Supporting Information

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SI Materials and Methods

Cell Culture and Transfection. The 293T and the mouse–adult erythroleukemic MEL cell lines (1) were cultured in DMEM (Invitrogen) containing 10% FBS (HyClone), 50 units/mL of penicillin, and 50 μg/mL of streptomycin (Invitrogen). The human leukemic cell line K562 (2) and the mouse–adult erythroleukemia cell line CB3 (3), a MEL derivative lacking the expression of p45, were cultured in RPMI medium 1640 (Invitrogen) containing the same ingredients as above. The stable transfectants of CB3 were selected in G418 (1.0 mg/mL; Gibco/BRL).

Antibodies and Reagents. Polyclonal anti-p45 rabbit antibody was generated in the laboratory. The anti-Myc mouse antibody, anti-NF–E2 rabbit antibody C-19, and anti-phospho-c-jun goat antibody A-20 were from Santa Cruz Inc. Anti-Flag (M2) and anti-tubulin (B-5-1-2) mouse antibodies were purchased from Sigma. Anti-phospho-SAPK/JNK (Thr183/Tyr185; G9) mouse antibody and anti-SAPK/JNK (56G8) rabbit antibody were purchased from Cell Signaling Tech. Anti-polyubiquitinated protein antibody (clone FK2) (4) was purchased from BIOMOL. Polyclonal anti-Bach1 rabbit antibody was kindly provided by Dr. Kazuhiko Igarashi (Graduate School of Medicine, Tohoku University, Sendai 980-8575, Japan.).

In Vitro Dephosphorylation. The whole cell extracts were prepared from MEL and K562 cells by lysis of the cell pellets containing 5×10^6 of PBS-washed cells in 300 μl of the M-PER Mammalian Protein Extraction Reagent (PIERCE) containing the protease inhibitor mixture from Roch. After incubation on ice for 15 min, the samples were centrifuged for 30 min at 13,000 rpm at 4°C, and the supernatants were kept as the cell lysates. The phosphatase assay was carried out at 30°C for 45 min in a reaction volume of 20 μl containing 2 μg of the cell lysate, the λ phosphatase mixture (50 mM Tris–HCl, pH 7.5, 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% Brij 35, and 2 mM $MnCl_2$), and 400 units of λ phosphatase (New England Biolabs). The reactions were stopped with 5x SDS sample buffer, and the samples were analyzed by WB with use of anti-p45.

Plasmid Construction. Plasmids pEF-GST-Myc, pEF-GST-p45-Myc, pEF-GST-p45(5KR-108K)-Myc, pEF-GST-p45(5KR-137K)-Myc, pEF-GST-p45(5KR-215K)-Myc, pEF-GST-p45(5KR-234K)-Myc, pEF-GST-p45(5KR-241K)-Myc, pEF-GST-p45(5KR-368K)-Myc, pEF-GST-p45(1-83)-Myc, pEF-GST-p45(□78)-Myc, pEF-GST-p45(78-206)-Myc, pEF-GST-p45(207-268)-Myc, pEF-GST-p45 (269-297)-Myc, pEF-GST-p45 (298-373)-Myc, pEF-GST-p45 (6KR)-Myc, pEF-GST-p45(S157A)-Myc, pEF-GST-p45(S346A)-Myc, and pEF-GST-p45(S157A/S346A)-Myc were constructed in the following way. The GST and GST-p45 DNA fragments encoding the GST and GST-p45 polypeptides, respectively, were amplified by PCR using pGEX-2TK-p45 (5) as the template. They were cloned in frame into the NcoI and NotI sites of the pEF-Myc vector (Invitrogen), resulting in pEF-GST-Myc and pEF-GST-p45-Myc, respectively. GST-p45 fragments containing different mutations were generated by PCR-directed mutagenesis and cloned into pEF-Myc in a similar way. pEF-Flag-Ub was constructed by cloning of a fragment that PCR amplified from a plasmid pMyc-Ub (Zee-Fen Chang, National Taiwan University, Taipei 100, Taiwan.) into the HindIII-NotI sites of pEF-Flag (Invitrogen).

