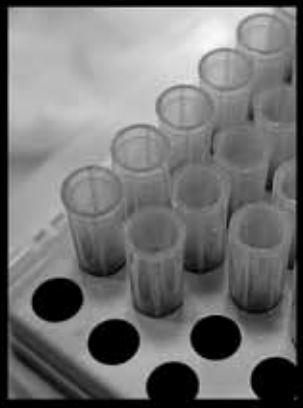
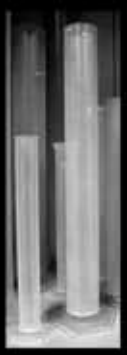


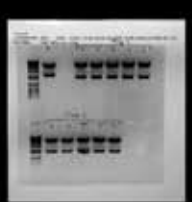
Departament de Bioquímica i Biologia Molecular
Facultat de Biologia - Universitat de Barcelona

ESTUDI FUNCIONAL DEL GEN DOR

Jordi Duran i Castells

Tesi Doctoral - Barcelona, 2007





Estudi funcional del gen DOR

Jordi Duran i Castells
TESI DOCTORAL
Barcelona, 2007

Programa de Doctorat de Biomedicina, Bienni 2002-2004
Departament de Bioquímica i Biologia Molecular
Facultat de Biologia
Universitat de Barcelona

Memòria per a optar al grau de
Doctor per la Universitat de Barcelona

Presentada per:

JORDI DURAN I CASTELLS

Vist i plau del director: L'interessat,

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annexos

La intenció d'aquesta secció és la d'incloure en aquesta tesi una sèrie d'informacions que poden ser útils a diferents nivells.

En el primer annex incloem l'article que actualment es troba en revisió per ser publicat; en aquest article la investigació realitzada en aquesta tesi es posa en context amb la resta d'investigació realitzada al nostre grup sobre DOR. Així s'hi poden trobar altres informacions que confirmarien el paper de DOR com a coactivador, com ara la seva interacció amb TRα, els estudis de CHIP o els canvis d'expressió que provoca la seva repressió.

El segon annex detalla el procediment seguit per aconseguir una eina que a dia d'avui encara no ha donat cap fruit però que de ben segur ho farà en un futur immediat: un ratolí transgènic que sobreexpressa DOR a nivell de múscul esquelètic.

La resta d'annexos mostren línies d'investigació engegades en el seu moment i que de moment no s'han seguit perquè se n'ha prioritzat altres que s'han considerat més interessants. La inclusió en aquest apartat té l'objectiu de deixar-ne constància, sobretot com a eina per a futures investigacions.

ANNEX 1. ARTICLE.

Identification of a novel modulator of thyroid hormone receptor-mediated action

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Running head: DOR regulates thyroid hormone action

SUMMARY.

Background: Diabetes is characterized by reduced thyroid function and altered myogenesis following muscle injury. In this study we identify a novel component of thyroid hormone action that is repressed in muscle in diabetes.

Methodology/Principal Findings: We have identified a gene, named *DOR*, abundantly expressed in insulin-sensitive tissues such as skeletal muscle and heart, whose expression is highly repressed in muscle from obese and diabetic rats. DOR expression is up-regulated during muscle differentiation and its loss-of-function negatively impacts on gene expression programmes linked to myogenesis or driven by thyroid hormones. In agreement with this, DOR functions as an enhancer of the transcriptional activity of thyroid hormone receptor TRα1. This function is mainly driven by the N-terminal part of the protein, whereas the C-terminal fragment of DOR exhibits an inhibitory activity. Moreover, DOR physically interacts with TR α1 and to T_3-responsive promoters as assessed by ChIP assays. T_3 stimulation also promotes a mobilization of DOR from its localization in nuclear PML bodies, suggesting a possible relationship between its nuclear localization and cellular function. Conclusions/Significance: Our data indicate that DOR modulates thyroid hormone function and controls myogenesis. *DOR* expression is down-regulated in skeletal muscle in diabetes, which may be relevant in the alterations in muscle function associated to this disease.

INTRODUCTION.

Thyroid hormones play a central role in metabolic homeostasis, development, differentiation and growth (Oppenheimer et al., 1987;Fowden, 1995;Yen, 2001). Disorders in thyroid function are among the most common endocrine diseases and affect 5-10% of individuals during their lifetime (Hollowell et al., 2002). Thyroid hormones stimulate basal metabolic rate and adaptive thermogenesis through effects on major metabolic tissues such as skeletal muscle, liver and adipose tissue. The major effects of thyroid hormones are mediated by modulation of gene transcription. Most of the thyroid response elements function in such a way that thyroid hormone receptors (TRs) repress gene transcription in the absence of ligand and are activated after binding to thyroid hormones. In the presence of T_3, TR undergoes a conformational change which results in the replacement of a corepressor by a coactivator complex, which in turns triggers the transcriptional activation of TR-regulated genes. Thyroid hormone response elements have been identified in muscle-specific genes such as myogenin, α-actin, or GLUT4 (Downes et al., 1993;Santalucia et al., 2001;Moreno et al., 2003). Several TR-regulated genes determine distinct aspects of muscle biology. Thus, thyroid hormones regulate muscle development and function by inducing myoblast cell cycle exit (Muscat et al., 1995). In addition, thyroid hormones exert substantial effects on myotube formation and muscle fiber composition by the regulation of the expression of several masters of differentiation such as MyoD or myogenin (Collie and Muscat, 1992;Carnac et al., 1992;Albagli-Curiel et al., 1993;Marchal et al., 1995) or by inducing muscle-specific genes such as the myosin heavy chain (Izumo et al., 1986;Schiaffino and Reggiani, 1996). Thyroid hormones also affect the outcome of repair in adult muscle. Thus, increased T_3 levels in vivo shorten the time myoblast spend in proliferative state and speed their transition to fusion, limiting the number of myotubes resulting during repair from an injury (Pernitsky et al., 1996). In contrast, decreased levels of T_3 in vivo slow myoblast proliferation and reduce the number of new myotubes formed in the course of repair (McIntosh et al., 1994). Here we identified a novel protein, DOR, which is abundantly expressed in insulinsensitive tissues and it is markedly repressed in diabetes. Since we also report that DOR regulates thyroid hormone action, our data suggest that DOR determines muscle development, function and metabolic response to hormonal cues through the modulation of the expression of TR-regulated genes.

RESULTS.

Identification of *DOR*, a gene that is abundantly expressed in skeletal muscle and heart and is down-regulated in obese-diabetic rats.

To identify potential risk factors responsible for the alterations associated with type 2 diabetes, we screened genes differentially expressed in Zucker obese-diabetic (ZDF) rats and non-diabetic lean rats (control) by PCR-select cDNA subtraction. After obtaining the subtracted cDNA library, we isolated several clones using differential screening by PCR-selection. One of these clones was selected and used as a probe which further allowed the detection of a 4.5 kb mRNA species in various tissues. A human heart cDNA library was then screened and the full-length cDNA of human *DOR* was isolated. This cDNA coincided with the predicted open reading frame C20orf110 (NM021202). Based on the criteria that had led to its identification, we named the gene as *DOR* for Diabetes- and Obesity Regulated (gene). The human DOR gene maps to

chromosome 20q11.22, close to loci linked to human obesity (Lembertas et al., 1997;Lee et al., 1999;Dong et al., 2003) and type 2 diabetes (Ghosh et al., 1999;Vionnet et al., 2000;Iwasaki et al., 2003). A comparison between the genomic and the cDNA product reveals an intronic-exonic distribution of four introns and five exons.
The protein coding region starts at exon 3 and generates an ORF of 672 nucleotides. Murine and rat *DOR* cDNAs were also amplified and sequenced. Human, rat and mouse DOR polypeptides are well conserved (84% identity human and mouse, 83% identity human and rat, 85% identity rat and mouse), and encode a protein of 220 (human) or 221 (mouse, rat) residues (Figure 1A). The only homologous protein described to date is a human p53-dependent apoptosis regulator named p53DINP1/TEAP/SIP, with 36% identity with human DOR (Okamura et al., 2001). DOR contains a strong positive charge in its C-terminal region, which is predicted to form an alpha-helix structure (Figure 1A) whereas the rest of the protein is predicted to be unstructured (GLOBPLOT 2).
The distribution of *DOR* mRNA in human and rat tissues was examined by Northern blot. In the two models, transcripts were predominant skeletal muscle and heart, while lower expression was detected in other tissues such as white fat, brain, kidney or liver (Figure 1B, 1C and data not shown). These data suggest a potential role of DOR in tissues with high metabolic requirements or which respond to insulin. *DOR* expression was also examined in skeletal muscle from ZDF rats, revealing a reduction of 77% in its expression (Figure 1D), and corroborating the original subtraction hybridization assay.
By the use of the differential screening, we thus identified a novel protein which is strongly repressed in obese-diabetic rats, and highly expressed in tissues involved in metabolic homeostasis. Next, we analyzed DOR cellular function in order to assign a posible role of the alterations of *DOR* expression in the pathophysiology of diabetes.

DOR is a nuclear protein that potentiates the activity of thyroid hormone receptors.

Several lines of evidence suggest that DOR may have a nuclear function, namely: a) DOR is predicted to be a nuclear protein (WoLF PSORT Prediction program), and b) DOR uniquely shows homology to a nuclear protein. To assess whether, in fact, DOR is a nuclear protein, HeLa cells were transfected with a plasmid encoding DOR ORF and the protein was detected by Western Blot or immunofluorescence. DOR migrated as a 40 kDa protein in SDS-PAGE (Figure 2B). By subcellular fractionation assays, DOR was detected in nuclear extracts (Figure 2B). Immunofluorescence data confirmed this observation since DOR was mainly localized in nuclei (Figure 2A). Within the nucleus, DOR colocalized with PML nuclear bodies (Figure 2C). This localization of DOR in PML nuclear bodies was not due to its over-expression in HeLa cells since endogenously expressed DOR was also detected in these bodies in murine 1C9 muscle cells (Figure 2C) derived from the immortomouse (Jat et al., 1991).
As theoretically predicted, DOR was localized within the nucleus. Based on this observation, and the fact that DOR is homologous to a nuclear protein involved in transcriptional regulation, we proposed that it may also regulate transcription. Furthermore, the high DOR expression in tissues characterized by high metabolic requirements made us to speculate on a regulatory role of DOR on thyroid hormone action. To this end, HeLa cells were transfected with DNA encoding thyroid hormone receptors ($TR\alpha 1$) and CAT or luciferase reporters gene fused to thyroid hormone receptor elements, in the presence or absence of DOR. $TR\alpha 1$ transactivated the reporter gene, whereas DOR alone showed a small stimulatory effect on reporter activity (Figure

3A). The cotransfection of DOR and TRα1 enhanced the transcriptional activity of the reporter gene in a dose-dependent manner (Figures 3A, 3B). This effect was specific of DOR expression and transfection with a plasmid encoding the xCT amino acid transporter did not cause any effect (data not shown). The effects of DOR were also detected when using luciferase as a reporter gene (data not shown). DOR did not cause any effect on the reporter activity induced by transcription factors p53 or c-Myc (Figure 3C and 3D). In addition, DOR did not stimulate the activity of the chimeric protein GAL4-VP16 generated by fusion of the GAL4 DNA-binding domain and the VP16 activation domain (Figure 3E). In all, these observations indicate that DOR specifically potentiates the activity of thyroid hormone receptors. The effect of DOR is not a consequence of a generalized stimulation of transcription since basal reporter activity, activity driven by c-Myc or p53, or activity of GAL4-VP16 remained unaltered.
The extent of the coactivation induced by DOR on TRα1 is relatively modest compared to other well-reported coactivators, which made us to analyze whether DOR contained different domains within its sequence. To this end, full-length DOR or distinct cDNA fragments were fused to a GAL4 DNA-binding domain (Gal4-DBD) and its transcriptional activity was assayed by cotransfection with a Gal4 reporter plasmid in HeLa cells. Gal4-DBD fused to full-length DOR caused a moderate increase (3-fold) in reporter activity (Figure 4A) and deletion of the C-terminal half of the protein (fragment 1-120) further increased this activity (8.5-fold) (Figure 4A). The fragment encompassing amino acid residues 31-111 showed the maximal stimulatory activity (47-fold) (Figure 4A). In contrast, the C-terminal half of DOR did not show any transcriptional activity. Similar data were obtained in HEK293T cells (Figure 4B).
These data suggest that the C-terminal half of DOR inhibits the transcriptional activity of the N-terminal stimulatory fragment, thus explaining the modest levels of transcriptional coactivation previously observed.

DOR loss-of-function reduces the action of thyroid hormones in muscle cells.

To determine whether DOR is required for thyroid hormone action, we generated lentiviral vectors encoding for siRNA to knock-down (KD) DOR expression in mouse cells. The siRNA lentiviral infection in C2C12 muscle cell markedly reduced DOR protein expression (80% reduction) compared to levels found in cells infected with scramble RNA (control group) (Figure 5A). Once validated the KD system, control and KD cells were transiently transfected with a reporter gene driven by a TRE, in the present or absence of TRα1 or T3. In control muscle cells, while thyroid hormone caused a 5-fold stimulation of reporter activity due to the activation of endogenous TR α1, the addition of exogenous TR increased up to a 10-fold the stimulation of transcriptional activity. (Figure 5B). DOR loss-of-function markedly reduced the effect of T3 and of TRα1 and T3 (Figure 5B).
Based on these data, next we tested whether the reduced DOR expression altered the effect of thyroid hormones on endogenous target genes. In control C2C12 muscle cells, the stimulation with T3 (100 nM for 48h) markedly enhanced the expression of genes such as myogenin, IGF-II, actin α1, caveolin-3, creatine kinase or UCP2 (Figure 5 CH).
Stimulation of gene expression for α-actin and myogenin in response to thyroid hormones has been previously reported (Collie *et al.*, 1992;Carnac *et al.*, 1992). Under these same conditions, DOR-KD cells markedly reduced the stimulatory effect of thyroid hormones on the expression of the same subset of genes (Figure 5 C-H). Based on these data, and the previous observation that DOR is able to enhance the

transcriptional activation of TRα1 (Figure 4), we suggest that DOR functions as a regulator of TR-mediated cellular responses.

Functional role of DOR in myogenic differentiation.

