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AIB1 Promotes DNA Replication by JNK Repression and AKT Activation during Cellular Stress

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Amplified in breast cancer 1 (AIB1) is a member of the p160 family of nuclear receptor coactivator protein. Recent studies have reported that high-level AIB1 production is involved in the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway for progression to malignant carcinoma in a steroid-independent manner. Here we demonstrate that, in AIB1-knockout DT40 chicken B-lymphocytes, loss of AIB1 results in induction of phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun, in addition to the inhibition of DNA replication. In contrast, high-level AIB1 production prevents proapoptotic activation of the JNK/c-Jun signal transduction pathway and induces DNA replication through phosphorylation of the Akt/p65 NF-κB subunit RelA under cellular stresses such as UV irradiation or serum deprivation. Moreover, we have found that AIB1 is essential for the phosphorylation of histone H3 at serine 10, which is associated with the signal transduction to chromatin, leading to the transient expression of immediate-early genes in response to UV stimulation. Our results therefore suggest that AIB1 directly links to cell cycle control mechanisms in concern with the balance between apoptosis and proliferation.

Key words: amplified in breast cancer 1, cellular stress, DNA replication, phosphorylation, signal transduction.

Abbreviations: AIB1, amplified in breast cancer 1; Akt, cellular homolog of v-akt oncogene; CARM1, coactivator-associated arginine methyltransferase 1; CBP, cyclic AMP response element binding protein; CDK, cyclin dependent kinase; ER, estrogen receptor; ERK, extracellular signal–regulated kinase; FCS, fetal cauf serum; FACS, fluorence-activated cell sorting; GSK3, glycogen synthase kinase 3; HAT, histone acetyltransferase; HER-2, human epidemal growth factor receptor-2; JNK, c-Jun amino-terminal kinase; MAPK, mitosis-activated protein kinase; NF-κB, nuclear factor-κB; PAS, Per/Arnt/Sim; PI3K, phosphatidylinositol 3 kinase; RSK2, ribosomal S6 kinase 2; SRC-1, steroid receptor coactivator-1; TIF2, transcriptional intermediary factor 2; TUNEL, terminal deoxynucleotidyl transferase–mediated nick end label.

The nuclear receptor coactivator known as AIB1 (also called p/CIP, ACTR, RAC3 and SRC-3) is a member of the p160 nuclear receptor coactivator family. This family contains SRC-1 (steroid receptor coactivator-1) and TIF2 (transcriptional intermediate factor-2) that interact with the general transcriptional coactivators CBP, p300 and p/CAF (*1–8*). These coactivator complexes possess intrinsic histone acetyl transferase activity and are responsible for the remodelling of chromatin and modification of components of the transcription machinery (*9*, *10*).

AIB1 increases estrogen-dependent transcriptional activation by interaction with estrogen receptor (ER) α in a ligand-dependent manner. Furthermore, AIB1 mRNA and protein have been shown to be amplified and overexpressed in primary human breast and ovarian cancer cell lines, in which transcription is upregulated and the AIB1 gene on chromosome 20q12 is amplified (*1*). Recent studies report that high levels of AIB1 production are relates to both a high DNA-synthesis phase fraction and HER-2/*neu* production with p53 mutations in breast cancer, which is a disease characterized by an imbalance between cell division and cell death (*11*, *12*). Her-2/*neu* protein activates the PI3K (phosphoinositide 3-kinase)/Akt (also known as protein kinase B, PKB) pathway, which, through NF-κB activation, plays an important role in preventing cells from undergoing apoptosis (*13*, *14*). More recently, it has been shown that overexpression of AIB1 in the mammary gland leads to activation of the PI3K/Akt pathway, with IGF-1 signaling (*15*). Because AIB1 (RAC-3) has also been shown to interact with NF-κB and enhance its transcriptional activity (*16*, *17*), it has been suggested that AIB1 is an altered regulator for the mechanism by which constitutive activity of an NF-κB–dependent promoter is involved in chemotherapeutic resistance in ER-negative cancer cells (*18*, *19*). However, the biological function of AIB1 in the signal transduction pathways influenced by complex cascades of phosphorylation events triggered by exposure to cellular stress is not completely understood. Therefore, we focused our attention on AIB1 activity, to determine whether this protein regulates the antiapoptotic process or perturbs signal integration in response to cellular stress.

Importantly, c-Jun N-terminal kinase (JNK) has also been shown to be a key regulator of programmed cell death and part of a subfamily of the mitogen-activated protein kinase (MAPK) superfamily (*20*). Recent studies indicate that JNK activation contributes to

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TNF-α–induced apoptosis in the absence of NF-κB activation, and that NF-κB–mediated inhibition on JNK activation is important for cell survival (*21–24*). Here we show that, in wild-type DT40 cells, high-level production of AIB1 suppresses the phosphorylation of JNK and c-Jun, the main physiological substrate of the JNK kinase, in response to cellular stress such as serum deprivation or UV irradiation. In contrast, loss of AIB1 leads to inhibition of activation of the Akt/p65 signaling pathway and suppresses DNA synthesis.

Finally, we found that AIB1 enhances the induction of phosphorylation of histone H3 at serine 10 but not the acetylation of histone H3 at lysine 9 or lysine 14 in response to UV stress. Phosphorylation of histones provides motifs for the recruitments of chromatin modifying or remodelling complexes, including coactivators which are linked to cellular processes such as transcription, DNA replication, DNA repair and apoptosis in the stress response (*25*, *26*). Because AIB1 enhances the induction of phosphorylation of histone H3 at serine 10, it plays a critical role as a transcriptional modifier that is recruited for chromatin remodelling in response to cellular stresses. These results indicate that changes in AIB1 production may determine cell fate in association with the balances between the Akt/p65 and JNK/c-Jun signaling pathways in cellular stress responses.

EXPERIMENTAL PROCEDURES

Cell Culture—DT40 cell lines were maintained in RPMI 1640 (Nikkenseibutsu) medium supplemented with 10% fetal calf serum (FCS; Gibco BRL) and 1% chicken serum (JRH Bioscience) at 39.5°C in a humidified atmosphere with 5% CO_2. Cell density was maintained at 0.1–1.0/10^6 ml by splitting the culture daily.

Construction of Targeting and Expression Vectors—A chicken AIB1 (*GdAIB1*) partial cDNA fragment from the bHLH/PAS domain was amplified from chicken brain cDNA by RT-PCR with primers (5′-aaggaaaaactatttccagtgagatgatgttc-3′, 5′-cgaattgtatcctaaagccaggtctcagg-3′). We then used 5′ and 3′ RACE on chicken brain cDNA to isolate the entire open reading frame of *GdAIB1*. To construct the *GdAIB1* expression vector, chicken *AIB1* cDNA was inserted into an expression vector containing the chicken β-actin promoter. We then isolated 6.5 kb of the partial chicken genomic *GdAIB1* locus from DT40 genomic DNA by long-range PCR. Chicken *AIB1* targeting constructs were made by replacing the genomic sequence containing the sequence encoding amino acids 122 to 156 with hygromycin or histidinol selection marker cassettes.

Generation of AIB1 −/− Clones—10^7 DT40 cells were suspended in 0.5 ml PBS containing 30 μg of linearized plasmid for the transfection and electroporated with a Gene Pulser apparatus (BioRad) at 550 V and 25 μF. Following electroporation, cells were transferred into 20 ml fresh medium and incubated for 24 h. Cells were then resuspended in 80 ml medium containing hygromycin (2.5 mg/ml, Calbiochem) or L-histidinol (1 mg/ml, Calbiochem) and divided into four 96-well plates. After 7 to 10 days, drug-resistant colonies were selected. Disruption of the gene was confirmed by Southern blot analysis of genomic DNA.

Cell Cycle Analysis—A total of of 2×10^5 cells were treated with 5-bromodeoxyuridine (BrdU; 10 μM, Sigma) for 10 min and the subconfluent cells harvested. After fixation with 70% ethanol, the cells were incubated overnight at −20°C. The next day, cells were collected and resuspended in 2 N HCl with 0.5% Triton X-100 for 30 min at room temperature; this was followed by neutralization with 0.1 M $Na_2B_4O_7$. Cells were then collected and incubated with anti-BrdU antibody (Becton-Dickinson) for 30 min in the dark at room temperature. The cells were washed with PBS and stained with FITC-labeled goat anti-mouse Abs (Jackson) for 30 min at room temperature in the dark. The cells were resuspended in PBS containing propidium iodide (5 μg/ml, Sigma). The filtered cells were analyzed by fluorescence-activated cell sorter (FACScan, Becton-Dickinson). The distribution of cells in each phase of the cell cycle was determined by using Cell Quest software (Becton-Dickinson).