GST Pull-Down Experiments. For GST pull-down experiments, the whole-cell extracts were incubated with the glutathione Sepharose 4B beads (GE Healthcare) at 4°C for 1 h. After the beads were washed three times with 20 volumes of PBS, the GST-fusion proteins were eluted with 10 mM reduced glutathione, resuspended in equal volumes of 2x SDS sample buffer, and analyzed by SDS/PAGE and WB.

In Vitro Phosphorylation Assay. To detect p45 phosphorylation by JNK, the pEF-GST-p45-Myc and its two mutant forms, pEF-GST-p45(1-83)-Myc and pEF-GST-p45(Δ78)-Myc, were individually transfected into 293T cells. The GST-fusion proteins were pulled down, and 0.5 μg of each protein were incubated in a 20-μl reaction mixture containing 50 ng of the active JNK (Upstate Biotechnology, Inc.) and 4μCi of [γ-^{32}P] ATP in the kinase buffer (50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 10 mM β-glycerophosphate, 0.4 mM Na_3VO_4, 0.4 mM DTT, 5 mM $MgCl_2$, and 0.2 mM ATP). After incubation at 30°C for 30 min, the reaction was stopped by 2x SDS sample buffer and boiling for 5 min. The phosphorylated protein products were separated by 8% SDS/PAGE and analyzed by x-ray autoradiography and WB.

Nanoscale Microcapillary Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry. High-resolution and high-mass accuracy nanoscale microcapillary liquid chromatography electrospray ionization tandem mass spectrometry (nano-LC-MS/MS) experiments were performed on a linear quadrupole ion trap-Fourier transform ion cyclotron resonance (LTQ-FT) hybrid mass spectrometer (Thermo Fisher Scientific, Inc.) equipped with a nanoelectrospray ion source (New Objective, Inc.), an Agilent 1100 series binary HPLCy pump (Agilent Technologies), and a Famos autosampler (LC Packings). The enzyme-digested peptide solution was injected on to a self-packed precolumn (150 μm I.D. × 20 mm, 5 μm, and 200 Å). Chromatographic separation was performed on a self-packed reversed phase C18 nano-column (75μm I.D. × 300 mm, 5μm, and 100 Å) using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in 80% acetonitrile (mobile phase B) and applying a linear gradient from 5% to 45% mobile phase B for 90 min at a flow rate of 300 nl/min provided across a flow splitter by the HPLC pumps. Electrospray voltage was applied at 2.0 kV, and ion transferring capillary temperature was set at 200°C.

A scan cycle was initiated with a full-scan survey MS spectrum (m/z 300–2000) performed on the Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer with resolution of 100,000 (at m/z 400). The three most abundant ions detected in this scan were subjected to a MS/MS experiment performed in the linear quadrupole ion trap (LTQ) mass spectrometer. If the phosphoric acid neutral loss was detected in MS/MS scan, an MS3 experiment will be triggered. Ion accumulation (Auto Gain Control target number) and maximal ion accumulation time for full-scan, MS/MS, and MS3 were set at 1×10^6 ions, 1,000 ms; 5×10^4 ions, 200 ms; and 5×10^4 ions, 200 ms, respectively. For parameters of collision-induced dissociation, the normalized collision energy was set to 35%, activation Q was 0.3, and activation time was 30 ms.

The MS/MS and MS3 spectra were converted to DTA format from the experimental RAW file by Bioworks (version 3.0, Thermo Fisher Scientific) and then, merged into a single file for Mascot (version 2.2, Matrix Science, Inc.) MS/MS ion search. The precursor ion error tolerance in MS/MS and the neutral loss triggered MS3 modes were 20 ppm and 2 Da, respectively. The MS/MS fragment ion error tolerance was 0.8 Da for both MS/MS and MS3 modes.

