BJ www.biochemj.org

Biochem. J. (2011) **436**, 661–669 (Printed in Great Britain) doi:10.1042/BJ20101031

online data

Phosphorylation of JDP2 on threonine-148 by the c-Jun N-terminal kinase targets it for proteosomal degradation

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JDP2 (c-Jun dimerization protein 2) is a member of the basic leucine zipper family of transcription factors that is ubiquitously expressed in all examined cell types. JDP2 is phosphorylated on Thr[148] by JNK (c-Jun N-terminal kinase) and p38 kinase, although the functional role of its phosphorylation is unknown. In the present paper we show that the JDP2 protein level is dramatically reduced in response to serum stimulation, anisomycin treatment, ultraviolet light irradiation and cycloheximide treatment, all of which activate the JNK pathway. In addition, endogenous and overexpressed JDP2 are phosphorylated in response to these stimuli. Replacement of Thr[148] with an alanine residue stabilizes ectopically expressed JDP2 in the presence of the stimuli; conversely, substitution with glutamic acid destabilizes it. Serum-induced phosphorylation and degradation of JDP2 are specific to JNK activation since a JNK inhibitor (SP600125) abolishes these effects, whereas p38 and MEK inhibitors (SB203580 and UO126) have no effect. In the presence of cycloheximide, JDP2 is rapidly phosphorylated and degraded due to the combined effects of protein synthesis inhibition and activation of JNK. Pre-treatment of cells with SP600125 prior to cycloheximide treatment significantly prolongs the half-life of JDP2 that is found mainly in the unphosphorylated form. Lastly, the proteasome inhibitor (MG132) rescues JDP2 degradation following cycloheximide treatment and increases the expression of the JDP2 phospho-mimetic T148E mutant. Collectively, these results suggest that phosphorylation of JDP2 on thr[148] by JNK targets it to the proteasome for degradation.

Key words: activator protein 1 (AP-1), c-Jun, c-Jun N-terminal kinase, c-Jun dimerization protein 2 (JDP2), phosphorylation, protein stability.

INTRODUCTION

JDP2 (c-Jun dimerization protein 2), is an 18 kDa bZIP protein that specifically interacts with c-Jun [1], CHOP10 [C/EBP (CCAAT/enhancer-binding protein)-homologous protein 10] [3], C/EBPγ [2] and ATF2 (activating transcription factor 2) [4] transcription factors. JDP2 binds TRE (PMA response DNA elements) as well as the CRE (cyclic AMP response element) as a homo- or heterodimer [1,4]. Upon dimerization with c-Jun, DNA binding is potentiated, but transcription is inhibited [1]. JDP2 inhibits transcription by multiple mechanisms [1,5], and is involved in cell differentiation processes, such as differentiation of skeletal muscle cells [6] and osteoclasts [7], and in cell response to ultraviolet irradiation [8]. It is well established that JDP2 counteracts AP-1 (activator protein 1) transcription [1], and thus may interfere with the oncogenic properties of c-Jun and ATF2. JDP2 has been found to inhibit cell proliferation [9] and cell transformation induced by Ras *in vitro* and in xenografts injected into SCID (severe combined immunodeficiency) mice [10]; on the other hand, it has been identified as a candidate oncogene in a high-throughput screen based on viral insertional mutagenesis in mice [11–13]. In addition, JDP2 overexpression potentiates hepatocellular carcinoma in mice [14].

JDP2 is most closely homologous to ATF3, a member of the ATF/CREB (CRE-binding protein) subfamily of the AP-1 group [3,15,16]. ATF3 is a stress-inducible gene, as its mRNA and protein levels greatly increase upon exposure of cells to stress signals (reviewed in [15,17]). Despite their high degree of similarity in this aspect, there appear to be considerable differences between JDP2 and ATF3 with regard to their regulation. JDP2 is ubiquitously expressed in all cells tested and its mRNA level remains unchanged following various stimuli [18]. Nevertheless, in a number of processes, such as differentiation of skeletal muscle cells [6] and osteoclasts [7], and following ultraviolet irradiation [8], a modest increase in JDP2 expression is observed. JDP2 undergoes phosphorylation by JNK (c-Jun N-terminal kinase) [19] and p38 kinase on Thr[148], but not by ERK (extracellular signal-regulated kinase)/MAPK (mitogen-activated protein kinase) [20]. The JNK docking domain within JDP2 is uniquely located C-terminally to the phospho-acceptor site and displays no sequence homology to the consensus D domain found in other JNK substrates [19]. The precise role of JDP2 phosphorylation on Thr[148] in terms of its biological function is unknown [18,21]. We observed that the JDP2 protein level is rapidly down-regulated following various cell stimuli. JDP2 destabilization is dependent on Thr[148] phosphorylation by JNK and is mediated by the proteasome.

MATERIALS AND METHODS

Tissue culture and cell lines

MEF (mouse embryonic fibroblast) cells, NIH-3T3 cells and HEK (human embryonic kidney)-293T cells were grown in DMEM (Dulbecco's modified Eagle's medium), supplemented with 4.5 g/l D-glucose containing 10 % fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin, grown in a 5 % CO_2 incubator.

Abbreviations used: AP-1, activator protein 1; ATF, activating transcription factor; C/EBP, CCAAT/enhancer-binding protein; CHX, cycloheximide; CRE, cyclic AMP response element; DTT, dithiothreitol, ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; JDP2, c-Jun dimerization protein 2; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryo fibroblast; MEK, MAPK/ERK; qRT–PCR, quantitative real-time PCR; TRE, PMA response DNA element.

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SV40 (simian-virus-40) large T-antigen immortalized MEF cells were established from 12–14 day mice embryo of C57BL/6 background of either wild-type or JDP2-disrupted allele [22] using the standard protocol [23].

Generation of stable JDP2-expressing cells

MEF cells harbouring JDP2-disrupted alleles (JDP2$^{-/-}$) were infected with replication-defective retroviruses to generate JDP2$^{-/-}$ puromycin-resistant cell lines overexpressing either the wild-type JDP2 or JDP2 mutant forms. The JDP2 mutants correspond with the substitution of Thr^{148} with either alanine (T148A) or glutamic acid (T148E) residues [18].

Wild-type and mutant full-length *JDP2* cDNA were inserted into the pCLBabe retroviral expression plasmid [24] by EcoRI digestion. Retroviruses expressing the above JDP2 isoforms or an empty vector (as control) were generated by transfection into a viral packaging cell line 293gp expressing the *gag* and *pol* genes [10]. The medium of transfected 293gp cells containing retroviruses was used to infect JDP2$^{-/-}$ MEF cells. The infected cells were selected with puromycin (2 μg/ml) and pooled following 2 weeks of selection.