TUNEL Assay—DT40 cells were harvested at the designated time points and fixed in 70% ethanol in PBS. The fixed cells were then incubated for 30 min at 4°C and permeabilized with 0.2% Triton X-100 in PBS for 5 min. For apoptosis analysis, the cells were examined by the TUNEL technique, as described in the instructions supplied with the apoptosis detection system (Takara). Localized green fluorescent apoptotic cells were detected by fluorescence microscopy. The percentage of FITC-positive cells in the apoptotic fraction was determined in a fluorescence-activated cell sorter (Becton Dickinson).

Western Blot Analysis—Cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.5, and 1% NP-40) supplemented with protease inhibitors, aprotinin (1 μg/ml), and leupeptin (2 μg/ml). Lysates were centrifuged to clear cell debris, and then 30 μg of the total protein were size-fractionated by SDS-PAGE gel (7.5% to 15%). After electrophoresis, proteins were transferred to PVDF membranes (BioRad), blocked in PBS containing 0.2% Tween 20 and 3% bovine serum albumin, and probed with first antibody in PBS containing 0.2% Tween 20 and 1% bovine serum albumin. Detection of the immune signal was performed with the chemiluminescence detection system (Amersham Biosciences) and then quantified using densitometry (Molecular Dynamics).

Antibodies—The amino-terminal portion of chicken AIB1 (amino acids 1 to 250) was expressed in *E. coli* as a histidine tagged fusion protein and purified by affinity chromatography using Ni-agarose (Qiagen). The purified protein was then injected into rabbits to prepare specific antiserum. Rabbit antibodies of anti-phospho-Akt, anti-Akt, anti-phospho-p65, anti-p65, anti-phospho-JNK, anti-JNK, anti-phospho-c-Jun, anti-c-Jun, anti-phospho-p38, and anti-phospho-p44/42 ERK1/2 were purchased from Cell Signaling Technology. Mouse antibodies of anti-p38 and anti-ERK1 were purchased from BD Bioscience. Rabbit anti-histone H3 phosphorylated at Ser10 or Ser28 and rabbit anti-histone H3 acetylated at Lys9 or Lys14 antibodies were purchased from Upstate Biotechnology.

Immunoprecipitations and In Vitro Akt Kinase Assay—Cells were disrupted with cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC, and 0.1% SDS) containing protease inhibitors, aprotinin (1 μg/ml) and leupeptin (2 μg/ml). Approximately 200 μg of protein lysate was incubated with anti-chicken AIB1

antibody and anti-Akt antibody overnight at 4°C with end-over-end rotation, followed by an additional 2 h of incubation with protein A sepharose beads (Amersham Biosciences). The beads were then washed three times with cold RIPA buffer before being boiled in SDS-PAGE sample buffer. *In vitro* Akt kinase assay was performed for 30 min at 30°C in 40 μl of reaction volume containing 30 μl of immunoprecipitates in kinase buffer with 200 μM ATP. GSK-3 fusion protein (Cell Signaling Technology) was used as a substrate for Akt kinase activity. The reactions were terminated with 20 μl of SDS sample buffer and subjected to Western blotting using anti-phospho-GSK antibody (Cell Signaling Technology).

Northern Blot Analysis—Total RNA was isolated with Trizol reagent (Invitrogen). For generation of each probe, 1 μg of total RNA was used in reverse transcription reactions, as described by the manufacturer. The resulting total cDNA was then used in the PCR to estimate mRNA levels. The mRNA level of GAPDH was used as internal control. PCR was carried out with Taq polymerase, and the conditions were as follows: pre-denaturing at 94°C for 3 min, then 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The oligonucleotide primers used to generate these probes were as follows: AIB1 (GenBank accession number: XM417385), 5′-attacctgcattcagagaagaat-3′ and 5′-tcttctctcattgtctacacaa-3′; chicken cyclin D1 (U40844), 5′-tttacaccgacaactccatc-3′ and 5′-gtgataggaaatgtgtgagg-3′; chicken cyclin D2 (U28980), 5′-ccatcaatgatagcaactgg-3′ and 5′-aaaataaaaggggtgggag-3′; chicken cyclin E (U28981), 5′-cttcaccgctaccaattctg-3′ and 5′-caaactggtgcaactttggt-3′; chicken E2F1 (X89245), 5′-ggatccccggcagaggggca-3′ and 5′-ctccaggacattggtgatgt-3′; GAPDH (NM204305), 5′-accactgtccatgccatcac-3′ and 5′-tccacaacacggttgctgta-3′. Each total RNA (30 μg) was run on a 1.0% formaldehyde gel and transferred to a Hybond N+ nylon membrane (Amersham) using a Turbo blotter system (Schleicher & Schuell). DNA probes were labeled with [α-^{32}P] dCTP (Amersham Biosciences) using a random labeling kit (Takara). The membrane was hybridized with labeled DNA probes by QuikHyb hybridization (Stratagene) at 65°C for 2 h and then developed for autoradiography.

Histone Extraction—Cells were centrifuged, the medium discarded and the cells washed twice with PBS (pH 7.4). The cells were suspended in 5 to 10 volumes of lysis buffer (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 1.5 mM PMSF). The samples were incubated on ice for 30 min and centrifuged at 1,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the histones in the pellets were extracted by 0.2 M HCl solution. The samples were centrifuged at 12,000 × *g* for 10 min at 4°C after incubation on ice for 30 min. The histones were then precipitated from acid solution with 5 volumes of cold acetone.