In Vivo Ubiquitination Assay. 293T cells were transfected with 5 μg of pEF-Flag-Ub, pEF-GST-p45-Myc, and derivatives of pEF-

GST-p45-Myc, respectively. At 48 h after transfection, the cells were lysed, and GST pull-down experiments were carried out. The GST pull-down samples were analyzed by SDS/PAGE and WB with use of the anti-Flag and anti-Myc antibodies.

Cycloheximide Chase Assay. The cells were treated with 20 μg/mL CHX (Sigma) for various time intervals and then lysed in 2x SDS sample buffer. The samples were analyzed by WB with use of different antibodies.

Northern Blot Analysis. The Northern blot hybridization procedures followed those of Liu et al. (6).

Preparation of Anti-P-p45(Ser157). A keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to residues 141–160 of the mouse p45 with phospho-Ser157 (DSGLSLNYSDAE-pS-LELEGMEAGRRR) was injected into rabbits to raise the anti-phospho-p45(Ser157) antiserum. After the tenth booster injection, the anti-P-p45(Ser157) polyclonal antibody was purified with use of an antigen peptide-coupled affinity column, followed by passage over a nonphosphorylated antigen peptide-coupled affinity column to remove antibodies against the nonphosphorylated p45.

ChIP. The procedures followed those described previously (7, 8). The primers used for PCR were from Mission Biotech: HS2, 5′-CAGCCTTGTGAGCCAGCAT-3′ and 5′-TTCCTCCTAGAGACCCAGATAGCA-3′; ALAS-E, 5′-TCAGAGCCCCAGTGCCTAGA-3′ and 5′-CTGTAGAACCATCCCTCTGACCTT-3′; Actin, 5‘-CGTGCGTGACATCAAAGAGAA-3′ and 5′-ACCGCTCGTTGCCAATAGTG-3′.

Quantitative RT-PCR. RNA was prepared using TRIzol reagent (Invitrogen) and reverse transcribed using oligo-dT primer and SuperScript II Reverse Transcriptase (RT) (Invitrogen) according to standard procedures. Quantitative PCR (qPCR) using the validated TaqMan assays was carried out on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) instrument under default cycling conditions (50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min for 40 cycles). The relative expression levels were determined from a standard curve of serial dilutions of the cDNA samples and then normalized to the β-actin expression levels. The control reactions lacking RT yielded little or no signal.

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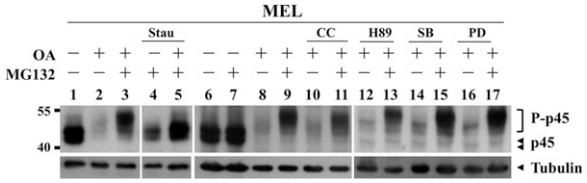

Fig. S1. Identification of the signaling pathway(s) contributes to the turnover of the p45. MEL cells were pretreated with 0.6 μM staurosporine (Stau), 2 μM Calphostin C (CC), 32 μM H89, 4 μM SB203580 (SB), or 50 μM PD98059 (PD) for 30 min before being treated with OA or (OA+MG132) for 2 h. The whole cell extracts (WCE) were then analyzed by WB with anti-p45. Tubulin was used as a loading control.