Based on the observation that DOR expression is markedly repressed in muscle from diabetic ZDF rats and that diabetes is linked to skeletal muscle atrophy (Chao et al., 1976;Klueber and Feczko, 1994;Greenman et al., 2005;Aughsteen et al., 2006), we next studied whether DOR participates in myogenesis. To this end, the expression of several genes and proteins in scramble or DOR siRNA C2C12 cells was studied along the myogenic differentiation process (from myoblasts to myotubes). Muscle differentiation in C2C12 cells caused a 3-fold stimulation of DOR gene expression (Figure 5A), which was blocked in DOR knockdown cells (Figure 6A). During C2C12 myoblast differentiation, several muscle-specific genes such as myogenin, creatine kinase, caveolin 3, actin α1 or IGF-II were markedly induced in control cells (from 10- to 20-fold) (Figure 6 B-F). Under these conditions, DOR-KD cells showed an altered induction in the expression of those genes (Figure 6 B-F). However, the pattern of every particular gene was not identical. Myogenin, a transcription factor which plays a unique function in the transition from a determined myoblast to a fully differentiated myotube (Hasty et al., 1993;Nabeshima et al., 1993), is rapidly induced at early stages of differentiation. While control cells normally induced myogenin mRNA levels (5- fold stimulation at day 1 of differentiation), DOR-KD cells showed a delay in that stimulation (Figure 6C). However, at day 3 of differentiation no differences were detected between control and KD cells (Figure 6C). In the case of the pattern shown by actin α1, creatine kinase or IGF-II, the inhibition of expression was larger at the onset of differentiation process (days 1 and 2) (Figure 6 D-F). Finally, the expression of muscle-specific genes at the protein level was also analyzed and this further confirmed that DOR siRNA reduced the abundance of myogenin, glycogen synthase or caveolin-3 compared to control cells (Figure 6 G).
In all, our results indicate that DOR plays a regulatory role in the myogenic programme, and more specifically, at early stages of muscle differentiation.

DOR physically binds TRα1.

Based on the observation that DOR functionally modulates thyroid hormone action, we also aimed to determine whether DOR and thyroid hormone receptors physically interact. To this end, chimeric fusion proteins TRα1-GST, RXR-GST, and DOR-His were produced. TR α1-GST bound DOR protein and the physical interaction in pulldown assays was independent of the presence of T_3 in the medium (Figure 7A). Under these conditions, neither GST nor RXR-GST bound DOR protein (Figure 7A and data not shown). To verify that the DOR-TR α1 interaction was also established *in vivo*, HeLa cells were transfected with DOR, TR α1 or both, in the presence or absence of T_3, and extracts were immunoprecipitated with an anti-DOR antibody. The bound proteins were eluted and analyzed by Western blot with polyclonal antibodies against TR α1 or DOR. As a result, we detected specific co-immunoprecipitation of TR α1 and DOR proteins both in the presence and absence of T_3 (Figure 7B).
Next, we aimed to determine whether this binding was also detected *in vivo* in the context of a T_3-responsive promoter of a gene transcribed in HeLa cells. Thus, we selected the human *dio1* gene promoter, since its mRNA has been reported to be expressed in this cell line (Sharma and Fondell, 2000). DOR-TR α1-transfected HeLa

cells, treated or not with T_3 for 1 h, were subjected to ChIP assays by using DOR, TR $\alpha 1$ or SRC-1 antibodies. The resulting precipitated genomic DNA was then analyzed by PCR using primers flanking the boundaries of the TREs located in the promoter region of *dio1* (Sharma and Fondell, 2002). Under these conditions, SRC-1 was recruited in the complex only after T_3 treatment (Figure 7C), while TR $\alpha 1$ was bound both in the presence and in the absence of T_3 (Figure 7C). The same pattern was detected with antibodies against DOR (Figure 7C), thus confirming the results obtained by Co-IP. ChIP assays in the absence of antibodies did not amplify any unspecific band (Figure 7C). Immunoprecipitates did not amplify a fragment of the interleukin-2 gene, used as a negative control (Figure 7C).
In all, we have observed either by CoIP or ChIP methods that DOR physically binds TR in a ligand-independent manner, while the functional activation is ligand-dependent. Based on these data, we hypothesize that the presence of other proteins of the TR complex might ultimately determine DOR function.

Thyroid hormones rapidly modulate the intranuclear localization of DOR.

Current models propose that key components of transcriptional complexes are functionally compartmentalized (Isogai and Tjian, 2003;Zaidi et al., 2005) so that the achievement of a transcriptional active status implies physical recruitment of chromatin and related proteins. Based on previous data indicating that DOR is localized in PML bodies, and that it fuctionally activates TR in the presence of T_3, we aimed to determine whether DOR positioning in PML was affected by the presence of ligands. In cells over-expressing both TR$\alpha 1$ and DOR, the addition of T_3 caused the intranuclear movement of DOR protein from its basal position in PML nuclear bodies (Figure 8A). These effects were not detected in cells that only over-expressed DOR (Figure 8A). To have a more precise view of the kinetics of the process, a DOR-GFP construct was generated and transfected in HeLa cells. The chimeric DOR-GFP protein retained the capacity to stimulate the transcriptional activity of TR $\alpha 1$ (Figure 8B) compared to the activity of wild-type DOR. Immunolocalization analysis indicated that DOR-GFP rapidly moved after T_3 exposure (already detectable at 5 min) (Figure 8C); the effects were transient and after 60 min of T_3, the extent of colocalization of DOR and PML was similar to what detected in basal cells (Figure 8C). Further time-lapse studies indicated that T_3 caused a very rapid change in the localization of DOR-GFP (detectable in less than 1 min) in HeLa cells (data not shown).
Based on these data, we postulate that DOR is localized in PML nuclear bodies mainly as a storage site in which it remains until needed to act. In this regard, TR-mediated responses act as a driving force to mobilize DOR from the PML bodies. The sensitivity of DOR to T_3 reinforces the notion that the cellular role of DOR is related to the regulation of TR function.

DISCUSSION.

In this study we have identified a novel protein, named DOR, as a result of a substractive hybridization screening aimed to detect genes down-regulated in skeletal muscle from obese-diabetic ZDF rats. *DOR* is abundantly expressed in tissues with high metabolic rates such as skeletal muscle and heart. The experimentally induced DOR repression in muscle cells (via si-RNA) markedly reduces the action of thyroid hormones and alters muscle differentiation. In this regard, it has been reported that type 2 diabetes is characterized by reduced thyroid function (Kabadi and Premachandra,

1984;Smithson, 1998;Chubb et al., 2005). In addition, skeletal muscle atrophy is a well-documented complication of diabetes and is characterized by a reduction in the diameter of the myofibers and a reduced number of myonuclei (Chao *et al.*, 1976;Klueber *et al.*, 1994;Greenman *et al.*, 2005;Aughsteen *et al.*, 2006). All these data together with the marked reduction of *DOR* expression in skeletal muscle from obese diabetic ZDF rats permits to propose a role in the pathophysiology of diabetes. The evidence we have obtained in this study indicates that DOR is a protein that resides in PML nuclear bodies and shows some properties characteristics of nuclear coactivators.

Thus, DOR has the capacity to moderately enhance the transcriptional activity (2.5- to 5-fold) of thyroid hormone receptors in a ligand-dependent manner and it acts as an activator when tethered to DNA. In addition, DOR binds to thyroid hormone receptors *in vitro* and *in vivo* conditions and to the thyroid hormone responsive *dio1* promoter, as shown by chromatin immunoprecipitation. This transcriptional activation capacity occurs through the N-half of the protein, while. The C-terminal half of DOR exerts an inhibitory role, which may explain the moderate extent of the co-activation of nuclear hormone receptors in over-expression studies. Whether DOR is a bona fide nuclear co-activator and whether it exerts additional cellular roles, requires further work.

More specifically, we have demonstrated that DOR is a protein that participates in thyroid hormone action. The evidence we have obtained is as follows: a) DOR overexpression enhances 4-fold the transcriptional activity of thyroid hormone receptor $TR_{\alpha 1}$, b) DOR loss-of-function represses the stimulatory effect of thyroid hormones on the expression of genes such as actin α1, caveolin-3, creatine kinase, IGF-II, UCP2 or myogenin in muscle cells. c) DOR binds to $TR_{\alpha 1}$ *in vitro* and *in vivo* in the context of a T_3-responsive promoter (human *dio1* promoter), and d) DOR undergoes a rapid and transient intranuclear movement from PML nuclear bodies in response to T_3. The rapid changes in the nuclear localization of DOR detected in response to T_3, may be relevant in the ligand-dependent DOR-mediated potentiation of $TR_{\alpha 1}$ thyroid hormone receptor activity.

DOR protein contains two functionally different regions. The N-terminal half with predicted random structure (GLOBPLOT 2) shows transcriptional activation capacity (mapped between amino acid residues 31 to 111). In this regard, DOR may belong to the group of proteins characterized by having sizeable regions that lack a predicted well-structured three-dimensional fold, which show high conservation among species (from mouse to human in the case of DOR) and which, contrary to the traditional view, the disordered region is functional (Dyson and Wright, 2005). The C-terminal region of DOR is predicted to form a positively charged alpha-helix structure and has no transcriptional activation capacity; rather, the C-terminal region inhibits the transcriptional activity of the N-terminal half supporting the existance of an intramolecular control of the transcriptional activity of DOR. Such an intramolecular control of transcriptional activity has been reported for other nuclear proteins such as ATF2 or NK-2 (Li and Green, 1996;Watada et al., 2000).

Thyroid hormones stimulate muscle development and muscle differentiation (Muscat *et al.*, 1995). Thyroid hormones stimulate myogenin, myotube formation in muscle cells (Collie *et al.*, 1992;Carnac *et al.*, 1992;Albagli-Curiel *et al.*, 1993;Muscat et al., 1994;Marchal *et al.*, 1995) and induce the expression of muscle-specific genes such as α-actin, or GLUT4 (Downes *et al.*, 1993;Santalucia *et al.*, 2001;Moreno *et al.*, 2003). In our study, we have demonstrated that thyroid hormones also potently stimulate in C2C12 muscle cells the expression of other genes such as caveolin-3, creatine kinase, IGF-II or UCP2. The induction of IGF-II may be particularly relevant since it has been reported to modulate the biology of muscle cells (Florini et al., 1996). In addition, and

more central to our study we have found that DOR loss-of-function markedly reduced the myogenic effect of thyroid hormones in muscle cells as assessed by the gene expression of myogenin, α-actin, caveolin-3, creatine kinase, IGF-II or UCP-2. Thus, our data implicate DOR in the specific stimulatory effects of thyroid hormones on muscle differentiation.
In fact, DOR loss-of-function also had an impact in the capacity of myoblasts to undergo through the myogenesis programme. C2C12 muscle cells knockdown for DOR showed a lower induction of myogenin gene expression, and a reduced expression of creatine kinase, α-actin and caveolin-3. These results indicate that DOR regulates muscle differentiation, at least in part, by controlling myogenin gene expression.
Based on the data presented in this study, we propose that *DOR* repression participates in a deficient response of muscle to thyroid hormones and in the alterations in muscle biology associated to the diabetic condition.

MATERIALS AND METHODS.

Subtractive hybridization and cDNA cloning.

Messenger RNA was extracted from *gastrocnemius* muscle of non-diabetic lean (fa/+) and Zucker diabetic (ZDF) rats with oligo(dT)20-cellulose columns, as described (Bach et al., 2003). Complementary DNA was prepared from 2 μg of mRNA using Superscript II (Life Technologies). PCR-Select cDNA Subtraction kit (Clontech) was used to select genes that are down-regulated in diabetic muscle (Bach *et al.*, 2003). The C42 260 bp fragment obtained from subtractive hybridization was used to screen a human heart λ-ZAP cDNA library (Stratagene). Five clones were isolated, one of which contained the full-length cDNA of human *DOR*. This cDNA clone was subcloned and the sequence of human *DOR* was obtained by sequencing both strands with a two-fold coverage minimum. To determine the murine 5'-cDNA sequence, a cDNA clone (AI95670R) covering 1.8 kb was sequenced. The 3′-cDNA was obtained by RT-PCR amplification. The rat *DOR* cDNA 5′-region was obtained by RT-PCR using heterologous primers from the mouse *DOR* sequence. GenBank accession numbers are AJ297792 Homo sapiens mRNA for DOR protein; AJ297793 Mus musculus mRNA for DOR protein; AJ297794 Rattus norvegicus partial mRNA for DOR protein. Mutated versions of *DOR* were generated by the Quick Change Site Directed Mutagenesis Kit (Stratagene). Full-length DOR cDNA, and cDNA fragments encompassing different amino acid fragments were PCR-amplified and cloned in the pGBKT7 vector containing the DNA binding domain of GAL4 (Clontech) and then cloned in pCDNA3.

RNA expression studies.

Total RNA extraction and treatment with DNase I were performed with Rneasy mini kit (Qiagen). Total RNA from tissue samples or from cells was stored at -80°C until further assay. RNA concentration was determined by spectrophotometry at an absorbance of 260 nm. Northern blot assays on 20 μg of total RNA were performed as described (Vinals et al., 1997) using the ^{32}P-labeled C42 cDNA fragment. The C42 rat cDNA fragment is homologous to the nucleotide sequence 2,912-3,172 of the human AJ297792 (GenBank). Real-time PCR was performed from 0.1 μg of total RNA from muscle cells, as described (Bach et al., 2005). Cyclophilin or HPRT mRNA were assayed as controls in real-time PCR assays.

Western blot.

A rabbit antibody against the DOR specific peptide PPPAPSLMDESWFVTPPAC (amino acid residues 63-81) was purchased from Research Genetics. Anti-β-actin antibodies were used as a control of loading. Proteins from total homogenates or fractions enriched in nuclear proteins were resolved in 10% SDS-PAGE and transferred to Immobilon sheets. Incubation with antibodies and ECL detection were performed as described (Enrique-Tarancon et al., 2000).

Cellular localization studies.

The full cDNA sequence of human DOR was amplified by PCR and cloned into the HindIII-BamHI sites of the pCDNA3 vector (Invitrogen). The murine cDNA was amplified by PCR and cloned into the pGEM-T Easy vector (Invitrogen). Recombinant GFP-DOR vectors were generated by cloning a PCR product spanning the murine *DOR*-ORF in-frame into the EcoRI and SalI sites of the pEGFP-C2 vector (Clontech). HeLa cells were transfected with the DOR expression vectors by the calcium phosphate precipitation method. In some studies, 36-h transfected cells were fixed with 3% paraformaldehyde and subjected to immunofluorescence microscopy with a confocal scanning microscope adapted to an inverted Leitz DM IRBE microscope to the sequential acquisition of double and triple cellular staining (Leica TCS SP2, Leica Lasertechnik GmbH, Manheim, Germany). No bleed-through was detected between channels. Samples were scanned using a 63x Leitz objective (oil) and a zoom ranging from 2.5 to 4 to analyse intracellular regions. The fluorochromes used (Hoestch, Oregon Green or GFP, Alexa-Fluor 546 and Cyanine 5) were respectively excited with the UV, 488, 543 and 633 laser lines. To avoid bleed-through effects in double or triple staining experiments, each dye was scanned independently. In some experiments, nuclear extracts from transfected cells were obtained as reported (Wu et al., 2001) and subjected to Western blot analysis with a specific anti-DOR antibody.