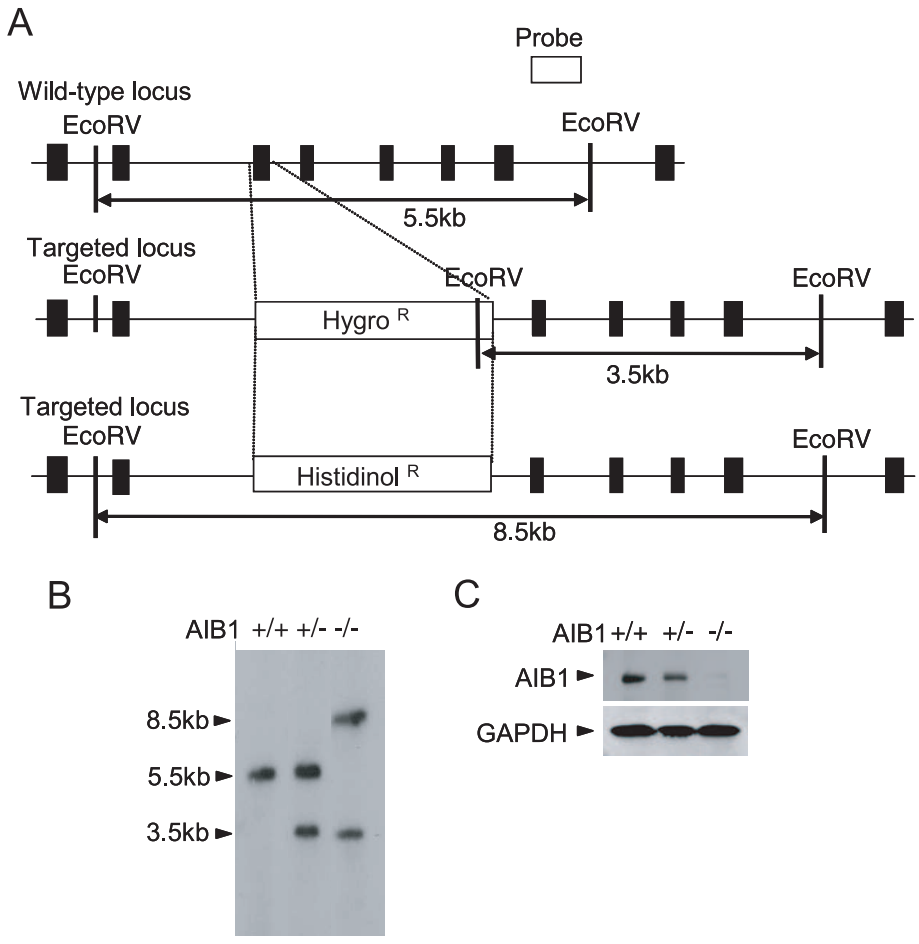
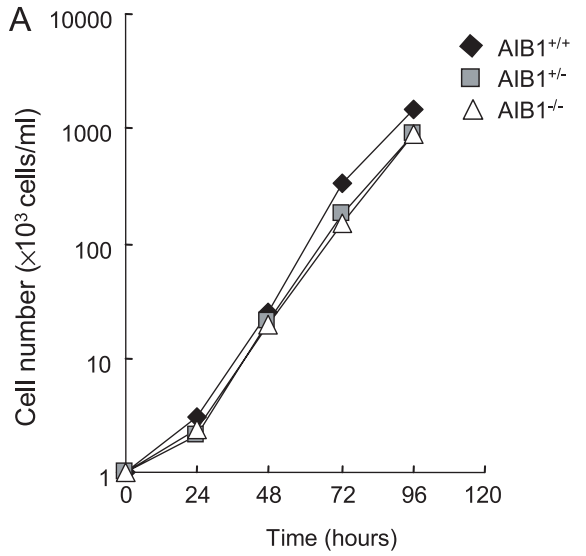
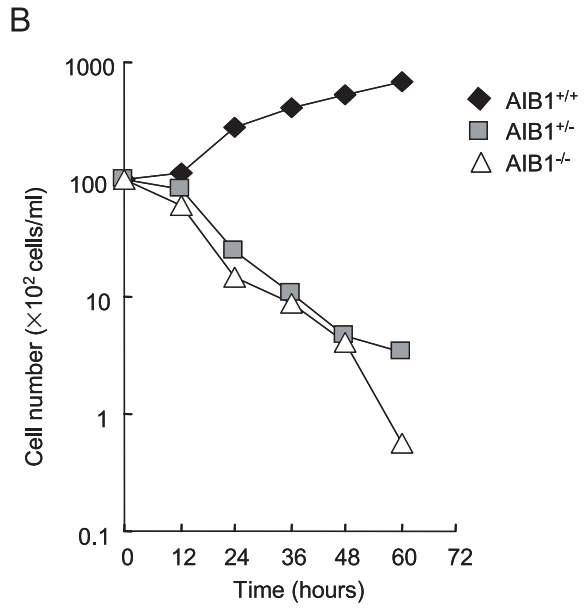
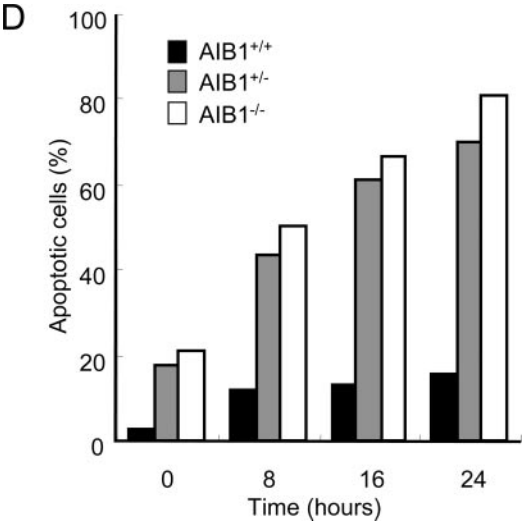
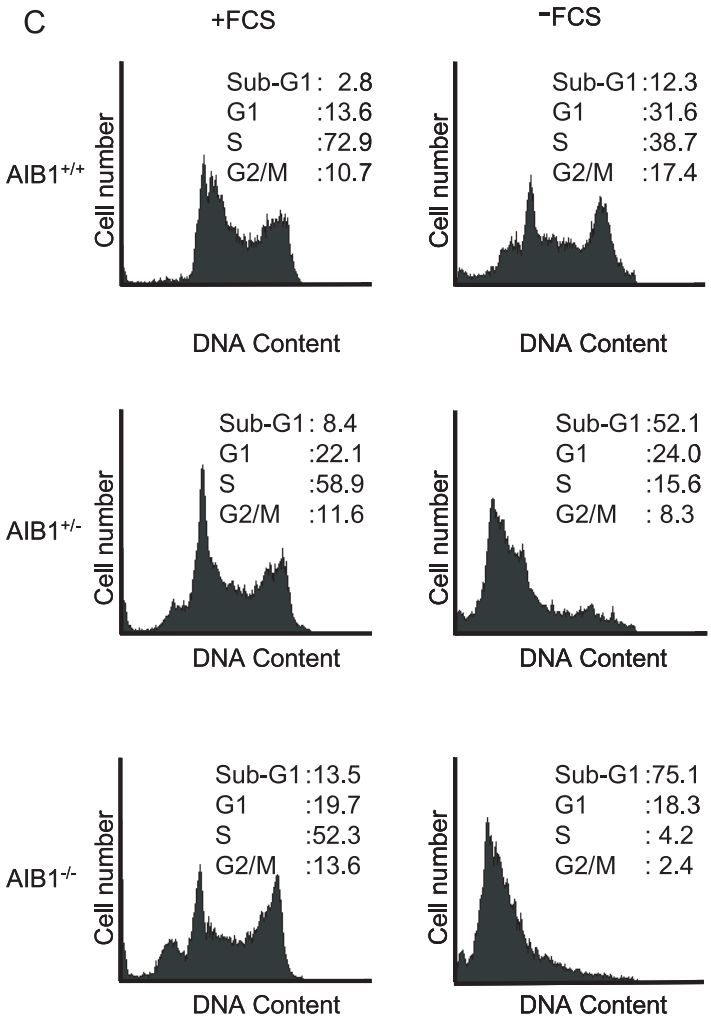


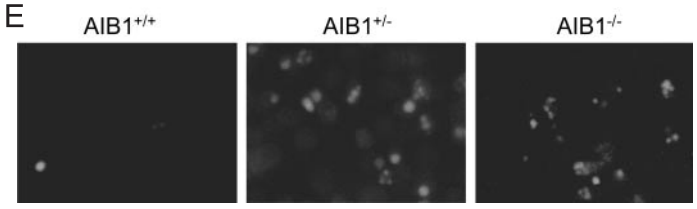
Fig. 1. **Generation of AIB1-knockout DT40 cells.** (A) Schematic representation of the targeting vectors. The configuration of the wild-type allele is shown at the top. In the targeting vectors, the exon encoding the Per-Arnt-Sim (PAS) domain is replaced by a hygromycin or histidinol resistance gene cassette. Solid boxes indicate positions of exons deduced from the cDNA sequence. The location of the external probe used to confirm correct targeted events and the location of the relevant *EcoRV* recognition sites are indicated. (B) Southern blot analysis of targeted integration. A DT40 cell in which one *AIB1* allele had been disrupted by the targeting construct of AIB1-hygromycin, was transfected with the second construct of AIB1-Histidinol. Genomic DNAs from untransfected DT40 cells (+/+) and doubly resistant clones (–/–) were digested with *EcoRV* and hybridized with the probe shown in (A). (C) Western blot analysis of wild-type, AIB1$^{+/-}$ and AIB1$^{-/-}$ DT40 cells. GAPDH protein was detected as an internal control.

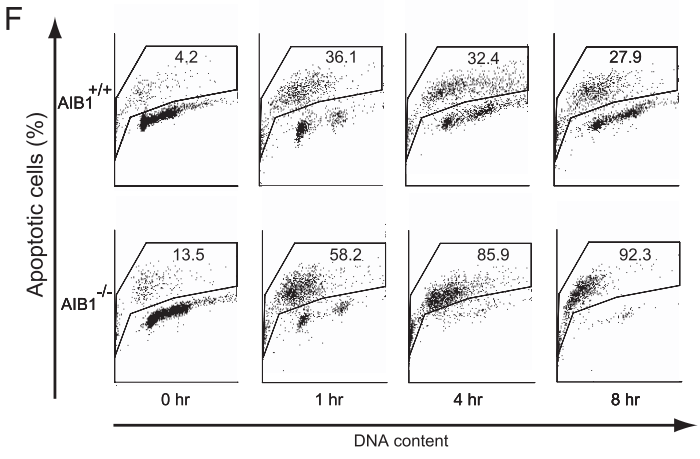












RESULTS

Generation of AIB1-Knockout DT40 Cells—To investigate the biological function of AIB1 in the signal transduction pathways triggered by cellular stress, we generated a model for the elimination of AIB1 production by using DT40 B-lymphocytes that constitutively express high levels of *c-myc* as a result of transformation by an avian leukosis virus (*27*). Because of their high rate of homologous recombination in vertebrate cells, DT40 cell lines have also been used as models for establishing strategies to identify genes that encode undiscovered components of a process or a pathway (*28*). We previously reported the cloning of a chicken homologue of AIB1 that exhibits 74.4% amino acid sequence similarity to human AIB1 (*29*). A full-length transcript of the gene encoding chicken AIB1 was isolated and found to encode a 1,399–amino acid protein. An AIB1 deletion construct was generated (Fig. 1A) and a portion of the AIB1 genomic locus was replaced with a hygromycin resistance gene and a histidinol resistance gene. The targeted homologous recombination in DT40 cells was confirmed by Southern blot analysis of genomic DNA (Fig. 1B). The deleted region encoded amino acid residues 122 to 156, including the PAS domain. AIB1-knockout DT40 cells were detected by Western blot analysis using anti-AIB1 antibody (Fig. 1C).