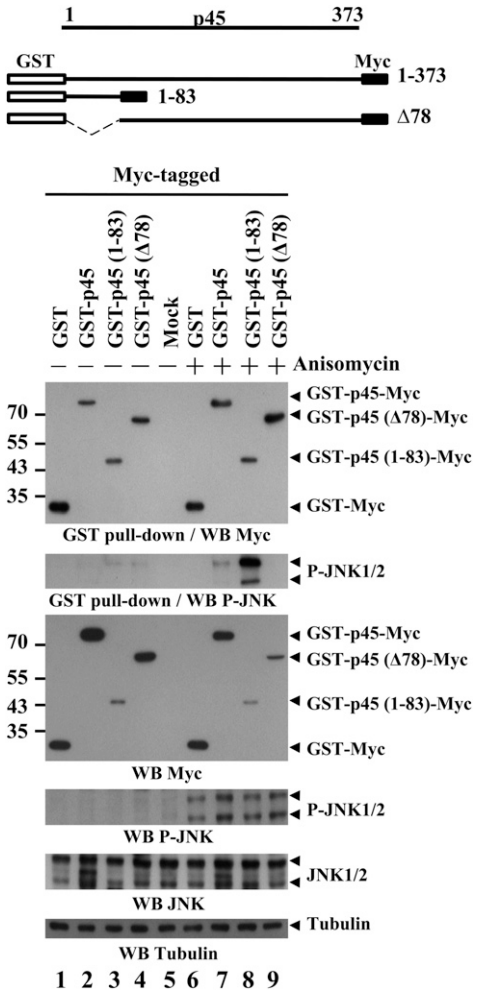

Fig. S2. Interaction between p45 and P-JNK in transfected cells. (*Top*) Maps of the WT and two deletion mutants of p45. 293T cells were transfected with 5 μg each of the plasmids pEF-GST-Myc, pEF-GST-p45-Myc, pEF-GST-p45(1-83)-Myc, and pEF-GST-p45(□78)-Myc, respectively. At 48 h posttransfection, the cells were treated with or without 5 μg/mL of anisomycin for 30 min, and the whole cell extracts were subjected to GST pull-down assay. The top two panels show pull-down samples that were analyzed by WB with use of anti-Myc and anti-P-JNK antibodies, respectively. WBs of the total extracts without GST pull-down assays are shown in the lower four panels. As seen in lanes 7 and 8 of the second panel, Myc-tagged GST-p45 and Myc-tagged GST-p45(1-83) interacted with P-JNK, with the latter interacting much more strongly. On the other hand, the Myc-tagged GST-p45(Δ78) (lane 9) could not interact with P-JNK.