Cell cultures and transcriptional activation assays.

HeLa, L6E9, 1C9, CH310T1/2 or C2C12 cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). For transient transfection assays, cells were typically plated onto 24-well plates 24 h prior to transfection by the Lipofectamine 2000 method (Invitrogen) as reported (Santalucia *et al.*, 2001). All transient transfections included 10% of the total DNA of expression vector for GFP (pEGFP, Clontech) to normalize for transfection efficiency. In a typical experiment, 150 ng of reporter plasmid, 75 ng of nuclear receptor expression plasmid and 100 to 300 ng of DOR expression vector were transfected. Ligands were dissolved in absolute ethanol (1 µM dexamethasone) or water (1 µM rosiglitazone or 100 nM T_3). Sixteen hours after transfection, cells were harvested and cell extracts were analyzed for CAT expression by specific CAT-Elisa® kit (Roche) or luciferase assay system (Promega). Transfection efficiency was analyzed by flow cytometric analysis of GFP expression. The reporter vector used to assay thyroid hormone receptor activation was previously described (Santalucia *et al.*, 2001), and consists of a functional thyroid hormone receptor element from the muscle-specific GLUT4 enhancer, cloned at 5' of a thymidine kinase basal promoter, controlling expression of the CAT reporter gene (TKCAT). An expression vector for the rat thyroid hormone receptor $TR_{\alpha 1}$ was also as previously described (Santalucia *et al.*, 2001). To express murine DOR ectopically in

cell lines, a PCR fragment spanning the murine ORF was cloned into the EcoRV and SalI sites of the pcDNA3 (Invitrogen) vector. A mutated version of *DOR* (mutDOR) was generated by the Quick Change Site Directed Mutagenesis Kit (Stratagene). Fulllength DOR cDNA, and cDNA fragments encompassing amino acid residues 1-120, 120-220 and 31-111 were PCR-amplified and cloned with NdeI and BamHI in the pGBKT7 vector containing the DNA binding domain of GAL4 (Clontech) and subsequently cloned in pCDNA3. The fragment of DOR cDNA encompassing amino acid residues 1-111 was generated by mutagenesis from construct 1-120 by generating a stop codon at position G112.

Protein binding assays.

Full-length *DOR* with an histidine-tagged N-terminus (DOR-His) was generated. The DOR-His and TRα1-GST fusion proteins were expressed and purified from *E.coli* on affinity beads. Two µg of extract GST or TRα1-GST and 2 µg of DOR-His were incubated in resuspension buffer (10 mM Tris/HCl, 200 mM NaCl, EDTA 0.2% pH 7.5 containing 10 mM PMSF,10 mM aprotinin, 1 mM pepstatin and 1 mM leupeptin). Proteins were incubated with glutathione-Sepharose beads (Pharmacia) for 1 h at 4ºC. The beads were then washed three times in 0.5 ml of resuspension buffer in the presence of 0.1 mM Mg_2Cl. Proteins were eluted in 200 µl of Laemmli sample buffer and subjected to SDS-PAGE. Proteins were then blotted. The DOR-His and the TRα1 expression vectors were transiently transfected in HeLa cells. Thirty-six hours after transfection, cells were exposed to T_3 for 1 h or were left untreated. Cells were then rinsed twice with ice-cold PBS containing 0.5 mM PMSF and cytosolic and nuclear fractions were obtained as described (Wu *et al.*, 2001). The nuclear soluble fraction was immunoprecipitated by means of a NI-NTA resin (Qiagen) (Ros-Baro et al., 2001). The immunocomplexes were resolved by SDS-PAGE and Western blot.

Chromatin immunoprecipitation (ChIP).

DOR and TRα1 expression vectors were transiently transfected in HeLa cells and 36 h later cells were exposed to T_3 for 1 h or left untreated. Cells were then treated with the cross-linking agent formaldehyde, lysed and chromatin was then sheared. Immunoprecipitation was performed with antibodies against TRα1, DOR or SRC-1. After ChIP, DNA was purified by phenol/chloroform extraction. Input (1% of total immunoprecipitated) and immunoprecipitated DNA were subjected to PCR analysis with primers flanking the TRE site on the promoter (*dio 1* promoter) (see primer sequences in supplementary methods) or flanking a region of the GAPDH or the IL-2 genes. The following primers were used for amplification of promoter regions: -the *dio 1* gene (forward: 5'-GAGGCCAAGGCGCGGGTAGGTCATCT-3'; reverse: 5'-CCGGGTCAGGGGAAGGAGTCAG-3'); -the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (forward: 5'- GCTCCAATTCCCCATCTCAG-3'; reverse: 5'-CCAGGCTCAGCCAGTCCCAG- 3'); -the interleukin-2 (IL-2) gene (forward: 5'-GTTCAGTGTAGTTTTAGGAC-3'; reverse: 5'-CTCTTCTGATGACTCTTTG-3').

Lentiviral infection and siRNA generation.

DOR siRNA was obtained from sFold software (http://sfold.wadsworth.org). Scrambled siRNA was obtained by scrambling a functional DOR siRNA sequence. Lentiviruses

encoding scramble or DOR siRNA were used as reported (Wiznerowicz and Trono, 2003). All HIV-1 derived lentiviral constructs (pLVTHM transfer vector, pCMVΔ8,74 helper packaging construct and pMD2G vector encoding for envelope protein) were kindly provided by Dr. Didier Trono from the Ecole Polytechnique Federale de Lausanne (Switzerland) and used as reported (Wiznerowicz *et al.*, 2003). The pLVTHM vector contains a GFP expression cassette and two restriction sites (*ClaI* and *MluI*) after the H1 promoter, allowing direct siRNA cloning. Lentiviruses encoding scrambled and DOR siRNA were produced by triple transient transfection of HEK 293T cells using the calcium-phosphate method. Subconfluent cells were transfected with 10 µg of pLVTHM encoding scrambled or DOR siRNA, 7 µg of pCMVΔ8,74 and 3 µg of pMD2G. Culture medium containing lentiviruses was harvested at 48 and 72 h after transfection. Lentiviruses were concentrated by ultracentrifugation (26,000 rpm, 1 h 30 min at 4ºC, using a 4 ml sucrose 20% cushion) and were resuspended in 100 µl fresh medium. We stored lentiviral working aliquots at -80ºC. Titration was performed transducing 10_5 HEK293T cells grown in 12-well plates with 1, 10 or 100 µl of a 1/100 dilution of the concentrated lentiviruses. After 48 h, the percentage of transduced HEK 293T cells (% GFP positive cells) was determined using an EPIC ® S XL flow cytometer (Beckman Coulter ®). Fifteen million C2C12 myoblasts grown on a 12-well plates were transduced at moi 100 and cells were amplified during 5-7 days. Transduced cells (GFP-positive) were then sorted with a MoFlo® flow cytometer (DakoCytomation®, Summit v 3.1 software), obtaining between 93%-99% GFP-positive cells.

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FIGURE LEGENDS.

Figure 1. DOR sequences and tissue distribution of *DOR* expression and downregulation in skeletal muscle from ZDF obese-diabetic rats

Panel A. Amino acid sequence of human, mouse and rat DOR proteins (sequences 1, 2 and 3, respectively). Multi-alignment done using the CLUSTALW Sequence Alignment program. Amino acids differing from the consensus are inverse. The amino acid residues used to generate the polyclonal antibodies are depicted in bold. The Cterminal basic motif is indicated by a line of "+" is predicted to form an alpha-helix structure whereas the N-terminal half is unstructured (GLOBPLOT 2). Panel B. Poly(A) RNA membrane containing human adult tissues was probed with ^{32}P-labeled rat *DOR* cDNA and washed in stringent conditions. The probe hybridises to a transcript of approximately 4.5 kb. Hybridisation with human β-actin cDNA was used as a control probe. Lane 1, brain; lane 2, heart; lane 3, skeletal muscle; lane 4, colon; lane 5, thymus; lane 6, spleen; lane 7, kidney; lane 8, liver; lane 9, small intestine; lane 10, placenta; lane 11, lung; lane 12, leukocytes. Panels C. Total RNA was purified from different rat tissues and subjected to Northern blot analysis. Ethidium bromide staining of the ribosomal 28S subunit was used as a control of the relative amounts of RNA loaded in each lane and to check the integrity of RNA in each sample. Panel D. Total RNA was purified from skeletal muscle from non-diabetic and ZDF rats, and RNA was subjected to Northern blot analysis. The mean+SEM of 6 separate observations are shown. * difference compared to the control group, at $P<0.01$.

Figure 2. DOR protein is localized in nuclear bodies.

Panel A. HeLa cells were transfected with a DOR expression vector or with the empty vector and with GFP. After 48 h, cells were fixed and stained to view DOR, GFP or with the DOR pre-immune serum (negative control). Cells were also counterstained with Hoescht. The arrow indicates a GFP-positive cell, also DOR-positive. DOR coloured in red; GFP in green; nuclei counterstained in blue. Panel B. After DOR transfection in HeLa cells (48 h), cytosolic (Cyt), nuclear soluble (NuS) or nuclear nonsoluble (NuM) fractions were obtained by an osmotic-shock method. Fractions were subjected to Western Blot assays with anti-DOR, anti-Np62 or anti- β-actin antibodies. Panel C. DOR-transfected HeLa cells or wild-type mouse 1C9 myoblasts were fixed and stained to view DOR and markers of subnuclear domains, such as the splicing speckles (SC-35), PML bodies (PML) or transcriptionally active sites (RNA Pol. II). DOR is shown in red in the left images, and SC-35, PML or RNA Polymerase II are shown in green in middle images. Merging is shown in the right images.

Figure 3. DOR transactivates nuclear hormone receptors.

Panels A. HeLa cells were transfected with expression plasmids encoding $TR_{\alpha 1}$ (TR), DOR, the empty vector pcDNA3 as a control vector, and the reporter vectors containing $TR_{\alpha 1}$ response elements linked to CAT. Cells were treated for 18 h in the presence or absence of ligands (100 nM T_3) and assayed for reporter expression. Results are mean ± SEM of triplicates and are representative of 6 independent experiments. * significant difference compared to the nuclear hormone receptor group, at $P<0.05$. Panels B. Reporter assays were done as in previous panels but different amounts of DOR (ranging

from 100 to 300 ng) were used for transfection and in the presence of ligands. Results are mean ± SEM of triplicates and are representative of 6 independent experiments. * significant difference compared to the nuclear hormone receptor group, at P<0.05. Panels C and D. HeLa cells were transfected with expression plasmids encoding p53 (panel C) or c-Myc (panel D), DOR expression vector, the empty vector pcDNA3 as a control vector, and the reporter vectors containing p53 or c-Myc response elements linked to luciferase. Results are mean ± SEM of triplicates and representative of 3 independent experiments. Differences between control and DOR transfected cells are statistically nonsignificant. Panel E. HeLa cells were transfected with different amounts of expression plasmids encoding DOR and with or without Gal4-VP16. Transcription was assayed with a reporter plasmid containing five copies of the UAS linked to luciferase. Results are mean ± SEM of triplicates and representative of 3 independent experiments.

Figure 4. DOR shows transcriptional activity when tethered to a target gene promoter.
DOR or fragments corresponding to the amino acids indicated were fused to the DNA binding domain of Gal4 (Gal4 DBD) and transfected in HeLa cells (panel A) or in HEK293T cells (panel B). Transcription was assayed with a reporter plasmid containing five copies of the UAS linked to luciferase. Results are mean ± SEM of triplicates and are representative of 6-27 independent experiments. * difference compared to the Gal4 DBD-DOR group, at P<0.05.

Figure 5. DOR loss-of-function in muscle cells.
Panel A. C2C12 myoblasts previously infected with lentiviruses encoding scramble (Scramble, open bar) RNA or DOR siRNA (siRNA, black bar) were cultured. Cell extracts and total RNA were obtained and DOR protein and mRNA levels were assayed by Western blot and real-time PCR. * difference compared to the Scramble group, at P<0.05. Panel B. Scramble (open bars) or DOR siRNA C2C12 muscle cells (black bars) were transfected with a reporter vector driven by a TRE, and with or without a expression vector for $TR\alpha 1$. Cells were then incubated in the presence or absence of thyroid hormone for 16 h. Results are mean ± SEM of triplicates and are representative of three independent experiments. * difference compared to the Scramble group, at P<0.05. Panels C-H. Scramble (open bars) or DOR siRNA C2C12 muscle cells (black bars) were incubated in 5% horse serum-containing medium either in the absence or in the presence of 100 nM T_3. Total RNA obtained at 48 h of T_3 were assayed by real-time PCR to measure the expression of different genes. Results are mean ± SEM of a representative experiment. * difference compared to the control group, at P<0.05.

Figure 6. DOR loss-of-function alters myogenesis.
Panels A-F. Confluent C2C12 myoblasts previously infected with lentiviruses encoding scramble (Sc, squares) RNA or DOR siRNA (Si, triangles) were allowed to differentiate in 5% horse serum-containing medium for 4 days. Total RNA was purified and the gene expression of DOR, myogenin, caveolin-3, actin α1, creatine kinase and IGF-II was assayed by real-time PCR. Results are mean ± SEM of four independent experiments. Sc and Si groups were significantly different as analyzed by ANOVA, at P<0.05. Panel G. DOR, and muscle-specific protein expression (myogenin, caveolin 3, and glycogen synthase) were analyzed by Western blot of total cell lysates (20 μg) from each condition. Relative amounts of proteins in each sample were checked by expression of the nonmuscle-specific protein β-actin.

