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ORIGINAL ARTICLE

Suppression of cell-cycle progression by Jun dimerization protein-2 (JDP2) involves downregulation of cyclin-A2

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We report here a novel role for Jun dimerization protein-2 (JDP2) as a regulator of the progression of normal cells through the cell cycle. To determine the role of JDP2 *in vivo*, we generated *Jdp2*-knockout (*Jdp2*KO) mice by targeting exon-1 to disrupt the site of initiation of transcription. The epidermal thickening of skin from the *Jdp2*KO mice after treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) proceeded more rapidly than that of control mice, and more proliferating cells were found at the epidermis. Fibroblasts derived from embryos of *Jdp2*KO mice proliferated faster and formed more colonies than fibroblasts from wild-type mice. JDP2 was recruited to the promoter of the gene for cyclin-A2 (*ccna2*) at the AP-1 site. Cells lacking *Jdp2* had elevated levels of cyclin-A2 mRNA. Furthermore, reintroduction of JDP2 resulted in the repression of transcription of *ccna2* and of cell-cycle progression. Thus, transcription of the gene for cyclin-A2 appears to be a direct target of JDP2 in the suppression of cell proliferation.
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Introduction

Progression of the cell cycle in mammalian cells is regulated by the interplay of protein kinase complexes, known as cyclin-dependent kinases (CDKs). CDKs are controlled by the levels of expression of their respective cyclin partners, which act as positive coactivators or as negative regulators in the case of the so-called CDK inhibitors (Grana and Reddy, 1995; Sherr and Roberts, 1999; Malumbres and Barbacid, 2009; Satyanarayana and Kaldis, 2009; Caldon and Musgrove, 2010). Although cyclin-D–cdk4/6 and cyclin-E–cdk2 control progression through the G_1 phase and cyclin-B–cdc2 appears to be necessary for entry into mitosis (Furuno *et al.,* 1999), cyclin-A is a rate-limiting component required for the initiation of DNA synthesis and entry into mitosis (Pagano *et al*., 1992; Chaudhry *et al*., 2004). There are two subtypes of cyclin-A: cyclin-A2, which is expressed almost ubiquitously, and cyclin-A1, the expression of which is restricted to the testis (Sweeney *et al*., 1996). In conditional cyclin-A-knockout mice, the functions of cyclin-A are essential for progression of hematopoietic cells and embryonic stem cells through the cell cycle; however, cyclin-A and cyclin-E have redundant roles in cell proliferation in fibroblasts (Kalaszczynska *et al*., 2009).

Jun dimerization protein-2 (JDP2), a member of the activation protein-1 (AP-1) family, forms homodimers, as well as heterodimers, with other members of the AP-1 family, such as c-Jun, JunB, JunD and ATF2, and with a member of the C/EBP family, C/EBPγ (Aronheim *et al*., 1997; Broder *et al*., 1998; Jin *et al*., 2001). Assays involving ectopic expression of JDP2 indicate that it can block transformation of NIH3T3 cells and of prostate cancer cell lines (Heinrich *et al*., 2004). JDP2 also induces the partial transformation of chick embryonic fibroblasts (Blazek *et al*., 2003) and functions as a cell-survival protein in several cell lines (Piu *et al*., 2001; Lerdrup *et al*., 2005). JDP2 inhibits the differentiation of embryonal carcinoma F9 cells (Jin *et al*., 2002) and adipocytes (Nakade *et al*., 2007), and even promotes the differentiation of osteoclasts (Kawaide *et al*., 2003), C2 myoblasts and rhabdomyosarcoma cells (Ostrovsky *et al*., 2002).

JDP2 most likely participates in the repression of transcription through multiple mechanisms, which include DNA-binding competition and inactivation of the formation of heterodimers with other members of the AP-1 family (Aronheim *et al*., 1997), recruitment of HDAC-3 (Jin *et al*., 2002), inhibition of histone acetylation and direct regulation of chromatin assembly (Jin *et al*., 2006). However, the details of the physiological role of JDP2 in cell fate remain unknown, and the

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mechanisms by which JDP2 acts as a regulator of the proliferation or transformation of cells are yet to be clarified. Recently, we generated *Jdp2*-deficient mice with a deletion in the promoter and non-coding exon-1 region of the *Jdp2* locus (*Jdp2*KO mice) and reported that 'knockout' of *Jdp2* affects adipocyte differentiation (Nakade *et al*., 2007) and resistance to replicative senescence (Nakade *et al*., 2009).

Although our *Jdp2*KO mice did not show any apparent abnormalities under standard breeding conditions, we report here that loss of JDP2 results in accelerated cell cycling by mouse embryonic fibroblasts (MEFs) and enhances the expressions of cyclin-A2. Increased expression of cyclin-A2 occurred through loss of direct binding to the promoter of cyclin-A2 gene at the AP-1 site. We also observed accelerated cell growth, which resulted in epidermal thickening in adult *Jdp2*KO mice after treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Our data indicate that the gene for cyclin-A2 is one of the targets of JDP2 in the repression of cell proliferation by JDP2.

Results

Epidermal thickness and scratch-wounding assays

To investigate the functional effect of loss of expression of the JDP2 gene *in vivo*, we used the epidermal thickness assay to compare the back skins from TPA-treated hairless skins of wild-type (WT) or *Jdp2*KO mice. Both WT and *Jdp2*KO mice have a thin back epidermis of only 2–3 cells thick (Figure 1). However, the epidermal thickness of the hairless skins from *Jdp2*KO mice increased by 1.4-fold compared with that of WT mice four days after treatment with TPA (Figures 1A and B). The expression of proliferation cell nuclear antigen (PCNA) in the epidermis from *Jdp2*KO mice was 1.3-fold higher than that in the epidermis of WT mice (Figures 1B and C). The results of wound-healing assay *in vivo* also indicated the higher healing potential in *Jdp2*KO mice (Supplementary Figure S1).

Control of the proliferation of Jdp2-*deficient cells*

We prepared MEFs from *Jdp2*KO embryos on day 12.5 post coitus. Northern blotting and western blotting of embryos and MEFs from *Jdp2*KO mice failed to show any signals of JDP2 mRNA or protein (Figures 2a and b). We next examined the proliferation of MEFs using three different assays, namely, Trypan blue dye exclusion, Alamar Blue assay and colony formation. *Jdp2*KO MEFs proliferated more rapidly than WT MEFs when plated at 5×10^5 cells per 10-cm dish in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal calf serum (FCS) (Trypan blue dye exclusion; Figure 2c). The results of the cell growth assay with Alamar Blue dye and colony formation assay also supported this conclusion (Figures 2d and e). These results indicated that JDP2 has a crucial role in growth control *in vitro* and *in vivo*.

Absence of a requirement for JDP2 in apoptosis

We found that UV-irradiated MEFs underwent apoptosis in a dose-dependent manner at doses of UVC from 20 to 60 J/m^2; however, we did not detect any obvious differences between WT and *Jdp2*KO MEFs in subsequent Trypan blue dye exclusion assays and assays of the activities of caspases 3 and 7 (Supplementary Figures S2A and B). When MEFs were exposed to UVC at 60 and 600 J/m^2, for 2 and 8 h, the level of expression of JDP2 fell significantly (Supplementary Figure S2D). The size of the sub-G_1 population of UV-irradiated MEFs from *Jdp2*KO mice was identical to that of MEFs from WT mice (Supplementary Figure S2C). All reagents of apoptosis inducers increased the activities of caspases 3 and 7 in a dose-dependent manner (data not shown); however, no significant differences in respective activities were found between WT and *Jdp2*KO MEFs (Supplementary Figure S3) except after application of cycloheximide. These data suggested that JDP2 has no significant effect on the apoptotic responses to UV and inducers of cell death in normal MEFs.

We next performed a cell-cycle re-entry assay, counting propidium iodide-stained MEFs by flow cytometry. A representative experiment after 18-h serum stimulation showed that 28% of *Jdp2*KO MEFs were in the S-phase, whereas only 20% of WT MEFs were in the S-phase (Figure 3a). The number of MEFs derived from *Jdp2*KO mice in the S-phase was considerably higher than that of WT MEFs (1.5-fold; Figure 3b). Proliferation of *Jdp2*KO MEFs was accelerated by the enhanced progression of cells from the G_1- or the G_0-phase to the S-phase, and not because of differential activity related to cell death.

