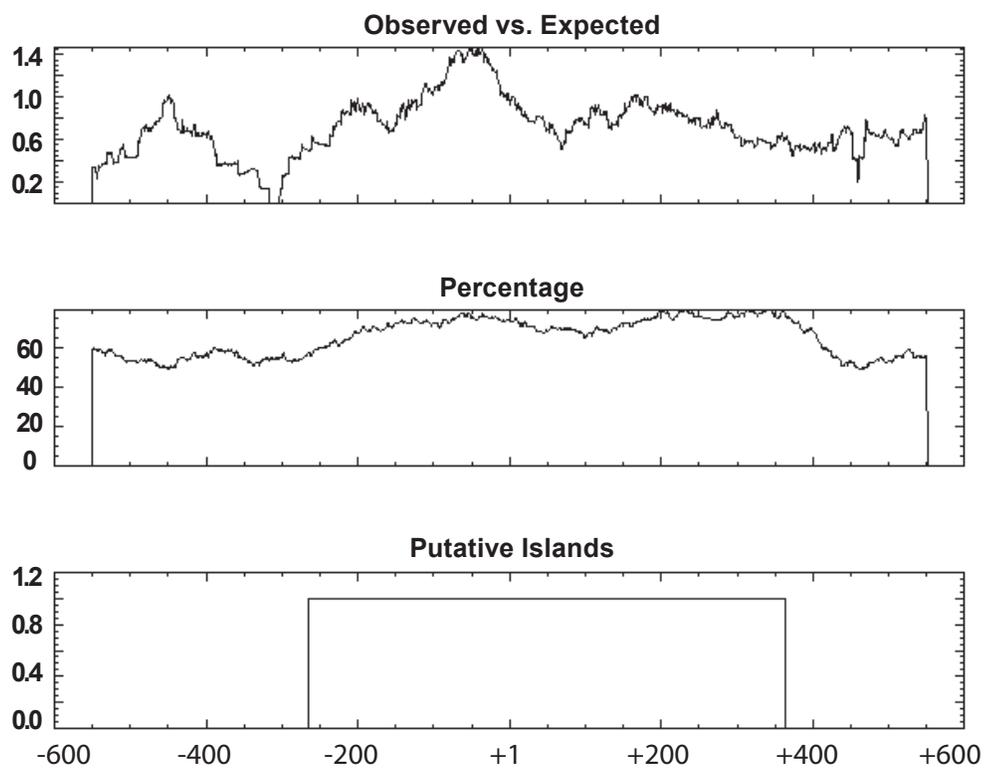
B) Six transcription start sites (TSS) were identified (TSS1 2 clones; TSS2 8 clones; TSS3 1 clone; TSS4 2 clones; TSS5 8 clones; TSS6 1 clone), three of them with homology with the consensus Inr element (shaded).

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-950     aatgtatttcatttagtcgagcgaatccaaagttatagcaacatatcaat
         GATA-1                                    C/EBP
-900     atgaaaacattagtaaggtagtttgcatttctttttaataataaatattg
                                                  C/EBP
-850     gggcatctctctacagcaactacatttcaggtcctcagtagctacaggtg
            NFκB
-800     gctggtgggtgttactacagagcctaggcttggagcactcccagcttggg
              C/EBP              Sp1
-750     gttgtatgctgtatcccagttcttggaacaaaataagaggccagtgaatg
-700     acatatgagcaactcatcatgccccgggtgtcctgggactgctgtgaaaa
-650     ttaaaaattgcatgttagatacccagctcaatgactgtcacctagtaggt
            C/EBP
-600     gcctggtgcactgtggctgctgttttgttgcctcaaaggcaactgaagga
                                 C/EBP    ERRa
-550     caattacttcaccctaacggtctgtttgttccctttgaagacacactttc
-500     aaattatgaaacgcaagtctggatttcaaagatgacaggtgttacagggt
                               C/EBP               AP-1
-450     catgttctggagcaacaacaacaaaagggcacgcaaacctctaatccgca
                                 Sp1
-400     cccttatcgtttttgtgccggtccccttctcctctgtccaccctctcccc
                               Sp1            Sp1
-350     tccactcccccccccttcccccttcccaacccctgcttgacagctcgaggct
             Sp1                 Sp1
-300     ccgcccctaccccaccctgccgggcgctggttcaaactacaactcccatga
         Sp1                 Sp1
-250     tgcgctgggagccccacccccacagcttacgtcatctgcataagacgcggg
                       Sp1                         Sp1
-200     acggttatcaggcacgcggaggctccttttggagcgcggagttcctcgac
                   Sp1                 Sp1
-150     actgtataggagtgatagggttgaagtgacggtgttcagaggccatcggt
                 GATA-1                        Sp1
-100     tcatccgggcttcagcaggctagtgacgagcgcagcccctccctccc
                       Sp1                        Sp1
 -50     gcttctctcaatgtggcgctggccagtgacgtgttgggtgtgatggccgc
                       Sp1
  +1     cgcgaggccgggaaggtgaaggtgagagggcgagcgcgctggggaggggg
 +51     aaccggcaggccccggccggagagtcctgagccacccggcttgggacgc
+101     tgggacgctggggcccctgggccttgggctcaggctccggtcatacgggc
+151     ttttcccctgctccggcctcgccgccgggccctcctcgtctctgaggagg
+201     gggcgggccacaggactcagggctgggaggtgggctccttggtctcattc
+251     ctgacccctgccccattcccggttttcatccagtacttttctttttctgtt
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SUPPLEMENTARY FIGURE 1

SUPPLEMENTARY FIGURE 2

A

B

			TSS1						TSS2

-60   gagtggcgcgctgAgacgccgctcgaagcgccgagtcgcggggcagcagaggcGTAAgga

                                              TSS3   TSS4   TSS5

+1   gtaggcggggcgagccggctgggctcagggtccaccAgctcacccgggtcgAggggcAat

+61   ctgaggcgactggtgacgcgcttatccacttccctcctcccgcctcccccctggggtggcg

       TSS6

+121   ctcGctggtgacgtagtgagtgtgatggccgccgcgaggccgggaaggtgaag

Inr: Py-Py(C)-A$_{+1}$-N-T/A-Py-Py

SUPPLEMENTARY FIGURE 3