AIB1 Promotes Cell Survival in Response to Cellular Stress in DT40 Cells—To determine the roles of AIB1 in mechanisms mediated by exposure to cellular stress in cancer cells, we examined whether loss of AIB1 was associated with changes in the regulatory mechanism of cell proliferation and cell death. Under standard culture conditions, we detected no significant difference in the rates of cell proliferation of wild-type and AIB1-knockout DT40 cells (Fig. 2A). However, AIB1-knockout cells were more susceptible to cell death than were wild-type cells in response to cellular stresses such as serum starvation. Usually, DT40 cells were maintained in medium with 10% FCS and 1% chicken serum. After deprivation of FCS, survival number of AIB1-knockout cells rapidly decreased in contrast with wild type cell (Fig. 2B). The heterozygote also manifested considerable decrease in cell number, suggesting that gene dosage of AIB1 is crucial for cell survival under serum starvation (Fig. 2B). FACS analysis revealed that 75.1% of the AIB1-knockout cells were in the sub-G1 fraction (reflecting cell death), as compared to 12.3% for the wild-type cells under serum starvation (Fig. 2C). In contrast, the S-fraction (indicating DNA synthesis) was greater in wild-type cells than in AIB1-knockout cells (Fig. 2C). In order to confirm that these cell death were apoptosis, we carried out TUNEL analysis. When cells were stained by the TUNEL after deprivation of FCS, the double-stained cells (reflecting apoptosis) increased much more in $AIB1^{+/-}$ and $AIB1^{-/-}$ DT40 cells than in wild-type DT40 cells (Fig. 2, D, E). We observed similar results in the responses to not only serum starvation but also other forms of cellular stress, such as UV irradiation (10 J/m^2) (Fig. 2F) and culture at low temperature (data not shown). These results revealed that loss of AIB1 increased apoptosis in AIB1-knockout cells under cellular stress conditions.

Deletion of AIB1 Enhances Stress-Induced JNK/c-Jun Activation and Prevents Akt/p65 Activation—Signal cascades in response to cell survival have been linked to cancer and inflammatory disease. Previous studies have shown that cellular responses are regulated by the signaling pathways that lie downstream of the Ras induced by cross talk between the Raf-MER-ERK and PI3K-Akt pathways in breast cancer cells (*21*, *22*). Moreover, the PI3K/Akt/NF-κB pathway plays an important role in preventing cells from undergoing apoptosis and contributes to the pathogenesis of malignancy (*23*). Recently, cell proliferation and survival were reported to require activation of the PI3K/Akt pathway, which has been implicated in the control of Myc protein stability (*24*). The most recent study in transgenic mice also implicated overexpression of the *AIB1* gene in the etiology of breast cancer (*15*). However, these findings and our results in Fig. 2 raise the possibility that increased AIB1 production serves to promote cell survival through the PI3K/Akt pathway in response to cellular stress. Cell survival in response to stressful stimuli has been implicated in the activation of many signal transduction pathways, such as the p38 mitogen-activated protein kinase (MAPK) pathway, the stress-activated JNK pathway, and the PI3K/Akt pathway (*30–32*). Therefore, we investigated how signal pathways might be involved in AIB1-mediated cell survival in response to cellular stress. For Western blot analysis, wild-type and AIB1-knockout DT40 cells were cultured under normal conditions or cellular stress conditions. At first, stressful stimuli such as serum starvation or UV irradiation induced phosphorylation of NF-κB subunit p65 and Akt in wild-type DT40 cells (Fig. 3A). In contrast, phosphorylation of p65 and Akt were abolished in AIB1-knockout cells (Fig. 3A). To confirm the Akt activation under stress conditions, Akt activity was detected by an

Fig. 2. **AIB1 promotes cell survival in response to serum starvation and UV irradiation.** (A) Growth curves of wild-type, $AIB1^{+/-}$ and $AIB1^{-/-}$ DT40 cells under standard conditions. Cells were cultured in RPM1640 medium supplemented with 10% fetal calf serum (FCS) and 1% chicken serum at 39.5′C. Representative growth curves correspond to the indicated cell cultures. The number of cells was counted every 24 h by FACS with polybeads as the internal standard. Each experiment was conducted three times, and each time point was determined in triplicate. (B) AIB1 was required for cell survival under serum-starved conditions. Cells were cultured at 39.5°C in fresh RPM1640 medium supplemented with 1% chicken serum, after being maintained continuously with 10% FCS and 1% chicken serum. The number of cells was counted every 12 h. (C) Cell cycle analyses of wild-type, $AIB1^{+/-}$, and $AIB1^{-/-}$ DT40 cells in the presence or absence of FCS over 24 h. The DNA content of the cultured cells was examined by propidium iodide staining. The populations of the sub-G1, G1, S, and G2/M fractions are indicated by percentages. (D) Wild-type DT40 cells were inhibited from undergoing apoptosis under serum starvation, as measured by TUNEL analysis. Cells were cultured in the absence of FCS for the periods indicated. The cells were then stained for TUNEL analysis. Double-stained cells were counted as apoptotic and are shown as the percentages in three independent experiments. (E) DNA breaks, characteristic of apoptosis, were detected under fluorescent microscope by TUNEL analysis. Cells were harvested after being cultured for 8 h in the absence of FCS. (F) DNA replication was induced in wild-type DT40 cells under the stimulation of UV-irradiation, as measured by TUNEL analysis. After UV-irradiation (10 J/m^2) the cells were cultured for the periods indicated and then stained for TUNEL analysis. Double-stained cells were counted as apoptotic and are shown as percentages from three independent experiments.