We also examined the incorporation of bromodeoxyuridine (BrdU) into MEFs. The percentages of BrdU-positive cells in the six chambers showed that *Jdp2*KO MEFs moved more frequently from a quiescent state to a proliferative state than did WT MEFs (3.5-fold; Figures 3c and d).

Regulation of cell-cycle-related genes by JDP2

To examine the role of JDP2 in the cell cycle, we analyzed the expression of individual cell-cycle-related genes. MEFs were arrested at the G_0/G_1-phase by deprivation of serum for 48 h and then re-entry into the cell cycle was triggered by re-plating in DMEM plus 15% FCS. No notable differences in protein levels, such as cyclin D1, cyclin D3, cyclin-dependent kinase-4 (cdk4), cdk6 and Rb were detected between WT and *Jdp2*KO MEFs (Figure 4a). However, p53 and p21 levels in *Jdp2*KO MEFs were lower than those in WT MEFs after induction by serum, suggesting JDP2 might affect the p53-p21 cascade, which is further discussed in the section Discussion (Vousden and Prives, 2009).

We next examined global gene expression in *Jdp2*KO MEFs using an oligo DNA microarray known as 'Mouse' (Agilent Technologies Inc., Santa Clara, CA, USA) (Supplementary Table 1; the entire data set from the microarray analysis is available at the Center

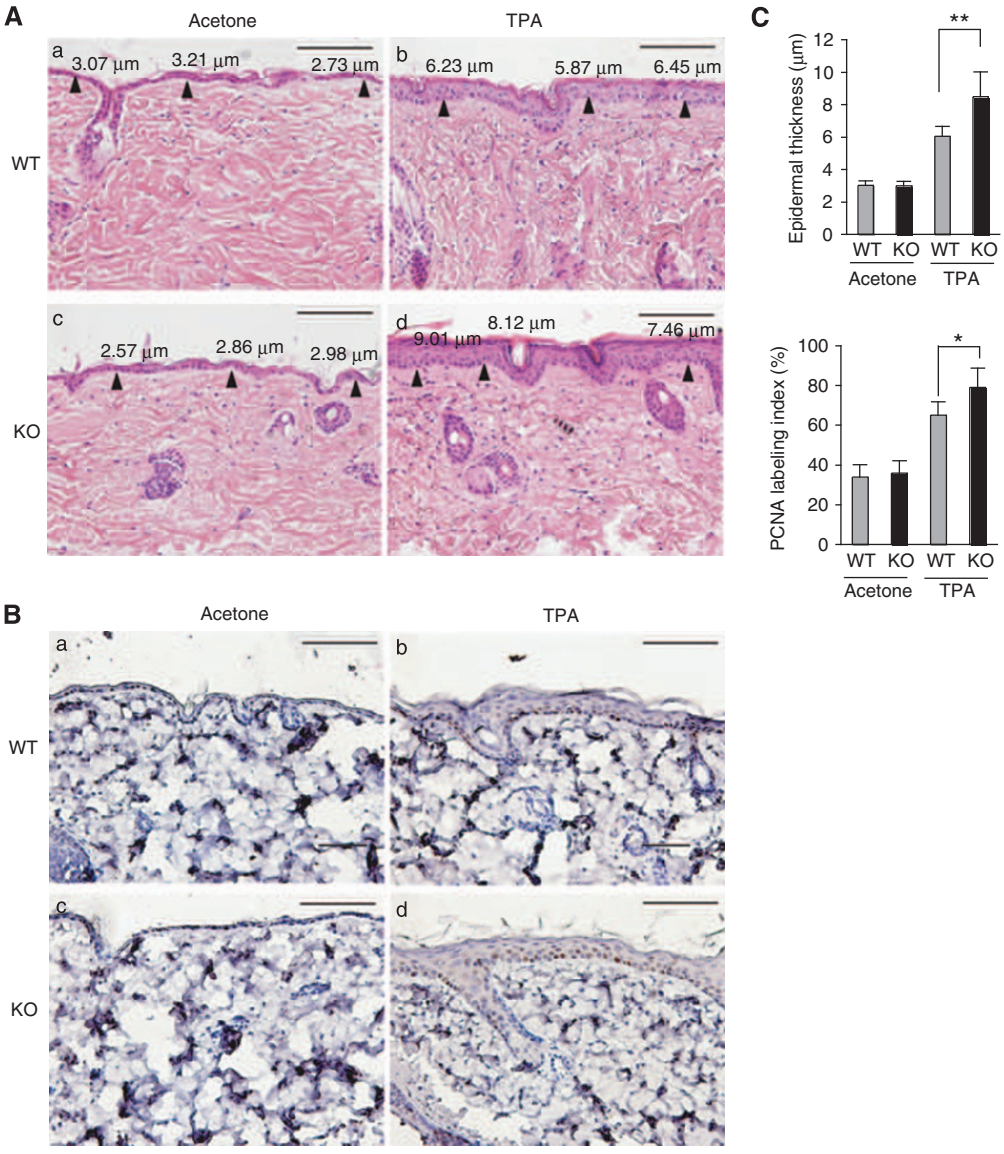


Figure 1 Proliferation of epidermal cells of WT and *Jdp2*KO mice in the TPA-induced epidermal thickness *in vivo*. (**A**) WT and *Jdp2*KO mice were shaved, painted four times with TPA or acetone and subjected to punch biopsy for pathology examination. Skins from WT mice and *Jdp2*KO mice were painted with acetone or 8.1 nmol TPA. Ten areas of skin in mid-power field were randomly selected in each section and assayed for their epidermal thickness ($n = 3$ each group, H&E; scale bar = 100 μm). (**B**) Immunostaining of the epidermis with antibodies against PCNA. (**C**) The thickness and PCNA-positive cell number of the epidermis was measured. Data were sketched in a chart showing the mean ± s.e. $^{*}P < 0.05$, $^{**}P < 0.001$. KO, *Jdp2*-knockout; PCNA, proliferation cell nuclear antigen; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; WT, wild type.

for Information Biology Gene Expression Database (CIBEX; DDBJ, Mishima, Japan; CIBEX accession, CBX101)). Higher levels of expression of genes for cyclin-A2 and cyclin-E2 were detected in *Jdp2*KO MEFs as compared with WT MEFs. Conversely, genes for the CDK inhibitors p21, p16 and p15, but not p27, were expressed at lower levels in *Jdp2*KO MEFs than in WT MEFs. However, the mRNA levels of p53, p107 and RB were not altered. The expression of genes from families of apoptosis-related genes and genes for other AP-1 and C/EBPs did not show any remarkable differences between *Jdp2*KO and WT MEFs, except for JDP2 and C/EBPδ. The expression of 21 known genes, including the Wnt/transforming growth factor-β (TGFβ) regulators Wisp2, Sfrp2 and Ltbp1, was enhanced in *Jdp2*KO MEFs as compared with WT MEFs, and the expression of another 20 known genes, including the Wnt target gene Lef1 and cAMP/Ca^{2+} regulators Cap-1 and Penk-1, was depressed in *Jdp2*KO MEFs as compared with WT MEFs (Supplementary Table 1).

As shown in Figure 4b, the correlation coefficient with respect to the relative change in levels of mRNAs between the two assays was higher than 0.8, indicating that the data from the microarrays were valid. We examined the levels of the transcripts of representative genes by real-time PCR and found that the results were consistent with those in the microarray assay (Figures 4c and d).