Cellular treatments

MEF cells were serum starved with medium containing 0.5 % serum for 24 h prior to cell stimulation.

UVC irradiation was applied using an 8 W 217 nm lamp. The medium was removed and the cells were exposed for 1 min to irradiation at a distance of 30 cm from the UVC light source. The medium was returned to the cells, and they were placed in the incubator for 1 h.

Either 20 % serum or anisomycin (50 ng/ml) was added to the serum-starved cells for 1 h prior to cell harvesting.

The p38 inhibitor SB203580 (10 μM, Calbiochem), the JNK inhibitor SP600125 (40 μM, Calbiochem), the MEK inhibitor UO126 (20 μM, Calbiochem) and the proteasome inhibitor MG132 (40 μM, Sigma–Aldrich) were added to the cells at the indicated concentrations 1 h prior to cellular treatment. CHX (cycloheximide) (Sigma–Aldrich) was added to the cells at a 10 μg/ml concentration.

HEK-293T cells were transfected with the appropriate expression plasmid (pBabe-JDP2, 2 μg) using the calcium phosphate (Ca_2PO_4) method [25]. The total amount of plasmid DNA was adjusted to 10 μg with an empty pBabe vector. The cells were replaced with fresh medium 4–5 h post-transfection and harvested 24 h thereafter.

Nuclear and total cell lysate preparation

Cells were washed twice in PBS followed by harvesting and centrifugation. Total cell lysate was prepared by dissolving the cell pellet directly in a 1× sample buffer, followed by 15 min sonication at maximal level with Bioruptor (UCD-200). For nuclear cell lysate preparation, the cell pellet was resuspended in 10 mM Hepes (pH 7.9) containing 10 mM KCl, 1 mM EDTA, 1 mM DTT (dithiothreitol), 1 mM PMSF and protease inhibitor cocktail (Sigma). The cells were allowed to swell on ice for 15 min. Subsequently, Nonidet P40 was added to final concentration of 0.625 % and the tube containing the lysate was vigorously vortexed for 10 s. The lysate was centrifuged for 30 s and the nuclear pellet was resuspended in 20 mM Hepes (pH 7.9) containing 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail. The isolated nuclei were rocked at 4 °C for 15 min followed by 5 min centrifugation at 16000 ***g***. The pellet was discarded and supernatant was designated as nuclear protein lysate.

Western blot analysis

Nuclear protein lysate (unless otherwise indicated) was separated by SDS/PAGE (12.5 % gel) followed by immobilization on to nitrocellulose membrane (Hybond-Amersham Biosciences). The membranes were preblocked and probed in 5 % non-fat dry milk (Carnation) in PBS. Washes were performed with PBS. The primary antibodies used were rabbit polyclonal anti-ATF3 (C-19, Santa Cruz Biotechnology) at a dilution of 1:500, mouse monoclonal anti-p-c-Jun (SC-822, Santa Cruz Biotechnology) at a dilution of 1:500, rabbit polyclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; FL-335, Santa Cruz Biotechnology) at a dilution of 1:200, mouse monoclonal anti-α-tubulin (T-9026, Sigma–Aldrich) at a dilution of 1:2000, and rabbit polyclonal anti-JDP2 at a dilution of 1:500 [1]. Proteins were detected using the corresponding HRP (horseradish peroxidase)-conjugated secondary antibodies obtained from Sigma–Aldrich (anti-rabbit A0545 and anti-mouse A0168) followed by ECL (enhanced chemiluminescence) reaction visualized by Fuji X-Ray film.

Protein quantification

Western blot X-Ray films were used to quantify proteins using the TotalLab TL100 software ver2006c (Nonlinear Dynamics). The JDP2 expression level was normalized with the indicated housekeeping proteins α-tubulin or GAPDH.

qRT–PCR (quantitative real-time PCR)

Total RNA was extracted from MEF cells using High Pure RNA Isolation Kit (Roche) according to the manufacturer's instructions. Single-stranded cDNA was synthesized with 200 ng of mRNA samples in a 20 μl of total reaction mix of Verso^{TM} cDNA kit (Thermo Scientific), with a mixture of random hexamer and oligo-dT primers at a ratio of 3:1 respectively.

qRT–PCR was performed using the Rotor-Gene 6000TM (Corbett) equipment with absolute blue SYBER green ROX mix (Thermo Scientific). Serial dilution of a standard sample was included for each gene to generate a standard curve. The values were normalized with β_2 microglobulin expression levels.

Primer sequence were: mJDP2-F, 5′-GCTGAAATACGCTG-ACATC-3′; mJDP2-R, 5′-CTCACTCTTCACGGGTTGG-3′; β2 microglobulin-F, 5′-TTCTGGTGCTTGTCTCACTGA-3′; and β2 microglobulin-R, 5′-CAGTATGTTCGGCTTCCCATTC-3′.

RESULTS

JDP2 expression level is reduced in response to various cellular stresses

JDP2 is known to undergo phosphorylation by the stress-activated kinases JNK and p38 in response to various stimuli [18,21]. The role of JDP2 phosphorylation is, however, currently unknown. To investigate the biological role of this phosphorylation, we first characterized endogenous JDP2 expression in immortalized MEF cells treated with various cellular stimuli. Nuclear extracts were prepared, and JDP2 protein levels were assessed by Western blot analysis using anti-JDP2 antibodies. Serum stimulation resulted in a decrease in JDP2 protein levels to approximately 50 % of the serum-starved cells. Treatment with a low level of the translation inhibitor anisomycin, as well as UV irradiation, resulted in a similar decrease (Figure 1A).

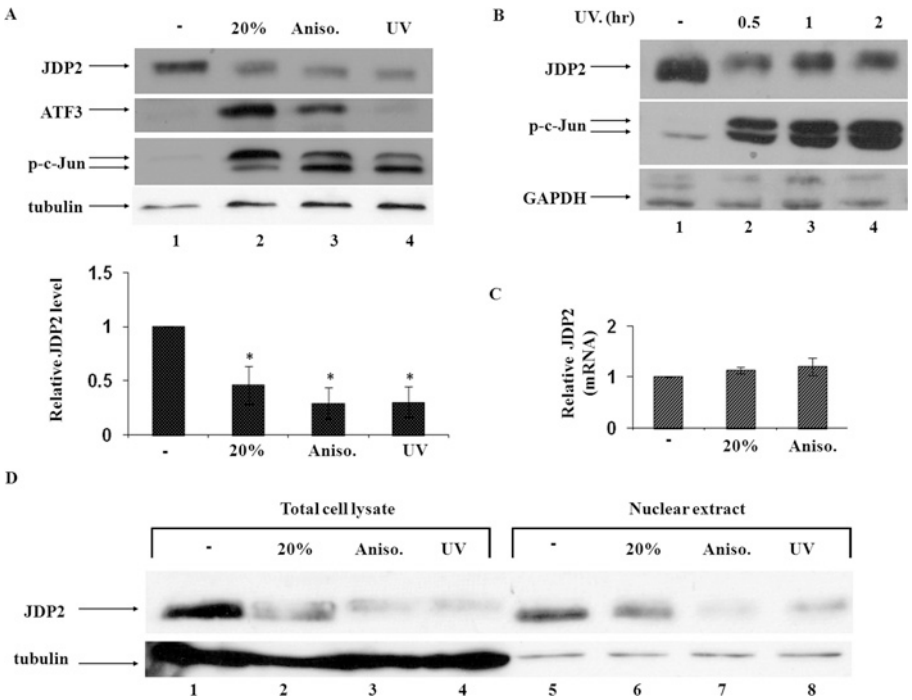

Figure 1 JDP2 protein level is rapidly reduced in response to different cell stimuli