Figure 7. DOR binds *in vitro* and i*n vivo* to thyroid hormone receptors.
Panel A. GST protein or $TR_{\alpha 1}$ fused to GST (TR-GST) were immobilized on glutathione sepharose beads and incubated with the DOR protein containing an Nterminal histidine tag (HisDOR), with or without the ligand (1 μM T_3). Bound proteins were eluted and resolved by SDS-PAGE and further Western blot using an antibody against the histidine tag (to visualize HisDOR) or against GST (to visualize GST or TR-GST). Panel B**.** HeLa cells over-expressing His-tagged DOR (left), TRα-1 (middle), or both (HisDOR + $TR_{\alpha 1}$) (right) were exposed to T_3 or were left untreated. After 1 h of treatment, cells were collected and DOR was immunoprecipitated from the nuclear fractions. The input control (10% input) and the immunoprecipitates (IP) were assayed by Western blot with specific antibodies. Panel C. ChIP analysis over a T_3 responsive promoter. DOR and $TR_{\alpha 1}$-transfected HeLa cells were treated with T_3 for 1 h or left untreated. Cross-linked chromatin prepared from cells was immunoprecipitated with the antibodies indicated. As a negative control, the samples were subjected to ChIP procedure in the absence of antibody. Aliquots of chromatin taken before immunoprecipitation (input) and the immunoprecipitates were subjected to PCR analysis with primers directed to the dio1 promoter.

Figure 8. DOR rapidly delocalizes from PML nuclear bodies in response to thyroid hormones.
Panel A. HeLa cells were transiently cotransfected with DOR and $TR_{\alpha 1}$ expression vectors. The intranuclear positioning of DOR relative to PML nuclear bodies was determined before and after T_3 addition. Antibodies and immunofluorescence legend: Anti-DOR, stained in red (column 1); anti-PML, stained in green (column 2); merged images (column 3). Panel B. Full length DOR was fused in frame with the fluorescent protein GFP. To determine whether DOR-GFP retained the capacity to coactivate thyroid hormone receptors $TR_{\alpha 1}$, experiments were done as in Figure 3A. Panel C. HeLa cells were transiently cotransfected with DOR-GFP and $TR_{\alpha 1}$ expression vectors. The intranuclear positioning of DOR relative to PML nuclear bodies and $TR_{\alpha 1}$ was determined before and after different times of T_3 addition. Antibodies and immunofluorescence legend: Anti-DOR, stained in red (column 1); anti-PML, stained in green (column 2), anti- $TR_{\alpha 1}$ in cyan. Merged images: DOR/PML (column 3), DOR/$TR_{\alpha 1}$ (column 4).

Figure 1:

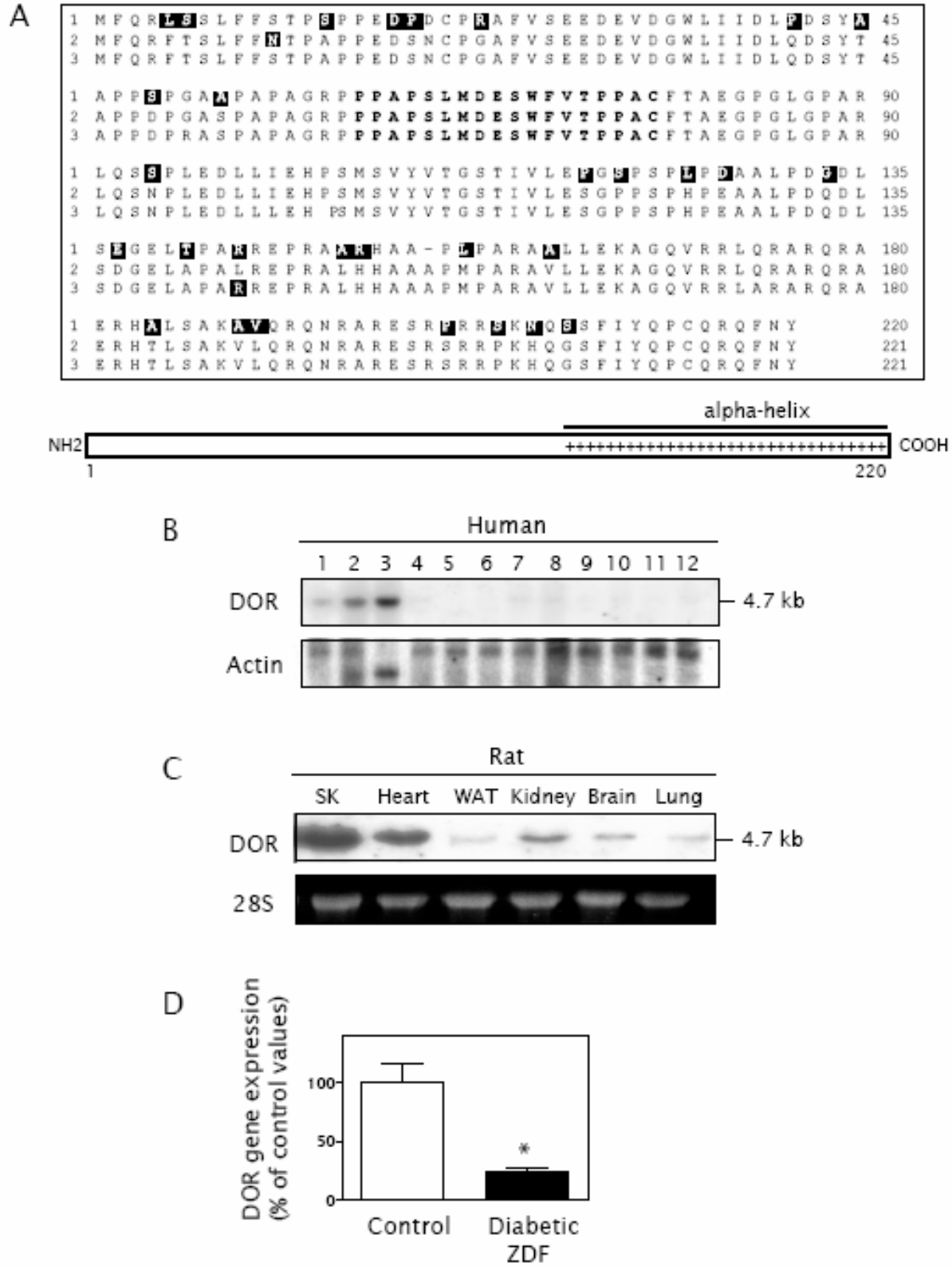

A
1 M F Q R L S S L F F S T P S P P E D P D C P R A F V S E E D E V D G W L I I D L P D S Y A 45
2 M F Q R F T S L F F N T P A P P E D S N C P G A F V S E E D E V D G W L I I D L Q D S Y T 45
3 M F Q R F T S L F F S T P A P P E D S N C P G A F V S E E D E V D G W L I I D L Q D S Y T 45
1 A P P S P G A A P A P A G R P P P A P S L M D E S W F V T P P A C F T A E G P G L G P A R 90
2 A P P D P G A S P A P A G R P P P A P S L M D E S W F V T P P A C F T A E G P G L G P A R 90
3 A P P D P R A S P A P A G R P P P A P S L M D E S W F V T P P A C F T A E G P G L G P A R 90
1 L Q S S P L E D L L I E H P S M S V Y V T G S T I V L E P G S P S P L P D A A L P D G D L 135
2 L Q S N P L E D L L I E H P S M S V Y V T G S T I V L E S G P P S P H P E A A L P D Q D L 135
3 L Q S N P L E D L L L E H P S M S V Y V T G S T I V L E S G P P S P H P E A A L P D Q D L 135
1 S E G E L T P A R R E P R A A R H A A - P L P A R A A L L E K A G Q V R R L Q R A R Q R A 180
2 S D G E L A P A L R E P R A L H H A A A P M P A R A V L L E K A G Q V R R L Q R A R Q R A 180
3 S D G E L A P A R R E P R A L H H A A A P M P A R A V L L E K A G Q V R R L A R A R Q R A 180
1 E R H A L S A K A V Q R Q N R A R E S R P R R S K R Q S S F I Y Q P C Q R Q F N Y 220
2 E R H T L S A K V L Q R Q N R A R E S R S R R P K H Q G S F I Y Q P C Q R Q F N Y 221
3 E R H T L S A K V L Q R Q N R A R E S R S R R P K H Q G S F I Y Q P C Q R Q F N Y 221
alpha-helix
NH2
COOH
1
220
B
Human
1 2 3 4 5 6 7 8 9 10 11 12
DOR
4.7 kb
Actin
C
Rat
SK
Heart
WAT
Kidney
Brain
Lung
DOR
4.7 kb
28S
D
DOR gene expression (% of control values)
100
50
0
*
Control
Diabetic ZDF

Figure 2:

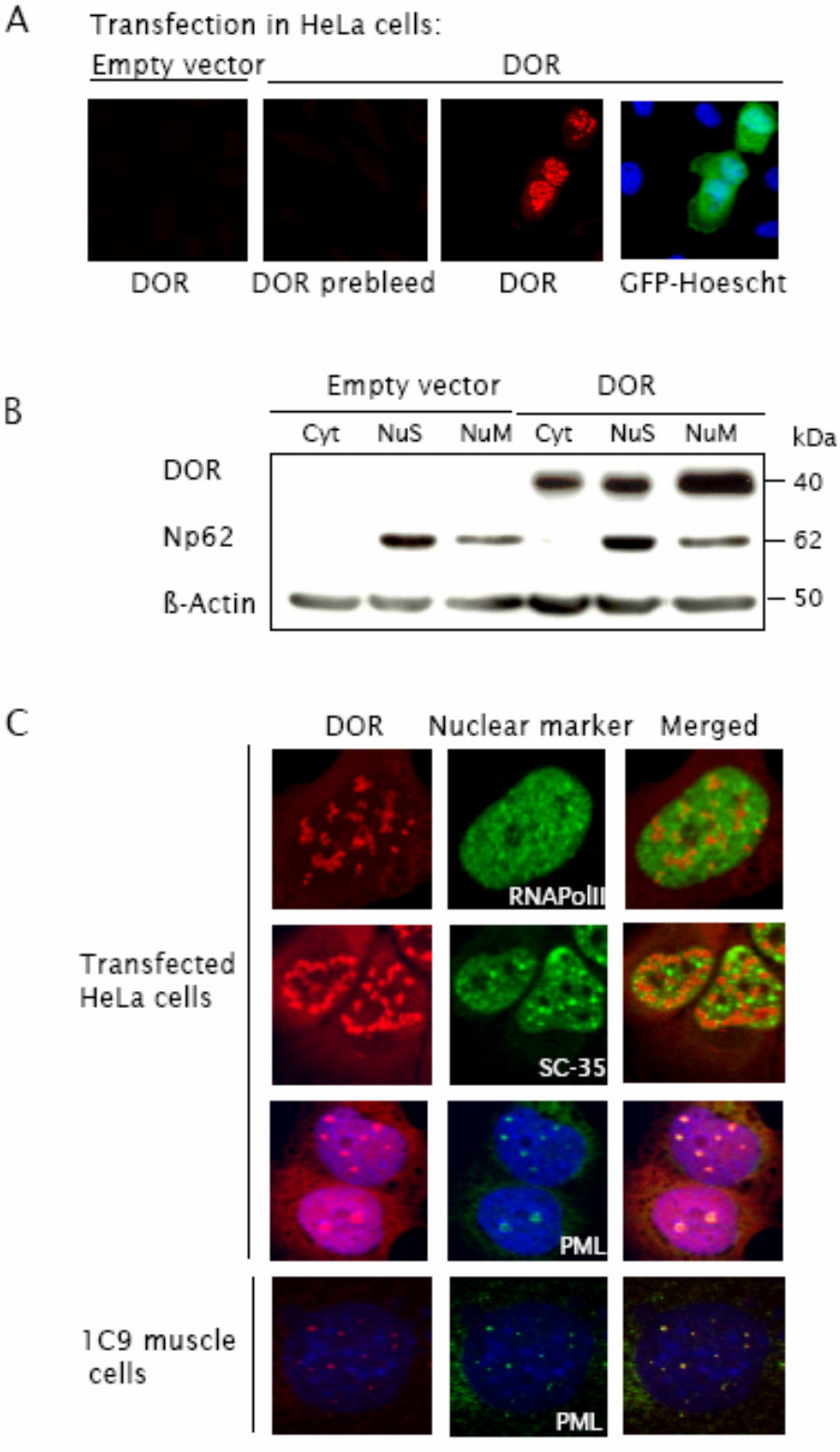

A
Transfection in HeLa cells:
Empty vector
DOR
DOR
DOR prebleed
DOR
GFP-Hoescht
B
Empty vector
DOR
Cyt
NuS
NuM
Cyt
NuS
NuM
kDa
DOR
Np62
ß-Actin
40
62
50
C
DOR
Nuclear marker
Merged
RNAPolII
SC-35
PML
PML
Transfected HeLa cells
1C9 muscle cells

Figure 3:

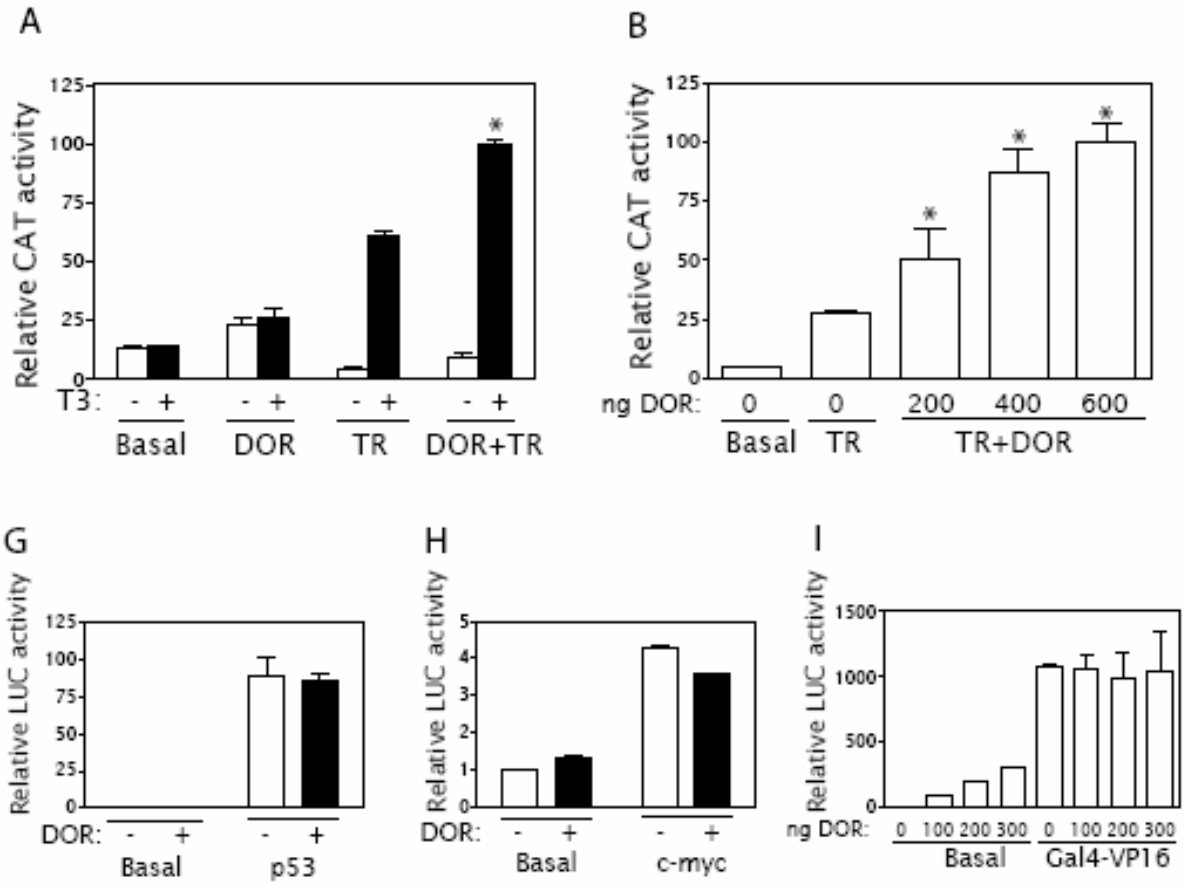

A
Relative CAT activity
125
100
75
50
25
0
T3: - + - + - + - +
Basal
DOR
TR
DOR+TR
B
Relative CAT activity
ng DOR: 0 0 200 400 600
Basal
TR
TR+DOR
G
Relative LUC activity
DOR: - + - +
Basal
p53
H
Relative LUC activity
DOR: - + - +
Basal
c-myc
I
Relative LUC activity
1500
1000
500
0
ng DOR: 0 100 200 300 0 100 200 300
Basal
Gal4-VP16

Figure 4:

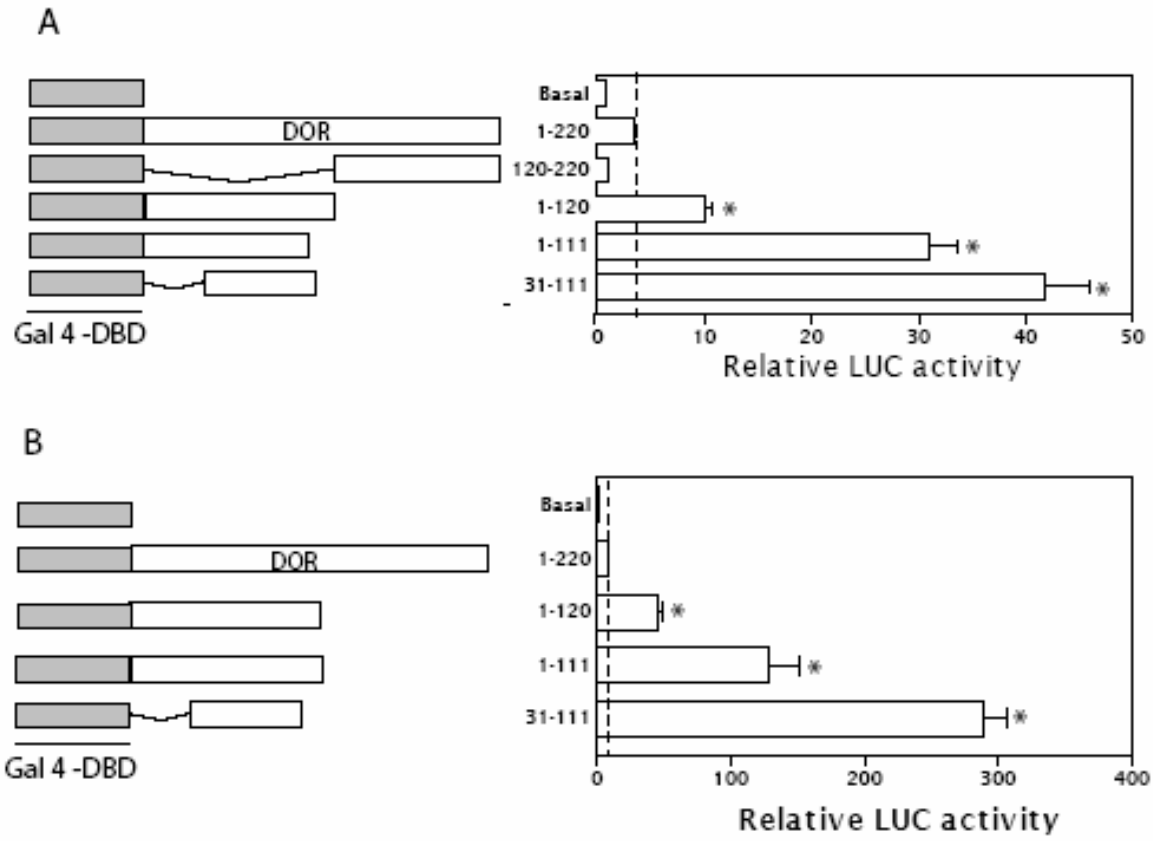

A
DOR
Gal 4 -DBD
Basal
1-220
120-220
1-120
1-111
31-111
0
10
20
30
40
50
Relative LUC activity
B
DOR
Gal 4 -DBD
Basal
1-220
1-120
1-111
31-111
0
100
200
300
400
Relative LUC activity

Figure 5:

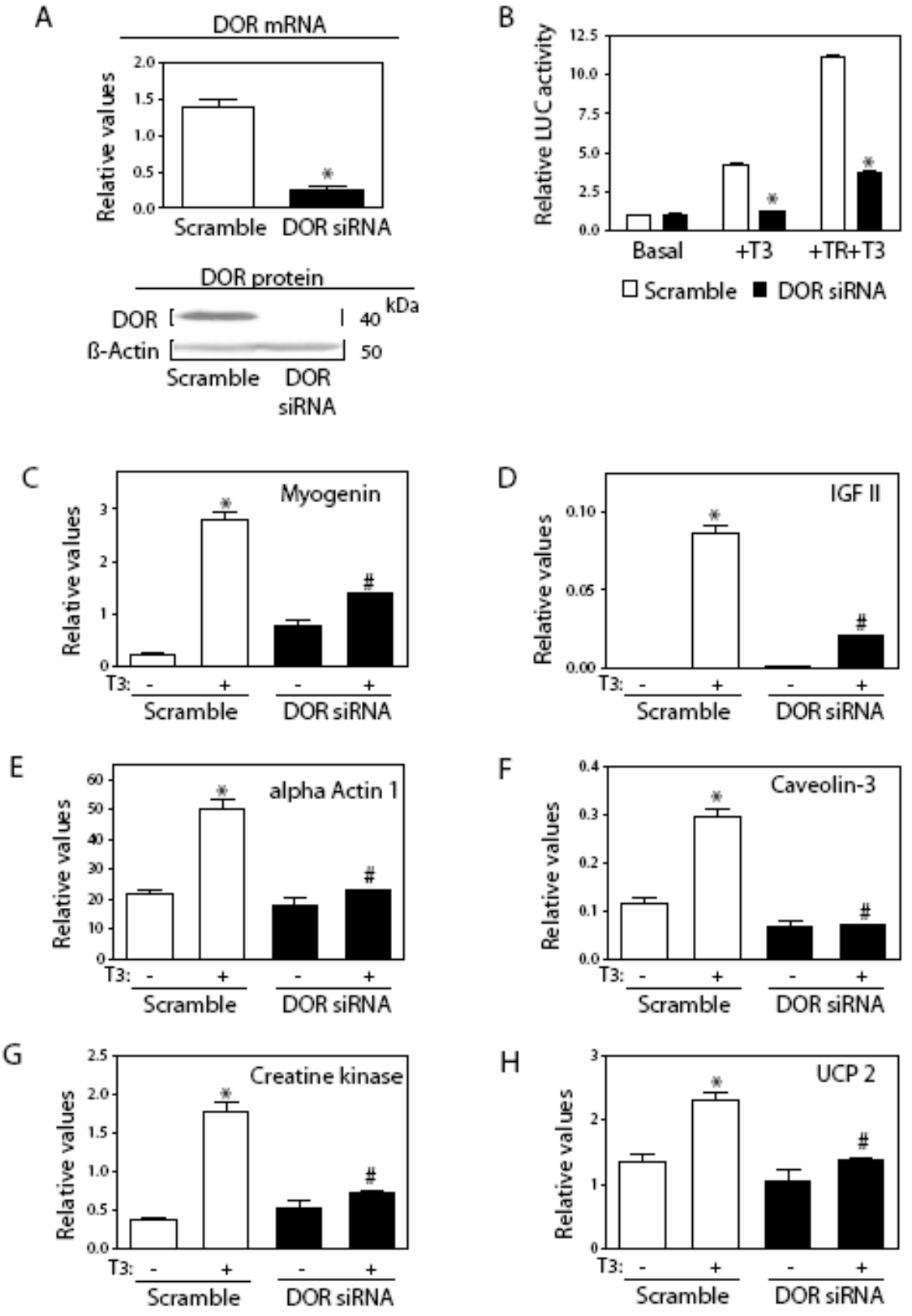

A
DOR mRNA
Relative values
2.0
1.5
1.0
0.5
0.0
*
Scramble
DOR siRNA
DOR protein
DOR
ß-Actin
40
50
kDa
Scramble
DOR siRNA
B
Relative LUC activity
12.5
10.0
7.5
5.0
2.5
0.0
*
*
*
Basal
+T3
+TR+T3
Scramble
DOR siRNA
C
Myogenin
Relative values
3
2
1
0
*
#
T3: - + - +
Scramble
DOR siRNA
D
IGF II
Relative values
0.10
0.05
0.00
*
#
T3: - + - +
Scramble
DOR siRNA
E
alpha Actin 1
Relative values
60
50
40
30
20
10
0
*
#
T3: - + - +
Scramble
DOR siRNA
F
Caveolin-3
Relative values
0.4
0.3
0.2
0.1
0.0
*
#
T3: - + - +
Scramble
DOR siRNA
G
Creatine kinase
Relative values
2.5
2.0
1.5
1.0
0.5
0.0
*
#
T3: - + - +
Scramble
DOR siRNA
H
UCP 2
Relative values
3
2
1
0
*
#
T3: - + - +
Scramble
DOR siRNA

Figure 6:

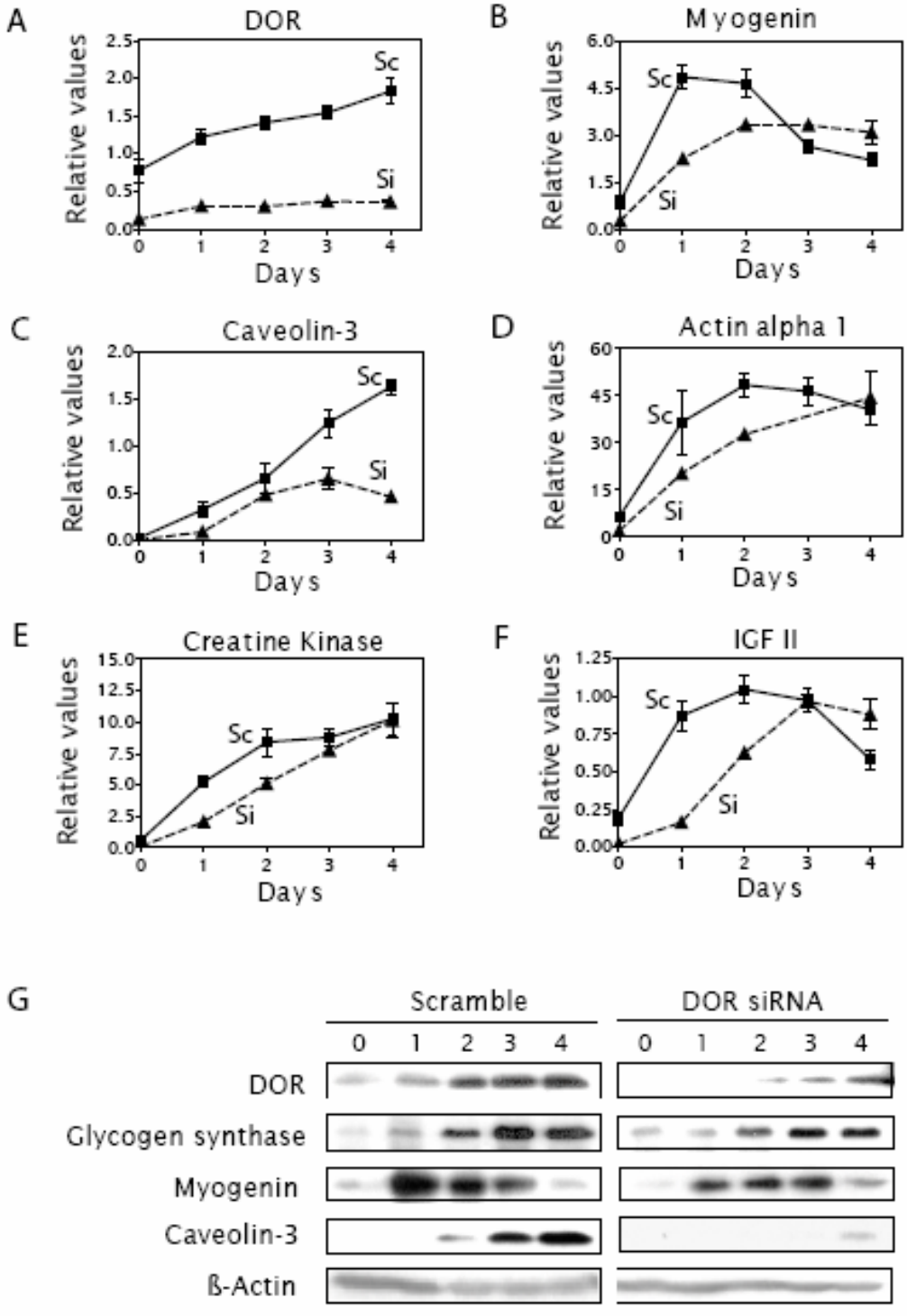

A
DOR
Relative values
Days
Sc
Si
B
Myogenin
C
Caveolin-3
D
Actin alpha 1
E
Creatine Kinase
F
IGF II
G
Scramble
DOR siRNA
DOR
Glycogen synthase
Myogenin
Caveolin-3
ß-Actin