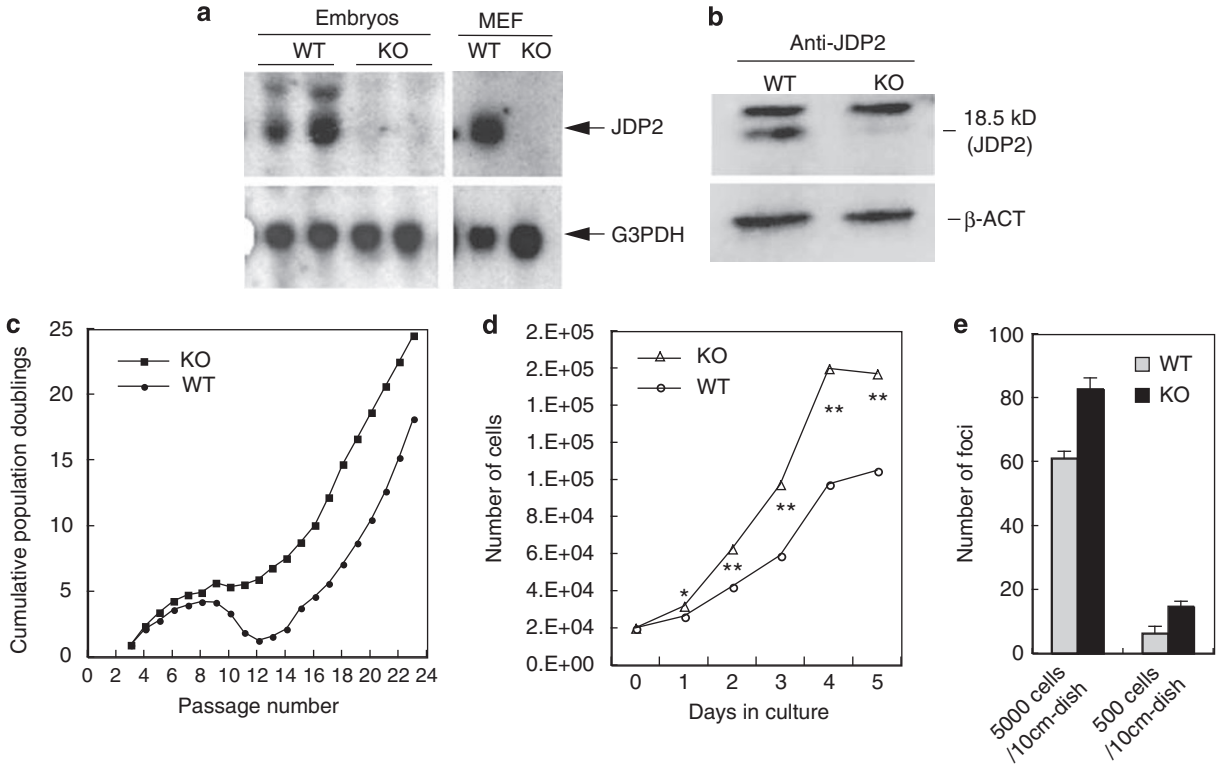


Figure 2 Proliferative properties of MEFs from WT and *Jdp2*KO mice. (**a**) The expression of JDP2 mRNA in embryos and in MEFs from WT and *Jdp2*KO mice was analyzed by northern blotting. The blot was re-probed for analysis of GPDH mRNA as loading control. (**b**) The expression of JDP2 in MEFs from WT and *Jdp2*KO mice was examined by western blotting. (**c**, **d**) Proliferation of MEFs from WT and *Jdp2*KO mice. MEFs were cultured in 10-cm dishes with DMEM plus 10% FCS for 3 days and then re-inoculated at the same density during 20 passages. Mean cell numbers from four independent plates were determined at each passage. A representative assay was shown (**c**; Trypan blue dye-exclusion test). Similar results were obtained in each experiment of three pairs of independent MEF clones. The number of population doublings (PD) in each passage was calculated as described elsewhere (Polisetty *et al*., 2008). Number of cell doubling (NCD) $=\log_{10}(N/N_0)/\log 10_2$, where '$N$' is the final density of the cells and 'N_0' is the initial seeding density of the cells. In panel **d**, MEFs were starved in DMEM plus 0.1% FCS for 36 h and then re-plated in DMEM plus 15% FCS and cultured for 5 days. MEFs were counted (six repeats; Alamar Blue assay). $^*P<0.05$; $^{**}P<0.001$. (**e**) MEFs from WT and *Jdp2*KO mice were plated in gelatin-coated dishes. Colonies (of more than 2 mm in diameter) were counted 2 weeks later. DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; JDP2, Jun dimerization protein-2; KO, *Jdp2*-knockout; MEF, murine embryonic fibroblast; WT, wild type.

Cyclin-A2 is a target of JDP2

We examined the levels of cyclin-A2 mRNA by real-time PCR after re-stimulation of MEF by serum. The expression of cyclin-A2 was enhanced by the addition of FCS in both WT and *Jdp2*KO MEF lines (Figure 5a). However, a more significant increase in the expression of cyclin-A2 was noted in *Jdp2*KO MEFs than in WT MEFs after stimulation (>1.8-fold; 12 h after serum re-stimulation). In parallel, we also measured the kinetics of changes in the levels of mRNA for JDP2 and c-fos. The expression of JDP2 mRNA decreased slightly after serum stimulation in WT MEFs. By contrast, expression of c-fos mRNA rose rapidly but transiently within 4 h after the start of stimulation, without any significant difference between the two lines of MEFs. As shown in Figure 5b, expression of mRNAs for cyclin-A2 and cyclin-E2 started earlier and were significantly higher and expressions of both genes started earlier in *Jdp2*KO MEFs than in WT MEFs after re-addition of serum. By contrast, expression of $p16^{Ink4a}$ decreased significantly for the first 6 h after the start of stimulation with serum in *Jdp2*KO MEFs.

Chromatin immunoprecipitation (ChIP) assay showed that PCR product that corresponded to the promoter regions of the genes for cyclin-A2 and $p16^{Ink4a}$ were detected in sonicated chromatin derived from WT MEFs but not in that from *Jdp2*KO MEFs (Figure 5c). In the case of cyclin-E2, no specific band was detected. Our data indicate that the genes for cyclin-A2 and $p16^{Ink4a}$ are the most likely targets of JDP2 in the progression of the cell cycle, even though they showed opposite responses upon serum stimulation of serum-starved cells.

Promoter analysis of the gene for cyclin-A2

We cloned an 1101-bp fragment of DNA from the promoter region of the mouse gene for cyclin-A2 and constructed a series of promoter deletion-luciferase genes (Figure 6a). We detected 2.5–4.5-fold higher luciferase activity in *Jdp2*KO MEFs than in WT MEFs when we tested this series of deletion constructs (Figures 6b and d). A point mutation at the AP-1 site of this *Sac*I–*Xho*I region enhanced luciferase activity in WT MEFs but not in *Jdp2*KO MEFs (Figure 6c).

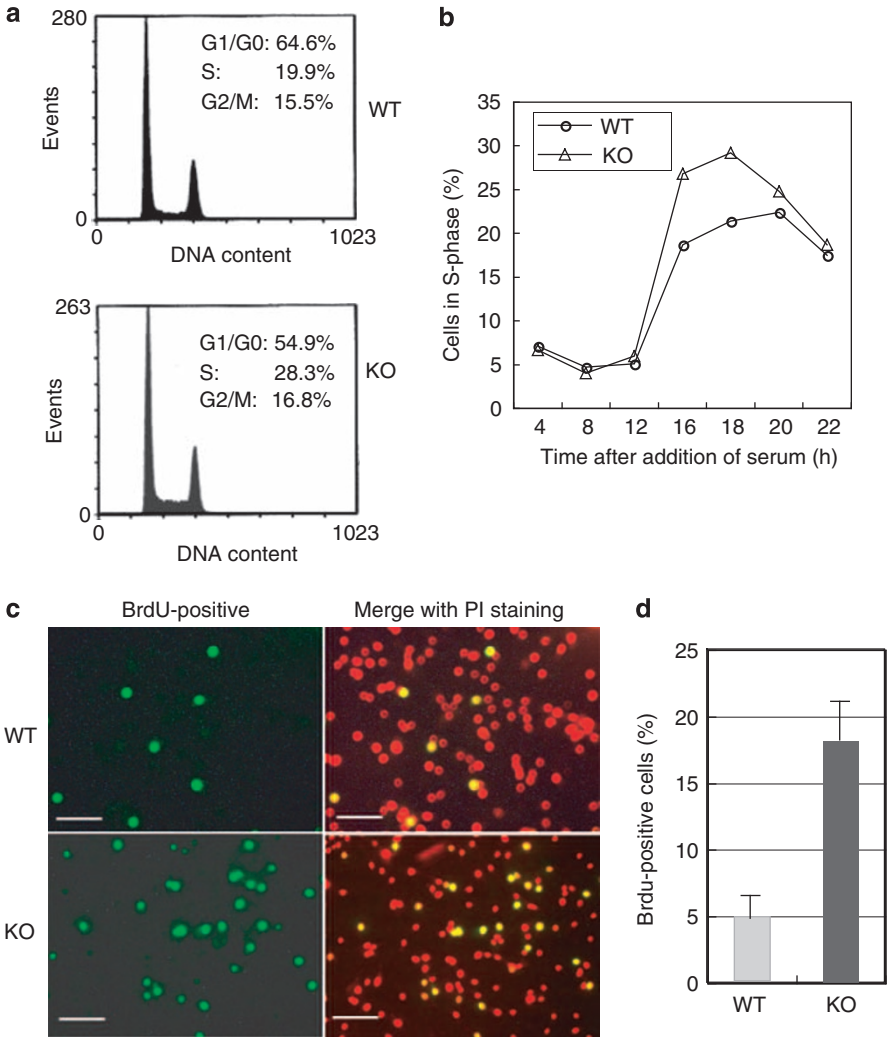
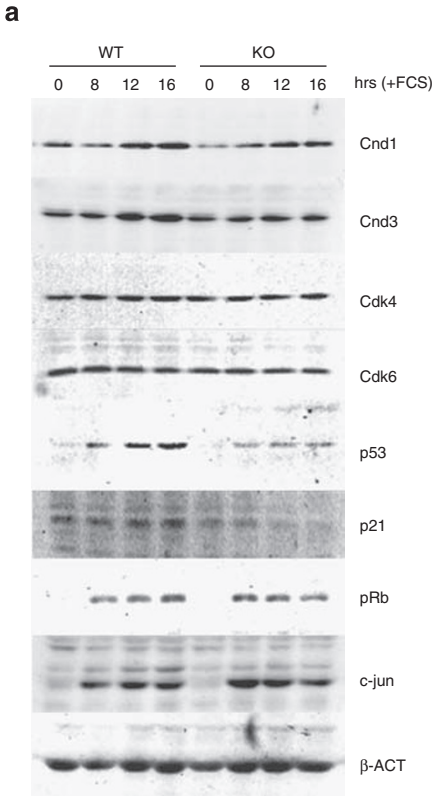


