

The ubiquitously expressed bZIP inhibitor, JDP2, suppresses the transcription of its homologue immediate early gene counterpart, ATF3

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ABSTRACT

JDP2 is a ubiquitously expressed bZIP repressor protein. JDP2 binds TPA response element and cyclic AMP response element located within various promoters. JDP2 displays a high degree of homology to the immediate early gene ATF3. ATF3 plays a crucial role in the cellular adaptive response to multiple stress insults as well as growth stimuli. We have identified ATF3 as a potential target gene for JDP2 repression. JDP2 regulates the ATF3 promoter potentially through binding to both the consensus ATF/CRE site and a non-consensus ATF3 auto-repression DNA-binding element. Expression of ATF3 protein in wild-type mouse embryo fibroblast (MEF) cells is below the detectable levels, whereas, JDP2 disrupted MEF cells display noticeable level of ATF3 protein. Following either serum or ER stress stimulation, ATF3 expression is potentiated in JDP2-KO fibroblast cells as compared with wild-type cells. Mice with either JDP2 over-expression or JDP2 disruption display undetectable level of ATF3 protein. However, ATF3 induction in response to either growth or stress signals is dependent on JDP2 expression level. ATF3 induction is attenuated in JDP2 over-expressing mice whereas is potentiated in JDP2-KO mice as compared with the corresponding wild-type mice. Collectively, the data presented strongly suggest that JDP2 plays a role in the determination of the ATF3 adaptive cellular threshold response to different stress insults and growth stimuli.

INTRODUCTION

c-Jun dimerization protein 2 (JDP2) is a bona fide member of the AP-1 family that was isolated because of its ability to interact with c-Jun (1) and ATF2 (2). JDP2 encodes an 18 kDa protein that is able to homodimerize, as well as to form heterodimers with other AP-1 members, such as c-Jun, JunB, JunD and ATF2, and members of the C/EBP family, C/EBP γ (3) and CHOP10 (4) and more recently, IRF2-binding protein (5). Dimerization occurs through a conserved leucine zipper domain found in all members of the AP-1 family. A basic domain located adjacent to the leucine zipper dimerization motif is responsible for the direct association with TRE as well as CRE DNA-binding sequences. JDP2 is a repressor of AP-1 protein family (1). The mechanism by which JDP2 represses AP-1 transcription involves competition for DNA binding (1), inactive heterodimer formation (1), indirect recruitment of histone deacetylase 3 (6), nucleosome assembly activity (7), inhibition of histone acetylation (7), and potential competition with JNK phosphorylation (8). JDP2 has been shown to play a role in the cellular differentiation of skeletal muscle (9), osteoclasts (10), adipocytes (11) and F9 cells (6).

JDP2 is most closely related to ATF3, a member of the ATF/CREB (CRE-binding protein), which is a subfamily of the AP-1 group (12). ATF3 is a stress inducible gene, as its mRNA level greatly increases upon exposure of cells to stress signals (12). ATF3 has been reported to activate transcription as a heterodimer with c-Jun, whereas it represses transcription as a homodimer (13). Both ATF3 and JDP2 are able to dimerize with CHOP10, which is a member of the C/EBP family. Whereas the JDP2-CHOP10 heterodimer forms a non-functional complex with CRE containing promoters, it strongly

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potentiates transcription from TRE containing promoters (4). JDP2 and ATF3 exhibit 61% overall homology. The bZIP domains of these two proteins share 90% homology, whereas, beyond the bZIP domain, JDP2 and ATF3 display very low similarity. Both JDP2 and ATF3 bind to TRE as well as to CRE DNA elements *in vitro*. It has recently been shown in our laboratory that transgenic mice with temporal cardiac expression of JDP2 exhibit a similar phenotype to ATF3 transgenic mice. This may suggest that these two proteins play a similar role in the heart (14,15). Despite the high degree of similarity in this aspect, it seems that there are considerable differences between JDP2 and ATF3 with regard to their transcription regulation. JDP2 is ubiquitously expressed and its level remains unchanged following various stimuli (8). Nevertheless, in a number of processes, such as differentiation of skeletal muscle cells (9) and osteoclasts (10), and ultraviolet irradiation (16), a modest increase in JDP2 expression is observed. JDP2 undergoes phosphorylation by c-Jun N-terminal kinase (JNK) and p38 kinase on threonine 148, but the precise role of this phosphorylation in terms of its biological function is currently unknown (8,17). Different studies suggest that JDP2 has a dual role in malignant transformation. On the one hand, it is well established that JDP2 efficiently counteracts AP-1 transcription (1), and thus may interfere with the oncogenic properties of c-Jun. JDP2 has been found to inhibit cell transformation induced by Ras *in vitro* and in prostate cancer cell line xenografts injected into SCID mice (18). On the other hand, JDP2 has been identified as a candidate oncogene in a high-throughput screen based on viral insertional mutagenesis in wild-type mice (19). It has also been associated with acceleration of multicentric lymphoma development, in collaboration with a loss of function mutation of the cell cycle inhibitor, p27, (20) and with the over-expression of c-Myc and Runx2 oncogenes (21).

In the present study, we identified ATF3 as a JDP2 target gene. In HeLa and MEF cells, JDP2 regulates ATF3 transcription by binding to the ATF3 promoter region involved in its auto-repression. In addition, JDP2 over-expression in mice results in attenuation of ATF3 expression in various model systems. Consistently, ablation of JDP2 expression results in the potentiation of the ATF3 expression in response to various signals both *in vitro* and *in vivo*. Collectively, JDP2 plays a role in the determination of the cellular adaptive response of the ATF3 expression level to various cellular stimuli.