Figure 7:

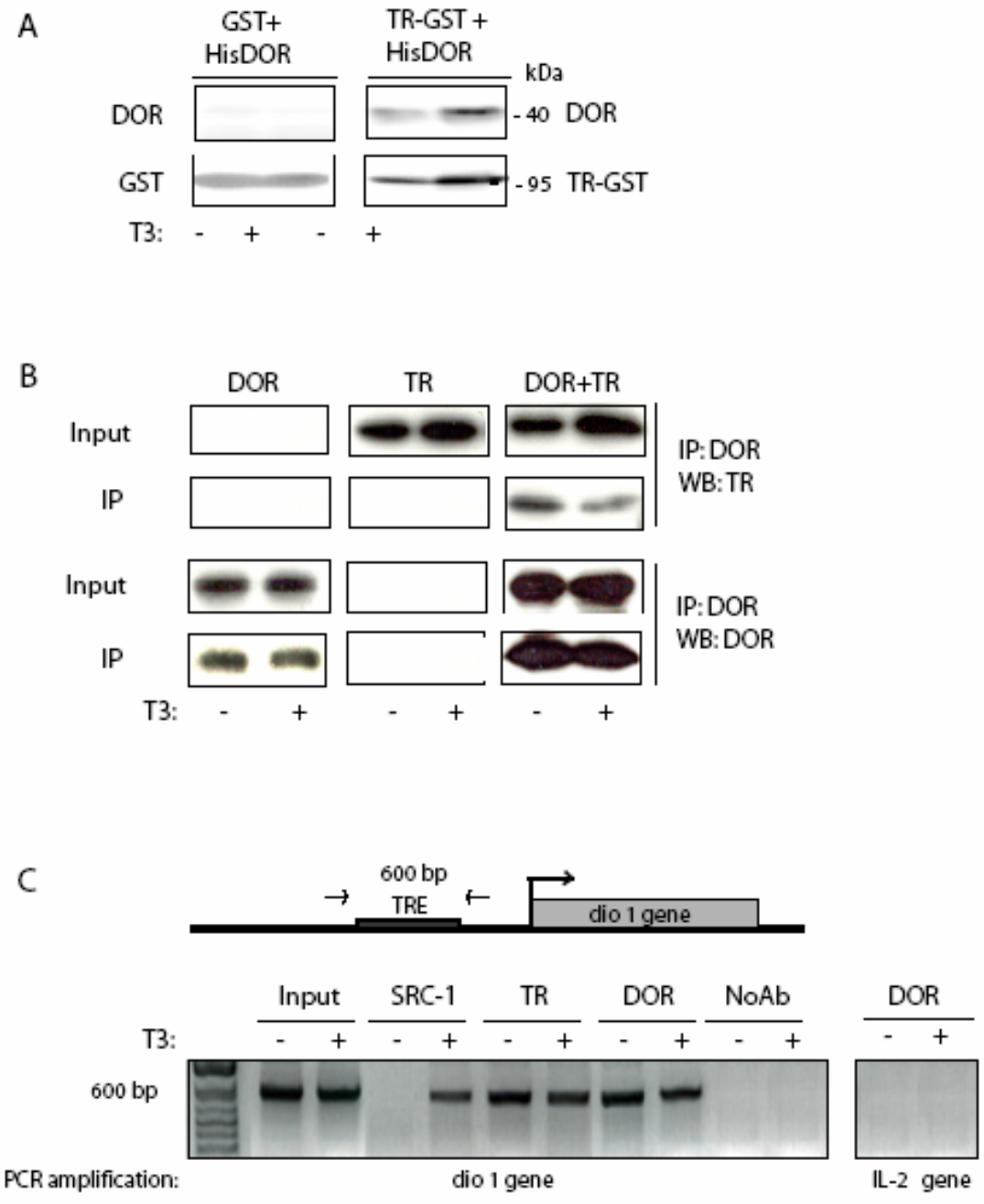
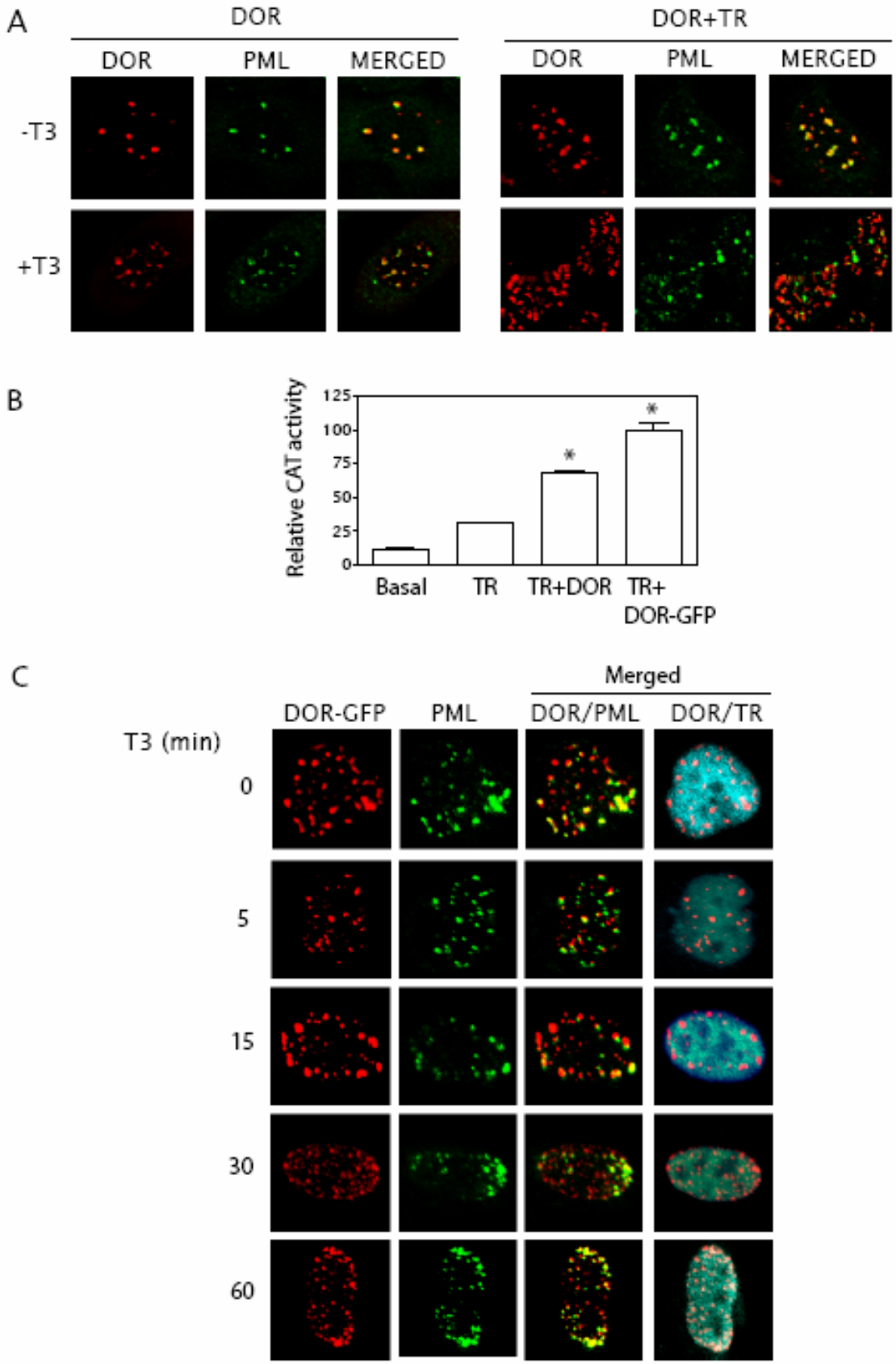

A
GST+
HisDOR
TR-GST +
HisDOR
kDa
DOR
- 40 DOR
GST
- 95 TR-GST
T3: - + - +
B
DOR
TR
DOR+TR
Input
IP
IP: DOR
WB: TR
Input
IP
IP: DOR
WB: DOR
T3: - + - + - +
C
600 bp
TRE
dio 1 gene
Input
SRC-1
TR
DOR
NoAb
DOR
T3: - + - + - + - + - + - +
600 bp
PCR amplification:
dio 1 gene
IL-2 gene

Figure 8:


A
DOR
DOR+TR
DOR
PML
MERGED
DOR
PML
MERGED
-T3
+T3
B
Relative CAT activity
125
100
75
50
25
0
*
*
Basal
TR
TR+DOR
TR+
DOR-GFP
C
Merged
DOR-GFP
PML
DOR/PML
DOR/TR
T3 (min)
0
5
15
30
60

ANNEX 2. GENERACIÓ D'UN RATOLÍ TRANSGÈNIC.

Per analitzar els efectes de DOR *in vivo* vam decidir generar un ratolí transgènic que sobre-expressés la proteïna. Per evitar efectes no desitjats derivats de l'expressió de DOR a teixits on en condicions normals no s'expressa vam decidir generar un transgènic de sobre-expressió exclusiva a múscul esquelètic, un dels principals teixits d'expressió de DOR. Per fer-ho vam clonar la pauta oberta de lectura del DOR de ratolí al vector pMDAF2, on la seva expressió es troba sota el control del promotor del gen de la Myosin-Light Chain 1 (MLC-1), un gen que s'expressa gairebé de forma exclusiva al múscul esquelètic (Rosenthal et al., 1989). Aquest vector també té elements a 3' com una regió de poliadenilació que dóna estabilitat al missatger i un enhancer específic de múscul (**figura 56**).

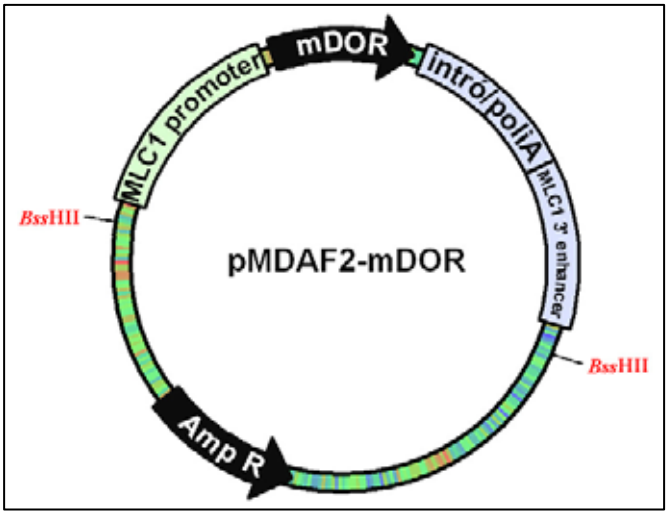


Figura 56. Clonació de DOR de ratolí al vector pMDAF2.

Després d'amplificar-lo el vector es va digerir amb l'enzim BssHII, de manera que es va aïllar el fragment d'interès de la resta del vector. Aquest fragment, contenint el gen DOR, el promotor i els elements 3', va ser el que es va usar per a la generació dels ratolins transgènics, que va portar a terme la Dra. Belén Pintado a l'INIA. Breument, el fragment del vector es va micro-injectar a pronuclis masculins d'embrions extrets de femelles donants. Un cop micro-injectats, els embrions es van implantar a femelles pseudo-gestants (muntades per mascles vasectomitzats).

La soca de ratolí en què es va realitzar el procediment és C57BL/6J; es tracta d'una soca utilitzada en nombroses àrees d'investigació incloent biologia cardiovascular, diabetis y

obesitat, genètica, immunologia, neurobiologia, i investigació sensorineural. El motiu principal de l'elecció d'aquesta soca per als nostres experiments és que els ratolins C57BL/6J alimentats amb una dieta rica en greixos desenvolupen obesitat, hiperglucèmia y hiper-insulinèmia. Tot i això, i degut a que aquesta soca respon malament a la super-ovulació i els mascles són poc fèrtils, es va realitzar la microinjecció també en embrions híbrids B6CBA F1, es a dir, híbrids de femelles C57BL6 creuades amb mascles CBA, per assegurar l'obtenció de ratolins transgènics.

Quan els animals nascuts tenien 3 setmanes d'edat es va obtenir un fragment de la cua del qual vam extreure DNA genòmic per analitzar la presència del transgèn. L'anàlisi es va realitzar per PCR amb primers específics de la regió codificant de DOR, dissenyats de manera que el primer 'forward' i el 'reverse' es troben a exons diferents. D'aquesta manera l'amplificació genera un fragment de diferent mida en funció de si s'amplificava DNA genòmic (que té introns) o el transgèn (que no en té) (**figura 57**). Així en la PCR dels ratolins no transgènics s'amplifica tan sols un fragment de mida gran, corresponent al DNA genòmic. En la PCR dels animals transgènics, en canvi, s'amplificaran dos fragments: el fragment gran del DNA genòmic i una fragment més petit corresponent al transgèn.

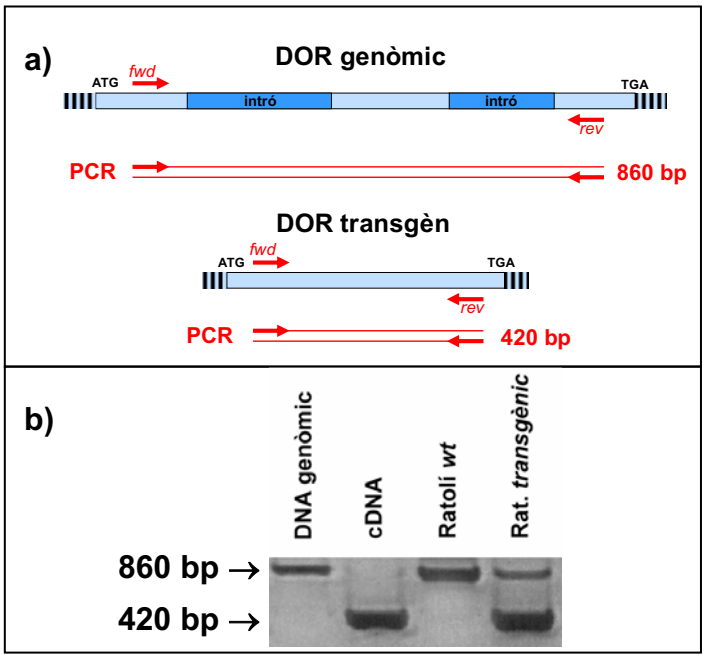


Figura 57. **a**) Esquema de la posició relativa dels primers en DNA genòmic i en el transgen. **b**) Exemple de genotipació.