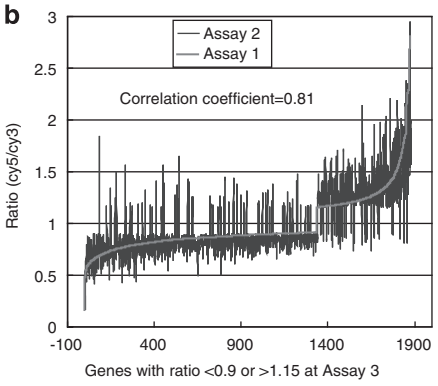
Figure 3 Proliferation of MEFs from WT and *Jdp2*KO mice. (**a**) Serum-starved cells (5×10^5) were cultured in DMEM plus 15% FCS for 18 h, stained with propidium iodide and subjected to FACS analysis to determine the percentage of cells in each phase of the cell cycle. (**b**) The percentage of MEFs in the S-phase was determined after re-stimulation in the 15% FCS at 4-h intervals, as in panel **a**. (**c**) MEFs were cultured in chamber slides in DMEM with 15% FCS for 12 h and then pulse-labeled with 10 μM BrdU for 3 h. Labeled cells were detected by immunostaining with BrdU-specific antibodies. Scale bars represent 100 μm. (**d**) Percentages of MEFs that were stained with BrdU-specific antibodies. Means of results of three experiments are shown. BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; JDP2, Jun dimerization protein-2; KO, *Jdp2*-knockout; MEF, murine embryonic fibroblast; WT, wild type.

To determine whether JDP2 represses the expression of the gene for cyclin-A by exploiting its histone modification activity, as described previously (Jin *et al*., 2006), we performed ChIP assays and found that the level of acetylation of histone-H4 in *Jdp2*KO MEFs was clearly higher than that in WT MEFs (Supplementary Figure S4).

Binding of JDP2 to the cyclin-A2 promoter

We performed electrophoretic mobility-shift assays to verify the DNA-binding activity of JDP2 using DNA probes that corresponded to the AP-1, the CRE and the AP-1-like sites (lanes 2–5 in Figure 7). The AP-1 and CRE probes but not the AP-1-like probe generated DNA–protein complexes with nuclear extracts (NEs) from either WT MEFs or *Jdp2*KO MEFs. The corresponding competitor AP-1 released [^{32}P]-AP-1 oligodeoxynucleotide from the DNA–protein complexes but the mutant competitor mAP-1 did not (lanes 9, 10, 11, 14, 15 versus lanes 12, 13, 16 in Figure 7a). When we added an antibody specific for JDP2 to the reaction mixture with the AP-1 probe, we detected super-shifted bands in the case of NEs from WT MEFs but not from *Jdp2*KO MEFs (lanes 2 and 6 versus lanes 4 and 8 in Figure 7b). Furthermore, the band in serum-stimulated WT MEFs was less intense than that from serum-starved WT MEFs, indicating that addition of serum suppressed the formation of a protein–DNA complex that included JDP2 under these conditions. In the case of the NEs from WT MEFs, the antibodies against JDP2 and JunD affected the migration and density of the protein–DNA complexes (lanes 5 and 9 in Figure 7c); however, antibodies against JunD and





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Symbol	Official gene name of gene product	KO / WT
Eif2s3y	Eukaryotic translation initiation factor 2, subunit 3	0.45
Hist1h2bc	Histone 1, H2bc	0.53
Fbln2	Fibulin 2	0.59
Ltbp1	Latent transforming growth factor beta-binding protein 1	1.80
Thbs4	Thrombospondin 4	2.27
Cxcl5	Chemokine (C-X-C motif) ligand 5	2.76
Cnd1	Cyclin D1	0.80
Cnc	Cyclin C	0.91
Cna1	Cyclin A1	0.96
Cnd3	Cyclin D3	0.98
Cne1	Cyclin E1	1.11
Cnb1	Cyclin B1	1.18
Cnb2	Cyclin B2	1.19
Cna2	Cyclin A2	1.32
Cne2	Cyclin E2	1.37
Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	0.66
Cdkn2a	Cyclin-dependent kinase inhibitor 2A (P16)	0.72
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (P21)	0.73
Cdkn1b	Cyclin-dependent kinase inhibitor 1B (P27)	1.00
Rb1	Retinoblastoma 1	0.96
Trp53	Transformation related protein 53	1.05
Rbl1	Retinoblastoma-like 1 (p107)	1.10

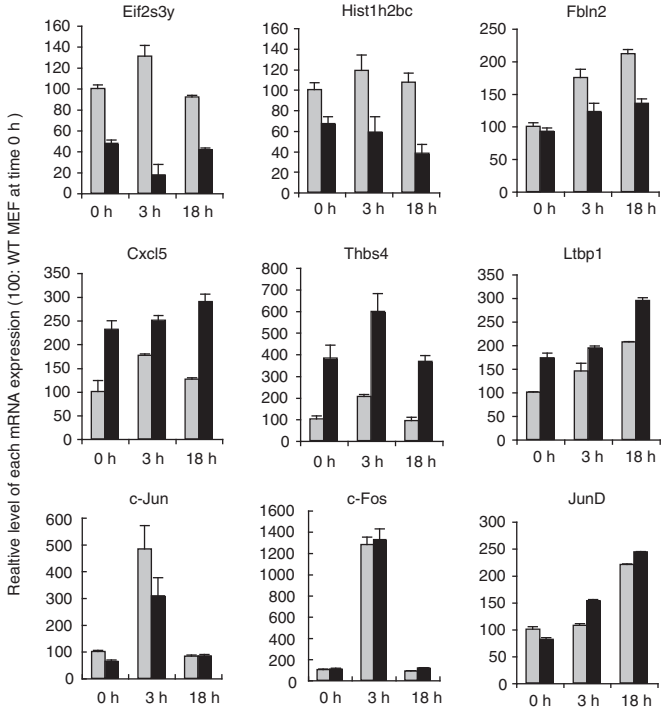

Figure 4 Gene expression in WT and *Jdp2*KO MEFs as determined by western blotting, microarray analysis and real-time PCR. (**a**) Western blots of cell-cycle-related proteins in MEFs from WT and *Jdp2*KO mice. MEFs were synchronized by serum starvation for 48 h and then re-stimulated with 15% FCS. MEFs were harvested 8, 12 and 16 h after addition of serum and cell lysates were prepared. (**b**) Summary of the microarray data obtained with mRNA from WT and *Jdp2*KO MEFs. (**c**) Validation of hybridization intensities. The correction coefficient with respect to relative changes in mRNA levels in two assays was higher than 0.8, indicating that the microarray data are valid. (**d**) Analysis by quantitative PCR of the expression of representative genes using mRNA from WT MEFs (gray lanes) and *Jdp2*KO MEFs (black lanes). MEFs were harvested after serum induction for 3 and 18 h, and then real-time PCR was performed with primer sets for the indicated genes, as described in the text. The level of each respective mRNA in unstimulated WT MEF was taken as 100. FCS, fetal calf serum; KO, *Jdp2*-knockout; MEF, murine embryonic fibroblast; WT, wild type.

c-Jun shifted the DNA–protein complexes in the NEs from *Jdp2*KO MEFs (lanes 12 and 14 in Figure 7c). By contrast, the CRE probe–protein complex was shifted by the addition of antibodies specific for JunD but not by antibodies specific for JDP2 (lane 9 in Figure 7d), indicating that the CRE site 'preferred' members of the AP-1 family other than JDP2. These data strongly suggest that JDP2 binds to the AP-1 site of the cyclin-A2 gene promoter.