(**A**) Upper panel: MEF cells were serum-starved (0.5 % for 24 h) and then either left untreated (−), or treated with 20 % serum (20 %), 50 ng/ml anisomycin (Aniso.) or UV irradiation (217 nm for 1 min; UV.). Nuclear extracts were was prepared 1 h following the respective stimulation. Lysates (50 ng) were separated by SDS/PAGE (12.5 % gel) followed by Western blotting with the indicated antibodies. Lower panel: the level of JDP2 protein was quantified from the Western blot and normalized to α-tubulin loading control. The level of JDP2 in each condition was calculated relative to the level in the untreated cells (designated a value of 1). The results represent the mean $\pm$ S.E.M. of four independent experiments. $^*P < 0.05$. (**B**) Representative Western blot analysis of nuclear extracts prepared at different time points following UV irradiation. GAPDH expression serves as loading control. (**C**) qRT–PCR was performed using cDNA prepared from mRNA derived from cells treated as in (**A**). JDP2 expression was normalized by dividing the mRNA level of the $\beta 2m$ housekeeping gene. The normalized JDP2 level of untreated cells was designated a value of 1, and the JDP2 levels of treated cells were expressed as relative values. The results represent the mean $\pm$ S.E.M. of three independent experiments. (**D**) Cells were treated as in (**A**) and the level of JDP2 in the nuclear extract or total cell lysate was determined by Western blot analysis. α-Tubulin serves as loading control.

JDP2 is most closely homologous to ATF3 [17]. They differ mainly in their mode of regulation [21]. To determine whether the effects of the three stimuli are specific to JDP2, we analysed the expression level of ATF3 under these conditions by probing the Western blot membrane with an anti-ATF3 antibody. The level of ATF3 was significantly increased upon serum stimulation, moderately increased with anisomycin treatment, and unaffected by UV irradiation (Figure 1A). Thus JDP2 and ATF3 differ in their response to the three stimuli. Whereas serum stimulation is known to activate mainly the ERK/MAPK pathway [20], anisomycin and UV irradiation activate the p38 [26] and JNK pathways [26,27].

Since JDP2 is known to be phosphorylated by JNK [18,21], we verified the activation of the JNK pathway in our system by probing the membrane with an anti-phospho-Jun antibody. As expected, serum-starved cells displayed no detectable phospho-c-Jun protein. In contrast, all three treatments resulted in a strong induction of c-Jun phosphorylation indicative of efficient JNK activation (Figure 1A). To determine whether these effects are not unique to MEF cells, the same set of experiments was performed in serum-starved NIH-3T3 cells, testing for JDP2 protein level and c-Jun phosphorylation in nuclear cell lysates. Indeed, the three stimuli resulted in an efficient increase in c-Jun phosphorylation accompanied by a drop in the JDP2 protein level (Supplementary Figure S1 at http://www.BiochemJ.org/bj/436/bj4360661add.htm).

The decrease in JDP2 protein level was found to be relatively rapid reaching its maximal level 20–30 minutes following UV irradiation (Figure 1B; see also Figure 2B). To determine whether the decrease in the JDP2 protein level is due to an alteration in *JDP2* mRNA levels, we performed qRT–PCR using cDNA derived from mRNA extracted from MEF cells. This analysis showed that the level of JDP2 mRNA was unaffected by serum stimulation and anisomycin treatment (Figure 1C). We were unable to obtain reliable qRT–PCR results with mRNA derived from UV-irradiated cells, possibly due to an mRNA irradiation-induced cross-link. On the basis of the qRT–PCR results and the rapid decrease in JDP2 protein level, we conclude that the reduction in JDP2 protein level observed following the three stimuli occurs post-transcriptionally.

A possible explanation for the reduced JDP2 protein level may be its translocation to a non-nuclear compartment. To address this possibility, we assessed JDP2 protein levels in nuclear and total cell lysates. The total cell lysates contained higher levels of JDP2 and α-tubulin (loading control) protein as compared with nuclear lysates. The JDP2 protein level in the total cell lysates was reduced in response to the three cellular treatments and the extent of the reduction was similar to that in the nuclear lysates (Figure 1D). This excludes the possibility that the observed reduction in JDP2 is due to its translocation out of the nucleus. Thus we conclude that the JDP2 protein level is reduced by these various stimuli.

Phosphorylation of Thr^{148} reduces JDP2 expression

Since the activation of the JNK pathway is common to the three stimuli that resulted in a decrease in JDP2 (Figure 1A) and

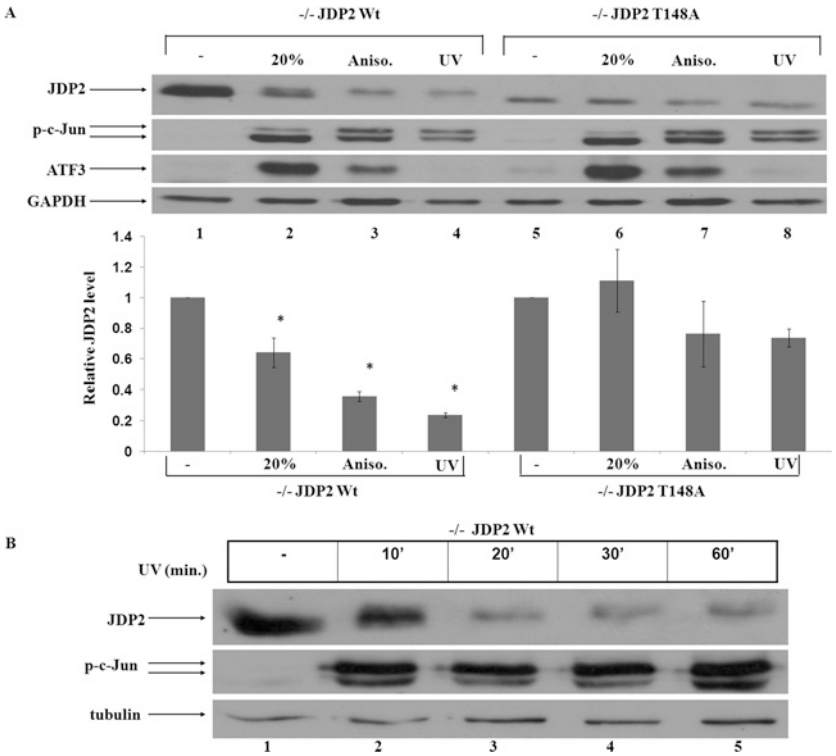

Figure 2 JDP2 Thr^{148} is responsible for reduced stability following various cell stimuli