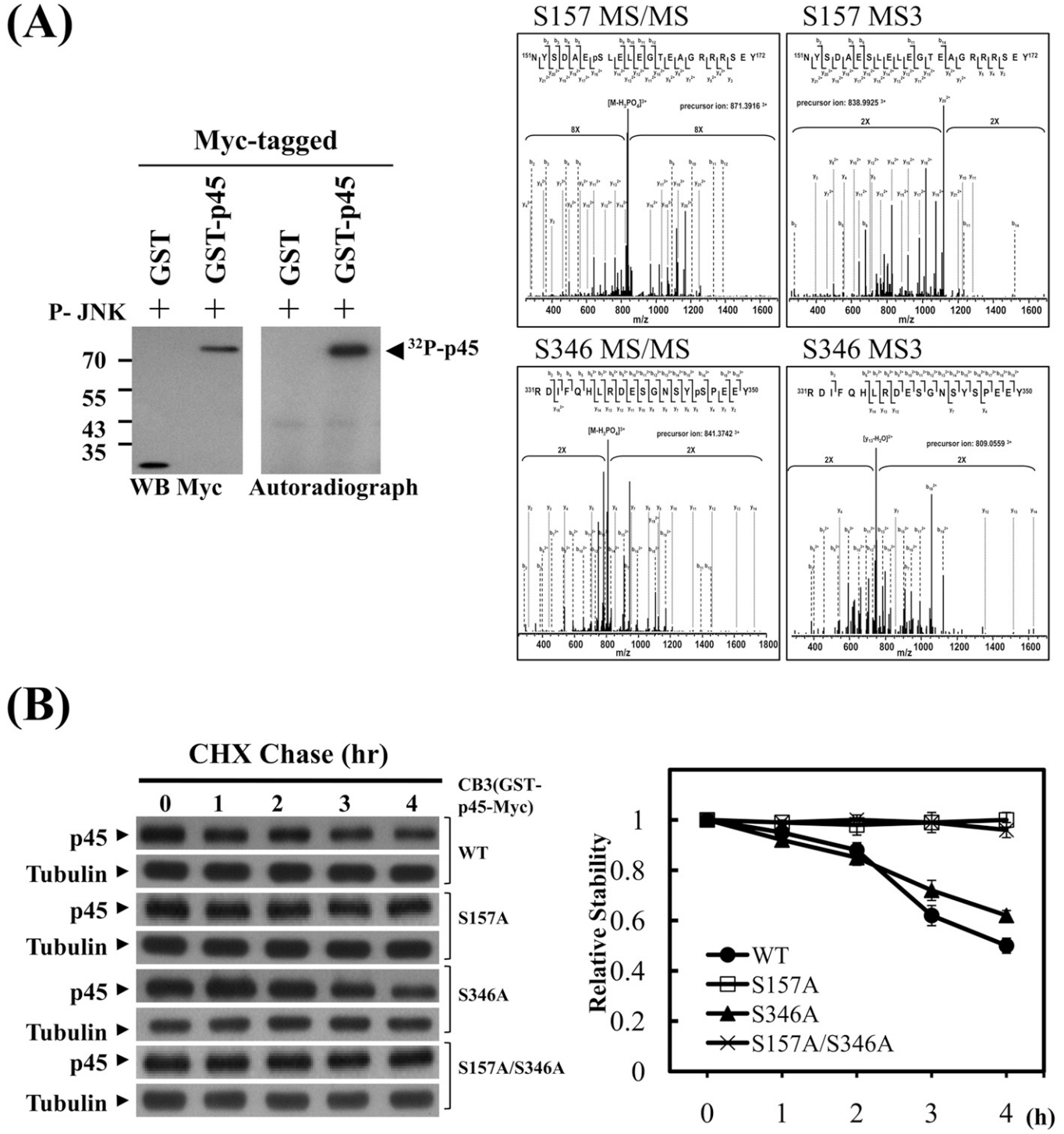

Fig. S3. Phosphorylation of p45 by active JNK. Myc-tagged GST or GST-p45 purified from 293T cells were incubated with the active JNK in in vitro phosphorylation reactions, and the products were analyzed either by WB and autoradiography in the left two panels. Proteomics analysis of phosphorylation sites of p45 was performed by activated JNK. The reaction products of in vitro phosphorylation reaction of GST-p45-Myc were separated by SDS PAGE, and the phosphorylated GST-p45-Myc band was cut from the gel, followed by the in-gel enzymatic digestion, and subjected to analysis by mass spectrometry. (*B*) Half-life measurements of Myc-tagged GST-p45 and its mutant derivatives in transfected CB3 cells. The half-lives of Myc-tagged GST-p45, GST-p45(S157A), GST-p45 (S346A), and GST-p45(S157A/S346A) in transfected CB3 cells were determined by the CHX chase experiments. Total cell lysates were prepared from the cells after their treatment with 50 μg/mL of CHX for 1–4 h and analyzed by WB. The band intensities on the blots (left panels) that were measured by densitometry tracing. The relative mean values of these intensities from two experiments were then calculated and plotted as a function of the time of CHX treatment.

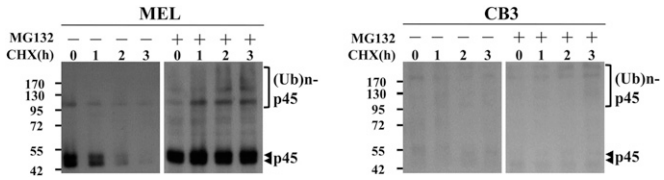

Fig. S4. Level of poly-ubiquitinated p45 in MEL and CB3 cells. MEL and CB3 cells were treated with 25 μg/mL CHX for 1–3 h with or without the presence of 20 μM MG132. WCE were then analyzed by WB with anti-p45 antibody.