Elevated levels of cyclin/cdk complex and cdk activity in Jdp2KO cells

The level of cyclin-A2 was fourfold higher in *Jdp2*KO MEFs than in WT MEFs at 8 h after stimulation with

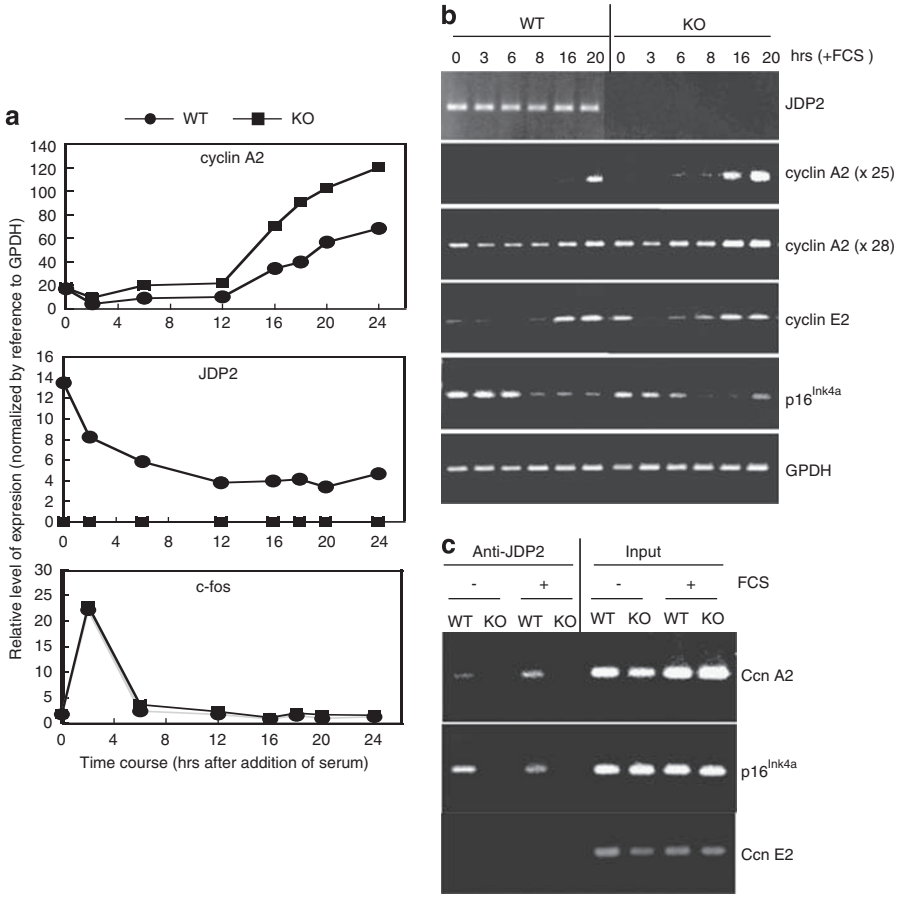

Figure 5 Kinetics of expression of cell-cycle-related genes in WT and *Jdp2*KO MEFs. (**a**) Relative levels of expression of genes for cyclin-A2, JDP2 and c-fos during cell cycling. Serum-starved MEFs from *Jdp2*KO (KO; black squares) and WT (black circles) mice were re-stimulated with serum for 24 h. The relative levels of respective mRNAs, normalized by reference to GPDH mRNA, were compared at the indicated time points. (**b**) Western blots of products of cell-cycle-related genes in lysates of MEFs from *Jdp2*KO and WT mice at the indicated time points. GPDH was used as control. (**c**) ChIP assays with JDP2-specific antibodies of lysates of *Jdp2*KO and WT MEFs with and without stimulation with serum. DNA fragments of genes for cyclin-A2, $p16^{Ink4a}$ and cyclin-E2 were detected by PCR with respective primers (Supplementary Table 2). ChIP, chromatin immunoprecipitation; JDP2, Jun dimerization protein-2; KO, *Jdp2*-knockout; MEF, murine embryonic fibroblast; WT, wild type.

serum (Figure 8a). We also found that no significant increases of cyclin-E2, cdk1 and cyclin-B1 proteins (Figure 8a, data not shown). We immunoprecipitated cdk2 from equal amounts of cell lysates prepared from WT and *Jdp2*KO MEFs 0, 8, 16 and 20 h after re-addition of FCS (Figure 8b). Immunoblotting of the immuprecipitates and/or cell lysates with antibodies specific for cyclin-A2, cyclin-E2, cdk2 and β-actin as a loading control showed that the amounts of cdk2-associated cyclin-A and of cdk2-associated cyclin-E2 were increased by serum stimulation (two- and fivefold, and six- and twofold, at 16 and 20 h, respectively), even though the level of cdk2 was almost constant.

In an attempt to measure the levels of cyclin-associated kinases, we prepared lysates from WT and *Jdp2*KO MEFs and subjected them to immunoprecipitation with antibodies against cyclin-A2 and cyclin-E2. We found that immunoprecipitates of cyclin-A2 and cyclin-E2 from *Jdp2*KO MEFs had 8- and 3-fold higher kinase activity, respectively, than those from WT MEFs (on the basis of radioactivity; Figure 8c). The increased kinase activity of cyclin-E2 was found 12 and 24 h after serum stimulation, and that of cyclin-A2 was detected 24 h after serum stimulation.

Repression of cell growth and the reporter activity of the cyclin-A2 promoter

We examined the effects of JDP2 on the proliferation of MEFs by overexpressing JDP2 in WT MEFs and re-expressing JDP2 in *Jdp2*KO MEFs. First, we introduced a pJDP2 vector into WT and *Jdp2*KO MEFs (two- to threefold increase of JDP2 mRNA than that of WT MEFs). Introduction of pJDP2 suppressed cell growth in both lines of MEFs (Figure 9a). We used the cyclin-A2 promoter–reporter construct pA2M and its AP-1 mutant pA2mAP-1 to examine the effect of re-expression of JDP2 in *Jdp2*KO MEFs. Transfection with pJDP2 significantly reduced the reporter luciferase activity in pA2M, but not in the mutant reporter pA2mAP1 (Figure 9b). These data suggested, yet again, that JDP2 represses the activity of the cyclin-A2