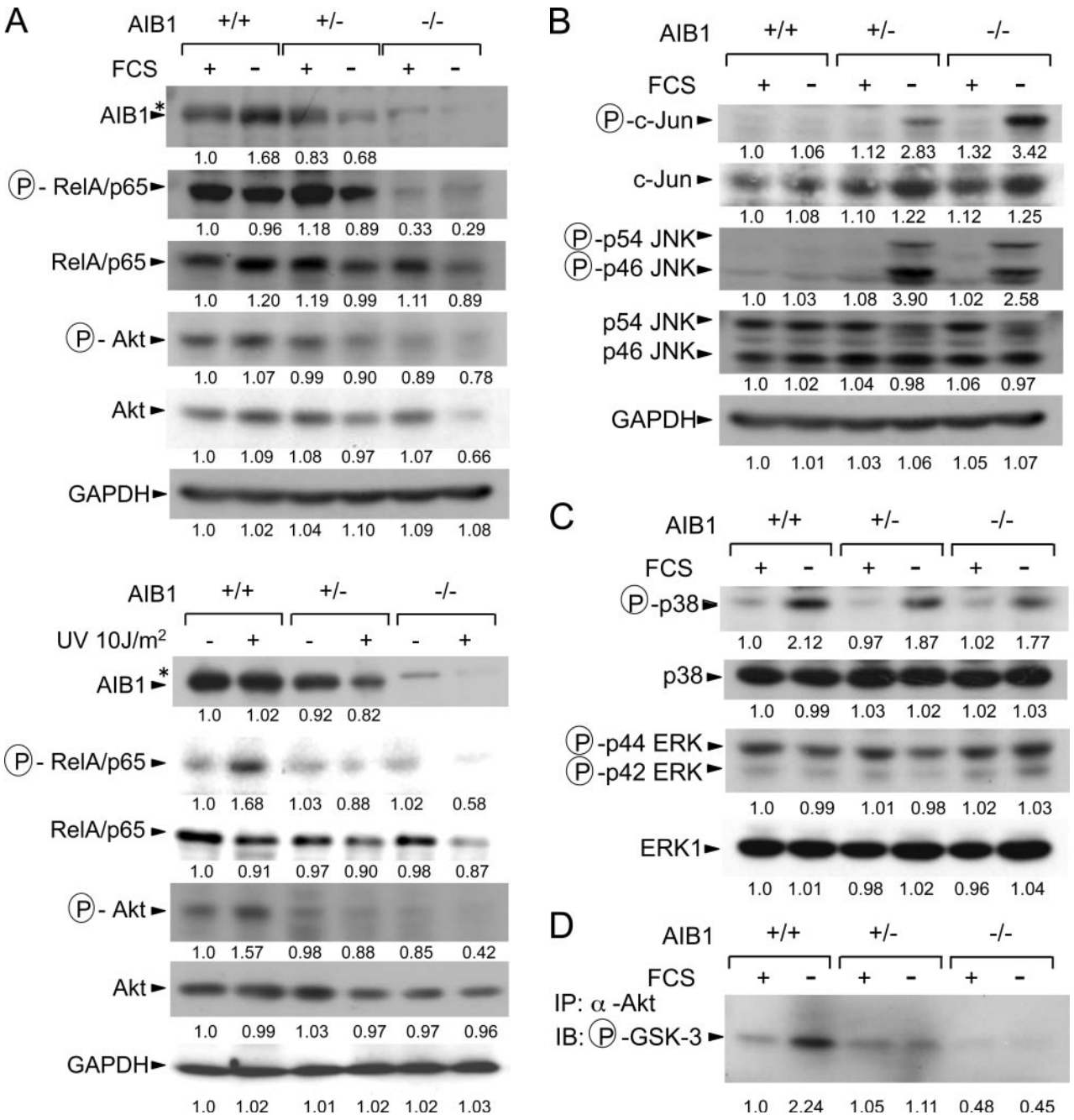


Fig. 3. **Activation of AIB1 triggers Akt survival pathway and blocks JNK-mediated cell death.** (A) Serum starvation and UV irradiation of wild-type DT40 cells induced Akt and p65 phosphorylation. The levels of production of proteins and phosphoproteins in the Akt/NF-κB cell-survival pathway were indicated by immunoblot analysis using 30 μg cell extracts prepared from wild-type, AIB1$^{+/-}$, or AIB1$^{-/-}$ DT40 cells cultured for 8 h in the presetnce or absence of FCS, with or without stimulation of UV-irradiation (10 J/m^2). The "P" in the circles indicates phosphorylation. The asterisk indicates that AIB1 antibody cross-reacted with the unspecific cytoplasmic protein. (B) Phosphorylation of JNK and c-Jun were induced by serum starvation in AIB1-knockout cells. The levels of production of protein and phosphoprotein in the JNK-mediated apoptosis pathway are indicated as shown in (A). (C) The presence of AIB1 protein did not affect the activation of the ERK or p38 MAPK pathways. (D) Serum starvation of wild-type DT40 cells induced Akt kinase activity. *In vitro* kinase assay was performed with immunoprecipitated Akt. Akt kinase activity is indicated by immunoblot analysis with glycogen synthase kinase-3 as a substrate. Western blots were quantified by densitometry and relative intensities of each band are shown.

in vitro assay of Akt-catalyzed phosphorylation of GSK3 (Fig. 3D). The catalytic activity of Akt was increased in wild-type DT40 cells by serum starvation. The activation of the PI3K/Akt pathway might be related to the cell survival of wild-type DT40 cells under stress conditions. Second, serum starvation induced phosphorylation of c-Jun and JNK in AIB1-deficient cells, not in wild type cells (Fig. 3B). Because both of AIB1$^{+/-}$ and AIB1$^{-/-}$ cells were killed by FCS deprivation (Fig. 2B), there are good correlation between the activation of JNK/c-Jun pathway

and the cell death. The stress-activated JNK pathway might be related to the cell death of AIB1-deficient cells under stress condition. On the other hand, there was no difference in the p38 MAPK/ERK pathway (Fig. 3C). Taken together, our data suggest that AIB1 acts as a molecular link between Akt/p65-induced cell survival and JNK/c-Jun-regulated cell death in response to cellular stress.

In Fig. 3A, a band was observed in the panel for AIB1$^{-/-}$ cells. This weak signal was found in all lanes using anti-AIB1 antibody, although it was difficult to identify because it was just above the specific band. Possibly, our AIB1-antibody might cross-react to the closely related protein such as TIF2 or SRC1.

Cellular Stress Induces Upregulation of AIB1 Gene Expression—To test directly whether cellular stress causes increased AIB1 gene expression by modulating Akt function or cell cycle regulators in GI/S transition, we examined the levels of expression of the mRNAs of various cell cycle regulators by Northern blotting in wild-type or AIB1-knockout DT40 cells after stimulation by serum starvation (Fig. 4). The mRNA levels of all the regulators investigated in AIB1-knockout cells were decreased efficiently by serum starvation. In contrast, the mRNA levels of AIB1, Akt, and RSK2 in wild-type DT40 cells were increased significantly by serum deprivation. No notable increases in mRNA levels of the G1/S-cell cycle regulators cyclin D1, cyclin D2, cyclin E and E2F1 were found in wild-type DT40 cells under serum starvation. These data indicate that, under cellular stresses such as serum starvation, high levels of AIB1 mRNA are mediated by Akt or RSK2, but not by activation of the regulators of cell cycle progression in the G1/S phase. Accordingly, cellular stress-induced AIB1 amplification may enhance cell survival and DNA replication through coordinated upregulation of the Akt signaling pathway.

AIB1 Is Essential for Induction of Akt-Dependent DNA Replication in Response to Cellular Stress—To assess the role of AIB1 in mediating Akt-activated DNA replication in response to cellular stress, we analyzed the contribution of phosphorylated Akt at designated time points after synchronization of the cell cycle by treatment with hydroxyurea, which arrests the cycle in the early S-phase. While we observed no differences in the low levels of Akt phosphorylation during synchronization, phosphorylation of Akt was blocked in AIB1-knockout DT40 cells, unlike in wild-type cells, after release of the cell cycle arrest. In contrast, induction of Akt phosphorylation was significantly increased in wild-type cells when they were released from arrest and progressed further into S phase (Fig. 5A). By treatment with the pharmacological agent LY294002, an inhibitor of PI3K/Akt kinase, we next investigated whether the requirement for AIB1 in DNA replication was dependent on Akt in stressed cells. Wild-type DT40 cells with LY294002 had marked decreased numbers of BrdU-positive cells (Fig. 5B), suggesting that, in wild-type cells, inhibition of cell cycle progression into S phase occurred by the inhibition of Akt, in the same way as in AIB1-knockout cells. Inhibition of MAPK activity by treatment with PD98059 did not lead to a marked decrease in DNA replication in response to serum starvation in wild-type DT40 cells (data not shown). Thus, these results indicate that induction of Akt phosphorylation as a consequence of progression into S phase caused a requirement for AIB1 in DNA replication in response to cellular stress.

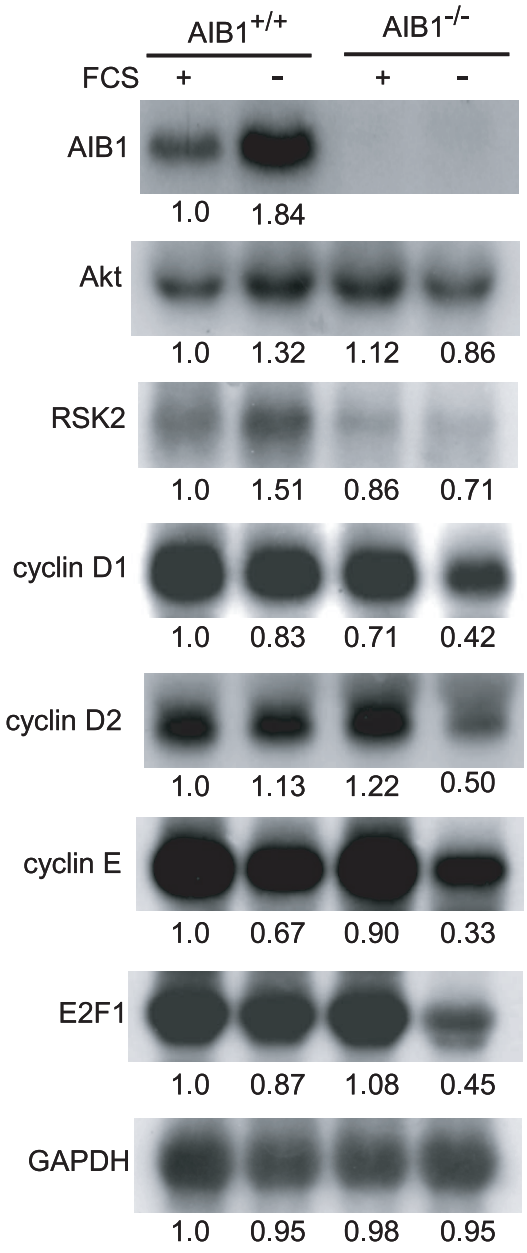


Fig. 4. **Relative levels of expression of mRNAs of cell cycle regulators.** Wild-type and AIB1-knockout DT40 cells were cultured for 8 h in the presence or absence of FCS. Total RNAs were prepared for Northern blot analysis. The radioactivities of the corresponding bands of cell cycle regulators and GAPDH mRNA were determined with an image analyzer as relative intensity, and the normalized intensity against levels of mRNA in wild-type DT40 cells with FCS are shown.