MATERIALS AND METHODS

Plasmids

pcDNA-JDP2 and pcDNA-VP16-JDP2 are mammalian expression plasmids encoding the wild-type JDP2 or JDP2 transcription activator, respectively. pGL2 basic luciferase reporter plasmid (Promega Inc.) was used to insert KpnI-XhoI fragments derived from the -1350 to +22 ATF3 promoter as indicated. The primers used in

this study are shown (sequences corresponding to the human ATF3 promoter are underlined):

-1350 KpnI top
5'-CGGGGTACCGTCATCCACGGGAGTAAGAA
-926 KpnI top
5'-GGGGTACCTCCTGGACTCCGATCTTTTC
-390 KpnI top
5'-GCGGGGTACCCAGCGCGGTGGAGTCA
-138 KpnI top
5'-CGGGGTACCTCCTCCGCTCCGTTCGG
+22 XhoI Bott
5'-CCGCTCGAGCGCGCCTGGCTGCGT

Cell lines

HeLa stable cell lines were generated by retroviral infections as described in (18). Following retroviral infections, puromycin resistant HeLa cells were pooled and used in all experiments. Transient transfections were carried out with JetPei reagent (PolyPlus transfection Inc.) according to the manufacturer's instructions. SV40 large T-antigen immortalized MEF cells were established from 12-14 days mice embryo of C57BL/6 background of either wild-type or JDP2 disrupted allele.

Reporter assay

Luciferase reporter assays were performed with luciferase kit (Promega Inc.) according to the manufacturer's instructions.

Cellular treatments

HeLa and MEF cells were serum starved 0.1% for 16 h; thereafter 20% serum was added for 1 h. Nuclear extracts were prepared as previously described (4). Exponentially growing MEF cells were treated with 1 μ M thapsigargin in 10% serum for the indicated time.

RT-PCR

Semi-quantitative RT-PCR was performed using Verso cDNA kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

The following primers were used:

human-ATF3-F	5'-ATGATGCTTCAACACCCAGGC
human-ATF3-R	5'-TTAGCTCTGCAATGTCCTTC
human-GSG1-F	5'-ATGGCCAAGATGGAGCTCTCG
human-GSG1-R	5'-CTCCCCTGGTCTTCCACAGT
human-GAPDH-F	5'-CATCACCATCTTCCAGGAGCGA
human-GAPDH-R	5'-GTCTTCTGGGTGGCAGTGATGG

Microarray

RNA extraction. Total RNA was isolated from an exponentially growing HeLa stable cell line and was prepared using TRI reagent solution (Sigma Chemicals Ltd). Cell lines represent vector, JDP2 and VP16-JDP2 expressing cells. RNA quality and integrity were determined using the Eukaryote Total RNA Nano 6000 assay (Agilent RNA 6000 Nano LabChip Kit, part number 5065-4476) on the Agilent Technologies 2100 Bioanalyzer, and quantified by measuring A260nm on a nanodrop

spectrophotometer (ND-1000, NanoDrop Technologies, Rockland, DE).

Generation of fluorescently labeled cRNA targets. Total RNA isolated was amplified and fluorescently labeled using the Agilent Technologies Low Input Linear Amplification Kit (part number 5184-3523) following the protocol described in the kit manual. The labeled cRNA was purified using Qiagen's RNeasy Kit (part number 74104).

Hybridization and scanning of microarrays. RNA samples were prepared from two biological replicate experiments for HeLa-JDP2 and HeLa-VP16-JDP2 to form technical replicates with the control HeLa vector cell line. For each hybridization, 750 ng of cyanine 3- and 750 ng of cyanine 5-labeled cRNA were fragmented and hybridized to an Agilent Technologies Whole Human Genome oligo Microarray (part number G4112F), using the Agilent Gene Expression hybridization kit (cat. number 5188-5242), as described in the manual. Following hybridization, all microarrays were washed and scanned using Agilent's dual laser G2505B microarray scanner. The data were then extracted from images by using Feature Extraction 9.5 software (Agilent Technologies).

Data analysis. The data from all arrays were analyzed using GeneSpring GX software (Agilent Technologies UK Limited, South Queensferry, UK).

Array normalization was done using the intensity-dependent Lowess normalization method. Analysis was performed on the log expression ratios that were produced in the normalization step. Biological replicates were treated as duplicate measurements, and their averages were calculated. Features that flagged absent in all the arrays and non-reliable features with a very low signal (<50 in both channels) were removed prior to further analysis. The normalized data were analyzed to identify genes whose expression appeared to be up- or down-regulated by an arbitrary cut off of at least 2-fold.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed with bacterially purified His-JDP2 as described in (1). The following ³²P-end-labeled DNA probes were used:

CRE	5' TCGATTGGCTGACGTCAGAGAGAG
-20 wt.	5' AAGGGGTGATGCAACGCTCTCCAAGCCACA
-20 mut	5' AAGGGGACCTGCAACGCTCTCCAAGCCACA

Chromatin immunoprecipitation (ChIP)

ChIP was performed using a ChIP assay kit (Upstate technologies) according to the manufacturer's instructions. PCR cycle was performed with an annealing temperature of 58°C and 32 cycles to obtain a signal in the linear range. The following oligonucleotides derived from the ATF3 promoter were used to amplify the corresponding genomic region. Numbers represent bp distance upstream (-) or downstream (+) from the ATF3 transcription start site according to (22).

Primers used for ChIP derived from the human ATF3 promoter:

ChIP-209-F	5'-CCGAACTTGCATCACCAGTGC
ChIP+34-R	5'-GAGCTGTGAGTGCAGCGCC
ChIP-928-F	5'-CCTCCTGGACTCCGATCTTTTC
ChIP-674-R	5'-TGCGGCGGGGACCGAGAA

Primers used for ChIP derived from the mouse ATF3 promoter:

ChIP-246-F	5'-GTCCCGGGCGTGCAGAGAA
ChIP+25-R	5'-GCGCGCGTCTCGCTGAGTG

Western blot analyses

Western blot analyses were performed with 5% dry milk in PBS. The primary antibodies used were rabbit polyclonal ATF3 (C-19, Santa Cruz), rabbit polyclonal anti-cJun (H-79, Santa Cruz), rabbit polyclonal anti-GAPDH (FL-335, Santa Cruz), mouse monoclonal anti- α -tubulin (T-9026, Sigma Aldrich), and rabbit polyclonal anti-JDP2 (1).