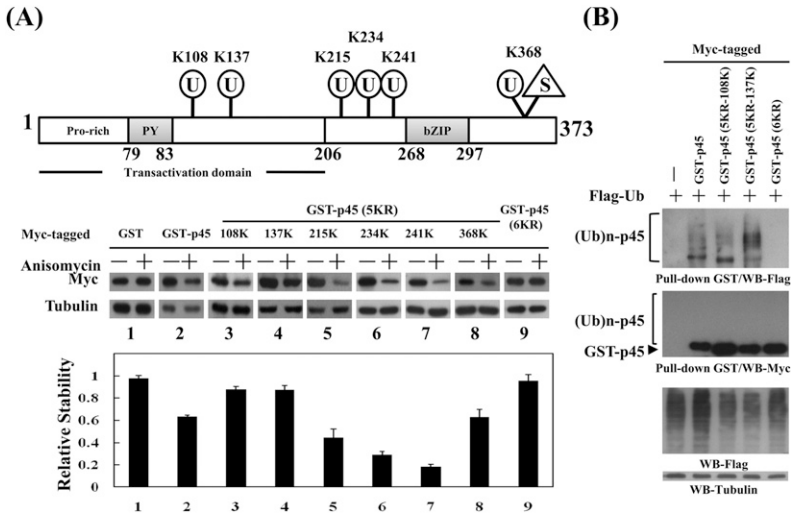

Fig. S5. Mapping of the ubiquitinated lysine residues of p45. (*A*) Mapping of the ubiquitinated lysine residues of p45 as induced by the active JNK. Shown on top is the p45 map with the six lysine (K) residues, the proline (Pro)-rich domain, the PY motif (PPxY), the transactivation domain, and the basic leucine zipper (bZIP) egion indicated. Note that K368 can be poly-ubiqitinated or sumoylated (see text). K562 cells were transfected with 5 μg each of the pEF-based plasmids expressing the different fusion proteins as indicated in the figure. Forty-eight hours after transfection, the cells were treated with 5 μg/mL anisomycin for 2 h. The total cell lysates were then analyzed by WB with anti-Myc or anti-tubulin. The ratios, as averaged from three independent experiments, of the band intensities on WB between samples with and without anisomycin treatment are shown in the lower bar histogram. (*B*) Poly-ubiquitination of the exogenous p45 in transfected 293T cells. Cells cotansfected with pEF-Flag-Ub and one of the four plasmids pEF-GST-p45-Myc, pEF-GST-p45(5KR-108K)-Myc, pEF-GST-p45 (5KR-137K)-Myc, and pEF-GST-p45(6KR)-Myc, were treated with 10 μM of MG132 for 3 h before harvesting at 48 h posttransfection. The GST-fusion proteins were purified by GST pull-down assay and then analyzed by WB with anti-Flag and anti-Myc, respectively (upper two panels). As control, the whole-cell extracts without GST pull-down assays were also analyzed by WB (lower two panels). Note the absence of the (Ub)n-p45 species when all six lysine residues were mutated to arginine, as in the lane of GST-p45(6KR).