Recent study showed that AIB1 overexpression enhanced the activation of PI3K/Akt pathway and AIB1 knockdown increased apoptosis (*15*). As mentioned above, the stress-activated JNK pathway was suppressed in the presence of AIB1 (Fig. 3B). In order to investigate if the JNK suppression is mediated by the PI3K/Akt pathway, we

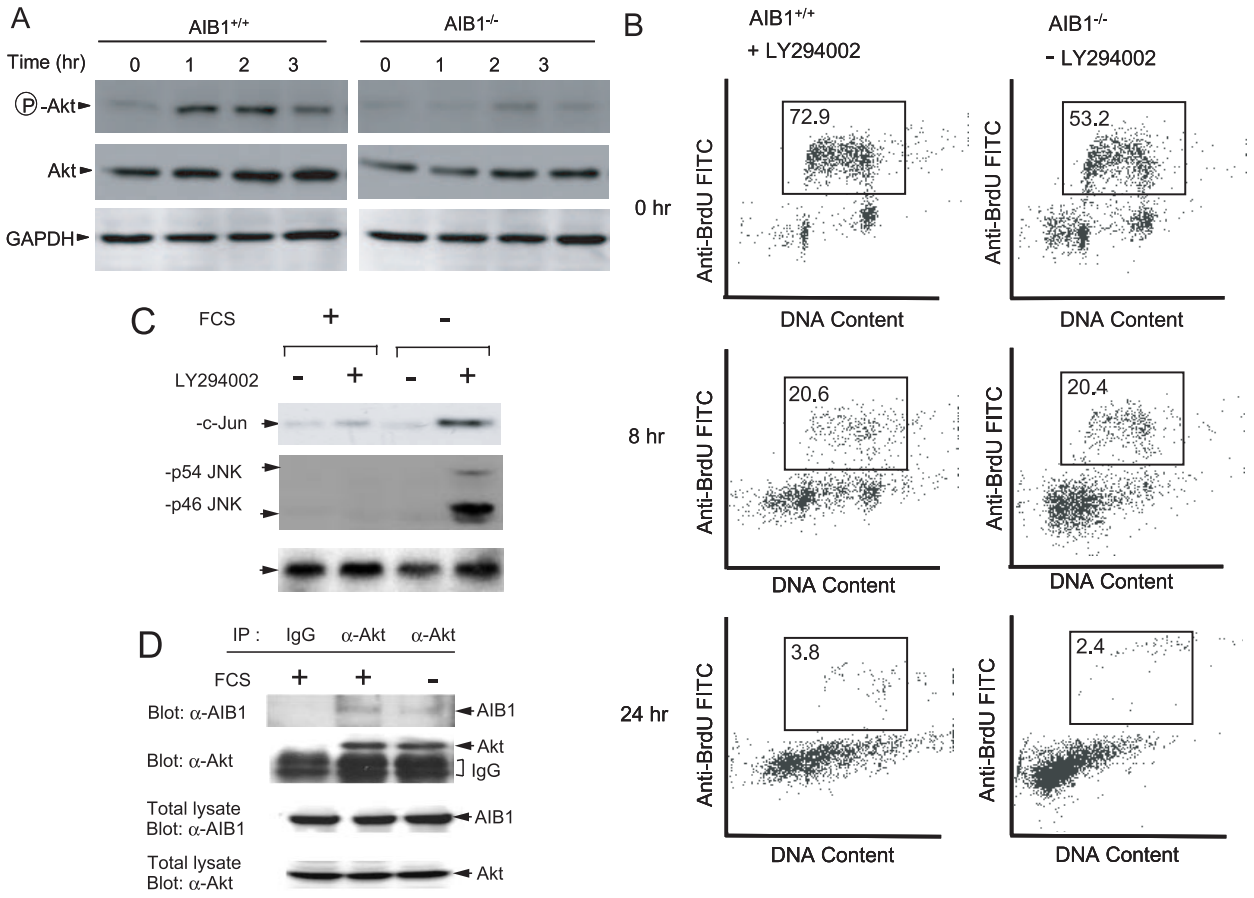


Fig. 5. **Inhibition of Akt or deficiency of AIB1 blocks DNA replication under stress conditions.** (A) Akt phosphorylation and AIB1 production were required for DNA replication. Cells were synchronized for 8 h with hydroxyurea (1 mM), which interfered with cell cycle progression by preventing DNA replication, and were harvested at the indicated points (0–3 h) following release from the cell cycle arrest. Production of phosphorylated Akt and total Akt was determined by immunoblot analysis using anti-phospho-Akt (Ser-473) and anti-Akt antibodies. (B) DNA replication in wild-type DT40 cells by in response to serum deprivation was blocked by LY294002, an inhibitor of PI3K/Akt. Wild-type cells or AIB1-knockout cells were cultured with LY294002 (50 μM) in the absence of FCS for 8 h or 24 h. The percentage BrdU positivity was determined by counting the number of cells at the BrdU-positive gates. (C) The JNK suppression in the AIB1-positive cells was blocked by LY294002. Wild-type cells were cultured with or without LY294002 (50 μM) in the presence or absence of FCS for 8 h. The levels of phosphoprotein in the JNK-mediated apoptosis pathway were determined by Western blot. (D) AIB1 is physically associated with Akt. The DT40 cell extracts were immunoprecipitated with anti-Akt antibody. For control, cell extract was precipitated with the IgG from non-immunized rabbit. The immunoprecipitates (IP) were subjected to Western blot analysis with the indicated antibodies.

examined the phosphorylation of c-Jun and JNK after LY294002 treatment. As a result, we found the marked increase of phospho-c-Jun and phospho-JNK by the LY294002 treatment in response to serum starvation (Fig. 5C), suggesting that the inhibition of PI3K/Akt pathway blocked the AIB1-mediated JNK suppression. Further, we showed the direct interaction between AIB1 and Akt (Fig. 5D). Collectively, these data suggested that AIB1 might be activated by Akt.

Loss of AIB1 Leads to Inhibition of UV-Induced Phosphorylation of Histone H3 at Serine 10—DNA replication is linked to chromatin modulation. This is associated with the modification of chromatin-associated proteins such as histones (H2A, H2B, H3, and H4) or remodeling cofactors, which are known to possess intrinsic histone acetyltransferase activity and are capable of chromatin modification by histone acetylation (*9*, *10*). We have shown here that AIB1 depletion causes a marked defect in the ability to induce DNA replication in response to cellular stress. Therefore, AIB1 may control the signal cascades for the remodeling of chromatin in response to cellular stress. We next examined whether AIB1 was required for phosphorylation of chromatin modulation in response to cellular stress. By Western blot analyses using cell extracts of acid-soluble proteins, we found that wild-type cells had a marked increase in the phosphorylation of histone H3 at serine 10 (Fig. 6), but not in the acetylation of histone H3 at lysine 9 or 14 (data not shown), very soon after treatment with UV-irradiation. This phosphorylation was completely eliminated under stress conditions by the loss of AIB1 in AIB1-knockout cells (Fig. 6). Although a previous study had shown that AIB1 possesses intrinsic histone acetyltransferase (HAT) activity for chromatin modification (*2*), we observed no difference in the levels of acetylation of histone H3 at lysine 9 and lysine 14 in AIB1-knockout DT40 cells compared with wild-type cells