Transgenic mice

All studies involving mice were regulated under the protocol approved by the Technion Animal Inspection Committee. The Technion holds an NIH animal approval license number A5026-01. The following mice lines were used in this study: Tet-JDP2 transgenic responder line, MHC α -tTA cardiomyocytes driver line, LAP-tTA liver driver line, C57BL/6 wild-type mice and C57BL/6 JDP2-KO mice.

Angiotensin treatments

Mice with the indicated genotype were injected intraperitoneally with either angiotensin II 1.5 mg/Kg or isoproterenol 2.5 mg/kg. Saline was used as the vehicle. Two hours following injection the mice hearts were harvested in RIPA buffer and subjected to SDS-PAGE followed by western blot analysis.

CCl₄ treatments

Mice were injected intraperitoneally with 0.5 μ l/Kg CCl₄ diluted in olive oil. Livers were harvested at the indicated time points and nuclear extract was subjected to SDS-PAGE followed by western blot analysis.

RESULTS

ATF3 is a JDP2 target gene

JDP2 is a well established bZIP repressor protein binding to either TPA or CRE DNA response elements. JDP2 can be turned into a transcription activator by fusing JDP2 to a strong activation domain derived from the herpes simplex virus protein VP16 (4). To identify JDP2 direct target genes, HeLa cell lines with ectopic expression of either JDP2 or VP16-JDP2 were established. We hypothesized that the transcription of a JDP2 target gene would be repressed in the JDP2 expressing cell line but potentiated in the VP16-JDP2 expressing cell line. To study the expression profile of the different HeLa cell lines, mRNA was extracted and was used as a template to generate

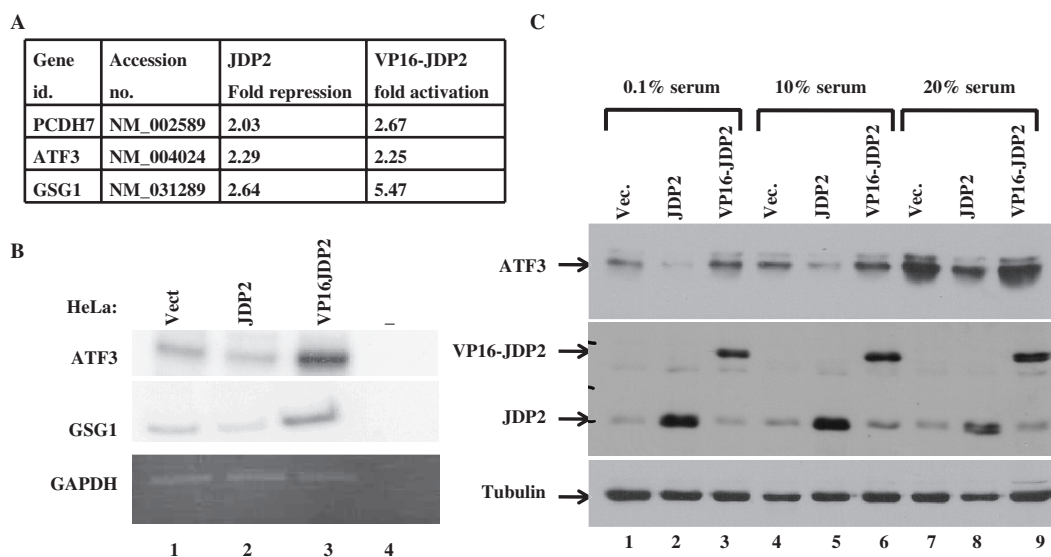


Figure 1. ATF3 is a JDP2 target gene in HeLa cells. (A) cDNA derived from HeLa cell lines with ectopic expression of either dominant active JDP2 (VP16-JDP2) or wild-type JDP2 (JDP2) was used to probe 4X44K microarray in duplicate. Average fold expression is determined relative to cDNA derived from HeLa-vector cell line. Genes displaying at least 2-fold reduced levels in HeLa-JDP2 cell line and a 2-fold increased level in HeLa-VP16-JDP2 cells are shown. Genebank accession number as well as gene annotation is indicated. (B) Validation of microarray results by RT-PCR. cDNA was prepared from mRNA derived from the indicated cell lines and was used as a template for semi quantitative RT-PCR. No cDNA was used for PCR control (-). ATF3 and GSG1 PCR reactions were performed in the presence of a trace amount of $\alpha^{32}\text{P}$ -dCTP. PCR reactions were separated on non-denaturing 10% PAGE dried and exposed to autoradiography (top and middle panel). The housekeeping gene GAPDH RT-PCR reaction was separated on 1.5% agarose gel followed by ethidium bromide staining and visualized by UV light (bottom panel). (C) Western blot analysis of nuclear extracts derived from the indicated HeLa cell lines grown in low serum (0.1% serum lanes 1–3), exponentially growing cells (10% serum, lanes 4–6) and cells induced with 20% serum for 1 h (lanes 7–9). Nuclear extract (50 μg) were separated on 12.5% SDS-PAGE transferred to nitrocellulose membrane and probed with anti-ATF3, anti-JDP2 and α -tubulin (loading control).

fluorescently labeled cRNA which was applied on human derived microarrays. cRNA derived from HeLa vector control cell line was used as a gene expression profile reference. A JDP2 candidate target gene was considered, if it displayed a differential expression of at least 2-fold repression and 2-fold activation with samples derived from either JDP2 or VP16-JDP2 expressing cells, respectively. Only three genes fulfilled this criterion: PCDH7, ATF3 and GSG1 (Figure 1A). To validate the microarray results, primers were designed to amplify the corresponding cDNA derived from the different HeLa cell lines. Consistent with the microarray data, both GSG1 and ATF3 displayed a reduced transcription in the HeLa-JDP2 cell line and a higher transcription in the VP16-JDP2 cell line (Figure 1B). In contrast, the PCDH7 transcription was found at similar levels irrespective of JDP2 expression (data not shown). GSG1 is a germ-cell-associated protein with an unknown function. The role of JDP2 in GSG1 regulation was not further pursued. Here, we further studied the role of JDP2 in ATF3 transcription regulation. ATF3 and JDP2 proteins are highly homologous in function (4,14) but differ mainly in their mode of regulation. While JDP2 is ubiquitously expressed (8), ATF3 is considered an immediate early gene. ATF3 protein expression is highly induced in response to various cell stresses and cellular signals (23).