(**A**) Upper panel: MEF JDP2$^{-/-}$ cells stably expressing either wild-type JDP2 ($-/-$ JDP2 Wt) or a JDP2 mutant in which Thr^{148} is converted to an alanine residue ($-/-$ JDP2 T148A) were serum-starved (0.5% for 24 h) and either left untreated ($-$), or treated with 20% serum (20%,), 50 ng/ml anisomycin (Aniso.) or UV irradiation (217 nm for 1 min, UV). Nuclear extracts were prepared 1 h following the respective stimulation and Western blotting was performed using the indicated antibodies. Lower panel: The level of JDP2 protein was quantified from the Western blot and normalized to the GAPDH loading control. The level of JDP2 in each condition was calculated relative to the level in the untreated cells (designated a value of 1). The results represent the mean $\pm$ S.E.M. of four independent experiments (bottom panel). *$P < 0.05$. (**B**) MEF JDP2$^{-/-}$ cells stably expressing JDP2 wild-type protein were either left untreated ($-$) or UV irradiated for the indicated time points. Nuclear extracts were prepared and Western blotting was performed using the indicated antibodies. α-Tubulin serves as loading control.

JDP2 is a known substrate of JNK [18,21], we hypothesized that phosphorylation of JDP2 may regulate the level of the protein. To investigate this hypothesis, we first verified that JDP2 is indeed phosphorylated in response to the three stimuli. Anti-phospho-JDP2-specific antibodies are currently unavailable. Nevertheless, phosphorylated JDP2 can be detected as a slower migrating band relative to the non-phosphorylated form when resolved on a long SDS/PAGE gel [18]. Using this technique, we detected JDP2 phosphorylation following all three stimuli (Supplementary Figure S2A, lanes 1–4 at http://www.BiochemJ.org/bj/436/bj4360661add.htm).

To examine the possibility that JDP2 phosphorylation results in its reduced level, we infected a JDP2$^{-/-}$ MEF cell line [16,22] with retrovirus encoding either wild-type JDP2 or mutant JDP2 in which Thr^{148} is mutated to an alanine residue (JDP2 T148A). Puromycin-resistant pooled cells were serum starved and exposed to different stimuli. JDP2 expression was assessed by Western blotting with anti-JDP2 antibody (Figure 2A). The level of ectopically expressed wild-type JDP2 was reduced upon serum stimulation, anisomycin treatment and UV irradiation (Figure 2A left-hand panel, lanes 1–4). The reduction in JDP2 levels was relatively rapid since the maximal decrease in JDP2 protein level already occurred 20 min after UV irradiation (Figure 2B). In contrast, the protein level of the T148A mutant was relatively unchanged following these stimuli (Figure 2A, right-hand panel, lanes 5–8). Consistently, resolving the proteins on a long SDS/PAGE gel revealed that JDP2 wild-type was phosphorylated in response to the three stimuli, whereas JDP2 T148A remained unchanged (Supplementary Figure S2B, lanes 5–8). Collectively, these results suggest that the phosphorylation of Thr^{148} may regulate JDP2 protein stability.

We then expressed a phosphomimetic JDP2 mutant in which Thr^{148} is mutated to a glutamic acid residue (JDP2 T148E) in the JDP2$^{-/-}$ cells and compared its expression to overexpressed wild-type JDP2 and JDP2 T148A, as well as to endogenous JDP2. The level of ectopically expressed wild-type JDP2 protein was approximately 3-fold that of endogenous JDP2 in wild-type MEF cells. The JDP2 T148A mutant protein was expressed to a lesser extent, whereas the T148E mutant protein was hardly detected (Supplementary Figure S3A at http://www.BiochemJ.org/bj/436/bj4360661add.htm). Using qRT–PCR, we showed that the decreased level of JDP2 T148A protein was due to lower mRNA levels. In contrast, JDP2 T148E mRNA levels were 30-fold higher than endogenous JDP2 and 3-fold higher than ectopically expressed wild-type JDP2 (Supplementary Figure S3B). Thus the level of JDP2 T148E protein is significantly decreased, despite the high level of mRNA. Collectively, the data suggest that phosphorylation at Thr^{148} plays a role in regulating the level of the JDP2 protein.

JNK activity is required for reduced expression of JDP2 following stress

To determine whether JNK activity is specifically required for JDP2 phosphorylation and its reduced expression, we assessed the effect of serum stimulation in the presence of a JNK inhibitor