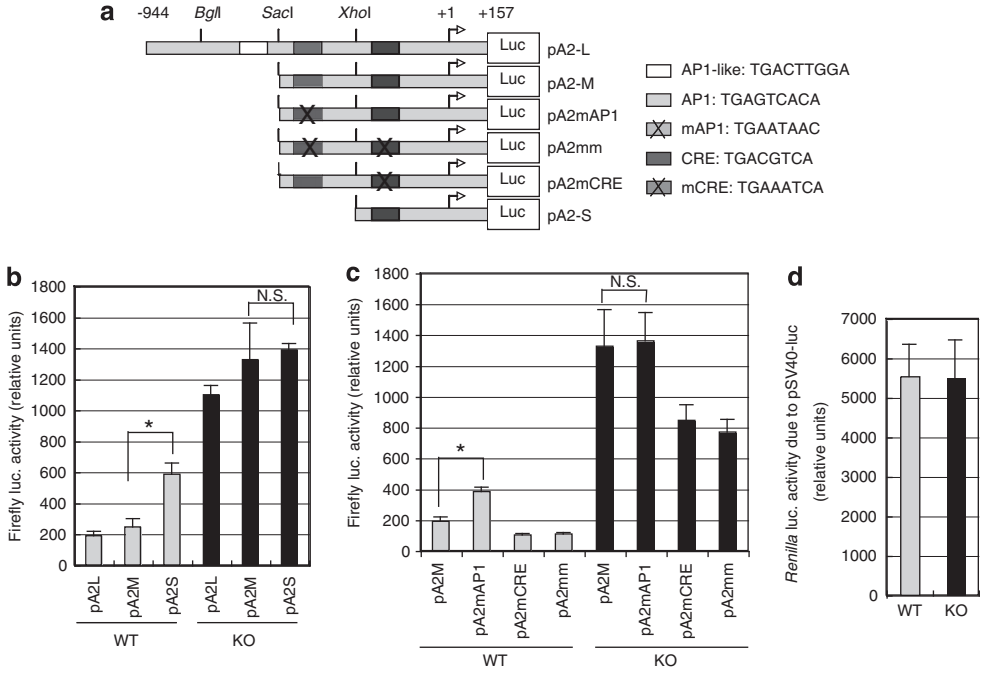

Figure 6 JDP2 negatively regulates the expression of the gene for cyclin-A2. (**a**) Schematic representation of the promoter of the cyclin-A2 gene (nt −944 to +157) fused with a gene for the luciferase. The series of deletion mutants of cyclin-A2-luciferase are described in the text. The AP-1-like motif, AP-1 motif and CRE are indicated by a white box, a gray box and a black box, respectively. Point mutations in each motif are also indicated. (**b**) Reporter activities of cyclin-A2-reporter deletion constructs pA2-L, pA2-M and pA2-N. A 1-μg weight of cyclin-A2 promoter-luciferase reporter plasmid and 200 ng of pSV-luc were used for transfection of 5×10^4 *Jdp2*KO MEFs. Six hours after transfection, MEFs were washed, incubated for another 48 h and assayed. $*P<0.001$. (**c**) Luciferase activities of the point mutants of the cyclin-A2 promoter-luciferase constructs were determined as in panel **b**. (**d**) The pSV-luc plasmid was used as control for transfections in comparisons of *Renilla* luciferase activities of MEFs from *Jdp2*KO and WT mice. AP, activation protein; JDP2, Jun dimerization protein-2; KO, *Jdp2*-knockout; MEF, murine embryonic fibroblast.

promoter, in a manner dependent specifically on the AP-1 site.

The infection with adenovirus JDP2 (Ad-JDP2) significantly suppressed cell growth in both WT and *Jdp2*KO MEFs, acting in a dose-dependent manner, even at a multiplicity of infection of 1 (Ad-JDP2 increased the expression of JDP2 by 50–100-fold; Nakade *et al*., 2009). Infection with the JDP2-expressing virus almost halved the number of WT and *Jdp2*KO MEFs by day 5 (Figure 9c). However, expression of cyclin-A2 was depressed in *Jdp2*KO MEFs (Figure 9d). In WT MEFs, two different JDP2–siRNA constructs reduced the expression level of JDP2 by as much as 50% (Figure 9f). These small interfering RNAs (siRNAs) accelerated the proliferation of WT MEFs but not of *Jdp2*KO MEFs (Figure 9e). Furthermore, treatment of WT MEFs with JDP2–siRNA increased the level of cyclin-A2 mRNA but reduced the level of cyclin-A2 mRNA in *Jdp2*KO MEFs (Figure 9f). These data strongly suggest that JDP2 represses the progression of the cell cycle through expression of cyclin-A2, thereby inhibiting the proliferation of MEFs at least partially.

Discussion

In this study, we show that loss of JDP2 accelerates cell-cycle progression with higher cyclin-associated cdk kinase activities in MEFs and cyclin-A2 is one of the molecular targets of JDP2. Thickening of epidermis after treatment with TPA showed the proliferation inhibition of JDP2 in mice skin derived from *Jdp2*KO and WT mice *in vivo*. Other assays *in vitro*, including population doubling, cell cycle and BrdU-incorporation, showed the elevated proliferative potential of *Jdp2*KO MEFs as compared with WT MEFs.

To identify the molecular targets of JDP2 on cell proliferation, we performed a microarray-quantitative PCR analysis and found genes encoding cyclin-A2, $p16^{Ink4a}$ and cyclin-E2 are the potential targets of JDP2. So far, only five JDP2 targets were identified, namely c-Jun, ATF-2, C/EBPδ, ATF3 and CHOP10 (Aronheim *et al*., 1997; Jin *et al*., 2001; Nakade *et al*., 2007; Cherasse *et al*., 2008; Weidenfeld-Baranboim *et al*., 2009). Here we found that JDP2 was recruited to the promoter of the gene for cyclin-A2 at the AP-1 site. A gel-shift assay and site-directed mutagenesis of the promoter also showed that the AP-1 site is crucial for JDP2-mediated repression of the cyclin-A2 promoter, suggesting that JDP2 is the major component in the regulation of the cyclin-A2 promoter. However, the endogenous partner with JDP2 to form heterodimer *in vivo* has not been identified yet.

A number of studies suggest that JDP2 has a dual role with pro- or anti-oncogenic properties in malignant transformation. JDP2 was found to inhibit cell-cycle