ATF3 protein level is dependent on JDP2 function

We first sought to verify that JDP2 regulation of ATF3 transcription observed at the mRNA level also reflects a

change in ATF3 protein expression level. The different HeLa cell lines were cultured in three different growth conditions representing exponentially growing cells (10% serum), serum starved cells (0.1% serum) and serum induced cells (20% serum). Nuclear cell lysate was subjected to SDS-PAGE and western blotting with anti-ATF3 antibody (Figure 1C, top panel). Whereas HeLa-vector control cells displayed a basal level of ATF3 protein, the HeLa-JDP2 expressing cell line exhibited a dramatically reduced ATF3 protein expression level. In contrast, high level of ATF3 protein was observed in lysate derived from HeLa-VP16-JDP2 cell line (Figure 1C, top panel). The differential expression profile was observed in cell lysate derived from cells grown in all three growth conditions examined. The expression level of the housekeeping gene α -tubulin confirmed similar protein lysate loadings (Figure 1C, bottom panel). In addition, the expression level of the ectopically expressed JDP2, VP16-JDP2, as well as the endogenous JDP2 expression level, is shown (Figure 1C, middle panel). Collectively, ATF3 protein expression levels present in the different HeLa cell lines are consistent with the changes observed at the transcriptional level. Thus, ATF3 expression is repressed in the presence of over-expression of JDP2, whereas it is induced following ectopic expression of the JDP2 transcriptional activator mutant.

Identifying JDP2 DNA responsive elements within the ATF3 promoter

We sought to map the JDP2 potential DNA-binding sequences within the human ATF3 promoter that are

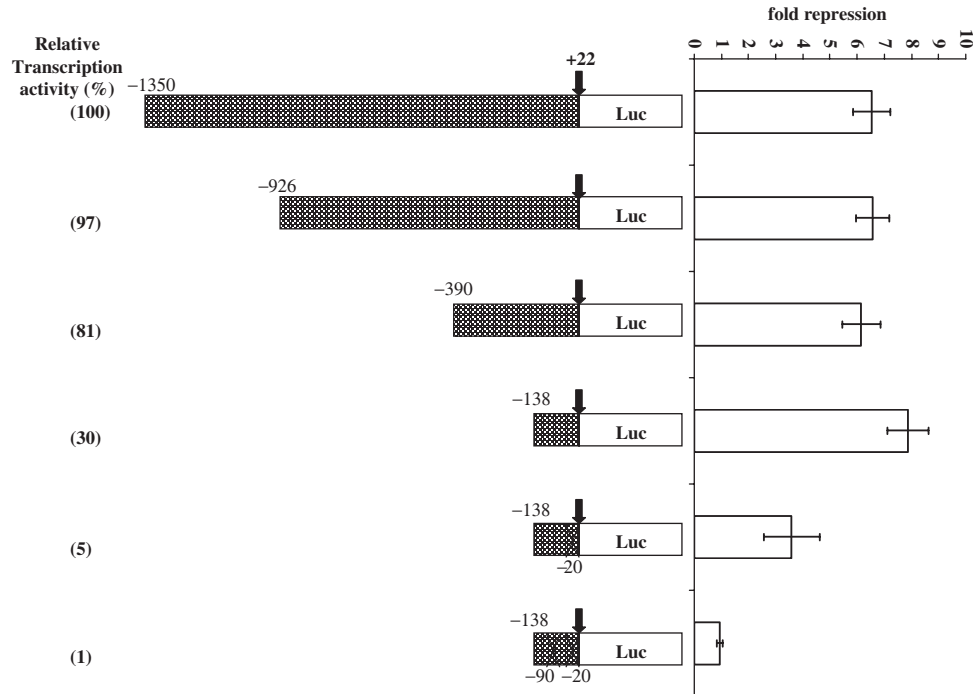


Figure 2. Mapping JDP2 repression activity within the ATF3 promoter. Schematic representation of the ATF3 promoter deletion mutants used. DNA fragments were subcloned upstream of the luciferase gene in the PGL2 basic luciferase reporter plasmid. The number on the left side of each construct represents the percentage of transcription activity obtained with the corresponding promoter fragment relative to the transcription activity obtained with the longest ATF3 fragment used (taken as 100%). The numbers at the top of each depicted construct correspond to the promoter length (bp) upstream (-) or downstream (+) in respect to the human ATF3 transcription start site. The position of the ATF3 promoter harboring mutations at positions -20 and -90 are indicated. HeLa cells were co-transfected with 1 μ g of PGL2 reporter in the presence or absence of 4 μ g of JDP2 expression vector. Luciferase activity was measured 24 h following transfection. Fold repression was calculated by dividing the luciferase activity obtained with the pcDNA empty expression vector to the luciferase activity obtained in the presence of JDP2 expression plasmid. The results represent the average and SEM of four independent experiments.

functionally important for the JDP2's transcription repression. To this end, we used reporter gene assays in HeLa cells. Since JDP2 strongly represses the empty PGL2-promoter luciferase reporter plasmid containing the simian virus 40 minimal early promoter (data not shown), the PGL2-basic reporter plasmid (Promega Inc.) was used. This plasmid harbors neither the promoter nor TATA element upstream of the luciferase gene (Figure 2). A fragment which consists of -1350 to +22 bp, relative to the ATF3 transcription start site, was designed to drive the expression of the luciferase reporter gene. This region contains multiple TRE and CRE potential consensus binding sites and a functional TATA element (22). HeLa cells were co-transfected with the luciferase reporter plasmid together with a JDP2 expression plasmid. Luciferase activity was determined 24 h following transfection. Indeed, JDP2 was able to repress significantly the luciferase reporter activity by 6–7 folds, relative to the luciferase reporter activity obtained in the absence of JDP2. To further map the JDP2 responsive elements within the ATF3 promoter, a series of 5' deletions of the promoter were designed (Figure 2). Each deleted mutant removed between two to three potential TRE or CRE consensus DNA elements and resulted in a reduced transcription relative to the longest ATF3 promoter fragment consisting of -1350 bp upstream to the transcription start site