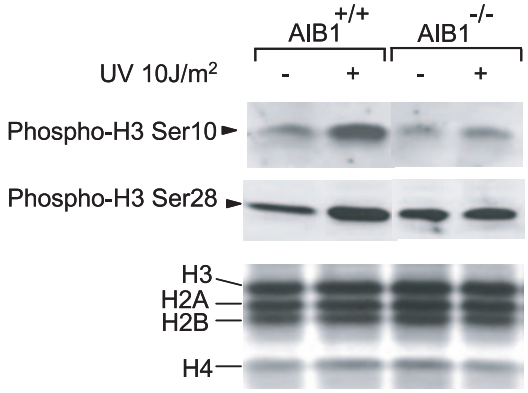


Fig. 6. **Knockout of AIB1 leads to inhibition of UV-induced phosphorylation of histone H3 at serine 10.** Western blot analyses are shown with the specific antibodies indicated on the left, in wild-type or AIB1-knockout cells either treated or untreated by UV irradiation (10 J/m^2). Coomassie Blue–stained 15% polyacrylamide SDS–containing gels indicated equal loading of proteins.

following UV irradiation (data not shown). These results suggest that AIB1 plays a critical role in the signaling cascade for both Akt activation and modulation of the phosphorylation of histone H3 at serine 10 in response to cellular stress.

DISCUSSION

Recent studies have demonstrated that AIB1 plays a pivotal role in activation of the intrinsic IGF-1–driven cell survival pathway, which is mediated through the PI3K/Akt pathway (*15*). Although the signal transduction pathways that lead to the positive control of AIB1 have been studied extensively, the critical targets of this kinase that mediate the stress response remain to be determined. We showed here that AIB1-deficient DT40 cells are extremely sensitive to be killed by cellular stresses such as serum deprivation and UV irradiation. This susceptibility was correlated with a reduction in the ability to restore DNA-synthesis levels under stress conditions. Moreover, we showed that, with serum deprivation or UV irradiation treatment, the induction of phosphorylation of both JNK and c-Jun in AIB1-knockout DT40 cells was much greater than in wild-type DT40 cells. These results are consistent with those of previous studies, which demonstrated that activation of the JNK/c-Jun pathway mediates the induction of cell death by DNA damage agents (*33*, *34*). Our results therefore indicate that the presence of AIB1 is required to suppress activation of the JNK/c-Jun signaling pathway in DNA replication under cellular stress conditions. On the other hand, in ER-negative cancer cells subjected to cellular stress, high levels of AIB1 production have been shown to promote activation of the Akt/p65 survival pathway (*15*). It has been shown that activation of c-Jun by the JNK apoptosis pathway is required to suppress NF-κB transcription (*35*, *36*). Our study is consistent with the result of a recent report, which demonstrated that Akt inhibits stress-activated JNK through activation of NF-κB (*36*). Collectively, these data indicate that AIB1 plays a key role as a mediator between the Akt/NF-κB and JNK/c-Jun pathways in controlling cell fate in response to cellular stress.

Importantly, various signal transduction pathways can modulate the interactions of specific coregulators with nuclear receptors or mediate their activities (*37*). Recent studies suggest that the transcriptional corepressors NCoR and SMRT interact with, and exert repressive effects on AP-1 or NF-κB (*38–41*). It has been proposed that the transactivation potential of c-Jun is repressed by histone deacetylase (HDAC) complexes and these repressor complexes are dissociated by JNK-mediated phosphorylation (*42*). As can be seen from our stress assays in DT40 cells, presence of AIB1 is an important key to modulate the switch from transcriptional repression to activation in association with the diverse protein kinase-dependent signalling pathways in response to cellular stress.

We have shown that, in addition to its role in signal ransduction pathways, AIB1 production is correlated with UV-induced phosphorylation of histone H3 at serine 10, but not with acetylation of histone H3 at lysine 9 or 14. These results suggest that AIB1 is essential in mediation of the phosphorylation of histone H3 in chromatin remodeling. Although it has been shown that the phosphorylation of histone H3 is mediated by the Aurora B kinases (*43*), IKK-*a* (*45*), MSK1 and MSK2 (*45*), and RSK-2 (*46*), we need to reveal by future studies a kinase that can phosphorylate histone H3 in association with AIB1 directly. Previous studies have reported that IκB kinase, a positive regulator of NF-κB activation, is activated by Akt (*13*, *14*). However, production of endogenous IKKα or IKKβ proteins did not change detectably in response to activated Akt in DT40 cells (data not shown). NF-κB activation by stress stimuli has also been shown to be independent of phosphorylation of IκBα at Ser 32/36, and to be IKK-independent (*47*). Moreover, NF-κB, which is usually maintained in an inactive state by protein–protein interaction with inhibitor IκBs, is constitutively active in ER-negative breast cancer cell lines (*48*). Thus, in DT40 cells AIB1 might be produced as one of the downstream targets interacting directly with Akt, independently of IKK.

Previous studies have found that, under conditions of stress, Akt interacts with JIP1 in primary neurons and thus inhibits JNK activation. Therefore, ectopic expression of Akt attenuated stress-induced apoptosis while Akt1 gene deletion rendered neurons more sensitive to stress stimulus than wild-type neurons (*49*). Moreover, recent studies have shown that activation of NF-κB is required for inhibition of JNK in response to TNF-α or UV stimulation (*21–24*). These previous studies and our findings suggest that the level of production of AIB1 is a key determinant of cell susceptibility to cellular stress, in association with phosphorylation cascades. Furthermore, we have elucidated the molecular mechanisms by which, in response to cellular stress, AIB1 plays a critical role in DNA replication or phosphorylation of histone H3 at serine 10, in association with active Akt/NF-κB pathway.