La genotipació dels animals nascuts dels embrions microinjectats va servir per determinar que havíem obtingut 5 línies diferents d'animals positius per a la inserció del transgèn. D'aquestes 5 línies, 2 eren animals purs C57BL/6J i les altres 3 eren híbrids B6CBA F1. Aquestes línies es van amplificar fent creuaments amb ratolins C57BL/6J.

També vam comprovar que les diferents línies tinguéssin més expressió de DOR a múscul esquelètic que els ratolins no transgènics. Per aquest motiu es van sacrificar animals de les diferents línies, dels quals es va extreure els músculs quadríceps i gastrocnemius. D'aquests es va extreure la proteïna is es va analitzar l'expressió de DOR per Western Blot (**figura 58**). Es va comprovar així que els ratolins transgènics de les diferents línies expressen més DOR al múscul esquelètic que els animals no transgènics.

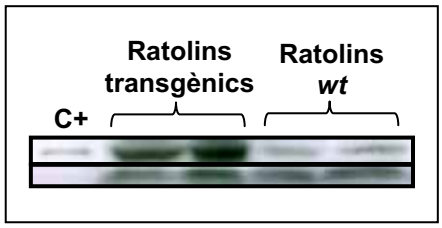


Figura 58. Exemple de la mesura de DOR per Western Blot a ratolins transgènics i *wild type*. C+: control positiu (cèl·lules HeLa transfectades amb DOR).

ANNEX 3. EFECTE DE VDUP1 SOBRE LA FUNCIÓ DE DOR.

Amb l'objectiu d'identificar proteïnes que interaccionen amb DOR es va realitzar al nostre laboratori un estudi de 'two-hybrid' amb una llibreria de cDNA de múscul esquelètic (M. Orpinell, Tesi Doctoral 2006, Universitat de Barcelona). Una de les proteïnes identificades en aquest estudi va ser VDUP1/TXNIP/TBP-2, una proteïna implicada en el sistema de la tioredoxina. Aquesta interacció física té lloc en la regió N-terminal de DOR (residus 1 a 120).

Quadre informatiu 4. Tioredoxina i VDUP1.

La tioredoxina forma part d'un sistema antioxidant que protegeix les cèl·lules davant d'estrès oxidatiu. Actua reduint proteïnes oxidades o espècies reactives d'oxigen. Per fer-ho la tioredoxina s'oxida, i és posteriorment regenerada per la Tioredoxina Reductasa en una reacció que implica el consum de NADPH (Powis et al., 2001; Nordberg et al., 2001). La proteïna VDUP1 inhibeix l'activitat de la Tioredoxina unint-se al lloc catalític d'aquesta en el seu estat reduït, competint així per la unió d'altres proteïnes i sensibilitzant les cèl·lules a estímuls apoptòtics (Junn et al., 2002; Wang et al., 2002). VDUP1 també impedeix l'entrada de tioredoxina al nucli (Schulze et al., 2002). Recentment la Tioredoxina i VDUP1 s'han relacionat amb la fisiopatologia del càncer i de la síndrome metabòlica (Kaimul et al., 2007).

Vam analitzar quin efecte tenia l'expressió deVDUP1 sobre la funció de DOR. Per aquest motiu vam realitzar estudis de coactivació de GRα en presència o en absència de VDUP1 (**figura 59a**). Els resultats mostren que en presència de VDUP1 l'activitat màxima de DOR coactivant GRα disminueix significativament. Vam analitzar també si aquesta inhibició de DOR per part de VDUP1 es dóna en el context dels constructes Gal4-DOR (**figura 59b**). La funció d'aquests constructes es veu clarament disminuïda per la presència de VDUP1, tant en el cas de la proteïna sencera com en el del fragment que provoca la màxima activació (fragment 1 a 111). Aquests resultats suggereixen que la unió de VDUP1 a la regió N-terminal de DOR té un efecte inhibidor sobre la seva capacitat d'activar la transcripció.

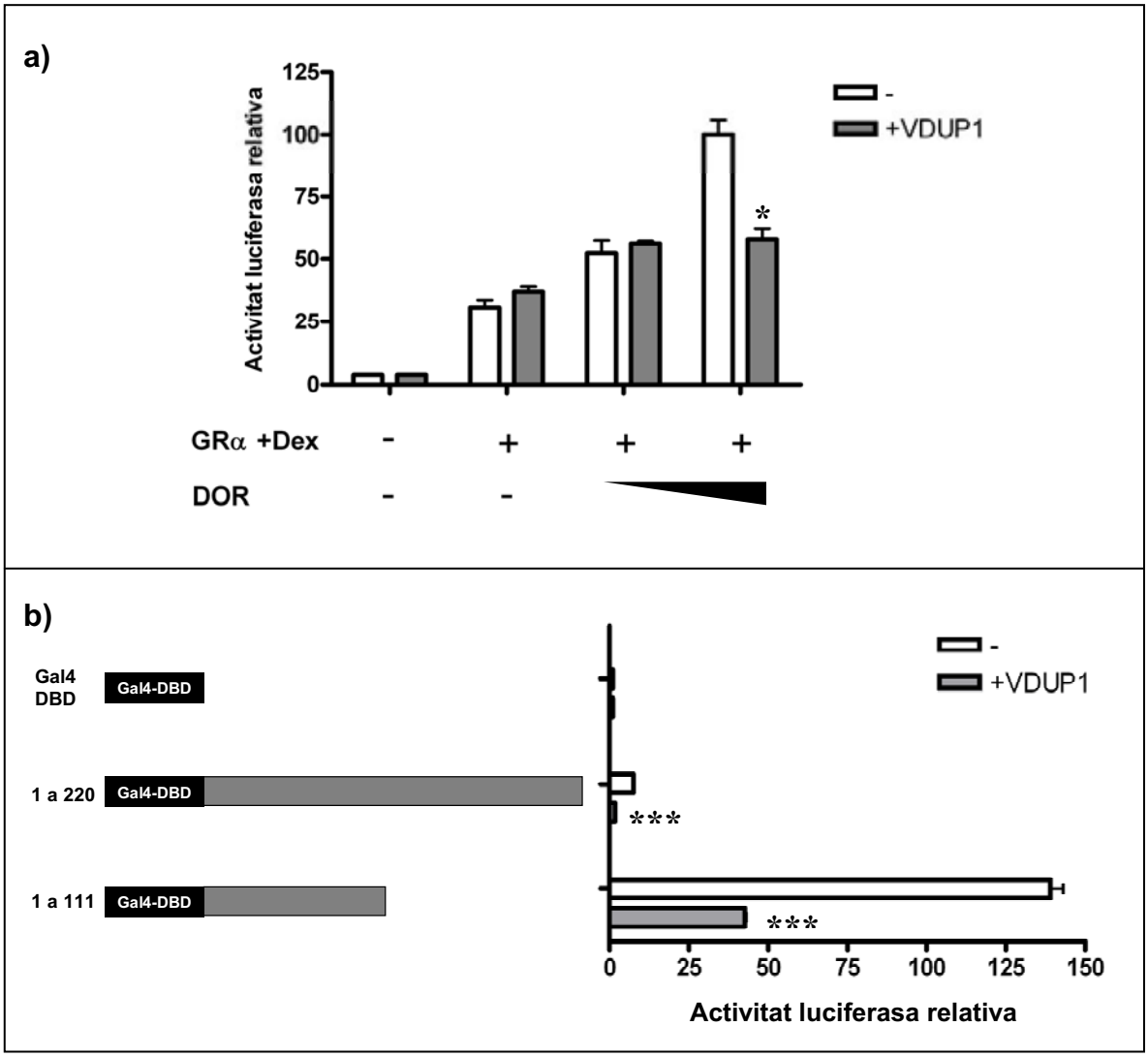


Figura 59. **a**) *Efecte de VDUP1 sobre la capacitat de DOR de coactivar GRα*. Es van transfectar cèl·lules HeLa amb vectors d'expressió per GRα, DOR, VDUP1, el vector buit pCDNA3 com a vector control i el vector reporter de GRα. Les cèl·lules es van tractar durant 18 h en presència del lligand (dexametasona 100 nM) i es va analitzar l'activitat luciferasa.
b) *efecte de VDUP1 sobre l'activitat dels constructes Gal4-DOR*. Es van transfectar cèl·lules HeLa amb el vector d'expressió del fragment de Gal4-DOR indicat i de VDUP1. A les 24 h es va analitzar l'activitat luciferasa.
Els resultats són la mitjana i l'error estàndard de triplicats i són representatius de 3 experiments independents. * : diferència significativa amb P<0,05 comparada amb el grup equivalent sense VDUP1; *** : P<0,001.

Les cèl·lules HeLa expressen abundantment VDUP1. Quan les cèl·lules són sotmeses a diversos estressos VDUP1 s'indueix (Junn et al., 2000). Per aquest motiu vam analitzar quin era l'efecte sobre la funció de DOR de sotmetre les cèl·lules a un estrès oxidatiu. Vam realitzar assajos de coactivació de PPARγ i GRα en presència o en absència de peròxid d'hidrogen 0'5 mM (**figura 60a**), concentració que indueix VDUP1 (Junn et al.,

2000). També vam analitzar l'efecte del peròxid d'hidrogen en el context de Gal4-DOR (**figura 60b**).

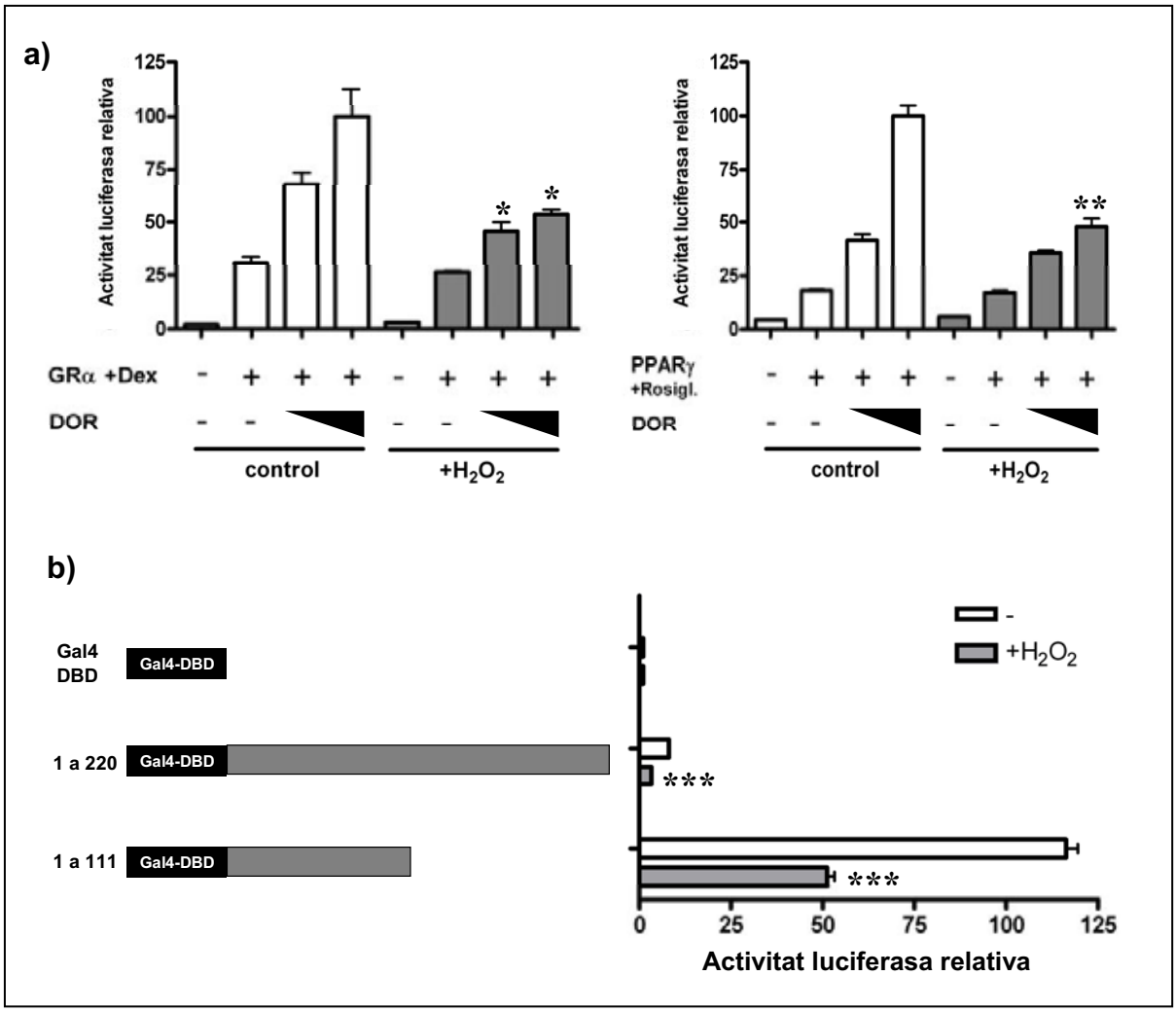


Figura 60. **a**) *Efecte de l'H_2O_2 sobre la capacitat de DOR de coactivar GRα i PPARγ.* Es van transfectar cèl·lules HeLa amb vectors d'expressió per GRα o PPARγ, DOR, el vector buit pCDNA3 com a vector control i el vector reporter de GR o PPAR en cada cas. Les cèl·lules es van tractar durant 18 h en presència del lligand (dexametasona 100 nM o rosiglitazona 100 nM) i en presència o en absència d'H_2O_2 (500 mM) i es va analitzar l'activitat luciferasa.
b) *efecte de l'H_2O_2 sobre l'activitat dels constructes Gal4-DOR*. Es van transfectar cèl·lules HeLa amb el vector d'expressió del fragment de Gal4-DOR indicat. A les 12 h es va afegir o no H_2O_2 (500 mM). A les 24 h es va analitzar l'activitat luciferasa.
Els resultats són la mitjana i l'error estàndard de triplicats i són representatius de 3 experiments independents. * : diferència significativa amb P<0,05 comparada amb el grup equivalent sense H_2O_2; ** : P<0.01; *** : P<0.001.

En tots els casos la presència del peròxid d'hidrogen redueix la capacitat de DOR d'activar la transcripció.