(Figure 2). Subsequently, the ability of JDP2 to repress the activity of the ATF3 promoter deleted mutants was examined in co-transfection experiments in HeLa cells. A promoter fragment, consisting of -138 bp to +22, derived from the ATF3 promoter, resulted in JDP2 repression activity similar to that obtained with the longest ATF3 promoter fragment (Figure 2). The remaining ATF3 promoter sequence (-138 till +22) consists of a consensus ATF/CRE site located at -90 bp as well as an ATF3 non-canonical auto-repression site located at -20 bp (22). To delineate which of these DNA-binding elements is responsible for ATF3 repression by JDP2, a luciferase reporter construct harboring a mutated -20-binding element (-138 Δ -20) was designed. The ability of JDP2 to repress the transcription of the mutated ATF3 promoter reporter plasmid was examined in HeLa cells. Although JDP2 was able to repress modestly the ATF3 reporter (-138 Δ -20), the repression level was only about 50%, in comparison with the wild-type construct (Figure 2). Once the -90 ATF/CRE site was mutated along with the -20 site, JDP2 repression was completely lost (Figure 2, -138 Δ both). This result indicates that JDP2 is able to repress ATF3 transcription via both the consensus ATF/CRE site and the ATF3 auto-repression element located at -90 and -20, respectively.

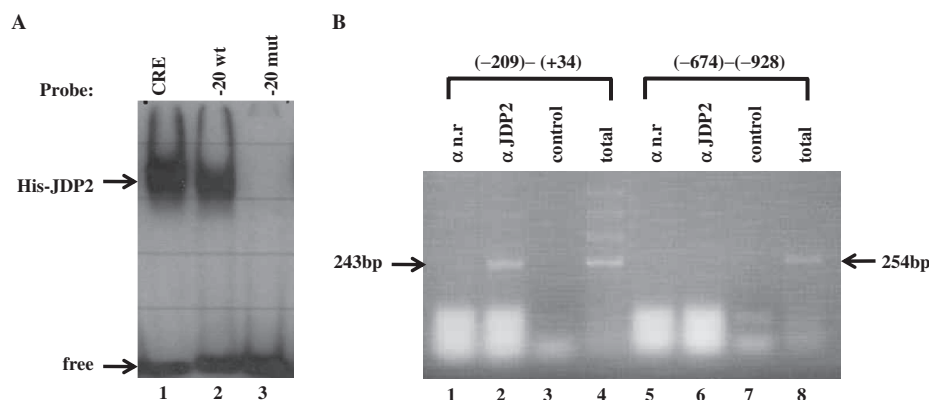


Figure 3. JDP2 associates with the consensus CRE and the non-canonical ATF3 autorepression site. (A) EMSA with bacterially purified His-JDP2 and the indicated DNA ^{32}P - γ ATP-labeled probe. The consensus CRE sequence (CRE, lane 1) as well the non-canonical ATF site located at -20 within the ATF3 promoter (-20 Wt., lane 2) and a mutated -20 site DNA-binding element (-20 mut, lane 3). (B) Chromatin immunoprecipitation with anti-JDP2 antibody. Formaldehyde fixed sheared lysate was either directly precipitated (total, lanes 4 and 8) or immunoprecipitated with either non-relevant antibody, anti-THTR1 (α n.r., lanes 1 and 4) or anti-JDP2 (α JDP2, lanes 3 and 6). Purified extracted DNA was amplified using appropriate primers corresponding to -209 till $+34$ and -674 till -928 (expected size 243 bp and 254 bp, respectively). PCR reaction with no added template (control, lanes 3 and 7). The amplified precipitated genomic DNA fragments were separated on 2% agarose gel stained with ethidium bromide and visualized by UV light.

JDP2 binds the ATF/CRE and the -20 DNA-binding sites *in vitro* and *in vivo*

Since the DNA element located at -20 is a non-canonical ATF/CRE site, we examined whether or not bacterially purified His-JDP2 protein is able to bind the -20 site using an electrophoretic mobility shift assay (EMSA). Indeed, bacterially purified His-JDP2 protein displayed strong binding to labeled CRE and the -20 DNA sequence elements but failed to associate with the labeled mutated -20 DNA-binding site (Figure 3A).

To examine further whether or not JDP2 is associated with the ATF3 promoter *in vivo*, we used chromatin immunoprecipitation (ChIP) in HeLa-JDP2 cell line. Cells were cross-linked with formaldehyde and the DNA was fragmented to 500–1000 bp by sonication. Immunoprecipitation was performed with either non-relevant serum (anti-thiamine transporter 1, THTR1) or with anti-JDP2 polyclonal antibodies. Following extensive washes, cross-linking was reversed and DNA was extracted. The precipitated purified DNA was used as a template for a PCR reaction with appropriate primers located at -209 to $+34$ within the human ATF3 promoter. As a control, we used PCR primers located at -928 to -674 within the ATF3 promoter. This region was found to be not associated with JDP2 repression activity. Whereas following 32 PCR cycles no fragment was detected with non-relevant serum, a specific amplified DNA fragment of the expected size (243 bp) was observed with DNA lysate derived from anti-JDP2 precipitate (Figure 3B). In addition, no specific amplification was observed using the control primers corresponding to ATF3 promoter located between -674 and -928 (Figure 3B). Since the sites located at -90 and -20 are in close proximity, it is impossible to resolve further the association of JDP2 with either or both of them *in vivo* using the ChIP assay. Collectively, these results strongly suggest that JDP2 regulates ATF3 promoter via direct

association with ATF/CRE and the non-canonical site located at -90 and -20 bp, relative to the ATF3 transcription initiation start site. The association of JDP2 with these sequences is demonstrated both *in vitro* and *in vivo*.