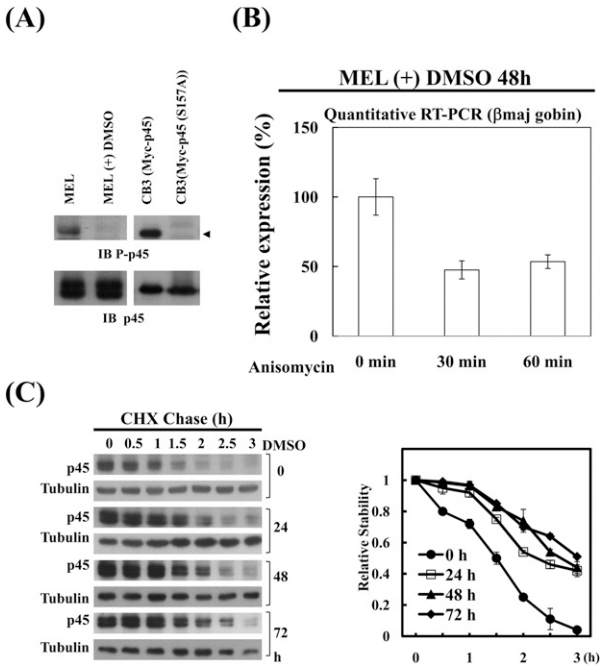

Fig. S6. (*A*) Validation of the in vivo specificity of anti-P-p45(Ser157) antibody or anti-p45. CB3 stable cell lines expressing Myc-p45 and Myc-p45(S157A), respectively, were lysed and analyzed by immunoblotting with the anti-P-p45 (upper panel) and anti-p45 (lower panel) antibodies, respectively. Note that anti-p45 detected the Myc-tagged p45 as well as Myc-p45(S157A) in the two CB3 extracts, whereas use of anti-P-p45 showed no signal in the CB3 [Myc-p45 (S157A)] lane. (*B*) Effect of anisomycin on the level of βmaj mRNA in DMSO-induced MEL cells. Anisomycin was added to MEL cells treated with DMSO for 48 h, and the levels of the βmaj globin RNA at different times (min) after addition of the drug were determined by quantitative RT-PCR. The level at 0 min of anisomycin treatment was taken as 100%. (*C*) Determination of the half-life of p45 in MEL cells during differentiation. The MEL cells were induced by DMSO to differentiate, and the half-life of the endogenous p45 at different hours postinduction was measured by the CHX chase experiments. The WB patterns are shown on the left, and the curves of the relative stabilities are plotted in the right panel. Two independent experiments were carried out.

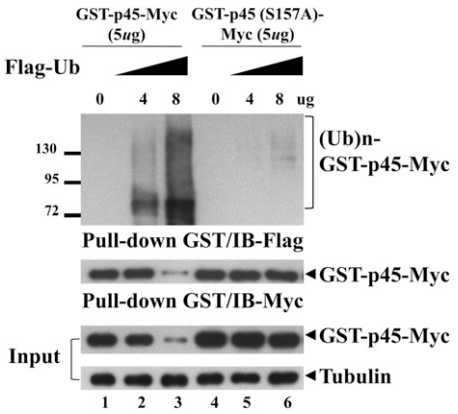

Fig. S7. Ser157 of p45 is important for its poly-ubiquitination. The cells were cotransfected with 5 μg of pEF-GST-p45-Myc (lanes 1–3) or pEF-GST-p45(S157A)-Myc (lanes 4–6) with different amounts (0 μg, 4 μg, and 8 μg) of pEF-Flag-Ub. At 45 h posttransfection, the cells were treated with 10 μM MG132 for 3 h. The extracts were then prepared, and the Myc-tagged GST-fusion proteins were pull-down assayed with use of the GST Sepharose 4B, eluted, and analyzed by WB with anti-Flag (top panel) and anti-Myc (second panel from top) antibodies, respectively. WBs of GST-p45-Myc and tubulin in the whole-cell extracts are shown in the lower two panels. As seen, the level of the mutated form GST-p45(S157A)-Myc (lanes 4, 5, and 6 in the second panel from bottom) is higher than GST-p45-Myc (lanes 1, 2, and 3 in the second panel from bottom), suggesting that the wild-type p45 is more accessible to proteasomal degradation than the mutant p45(S157A). Also, the wild-type GST-p45-Myc molecules consist of more poly-ubiquitinated forms than GST-p45(S157A)-Myc (compare lanes 2 and 3 with lanes 5 and 6 in the top panel).