ANNEX 4. ACCIÓ DE LA DISPONIBILITAT DE GLUCOSA SOBRE LA FUNCIÓ DE DOR.

L'anàlisi de la seqüència de la proteïna amb el programa YinOYang (Gupta et al, 2002) va servir per detectar la presència a DOR de diversos llocs de possible modificació per O-β-gluconoacetilació (**figura 61**).

Residue		O-GlcNAc result	Potential	Thresh. (1)	Thresh. (2)
7	S	+	0.5093	0.4598	0.5701
43	S	++	0.5323	0.3927	0.4797
49	S	+++	0.5470	0.3380	0.4060
93	S	+	0.3994	0.3825	0.4660
121	S	+	0.3862	0.3408	0.4098
198	S	+	0.3889	0.3341	0.4007
203	S	+++	0.5162	0.3218	0.3842

Figura 61. Resultats de l'anàlisi de la forma humana de DOR amb el programa YinOYang.

Aquesta predicció i la relació d'aquesta modificació post-traduccional amb l'etiopatologia de la diabetes mellitus de tipus II ens va portar a analitzar si la proteïna DOR està regulada d'aquesta manera.

Quadre informatiu 5. O-β-gluconoacetilació.

Moltes proteïnes nuclears i citoplasmàtiques són modificades per O-β-gluconoacetilació, és a dir, per addició de N-acetilgucosamina a residus de Serina o Treonina. Aquesta modificació pot actuar conjuntament amb la fosforilació per regular la interacció entre proteïnes, l'activitat enzimàtica, la localització sub-cel·lular o la vida mitja de les proteïnes. La maquinària de l'O-β-gluconoacetilació actua com un sensor nutricional de la cèl·lula, ja que els nivell d'UDP-N-acetilglucosamina, el substrat que s'utilitza en la reacció, estan modulats per la disponibilitat de glucosa. Així, canvis en els nivells extracel·lulars de glucosa regulen la modificació de proteïnes per O-β-gluconoacetilació. (Comer et al, 1999; Hanover, 2001; Zachara et al., 2004). Recentment l'O-β-gluconoacetilació s'ha implicat en l'etiopatologia de la diabetis mellitus de tipus II (Akimoto et al., 2005).

Primerament vam mesurar la capacitat de les construccions Gal4-DOR per activar la transcripció quan manteníem les cèl·lules després de la transfecció a diferents concentracions de glucosa, des de 25 mM (la concentració usada de rutina) a l'absència total de glucosa ('0 mM'). L'osmolaritat es va mantenir afegint la concentració necessària de manitol al medi (**figura 62**).

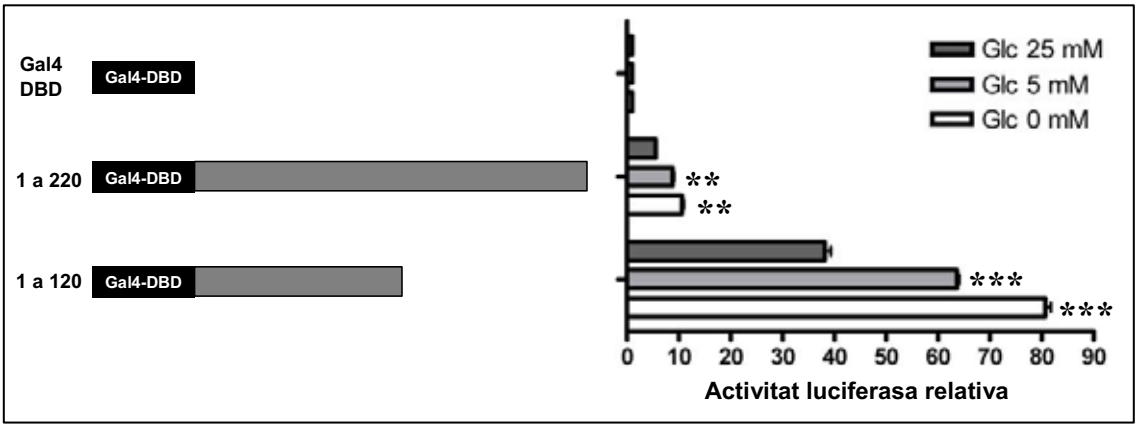


Figura 62. *Activitat de DOR i del fragment 1 a 120 units a Gal4 a diferents concentracions de glucosa*. Es van transfectar cèl·lules HeLa amb el vector d'expressió del fragment de Gal4-DOR indicat. A les 4 h es va canviar el medi per un medi amb la concentració de glucosa indicada. L'osmolaritat del medi es va mantenir per addició de manitol. A les 24 h es va analitzar l'activitat luciferasa. Els resultats són la mitjana i l'error estàndard de triplicats i són representatius de 3 experiments independents. ** : diferència significativa amb P<0,01 comparada amb el grup equivalent amb glucosa 25 mM; *** : P<0.001.

L'activitat de les proteïnes de fusió va ser més gran com menor era la concentració de glucosa del medi. Vam analitzar l'efecte en les mateixes construccions de l'addició al medi de benzil-2-acetamido-2-deoxi-α-D-galactopiranòsid (BADG), un inhibidor de la OGT, l'enzim que catalitza la O-β-gluconoacetilació (Sutton-McDowall et al., 2006) (**figura 63**).

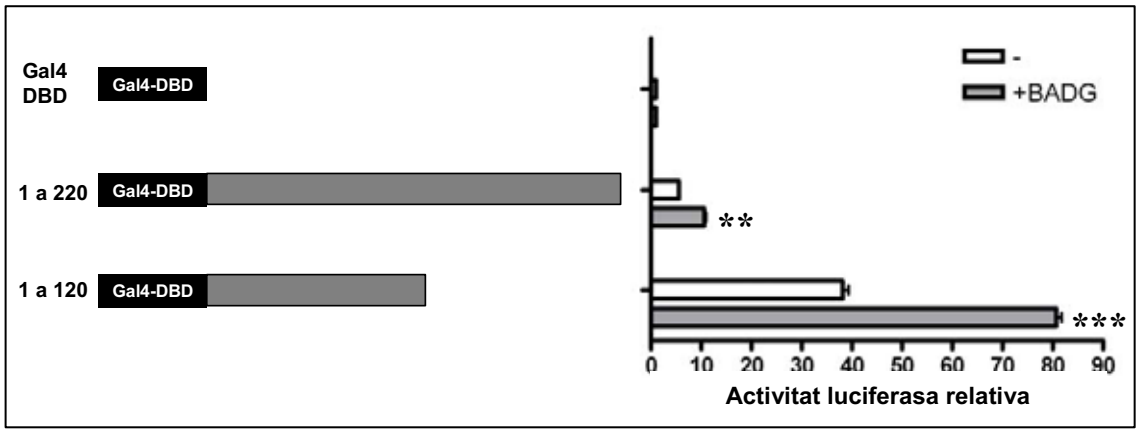


Figura 63. *Activitat de DOR i del fragment 1 a 120 units a Gal4 en presència o no de BADG*. Es van transfectar cèl·lules HeLa amb el vector d'expressió del fragment de Gal4-DOR indicat. A les 4 h es va canviar el medi per un medi amb glucosa 25 mM i amb o sense BADG (0.1 mM). A les 24 h es va analitzar l'activitat luciferasa. Els resultats són la mitjana i l'error estàndard de triplicats i són representatius de 3 experiments independents. ** : diferència significativa amb P<0,01 comparada amb el grup equivalent sense BADG; *** : P<0.001.

També vam analitzar com afectava a l'activitat de DOR el fet de substituir la glucosa (25 mM) per Lactat (5 mM). De nou la diferent osmolaritat dels medis s'igualava amb la concentració necessària de manitol. Vam analitzar aquest efecte tant en el context de Gal4-DOR (**figura 64a**) com en la coactivació de DOR sobre GRα (**figura 64b**).

En tots dos casos l'activitat obtinguda quan el medi té lactat és major que l'obtinguda en presència de glucosa.

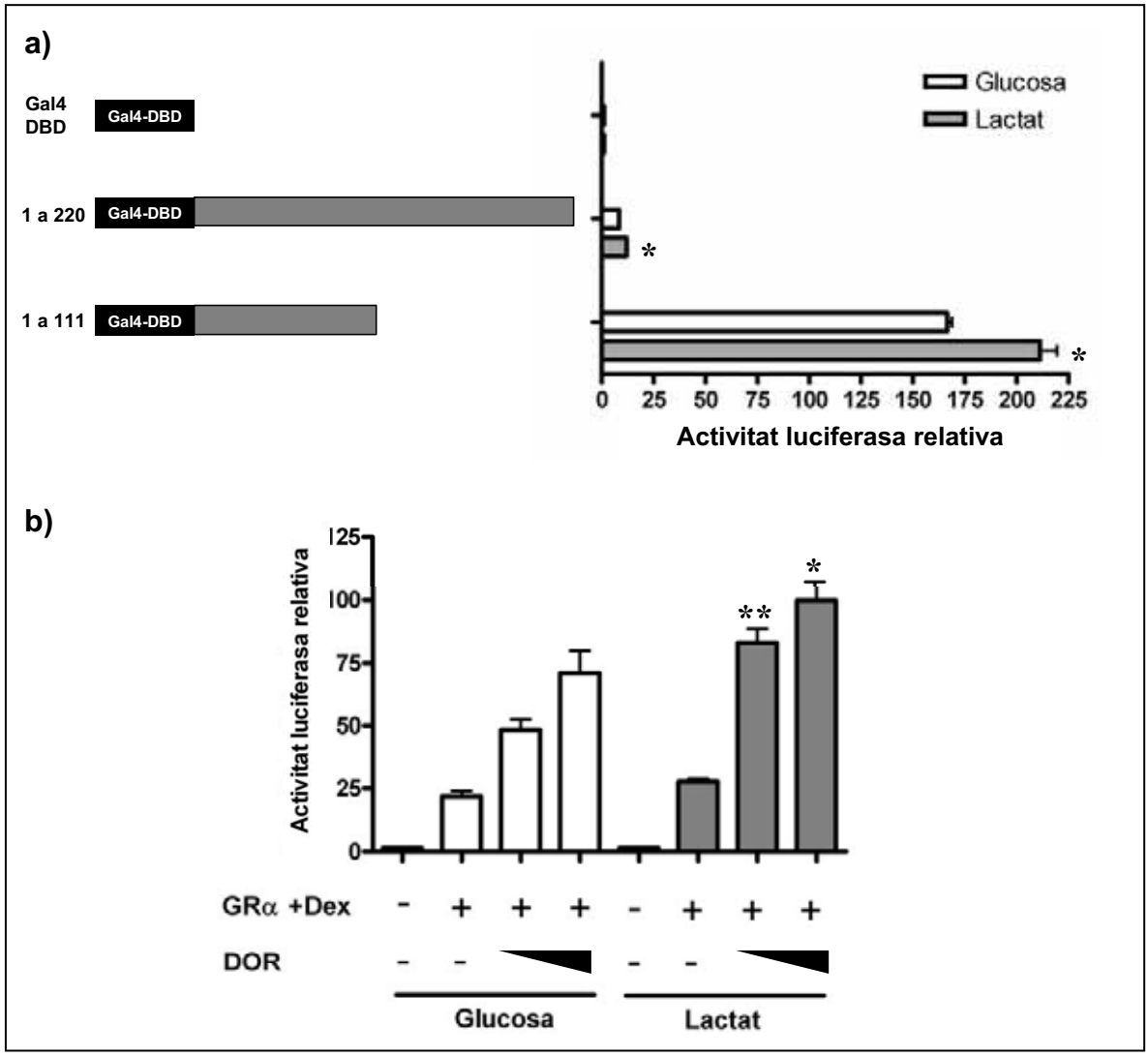


Figura 64. **a**) *Activitat dels constructes Gal4-DOR en presència de glucosa o de lactat*. Es van transfectar cèl·lules HeLa amb el vector d'expressió del fragment de Gal4-DOR indicat. A les 4 h es va canviar el medi per medi normal (glucosa 25 mM) o amb lactat (5 mM). A les 24 h es va analitzar l'activitat luciferasa. **b**) coactivació de DOR sobre GRα en presència de glucosa o de lactat. Es van transfectar cèl·lules HeLa amb vectors d'expressió per GRα, DOR, el vector buit pCDNA3 com a vector control i el vector reporter de GR. Les cèl·lules es van tractar durant 18 h en presència del lligand (dexametasona 100 nM o rosiglitazona 100 nM) i en medi amb glucosa (25 mM) o lactat (5 mM) i es va analitzar l'activitat luciferasa.
Els resultats són la mitjana i l'error estàndard de triplicats i són representatius de 3 experiments independents. * : diferència significativa amb P<0,05 comparada amb el grup equivalent amb glucosa; ** : P<0.01.

Tots aquests resultats ens van portar a pensar que DOR podia ser regulat per O-β-gluconoacetilació. Per analitzar aquesta possibilitat vam fer ús d'un anticòs que reconeix específicament aquesta modificació (anti- O-linked N-acetylglucosamine, *Affinity Bioreagents*). Vam transfectar una construcció de DOR que té una cua

d'histidines, i vam usar columnes amb boles de níquel per purificar la proteïna. Amb la proteïna purificada vam fer un Western Blot detectant amb l'anticòs per la modificació, però no vam detectar senyal a l'alçada corresponent a DOR. Per confirmar la presència de DOR vam realitzar un 'stripping' de la membrana i vam re-incubar-la amb l'anticòs anti-DOR (**figura 65**). Aquest experiment, per tant, no ens va servir per detectar la presència d'aquesta modificació a DOR.

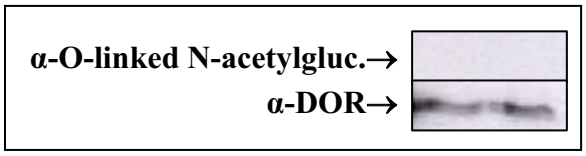


Figura 65. *Western Blot per detectar la modificació a DOR*. Es van transfectar cèl·lules HeLa amb un vector per a l'expressió de DOR unit a una cua d'histidines i es va mantenir les cèl·lules en un medi amb una alta concentració de glucosa (50 mM). A les 36 h es va obtenir la proteïna i es va purificar DOR fent passar la proteïna per columnes de níquel. Amb l'eluït final es va realitzar un assaig de western blot amb l'anticòs anti-O-linked N-acetylglucosamine. Després de fer un 'stripping' es va re-incubar la membrana amb l'anticòs anti-DOR per confirmar-ne la presència.