JDP2 expression level mediates ATF3 expression level

The role of JDP2 in determining ATF3 basal expression level was demonstrated so far in HeLa cell line with ectopic expression of either wild-type JDP2 or the JDP2 transcriptional activator mutant (VP16-JDP2). To examine whether or not loss of JDP2 expression affects ATF3 expression level, we took advantage of a mouse embryo fibroblast (MEF) cell line derived from mice harboring homozygous JDP2 disrupted alleles (11). Whereas, ATF3 expression is below the detectable level in nuclear cell lysate derived from exponentially growing wild-type MEF (Figure 4, top panel, lane 1), lysate derived from JDP2-KO MEF cells displayed low but detectable ATF3 expression (Figure 4A, top panel, lane 6). Moreover, upon either serum induction (20% serum) or thapsigargin treatment, cell lysate derived from JDP2-KO MEF resulted in higher induction of ATF3 protein as compared with wild-type MEF lysate (Figure 4A, top panel, compare lanes 2–5 with 7–10). The level of α -tubulin loading control displays comparable to lower levels of expression in JDP2-KO MEFs, suggesting that the differences observed represent an under-estimate of the actual differences in ATF3 protein level. Collectively, these results suggest that JDP2 moderately alter ATF3 basal expression level but mainly affect the expression of ATF3 in response to environmental changes.

We next examined the binding of the endogenous JDP2 to the ATF3 promoter in untreated and thapsigargin treated MEF cells using ChIP with anti-JDP2 antibodies (Figure 4B). In untreated wild-type MEF cells, endogenous JDP2 protein is found to be associated with the mouse ATF3 promoter region corresponding to -246 to $+25$.

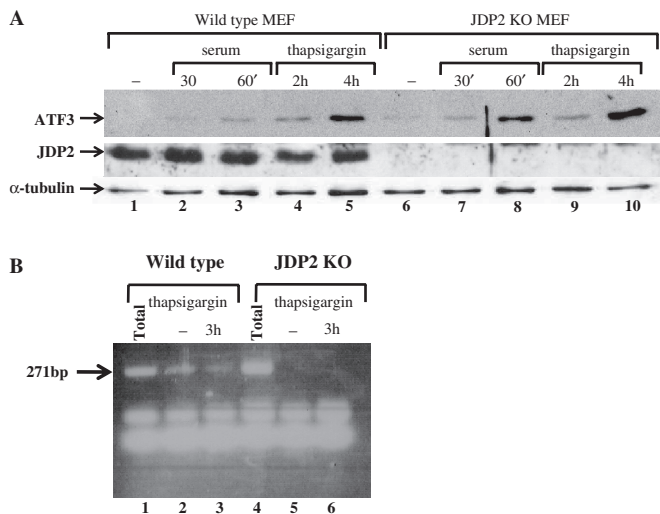


Figure 4. The extent of ATF3 induction to cellular stimulation is JDP2 dependent. **(A)** Western blot analysis with nuclear extract derived from either wild-type MEF or JDP2-KO MEF cells. Cell lysate was prepared at the indicated time from exponentially growing cells (-, lanes 1 and 6), 20% serum stimulated (serum, lanes 2-3 and 7-8) and 1 μ g/ml thapsigargin treated (thapsigargin, lanes 4-5 and 9-10). The expression level of ATF3 (top panel), JDP2 (middle panel) and α -tubulin (bottom panel) was examined with the corresponding antibodies. **(B)** ChIP with anti-JDP2 antibody with either wild-type or JDP2-KO MEF cells. Cells were either untreated or treated with thapsigargin for 3h. Formaldehyde fixed sheared lysate was either precipitated directly (total lanes 1 and 4) or precipitated with anti-JDP2 antibody. Purified extracted DNA was amplified using appropriate primers corresponding to -246 to +25 genomic (expected size 271 bp). The amplified precipitated genomic DNA fragments were separated on 2% agarose gel stained with ethidium bromide and visualized by UV light.

Upon thapsigargin stimulation, a significant reduction in the level of ATF3 promoter bound to JDP2 is observed (Figure 4B, lanes 2-3). To demonstrate the specificity of the anti-JDP2 antibody, we performed ChIP with JDP2-KO MEF cells. Indeed, in the absence of JDP2 protein, no amplification of the ATF3 promoter fragment is observed (Figure 4B, lanes 5-6).

JDP2 mediates ATF3 response to angiotensin II and isoproterenol in the heart

To examine whether or not JDP2 regulates ATF3 expression *in vivo*, we examined the ATF3 expression in transgenic mice with either ectopic or knockout expression of JDP2. Previously we have shown that intraperitoneal injection of angiotensin II into mice highly induces ATF3 expression in the heart. We used the JDP2 transgenic responder mice strain with temporal expression of JDP2 based on the tet-off transgenic system (24). Upon crossing of the JDP2-responder mice with MHC α tetracycline activator driver mice, double transgenic mice highly express HA-JDP2 in cardiomyocytes (15). To compare the transcription of ATF3 in response to neuroendocrine stimulation, control (driver mice, Wt) and JDP2-expressing (double transgenic, JDP2) mice were injected with a single dose of either angiotensin II (1.5 mg/kg) or β -adrenergic agonist isoproterenol (2.5 mg/kg). Mouse hearts were collected 2h following injections and ATF3