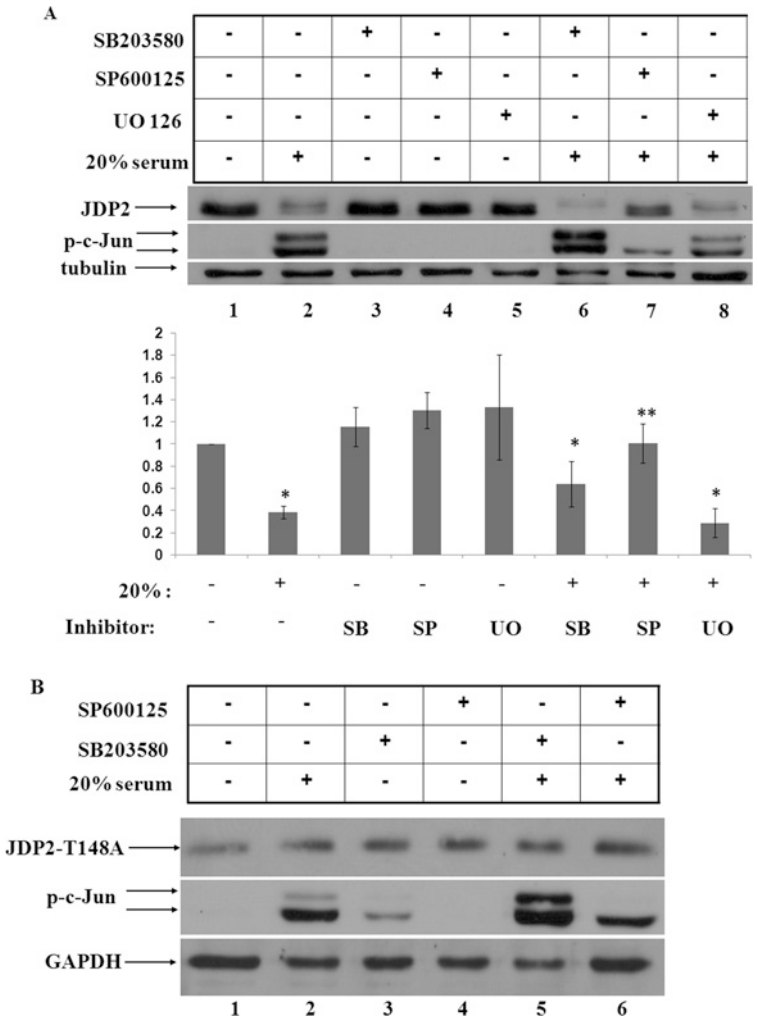

Figure 3 JNK activity is required for the serum-induced reduction in JDP2

(**A**) Upper panel: MEF cells were serum-starved (0.5 % for 24 h) and then treated with p38 inhibitor (10 μM; SB), JNK inhibitor (40 μM; SP) or MEK inhibitor (20 μM; UO) for 1 h, followed by addition of 20 % serum, where indicated, for an additional 1 h. Nuclear extracts were prepared and Western blotting was performed with the indicated antibodies. α-tubulin served as the loading control. Lower panel: The level of JDP2 protein was quantified from the Western blot and normalized to an α-tubulin loading control. The level of JDP2 in each condition was calculated relative to the level in the untreated cells (designated a value of 1). The results represent the mean $\pm$ S.E.M. of 6 independent experiment. $^{*}P < 0.05$, compared with untreated cells (lane 1). $^{**}P < 0.05$ compared with serum-treated cells $\pm$ SP600125 (comparing lane 2 with lane 7). (**B**) MEF JDP2$^{-/-}$ cells stably expressing the JDP2 T148A mutant were serum-starved (0.5 % for 24 h) and then treated with the indicated inhibitors as in (**A**) followed by addition of 20 % serum where indicated for an additional 1 h. Nuclear extracts were prepared and Western blotting was performed with the indicated antibodies. GAPDH served as the loading control.

(SP600125). As controls for specificity we used a p38 inhibitor (SB203580) and a MEK inhibitor (UO126). As expected, the JNK inhibitor abolished the serum-induced phosphorylation of c-Jun (Figure 3A, compare lanes 2 and 7), whereas the p38 and MEK (MAPK/ERK) inhibitors had no effect (Figure 3A, lanes 6 and 8). Importantly, the serum-induced reduction in the level of JDP2 was abolished in the presence of the JNK inhibitor, and was unchanged in the presence of the p38 and MEK inhibitors. In addition, serum-induced phosphorylation was abolished in the presence of the JNK inhibitor (Supplementary Figure S2A). Moreover, either JNK or p38 inhibitors did not affect the level of the JDP2 T148A mutant protein following serum stimulation (Figure 3B). Collectively, the data suggest that JNK activation is required for reducing the expression level of JDP2 in response to serum stimulation.

Phosphorylated JDP2 undergoes proteasomal degradation

The alteration of JDP2 protein levels can result from either decreased protein synthesis or increased protein degradation. In order to distinguish between these possibilities, we made use of the translation inhibitor, CHX. Serum-starved MEF cells were exposed to serum stimulation in the presence or absence of CHX for different time points following serum addition (Figure 4A). In the absence of CHX, JDP2 levels were greatly reduced by 30 and 60 min and returned to almost normal levels 2 h following serum stimulation. The decrease in JDP2 level was accompanied by an increase in ATF3 expression and JNK activation that peaked 1 h and 30 min following the addition of serum respectively (Figure 4A). In the presence of CHX, the addition of serum resulted in a complete loss of JDP2 expression, accompanied by enhanced JNK activation. In contrast, the increase in ATF3 expression in response to serum was completely abrogated in the presence of CHX (Figure 4A, lanes 5–8), reflecting the complete inhibition of protein synthesis under these experimental conditions.

Since CHX efficiently blocks protein synthesis and activates JNK simultaneously, we decided to examine the rate of JDP2 degradation in cells treated with CHX (Figure 4B). MEF cells

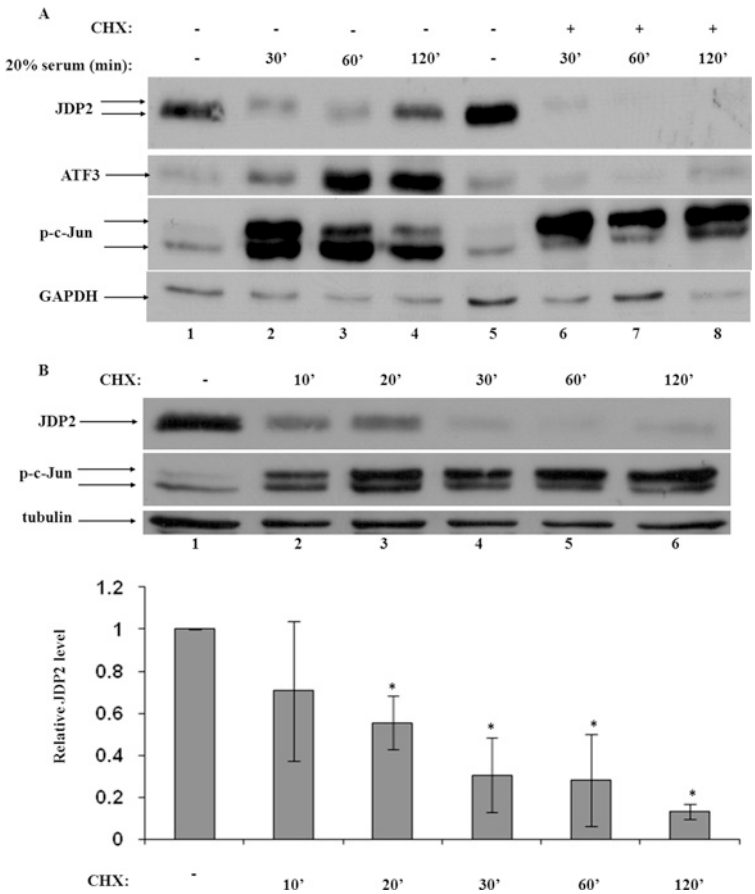

Figure 4 Serum–induced reduction of JDP2 protein is independent of protein synthesis