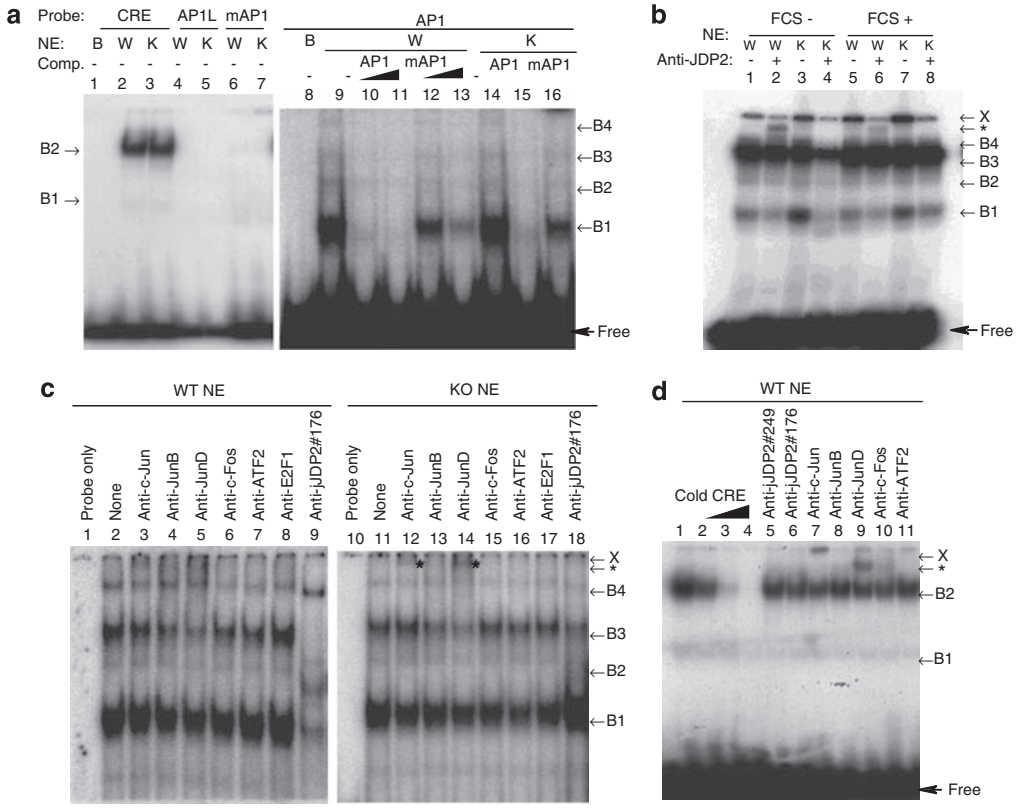

Figure 7 Electrophoretic mobility-shift assays with NEs with MEFs from WT and *Jdp2*KO mice. (**a**) NEs from *Jdp2*KO MEFs (indicated as K), WT MEFs (indicated as W) and bovine serum albumin (BSA; indicated as B were incubated with probes corresponding to the CRE (lanes 1–3), AP1-like sequence (AP1L; lanes 4 and 5), the AP-1 point mutant (mAP1, lanes 6 and 7) and AP-1 (lanes 8–16) in the presence of 1 and 2 pmol of unlabeled mAP1 oligodeoxynucleotide (lanes 12 and 13) or AP-1 oligodeoxynucleotide (lanes 10 and 11) as a competitor. The positions of shifted bands (B1–B4) and free DNA probes (Free) are indicated. (**b**) Super-shifting electrophoretic mobility-shift assay using NEs from serum-starved MEFs (FCS−) or 15% serum-induced MEFs (FCS+) and the DNA probe that corresponded to AP-1. NEs from WT MEFs (W) and *Jdp2*KO MEFs (K) were incubated without (odd lanes) or with (even lanes) antibodies specific for JDP2. '*' indicates the super-shifted bands and X indicates aggregated DNA–protein complexes that remained in the wells of the gel. B1–B4 and Free indicate protein–DNA complexes and free DNA probes. (**c**) Competition and super-shifting assays with the AP-1 DNA probe. Reaction mixtures containing NEs from WT MEFs (lanes 1–9) and *Jdp2*KO MEFs (lanes 10–18) were prepared with antibodies specific for c-Jun, JunB, JunD, c-Fos, ATF-2, E2F1 and JDP2 (lanes 1–18), as indicated. '*' indicates super-shifted bands of antibody–DNA–protein complexes. B1–B4 and Free indicate DNA–protein complexes and the unbound AP-1 probe. (**d**) Competition and super-shifting assays using NEs of WT MEFs and the CRE DNA probe. Reaction mixtures containing NEs from WT MEFs were prepared without (lane 1) and with 0.1, 1 and 10 pmol of unlabeled (cold) CRE probe (lanes 2–4) and antibodies specific for JDP2, pJDP2, c-Jun, JunB, JunD, c-Fos and ATF-2 (lanes 5–11), as indicated. B2 indicates super-shifted bands of antibody–DNA–protein complexes. B1 and Free indicate DNA–protein complexes and the unbound CRE probe. AP, activation protein; FCS, fetal calf serum; JDP2, Jun dimerization protein-2; KO, *Jdp2*-knockout; MEF, murine embryonic fibroblast; NE, nuclear extract; WT, wild type.

progression (Ostrovsky *et al*., 2002), Ras-dependent cell transformation and tumor formation in xenografts (Heinrich *et al*., 2004). Conversely, overexpression of JDP2 in chicken embryo fibroblasts imparts a partial oncogenic phenotype (Blazek *et al*., 2003). Furthermore, viral integration sites were identified within the genome of JDP2 resulting in T-cell lymphoma (Hwang *et al*., 2002; Rasmussen *et al*., 2005, 2009; Stewart *et al*., 2007). Recent publication showed that JDP2 potentiates the chemical carcinogenesis of liver cancer (Bitton-Worms *et al*., 2010). JDP2 acts at the promotion stage in which full blown inflammation is evident. Furthermore, multiple members of the bZIP family are highly expressed at this stage, including CHOP10. Heterodimerization between CHOP10 with either ATF3 or overexpressed JDP2 transgene may result in the transcriptional activation (Weidenfeld-Baranboim *et al*., 2008) of otherwise suppressed JDP2 target genes involved in cell-cycle progression such as cyclin-A2, cyclin-E2 or $p16^{Ink4a}$, which were identified in this report. These data indicate that JDP2 acts as a transcriptional activator or a repressor depending on the bZIP protein at each stage of cancer progression with which it is associated. However, the role of JDP2 in cancer progression mediated through regulation of cyclin-A2 transcription has not been determined yet; further investigations are necessary to clarify the JDP2 partner to regulate cell-cycle progression in response to various signals.

We show here that transcriptional regulation is the major mechanism of the JDP2-mediated expression of the cyclin-A2 promoter. Other possible regulations described below cannot be ruled out. First JDP2-mediated inhibitions of histone acetylation at H3 and H4 (Jin *et al*., 2006; Nakade *et al*., 2007) and histone

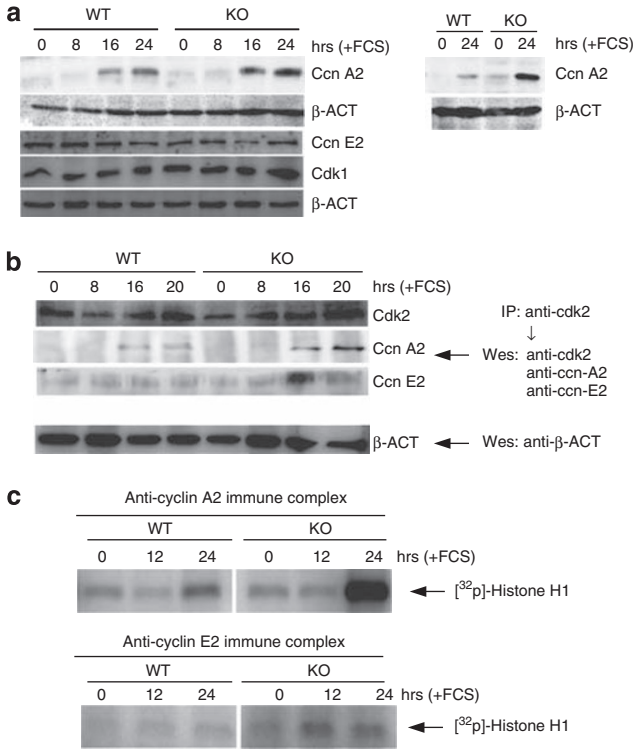

Figure 8 Enhanced cyclin–cdk activity in *Jdp2*KO MEFs. Cell growth was synchronized and cells were stimulated as described in the text. (**a**) Western blots (Wes) of cyclin-A2, cyclin-E2, cdk1 and β-actin in whole-cell lysates (left panel) and NEs (right panel). (**b**) Levels of the cdk-associated cyclin family. Whole-cell extracts from WT and *Jdp2*KO MEFs were immunoprecipitated (IP) with antibodies specific for cdk2 and then immunoprecipitates were subjected to sodium dodecyl sulfate-PAGE (10% acrylamide) and blotted with antibodies against cyclin-A2, cyclin-E2 and cdk2. Control of western blot using β-actin is indicated (lower panel). (**c**) Induction of cyclin–cdk phosphorylation of histone-H1. Immunoprecipitates containing the complexes with cyclin-A2 and cyclin-E2 were isolated from whole-cell extracts (400 μg total protein) using cyclin-A2- and cyclin-E2-specific antibodies and histone-H1-phosphorylating activity was determined *in vitro* as described in the text. cdk, cyclin-dependent kinase; KO, *Jdp2*-knockout; MEF, murine embryonic fibroblast; NE, nuclear extract; WT, wild type.

methylation at H3K27 at $p16^{Ink4a}$ locus (Nakade *et al*., 2009) are possible. Second, the stability of cyclin-A2 might be controlled by JDP2 (Mateo *et al*., 2009; data not shown). In fact, we found that JDP2 colocalized with cyclin-A in the nucleus (Supplementary Figure S6).

In the case of cyclin-E2 the mRNA and protein levels after serum induction were not coincident each other and, however, the cdk2–cyclin-E2 complex showed slightly higher cyclin-associated cdk kinase activity in *Jdp2*KO MEFs as compared with that in WT MEFs. The specific recruitment of JDP2 to the promoter of cyclin-E2 was not detected and no AP-1/CRE elements were found in the promoter of the cyclin-E2 gene. Thus, regulation of cyclin-E2 by JDP2 might not be direct transcriptional regulation by JDP2. Another indirect regulation like JDP2-induced $p16^{Ink4a}$-Rb-E2F regulation of cyclin-E2 gene might be possible (Nakade *et al*., 2009; Polager and Ginsberg, 2009).

The increase in the protein levels of p53 and p21 proteins was less significant in *Jdp2*KO MEFs after stimulation by serum as compared with that in WT MEFs. We generated p53-knockdown MEFs by using a short-hairpin RNA against p53 (shp53) and introduced lentivirus vector-encoded JDP2 (Supplementary Figure S5A). In MEFs in which p53 was downregulated completely, JDP2 still inhibited cell proliferation significantly ($>P=0.0075$; Supplementary Figures S5B and S5C). In addition, expression of p53 mRNA was increased by introducing JDP2 the level of which is comparable with the results in Figure 4a. These observations indicate that JDP2 may inhibit cell proliferation in p53-dependent and p53-independent manners, in the latter case, at least partially, by suppression of cyclin-A2 gene.