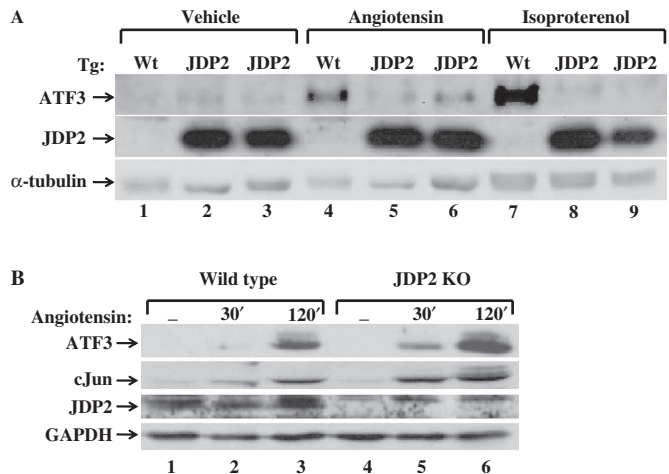


Figure 5. ATF3 induction to humoral stimulation is dependent on JDP2 expression level in the heart. **(A)** Western blot analysis with cell lysate derived from hearts of MHC α -tTA transgenic mice (Wt.) and JDP2-expressing double transgenic mice (JDP2) treated for 2h with saline (vehicle, lanes 1-3), 1.5 mg/kg angiotensin II (angiotensin II, lanes 4-6) and 2.5 mg/kg isoproterenol (isoproterenol, lanes 7-9). The expression levels of ATF3 (top panel), JDP2 (middle panel) and α -tubulin (bottom panel) were examined with the corresponding antibodies. **(B)** Western blot analysis with cell lysate derived from atria of either wild-type mice or JDP2 KO mice treated with 1.5 mg/kg angiotensin II for the indicated time. The expression level of ATF3 (top panel), c-Jun (second top panel), JDP2 (third top panel) and GAPDH (bottom panel) was examined with the corresponding antibodies.

expression was examined by western blot. Whereas vehicle injected mice displayed a low basal level of ATF3 in both genotypes, angiotensin II and isoproterenol injections resulted in high ATF3 expression in the hearts of the control mice (Figure 5, top panel, lanes 1, 4 and 7). In contrast, JDP2 expressing mice displayed a barely detectable induction of ATF3 protein (Figure 5, top panel, compare lanes 4 and 7 with 5-6 and 8-9, respectively). JDP2 expression in double transgenic mice as well as α -tubulin loading control are consistent with the finding that JDP2 overexpression strongly suppressed the expression of the ATF3 protein in response to angiotensin II and isoproterenol injections.

We wished to examine whether or not JDP2 protein is capable in regulating the ATF3 promoter also under physiological JDP2 expression level. Towards this end, we used wild type and JDP2-KO mice injected with angiotensin II and followed the induction of ATF3 protein level during a course of 2h. The induction of ATF3 protein already appeared 30 min following angiotensin II injection into JDP2-KO mice (Figure 5B). On the other hand, ATF3 induction in wild-type mice is apparent only 2h following angiotensin II injection. Moreover, ATF3 expression level is higher in JDP2-KO mice 2h following angiotensin II injection. Interestingly, c-Jun, a previously described JDP2 target gene (6), displayed a similar profile of expression as compared with ATF3 protein. Namely, earlier induction time and higher expression level in response to angiotensin II stimulation in JDP2-KO mice as compared with wild-type mice

(Figure 5B, middle panel). JDP2 expression level is unchanged following angiotensin II treatment and is absent in heart derived from JDP2-KO mice.

JDP2 mediates ATF3 response to liver injury by carbon tetrachloride, CCl₄

To examine whether or not JDP2 controls ATF3 expression also following cellular stress insult in mice, we used a single injection of CCl₄. CCl₄ is a known hepato-toxin that triggers lipid peroxidation and liver damage (25). ATF3 protein was shown to be induced around the central veins following CCl₄ injection. Therefore, we followed ATF3 expression in the liver of JDP2 transgenic, JDP2 KO and their respective control mice following a single injection of CCl₄. To induce JDP2 transgene expression in the liver, we crossed the LAP-tTA driver line with Tet-JDP2 transgene. Double transgenic mice highly express HA-JDP2 specifically in the liver (Figure 6A, middle panel, lanes 2 and 5–7). In wild-type mice, ATF3 is induced 4 h following CCl₄ injection. In contrast, ATF3 expression is barely detected in CCl₄-injected JDP2 expressing mice (Figure 6A, compare lanes 3–4 with 5–7).

To examine ATF3 expression following liver injury in the absence of JDP2 protein, we injected JDP2-KO mice and the respective C57BL/6 control mice with CCl₄. The ATF3 expression level in the liver was assessed at one and 3 h following CCl₄ injection (Figure 6B). JDP2-KO mice display higher induction level of ATF3 protein at 3 h following CCl₄ injection (Figure 6B). Consistently, the two other known JDP2 target genes, c-Jun and Chop10, are induced to higher levels at 3 h following CCl₄ injection in JDP2-KO mice. These results strongly suggest that alteration in the JDP2 expression level determines the cellular response towards multiple cellular stresses by controlling the expression level of the immediate early gene ATF3.

DISCUSSION

ATF3 is an immediate early gene, thus its mRNA and protein levels are highly induced following multiple cellular insults. In addition, overwhelming evidence from DNA microarray added numerous non-stress signals that affect ATF3 expression (23). ATF3 expression is not cell type-specific and represents part of the global cellular response to environmental changes. Yet, the cellular fate in response to environmental signals is dependent on the spatial expression of the immediate early genes that are being induced by the specific signal. Much information is available regarding the signaling pathways that are involved in the induction of the ATF3 gene. These include the MAPK cascades ERK, JNK and p38 (26) and multiple transcription factors such as c-Jun/AP-1, ATF2 and CREB, as well as others such as Myc, C/EBP β , Erg-1 and more (27,28). In the current study, we have identified ATF3 as a target gene for the AP-1 repressor protein, JDP2. Unlike the known induction pathways, JDP2 is shown to be involved in the cellular threshold response. Namely, the expression of the ATF3 is dependent on the JDP2 expression level. This was demonstrated in both JDP2 over-expression models (Figure 1C) as well as with models in which