(**A**) MEF cells were serum-starved (0.5 % for 24 h) and then treated with 20 % serum in the absence or presence of CHX (10 μg/ml) where indicated (+). Cells were harvested at the indicated time points and nuclear extracts prepared. Western blotting was performed with the indicated antibodies. GAPDH served as loading control. (**B**) Upper panel: MEF cells were either untreated (−) or treated with CHX for the indicated times and nuclear extracts were prepared. Western blotting was performed with the indicated antibodies. Lower panel: the level of JDP2 protein was quantified from the Western blot and normalized to α-tubulin loading control. The level of JDP2 at each time point was calculated relative to the JDP2 level in the absence of CHX (designated a value of 1). The results represent the mean $\pm$ S.E.M. of three independent experiments. $^*P < 0.05$.

were exposed to CHX for different time lengths and nuclear cell lysates were subjected to Western blot analysis. JNK activity was strongly induced 10 min following CHX addition and persisted for 2 h. As expected, the JDP2 expression level was reduced with time following exposure to CHX. Indeed, JDP2 phosphorylation was readily observed in cell lysates derived from CHX-treated cells (Supplementary Figure S2A, lanes 8 and 9). Thus the JDP2 half-life observed between 20–30 min corresponds mainly with the JDP2 protein found in the phosphorylated form. To measure the JDP2 half-life of the unphosphorylated form in the absence of protein synthesis, the cells were treated with the JNK inhibitor (SP600125) prior to the addition of CHX (Figure 5A). In the presence of the JNK inhibitor, c-Jun phosphorylation was completely inhibited, indicating efficient JNK inhibition under the experimental conditions used. Consistently, the JDP2 level remained unchanged for 2 h following the addition of CHX (Figure 5A). Similarly, the JDP2 mutant protein (T148A) that fails to be phosphorylated by JNK displayed a relatively long half-life during the course of 2 h following the addition of CHX (Figure 5B).

In order to determine whether JDP2 degradation is proteasome dependent, cells were preincubated with the proteasome inhibitor MG132 prior to treatment with CHX. Significantly, proteasome inhibition prolonged the half-life of JDP2 (Figure 6A), suggesting that JDP2 undergoes proteasomal degradation.

Finally, wild-type JDP2 and the phosphomimetic T148E mutant were overexpressed in the highly transfectable cell line, HEK-293T. MG132 was added 24 h after transfection, and the JDP2 level was measured. In untreated cells, JDP2 T148E was expressed at a low level compared with the transfected wild-type JDP2 (Figure 6B; compare lane 2 with lane 6). However, addition of MG132 increased the expression level of the T148E mutant to that of the transfected wild-type JDP2.

Collectively, these results suggest that JDP2 phosphorylation by JNK on Thr^{148} results in rapid proteasomal-dependent degradation.

DISCUSSION

The AP-1 transcription factor is controlled at transcriptional, translational and post-translational levels in response to various cell insults [28]. At the protein level, JNK phosphorylates Ser^{63}/Ser^{73} within the activation domain of c-Jun. This facilitates the association of c-Jun with CBP (CREB-binding protein)/p300 and thus potentiates its transcription ability [29]. Phosphorylation of c-Jun inhibits its proteasome-dependent degradation, resulting in an increase in c-Jun expression levels following stress stimuli [30]. In addition, c-Jun phosphorylation relieves HDAC (histone deacetylase) 3 suppression of c-Jun transcription activity [31].

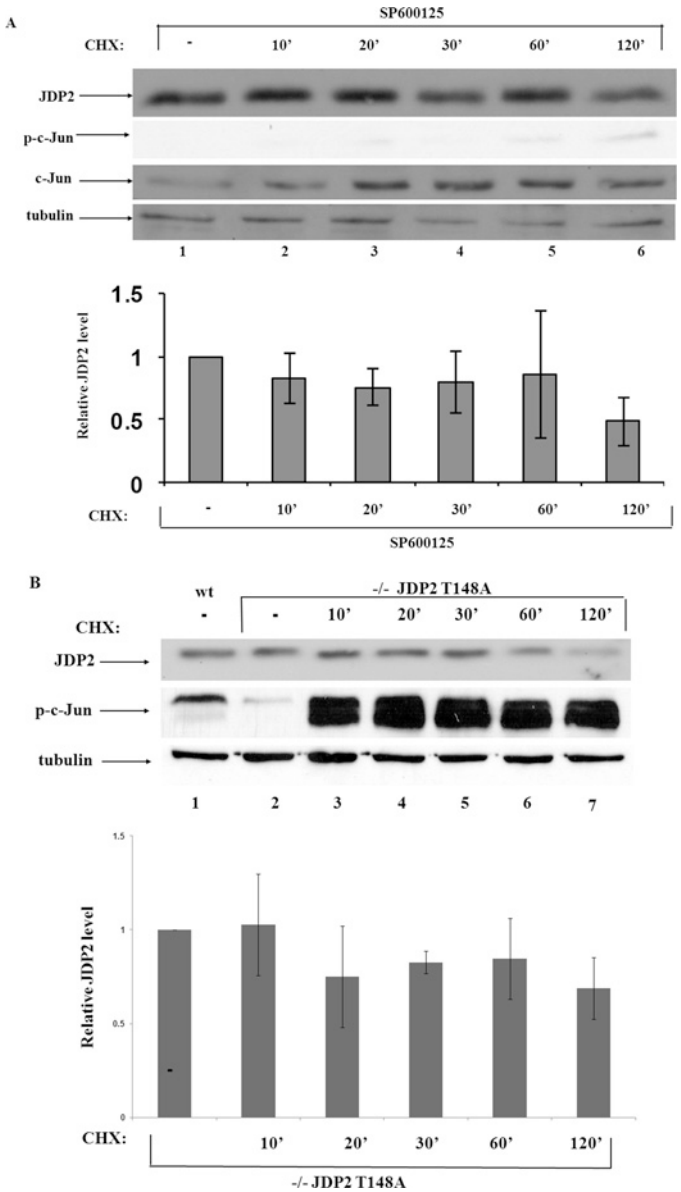

Figure 5 Phosphorylation on Thr^{148} by JNK is responsible for JDP2 degradation

Upper panels: serum-starved wild-type MEF cells were pre-treated with the JNK inhibitor SP600125 for 1 h (**A**) or JDP2$^{-/-}$ MEF cells expressing either wild-type (we) JDP2 or the T148A mutant (**B**). CHX was then added for the indicated times and nuclear extracts prepared. Western blotting was performed with the indicated antibodies. Lower panels: the level of JDP2 protein was quantified from the Western blot and normalized to the α-tubulin loading control. The level of JDP2 at each time point was calculated relative to the JDP2 level in the absence of CHX (designated a value of 1). The results represent the mean $\pm$ S.E.M. of three independent experiments.