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REFERENCES

1. Anzick, S.L., Kononen, J., Walker, R.L., Azorsa, D.O., Tanner, M.M., Guan, X.Y., Sauter, G., Kallioniemi, O.P., Trent, J.M., and Meltzer, P.S. (1997) AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* **277**, 965–968
2. Chen, H., Lin, R.J., Schiltz, R.L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M.L., Nakatani, Y., and Evans, R.M. (1997) Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* **90**, 569–580
3. Torchia, J., Rose, D.W., Inostroza, J., Kamei, Y., Westin, S., Glass, C.K., and Rosenfeld, M.G. (1997) The transcriptional co-activator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* **387**, 677–684
4. Takeshita, A., Cardona, G.R., Koibuchi, N., Suen, C.S., and Chin, W.W. (1997) TRAM-1, a novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator 1. *J. Biol. Chem.* **272**, 27629–27634
5. Li, H., Gomes, P.J., and Chen, J.D. (1997) RAC3, a steroid/nuclear receptor associated coactivator that is related to SRC-1 and TIF2. *Proc. Natl. Acad. Sci. USA* **94**, 8479–8484
6. Onate, S.A., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor supperfamily. *Science* **270**, 1354–1357
7. Voegel, J.J., Heine, M.J., Zechel, C., Chambon, P., and Gronemeyer H. (1996) TIF2, a 160 kDa transcriptional coactivator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J.* **15**, 3667–3675
8. Hong, H., Kohli, K., Garabedian, M.J., and Stallcup, M.R. (1997) . GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. *Mol. Cell Biol.* **17**, 2735–2744
9. Huang, Z.Q., Li, J., Sachs, L.M., Cole, P.A., and Wong, J. (2003) A role for cofactor-cofactor and cofactor-histone interactions in targeting p300, SWI/SNF and Mediator for transcription *EMBO J.* **22**, 2146–2155
10. Dilworth, F.J., Fromental-Ramain, C., Yamamoto, K., and Chambon, P. (2000) ATP-driven chromatin remodeling activity and histone acetyltransferases act sequentially during transactivation by RAR/RXR In vitro. *Mol. Cell* **6**, 1049–1058
11. Osborne, C.K., Bardou, V., Hopp, T.A., Chamness, G.C., Hilsenbeck, S.G., Fuqua, S.A., Wong, J., Allred, D.C., Clark, G.M., and Schiff R. (2003) Overexpression of the steroid receptor coactivator AIB1 in breast cancer correlates with the absence of estrogen and progesterone receptors and positivity for p53 and HER2/neu. *J. Nat. Cancer Inst.* **95**, 353–361
12. Bouras, T., Southey, M.C., and Venter, D.J. (2001) Overexpression of the steroid receptor coactivator AIB1 in breast cancer correlates with the absence of estrogen and progesterone receptors and positivity for p53 and HER2/neu. *Cancer Res.* **61**, 903–907
13. Ozes. O.N., Mayo, J.A., Gustin, S.R., Pfeffer, L.M., and Donner, D.B. (1999) NF-κB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* **401**, 82–85
14. Romashkova, J.A. and Makarov, S.S. (1999) NF-κB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* **401**, 86–90
15. Torres-Arzayus, M.I., De Mora, J.F., Yuan, J., Vazquez, F., Bronson, R., Rue. M., Sellers, W.R., and Brown, M. (2004) High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene. *Cancer Cell* **6**, 263–274
16. Werbajh S, Nojek I, Lanz R., and Costas, M.A. (2000) RAC-3 is a NF-κB coactivator. *FEBS Lett.* **485**, 195–199
17. Wu, R.C., Qin, J., Hashimoto, Y., Wong, J., Xu, J., Tsai, S.Y., Tsai, M.J., and O'Malley, B.W. (2002) Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) Coactivator activity by I kappa B kinase. *Mol. Cell Biol.* **22**, 3549–3561
18. Nakshatri, H., Bhat-Nakshatri, P., Martin, D.A., Goulet, JR, R.J., and Sledge, JR., G.W. (1997) Constitutive activation of NF-κB during progression of breast cancer to hormone-independent growth. *Mol. Cell Biol.* **17**, 3629–3639
19. Sheppard, K.A., Rose, D.W., Haque, Z.K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M.G., Glass, C.K., and Collins, T. (1999) Transcriptional activation by NF-κB requires multiple coactivators. *Mol. Cell Biol.* **19**, 6367–6378
20. Davis, R.J. (2000) Signal transduction by the JNK group of MAP kinases. *Cell* **103**, 239–252
21. Liu, J. and Lin, A. (2005) Role of JNK activation in apoptosis: a double-edged sword. *Cell Res.* **15**, 36–42
22. Tang, G., Minemoto, Y. Dibling, B., Purcell, N.H. Li, Z., Karin, M., and Lin, A. (2001) Inhibition of JNK activation through NF-kappaB target genes. *Nature* **414**, 313–317
23. Tang, F., Tang, G., Xiang, J., Dai, Q., Rosner, M.R., and Lin, A. (2002) The absence of NF-kappaB-mediated inhibition of c-Jun N-terminal kinase activation contributes to tumor necrosis factor alpha-induced apoptosis. *Mol. Cell Biol.* **22**, 8571–8579
24. Lin, A. (2003) Activation of the JNK signaling pathway: breaking the brake on apoptosis. *Bioessays* **25**, 17–24
25. Verger, A. and Crossley, M. (2004) Chromatin modifiers in transcription and DNA repair. *Cell. Mol. Life Sci.* **61**, 2154–2162
26. Nowak, S.J. and Corces, V.G. (2004) Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation. *Trends Genet.* **20**, 214–220
27. Hueber, A.O. and Evan, G. (1998) Traps to catch unwary oncogenes. *Trends Genet.* **14**, 364–367
28. Baba, T.W., Giroir, B.P., and Humphries, E.H. (1985) Cell lines derived from avian lymphomas exhibit two distinct phenotypes. *Virology* **144**, 139–151
29. Arai, S., Ogawa, K., Yamachika, S., Nishihara, T., and Nishikawa, J. (2001) Cloning and functional characterization of chicken p160 coactivator family members. *Biochim. Biophys. Acta* **1518**, 7–18
30. Karin, M. (1998) Mitogen-activated protein kinase cascades as regulators of stress responses. *Ann. N.Y. Acad. Sci.* **851**, 139–146
31. Tournier, C., Hess, P., Yang, D.D., Xu, J., Turner, T.K., Nimual, A., Bar-Sagi, D., Jones, S.N., Flavell, R.A., Davis, R.J. (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* **288**, 870–874
32. Chang, L. and Karin, M. (2001) Mammalian MAP kinase signalling cascades. *Nature* **410**, 37–40
33. Chen, Y.R., Wang, X., Templeton, D., Davis, R.J., and Tan, T.H. (1996) The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J. Biol. Chem.* **271**, 31929–31936
34. Faris, M., Kokot, N., Latiinis, K., Kasibhatla, S., Green, D.R., Koretzky, G.A., and Nel, A. (1998) The c-Jun N-terminal kinase cascade plays a role in stress-induced apoptosis in Jurkat cells by up-regulating Fas ligand expression. *J. Immunol.* **160**, 134–144
35. Reuther-Madrid, J.Y., Kashatus, D., Chen, S., Li, X., Westwick, J., Davis, R.J., Earp, H.S., Wang, C-Y., and Baldwin Jr., A.S. (2002) The p65/RelA subunit of NF-kappaB suppresses the sustained, antiapoptotic activity of Jun kinase induced by tumor necrosis factor. *Mol. Cell Biol.* **22**, 8175–8183
36. Yuan, Z., Feldman, R.I., Sun, M., Olashaw, N.E. Coppola, D., Sussman, G.E., Shelley, S.A., Nicosia, S.V., and Cheng, Q. (2002) Inhibition of JNK by cellular stress- and tumor necrosis factor alpha-induced AKT2 through activation of the NF kappa B pathway in human epithelial Cells. *J. Biol. Chem.* **277**, 29973–29982

37. Rosenfeld, M.G. and Glass, C.K. (2001) Coregulator codes of transcriptional regulation by nuclear receptors. *J. Biol. Chem.* **276**, 36865–36868
38. Zhang, J., Kalkum, M., Chait, B.T., and Roeder, R.G. (2002) The N-CoR-HDAC3 nuclear receptor corepressor complex inhibits the JNK pathway through the integral subunit GPS2. *Mol. Cell* **9**, 611–623
39. Perissi, V., Aggarwal, A., Glass, C.K., Rase, D.W., and Rosenfeld, M.G. (2004) A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. *Cell* **116**, 511–526
40. Lee, S.K., Kim, J.H., Lee, Y.C., Cheong, J. and Lee, J.W. (2000) Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule of activating protein-1, nuclear factor-kappaB, and serum response factor. *J. Biol. Chem.* **275**, 12470–12474
41. Baek, S.H., Ohgi, K.A., Rose, D.W., Koo, E.H., Glass, C.K., and Rosenfeld, M.G. (2002) Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. *Cell* **110**, 55–67
42. Weiss, C., Schneider, S., Wagner, E.F., Zhang, X., Seto, E., and Bohmann, D. (2003) JNK phosphorylation relieves HDAC3-dependent suppression of the transcriptional activity of c-Jun. *EMBO J.* **22**, 3686–3695
43. Clayton, A.L. and Mahadevan, L.C. (2003) MAP kinase-mediated phosphoacetylation of histone H3 and inducible gene regulation. *FEBS Lett.* **546**, 51–58
44. Yamamoto, Y., Verma, U.N., Prajapati, S., Kwak, Y.T., and Gaynor, R.B. (2003) Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. *Nature* **423**, 655–659
45. Soloaga, A., Thomson, S., Wiggin, G.R., Rampersaud, N., Dyson, M.H., Hazzalin, C.A., Mahadevan, L.C., and Arthur, J.S. (2003) MSK2 and MSK1 mediate the mitogen- and stress-induced phosphorylation of histone H3 and HMG-14. *EMBO J.* **22**, 2788–2797
46. Sassone-Corsi, P., Mizzen, C.A., Cheung, P., Crosio, C., Monaco, L., Jacquot, S., Hanauer, A., and Allis, C.D. (1999) Requirement of Rsk-2 for epidermal growth factor-activated phosphorylation of histone H3. *Science* **285**, 886–891
47. Li, N. and Karin, M. (1998) Ionizing radiation and short wavelength UV activate NF-kappaB through two distinct mechanisms. *Proc. Natl. Acad. Sci. USA* **95**, 13012–13017
48. Tergaonkar, V., Bottero, V., Ikawa, M., Li, Q., and Verma, I.M. (2003) IkappaB kinase-independent IkappaBalpha degradation pathway: functional NF-kappaB activity and implications for cancer therapy. *Mol. Cell Biol.* **23**, 8070–8083
49. Kim, A.H., Yano, H., Cho, H., Meyer, D., Monks, B., Margolis, B., Birnbaum, M.J., and Chao, M. V. (2002) Akt1 regulates a JNK scaffold during excitotoxic apoptosis. *Neuron* **35**, 697–709