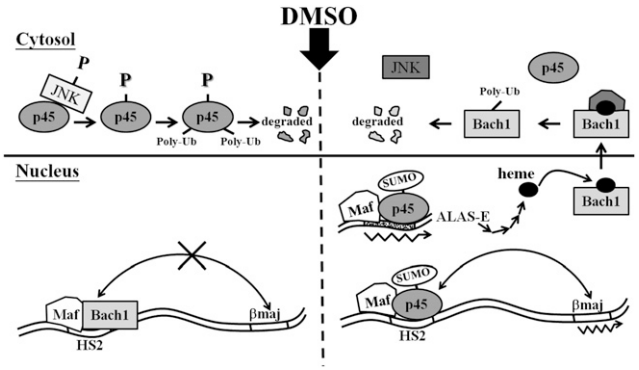

Fig. S8. Homeostatic regulation of gene expression in erythroid cells by P-JNK. A cartoon model is provided for the regulation of the p45 protein turnover in MEL cells, based on the data of this study and the relevant literature. Before DMSO-induced differentiation (Left), the p45 protein is kept at a basal level through its phosphorylation at S157 by P-JNK and consequently, poly-ubiquitination and degradation by the 26S proteasome. However, the Bach1-MafK heterodimer binds at the regulatory regions (e.g., β-LCR) and represses transcription of the erythroid genes (e.g., the βmaj globin). After differentiation, the JNK was inactivated by dephosphorylation. The stabilized p45, presumably sumoylated at K368, binds to and activates promoters of the heme-synthesis genes including ALAS-E. Heme binding to Bach1 causes export of the factor to the cytosol for proteasome degradation. The reciprocal changes of the stabilities of p45 and Bach1, and consequently, their cellular concentrations, leads to replacement of the Bach1-MafK on HS2 of β-LCR and possibly other erythroid regulatory elements and NF-E2, thus turning on the βmaj globin gene transcription. See text for more descriptions of the model.

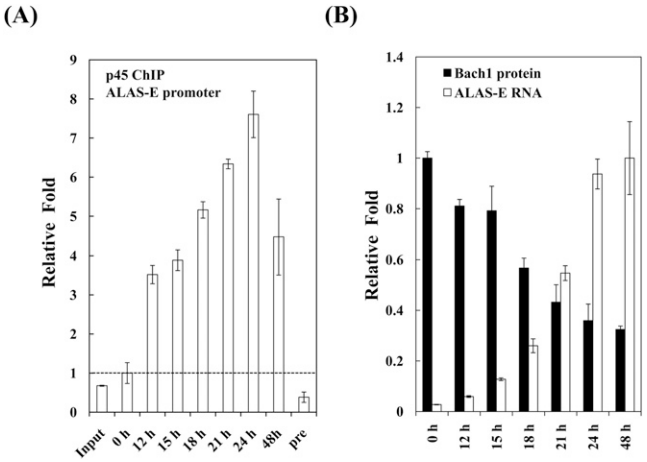

Fig. S9. (*A*) The chromatin binding of p45 at ALAS-E promoter. The ChIP assay was used to examine the chromatin binding of p45 at the ALAS-E promoter in MEL cells at different times after DMSO induction. The DNA samples from ChIP were analyzed by real-time PCR and standardized to the input, preimmune (pre) samples, and β-actin gene. The relative intensities were then calculated with that at 0 h as 1. (*B*) Expression levels of the ALAS-E mRNA and Bach1 protein during DMSO-induced MEL differentiation. The ALAS-E mRNA levels were measured by real-time PCR. That of the GAPDH mRNA was analyzed as the control. The levels of Bach1 protein were analyzed by WB with the anti-Bach1 antibody. The tubulin was used as a loading control for the WB. The relative levels of the ALAS-E mRNA and Bach1 protein at different times of DMSO induction were calculated with the Bach1 protein levels at 0 h as 1 and the ALAS-E mRNA level at 48 h as 1, and these levels are listed in the histogram. Three independent experiments were carried out.