In summary, our data indicate that JDP2 has a crucial role in the suppression of cyclin-A2 expression, with subsequent inhibition of cyclin-associated cdk kinase activity, and in the suppression of cell proliferation. This hypothesis is also supported by overexpression of JDP2 encoded by a recombinant adenovirus and by gene suppression experiments with siRNA. The expression of cyclin-A2 is controlled at the transcription level by JDP2. It is clear that JDP2 interferes with progression of the cell cycle at least partially by downregulation of cyclin-A2 but not apoptosis. The control of cell cycle by JDP2 may then lead to the commitments of cell differentiation, cellular senescence or cell fate determination possibly through the signal cascades of RB-E2F or p53-p21 or Wnt/TGFβ as indicated by the results of microarray and qPCR.

Materials and methods

Cell culture

MEFs were prepared from embryos at embryonic day 12.5 (E12.5) with a mixed genetic background of B6 and 129 as described previously (Nakade *et al*., 2007). For serum starvation experiments, MEFs were incubated in DMEM containing 0.1% FCS for 48 h, at confluence; then they were re-plated in DMEM that contained 15% FCS.

TPA-induced epidermal thickening assay

The assay for epidermal thickening was measured as described previously with a slight modification (Nakamura *et al*., 1998). Briefly, the dorsal skin of each mouse (six pairs of WT and *Jdp2*KO) was shaved before treatment with TPA. TPA or acetone as solvent control was administered four times to each animal at an interval of 24 h (8.1 nmol in 100 μl acetone; Sigma-Aldrich Co., St Louis, MO, USA). Mouse skin was obtained 1 h after the fourth application of TPA or acetone. Tissues were frozen and sectioned at 5 ìm thickness. The epidermal thickness of the skin was measured at 10 randomly selected sites using an Olympus microscope (Olympus, Tokyo, Japan). The vertical thickness of the epidermis was defined as the distance from the stratum basal to the stratum corneum. After incubation overnight at room temperature with or without mouse monoclonal antibody against PCNA (PC10; Abcom, Cambridge, MA, USA), slides were incubated with goat anti-mouse IgG 1 h at room temperature. PCNA labeling index was calculated by immunoreactive nuclei per total nuclei in epidermis.

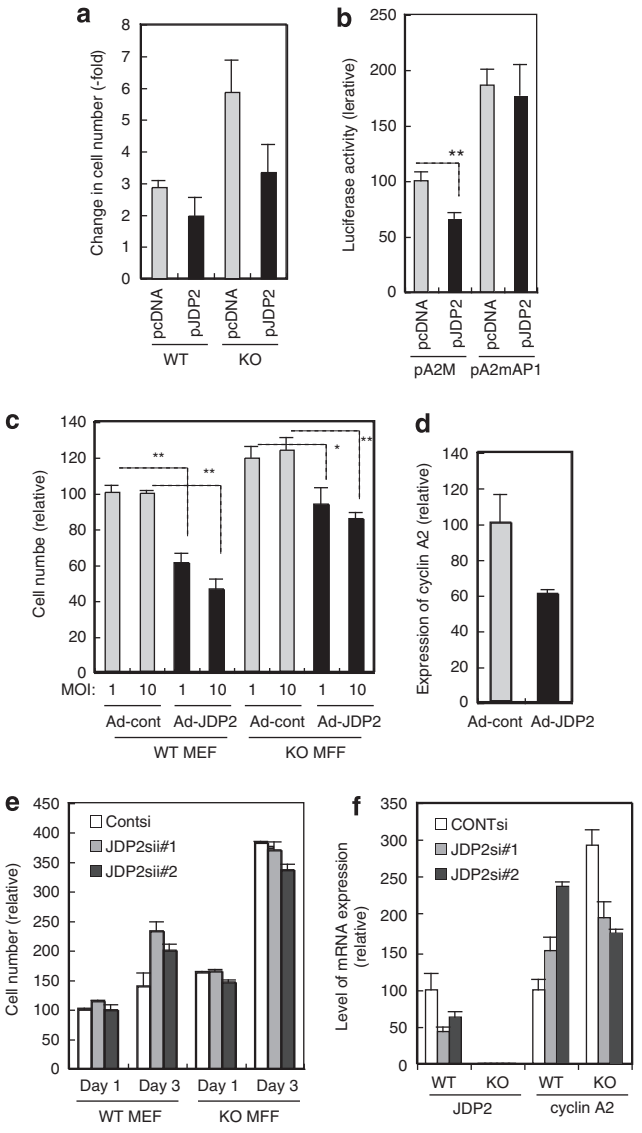

Figure 9 Effects of ectopic JDP2 and siRNA specific for JDP2 on proliferation and expression of cyclin-A2. (**a**) Reduced proliferation of WT and *Jdp2*KO MEFs upon expression of the JDP2 expression vector. 2×10^4 WT and *Jdp2*KO MEFs were transfected with 600 ng of pcDNA4-JDP2 or pcDNA and cells were counted 3 days later by the Alamar Blue method. The relative cell number on day 0 is given as one. The means of the results of three experiments ± s.d. are shown. (**b**) Depressed cyclin-A2 reporter activity upon introduction of the JDP2 expression vector. We introduced 600 ng of pJDP2 or pcDNA4, and 400 ng of pA2M or pA2mAP1 into 5×10^4 *Jdp2*KO MEFs, incubated the cells for 30 h and then measured luciferase activities. (**c**) Depressed proliferation of MEFs upon infection with recombinant adenovirus encoding JDP2 (Adeno-JDP2). WT and *Jdp2*KO MEFs (2×10^4) were infected with Ad-JDP2 or β-galactosidase-encoding cDNA (Ad-cont) at a multiplicity of infection of 1 and 10. Cells growth was counted 5 days later. (**d**) Depressed expression of mRNA for cyclin-A2 in *Jdp2*KO MEFs infected with Ad-JDP2. The culture and infection of cells are described in the legend to panel **c**. The expression of mRNA specific for cyclin-A2 was quantitated by real-time PCR as described in the legend to Figure 7a. (**e**, **f**) Effects of siRNA specific for JDP2 on cell proliferation and expression of cyclin-A2. WT MEFs (5×10^5 cells) were transfected with 30 pmol of siRNA specific for JDP2 (JDP2si#1 and #2) and non-specific double-stranded RNA (CONTsi). Three days after transfection, cells were harvested and counted (**e**), and total RNA was subjected to real-time PCR using primers specific for JDP2 or cyclin-A2 (**f**). The levels of expression were normalized by reference to that of GPDH mRNA. Data are shown as percentages, relative to results for WT MEFs as negative control. JDP2, Jun dimerization protein-2; KO, *Jdp2*-knockout; MEF, murine embryonic fibroblast; siRNA, small interfering RNA; WT, wild type.

Promoter-reporter assays

Cyclin-A2 promoter-reporter DNAs were transfected into WT and *Jdp2*KO MEFs with or without pcDNA3-JDP2 or pcDNA3 empty vector as 1 μg of total DNA per well of a 24-well plate (5×10^4 cells/well) using 2 μl of Lipofectamine-2000 reagent (Invitrogen, Paisley, UK). A series of JDP2 promoter-reporters were transfected as described elsewhere (Jin *et al*., 2002; Nakade *et al*., 2007).

Assay of cyclin-associated kinase activity

We immunoprecipitated total cellular proteins with antibodies against cyclins as indicated and using protein-A- and protein-G-Sepharose beads. Beads with bound immune complexes were mixed with [^{32}P]-ATP and histone H1 peptide substrate (cat. no. 14–155; Upstate Biotechnology Inc., Charlottesville, VA, USA) in a kinase assay buffer (50 mM HEPES (pH 7.0), 10 mM $MgCl_2$, and 1 mM dithiothreitol) for 30 min at 30 °C. Reaction products were resolved by sodium dodecyl sulfate-PAGE (12% polyacrylamide) and phosphorylated histone H1 peptide was detected by autoradiography.

Analysis of data

Results are expressed as the means ± s.d. of the results for each set of replicates. Statistical comparison of single parameters between two groups was performed by paired Student's *t*-test. *P*-values less than 0.05 or 0.01 were considered significant.

Plasmid constructions, generation and characterization of JDP2-deficient mice, skin wound healing, cell cycle, isolation of RNA, microarrays and real-time quantitative reverse transcription–PCR, recombinant virus infection and siRNA transfection, cell proliferation and apoptosis, electrophoretic mobility-shift assays, immunoprecipitation-western blotting, ChIP assay and immunofluorescence are described in the Supplementary Information.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)