In the present paper we describe an additional level of control of c-Jun net activity mediated by JNK, involving proteasome-dependent degradation of its inhibitory transcription repressor protein JDP2. Although JDP2 represents a bona fide JNK substrate [18,19,21], no biological function has been assigned to JDP2 phosphorylation. In the present paper we show that JDP2 undergoes a rapid reduction in its expression level following three different cellular stimuli: serum, anisomycin and UV irradiation. The JDP2 mutant protein in which Thr^{148} is substituted with alanine displays stable expression in the presence of the three stimuli. Conversely, JDP2 phosphomimetic mutant protein in which Thr^{148} is substituted with an glutamic acid residue is unstable and its expression in cells is undetectable despite high mRNA levels. Rapid reduction in JDP2 expression levels allows the efficient activation of the AP-1 complex following cell stress. Interestingly, JDP2 T148A stable cell lines always display a low level of expression, which reflects the reduced mRNA levels following long-term selection. We hypothesize that during the puromycin selection process, cells with a lower level of expression of the JDP2 T148A display a growth advantage compared with cells displaying higher levels of JDP2 T148A expression.

In the present paper we demonstrate that serum activates the JNK signalling pathway in MEF cells very efficiently. Serum typically activates the ERK/MAPK pathway as opposed to the JNK pathway [32]. However, JNK activation by serum has been shown in MEF cells in previous studies [33]. The use of the protein synthesis inhibitor CHX allowed us to determine the JDP2 half-life in its phosphorylated and unphosphorylated form. While the non-phosphorylated JDP2 is stable for a period of 2 h, the phosphorylated form displays a significantly shortened

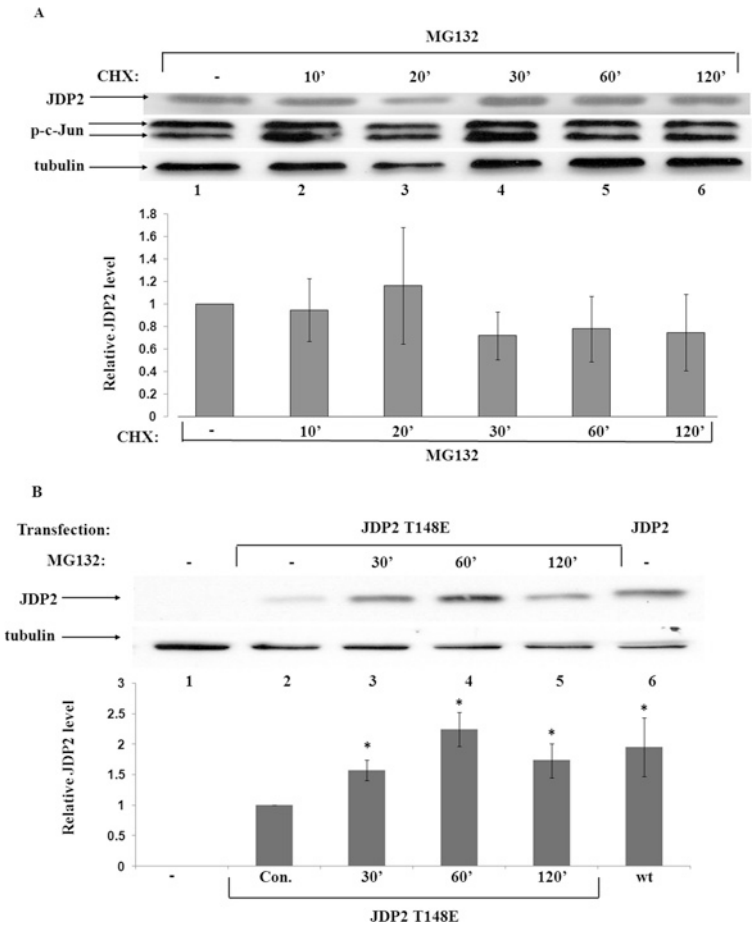

Figure 6 JDP2 degradation is proteasome-dependent

(**A**) Upper panel: serum-starved wild-type MEF cells were pre-treated with the proteasome inhibitor MG132 (40 μM) for 1 h. CHX was then added for the indicated times and nuclear extracts prepared. Western blotting was performed with the indicated antibodies. Lower panel: the level of JDP2 protein was quantified from the Western blot and normalized to the α-tubulin loading control. The level of JDP2 at each time point was calculated relative to the JDP2 level in the absence of CHX (designated a value of 1). The results represent the mean $\pm$ S.E.M. of three independent experiments. (**B**) Upper panel: HEK-293T cells were transfected with plasmids encoding either wild-type JDP2 or a T148E mutant. The cells were either untreated ($-$) or treated with MG132 (40 μM) for the indicated times prior to cell harvesting. Nuclear extracts were prepared and Western blotting was performed with the indicated antibodies. Lower panel: the level of JDP2 protein was quantified from the Western blot and normalized to α-tubulin loading control. The level of JDP2 at each time point was calculated relative to the level of JDP2 T148E in the absence of MG132 (designated a value of 1). The results represent the mean $\pm$ S.E.M. of three independent experiments. $^*P < 0.05$.

half-life of 20–30 min. The use of the proteasome inhibitor MG132 in the presence of the protein synthesis inhibitor completely abrogated JDP2 degradation, suggesting that the JDP2-dependent degradation is mediated by the proteasome.

Collectively, we show that JDP2 phosphorylation by JNK results in rapid JDP2 destabilization and reduced expression levels following various cell stimuli. We suggest that JDP2 degradation represents an additional level of control for the derepression of cellular inhibitory mechanisms leading to the activation of the c-Jun/AP-1 transcription factor following cellular insults.

AUTHOR CONTRIBUTION

Keren Weidenfeld-Baranboim carried out the experiments, performed data analysis and helped with figure preparation. Lilach Koren carried out the experiments, performed data analysis and helped with figure preparation. Ami Aronheim designed, interpreted and wrote the manuscript. All authors read and approved the final manuscript prior to publication.

ACKNOWLEDGEMENTS

The authors wish to thank Dr Ariel Stanhill for various reagents and M. Aviva Cohen for her excellent technical assistance.

FUNDING

This work was partially supported by the Israeli Ministry of Health [grant number 3-3955] to A.A.