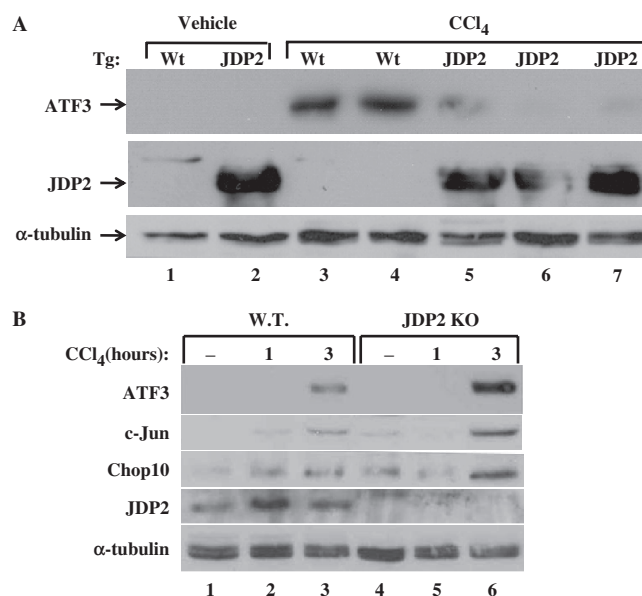


Figure 6. ATF3 induction in response to CCl₄ is dependent on JDP2 expression level in the liver. (A) Western blot analysis with nuclear extract derived from the liver of LAP-tTA transgenic mice (Wt.) and JDP2 expressing double transgenic mice (JDP2) treated for 4 h with either olive oil (Vehicle) or 0.5 ml/kg CCl₄. The expression level of ATF3 (top panel), JDP2 (middle panel) and α -tubulin (bottom panel) was examined with the corresponding antibodies. (B) Western blot analysis with nuclear cell lysate derived from the liver of either wild-type mice or JDP2 KO mice treated with CCl₄ 0.5 ml/kg for the indicated hours. The expression levels of ATF3, c-Jun, Chop10, JDP2 and α -tubulin were examined with the corresponding antibodies.

JDP2 expression is disrupted (Figures 4–6). In JDP2-KO MEF cells a moderate increase in ATF3 protein basal expression level is observed (Figure 4A). In contrast, in non-treated JDP2-KO mice, ATF3 protein is undetectable in the liver, heart, spleen and kidney (Figure 5 and 6 and data not shown). Yet, we cannot exclude the possibility that enhanced basal ATF3 protein expression in other organs of JDP2-KO mice may be affected. For example, in the liver the basal protein expression levels of JDP2 target genes such as c-Jun, CHOP10 is higher in JDP2-KO mice (Figure 6). On the other hand, no such change in c-Jun expression level is observed in the heart (Figure 5B). Previously it was shown, that HeLa cells transiently transfected with JDP2 SiRNA resulted in a higher basal level of CHOP10 mRNA (29).

Interestingly, in the heart of JDP2-KO mice, c-Jun displayed a similar kinetic as compared with ATF3 in response to angiotensin II stimulation, i.e. an earlier induction time and higher expression level as compared with wild-type mice. In addition, the expression of CHOP10 and c-Jun in response to CCl₄ injection was observed to follow ATF3 induction in the liver of JDP2-KO mice (Figure 6B). Collectively, the data suggests, that similar regulatory mechanisms may coordinate the spatial expression of these genes. Unexpectedly, a transient increase in JDP2 protein expression level 1 h following CCl₄ injection is observed. JDP2 is a ubiquitously expressed protein and is not transcriptionally regulated

following a large variety of stresses (8). Therefore, we hypothesize that the transient increase in JDP2 expression may represent dissociated JDP2 protein from the chromatin. This is consistent with reduced level of JDP2 that is found bound to ATF3 promoter 3 h following thapsigargin stimulation (Figure 4B).

Promoter deletion mutants suggested that although the ATF3 distal promoter harbors multiple CRE and TRE elements between -1350 and -138, none of these were regulated by JDP2. JDP2 is associated with CRE/ATF site and a non-canonical site that are located within 100 bp of the ATF3 proximal promoter region. So far, only three JDP2 target genes were identified: c-Jun, C/EBP δ and CHOP10. Interestingly, JDP2 was found to be associated with sequences located at the close proximity of the promoters of these genes (2,6,11,29). The identification of additional JDP2 target genes will be important in determining whether or not JDP2 binding to the proximal promoter region is a general rule or just a coincidence.

The approach we took to identify JDP2 target genes by microarray is clearly not comprehensive and additional target genes will be recognized in the future. The criteria we set for the identification of a bona fide JDP2 target gene demanded a requisite of a basal level of expression in the HeLa parental cell line. Luckily, HeLa cells are relatively unique cell lines that display basal ATF3 protein level even when grown in low serum medium (Figure 1C). Typically, the basal level of ATF3 protein is below the detectable levels in the cells. These results imply that lack of activity of positive transcription factors in resting, un-stimulated cells is responsible for the ATF3 low expression level.

The DNA elements within the ATF3 promoter, which are JDP2-responsive, were identified as the ATF/CRE consensus site and an ATF3 auto-regulatory element located at -90 and -20 respectively. A detailed analysis towards understanding the mechanism by which JDP2 dissociates from the promoter and allows ATF3 transcription and subsequent auto-inhibition is currently under way. Apparently, most of the signals that induce ATF3 expression may result in JDP2 phosphorylation. Since the precise role of JDP2 phosphorylation is currently unknown, it would be interesting to examine its role using the knock-in approach in JDP2-KO MEF cells.

The regulation of ATF3 transcription may have importance in cancer pathophysiology, since ATF3 was shown to have a dual role in carcinogenesis. On the one hand, ATF3 was found to inhibit Ras-mediated transformation (30). However, on the other hand, ATF3 was found to protect breast cancer cells from stress-induced cell cycle arrest and promote their cell motility and invasiveness (31). In addition, ATF3 over-expression is a mediator of cell proliferation in classical Hodgkin's lymphoma (32). Interestingly, JDP2 was found to inhibit cell cycle progression (9), Ras mediated cell transformation and tumor formation in xenografts (18). In contrast, JDP2 was also found to play a role in tumorigenesis under certain genetic circumstances (19-21). Whether or not the role of JDP2 in cancer progression is mediated via the regulation of ATF3 transcription is yet to be determined.

Importantly, ATF3 was found to mediate the pro-apoptotic effects of anti-cancer drugs such curcumin (33) and the PI3K inhibitor, LY294002 (34). Therefore, conditions that would enhance ATF3 expression level, resulting in potentiation of ATF3 pro-apoptotic activity in response to anti cancer drugs, will be very useful in reaching higher efficiency of the drugs with lower toxicity.

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