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Received 12 July 2010/21 March 2011; accepted 4 April 2011
Published as BJ Immediate Publication 4 April 2011, doi:10.1042/BJ20101031

Biochem. J. (2011) **436**, 661–669 (Printed in Great Britain) doi:10.1042/BJ20101031

online data

SUPPLEMENTARY ONLINE DATA

Phosphorylation of JDP2 on threonine-148 by the c-Jun N-terminal kinase targets it for proteosomal degradation

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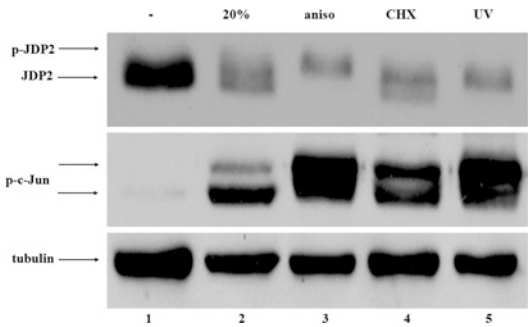

Figure S1 JDP2 protein level is rapidly reduced in response to different cell stimuli in NIH-3T3 cells

NIH-3T3 cells were serum-starved (0.5 % for 24 h) and then either left untreated (—), or treated with 20 % serum (20 %), 50 ng/ml anisomycin (Aniso.), 10 μg/ml CHX or UV irradiation (217 nm for 1 min; UV). Nuclear extract was prepared 1 h following the respective stimulation. Lysates (50 ng) were separated by SDS/PAGE (12.5 % gel) followed by Western blotting with the indicated antibodies.

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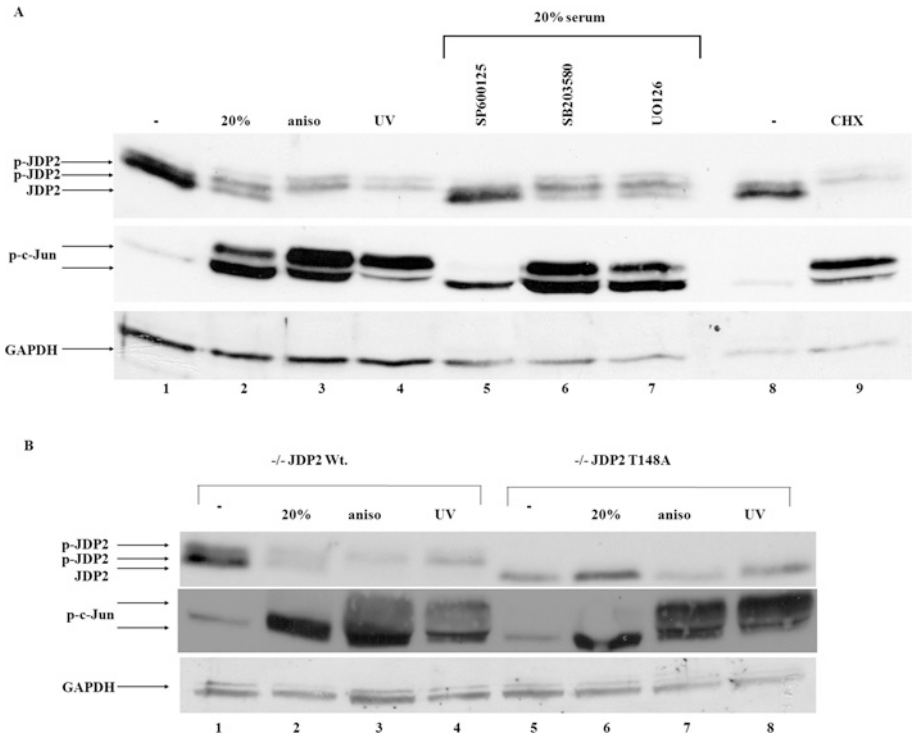

Figure S2 JDP2 is phosphorylated by JNK on thr^{148} in response to various cellular stimuli

(**A**) MEF wild-type cells were serum-starved (0.5 % for 24 h) and then treated with 50 ng/ml anisomycin, UV irradiation, p38 inhibitor (10 μM, SB203580), JNK inhibitor (40 μM; SP600125), MEK inhibitor (20 μM; UO126) or CHX (10 μg/ml; CHX) for 1 h. Serum was then added (20 %) for an additional 1 h, as indicated. Nuclear extracts were prepared and resolved on a 15-cm long SDS/PAGE gel followed by Western blotting with the indicated antibodies. This technique allows the phosphorylated form of JDP2 to be separated from the unphosphorylated form. JDP2 appears as a double band in lysates from serum-starved cells. Serum stimulation, anisomycin treatment, UV irradiation and CHX treatments result in super-shifting of the double band to a triple band. (**B**) MEF JDP2$^{-/-}$ cells stably expressing either wild-type JDP2 or JDP2 T148A were serum-starved (0.5 % for 24 h) and then treated with 20 % serum, 50 ng/ml anisomycin or UV irradiation for 1 h. Nuclear extracts were prepared and resolved as in (**A**). Western blotting was performed with the indicated antibodies.

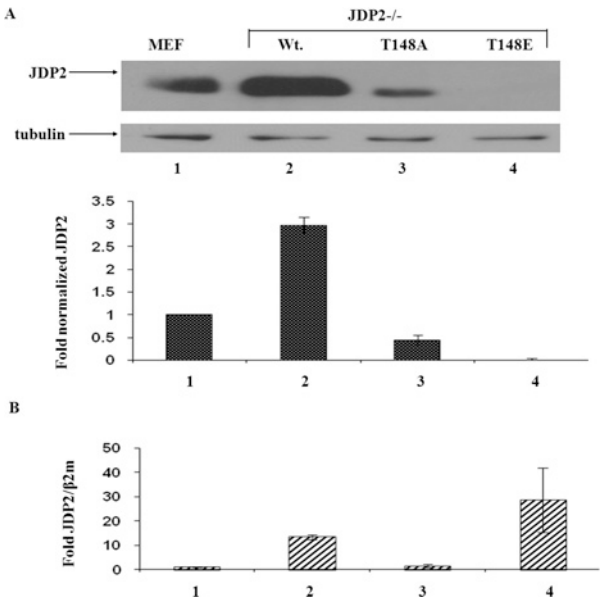

Figure S3 Phosphomimetic T148E JDP2 mutant is an unstable protein

(**A**) Upper panel: nuclear extracts were prepared from wild-type MEF cells and MEF JDP2$^{-/-}$ cells stably transfected with either JDP2 wild-type (Wt), JDP2 T148A or JDP2 T148E Western blotting was performed with the indicated antibodies. α-Tubulin serves as a loading control. Lower panel: The level of JDP2 protein was quantified from the Western blot and normalized to α-tubulin loading control. The level of JDP2 in each cell line was calculated relative to the level of the wild-type cell line (designated a value of 1). The results represent the mean $\pm$ S.E.M. of three independent experiments. (**B**) qRT–PCR with cDNA prepared from mRNA derived from cells as described in (**A**). JDP2 expression was normalized by dividing by the mRNA level of *β2m* housekeeping gene. The mRNA level in each cell line was calculated relative to the level of the wild-type cell line (designated a value of 1). The results represent the mean $\pm$ S.E.M. of three independent experiments.

Received 12 July 2010/21 March 2011; accepted 4 April 2011
Published as BJ Immediate Publication 4 April 2011, doi:10.1042/BJ20101031