MafB protein stability is regulated by the JNK and ubiquitin-proteasome pathways

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Abstract

MafB is a basic leucine zipper transcription factor that plays important roles in development and differentiation processes. During osteoclastogenesis, its expression is downregulated at the transcriptional level via the JNK and p38 MAP kinase pathways. In the present study, we demonstrated that MafB protein stability is regulated by JNK and identified a phosphorylation site, Thr62. The expression of a constitutively active form of JNK (a fusion protein MKK7α1-JNK1β1) promoted the degradation of MafB in COS7 cells, and a T62A substitution significantly reduced the instability of MafB. The introduction of a four-fold (T58A/T62A/S70A/S74A) substitution in an acidic transcription-activating domain almost protected the instability resulting from the activation of JNK. Furthermore, treatment with proteasome inhibitors increased the MafB level, and a high-molecular-weight smear, characteristic of polyubiquitination, was observed in lysates from cells in which MafB, ubiquitin, and MKK7α1-JNK1β1 were co-expressed. These results suggest that phosphorylation of MafB by JNK confers

susceptibility to proteasomal degradation.

Keywords: JNK; MafB; phosphorylation; proteasome; stability; ubiquitin

Introduction

Maf family proteins are novel transcription factors and share a conserved basic region and leucine zipper (bZIP) motif that mediate DNA binding and dimer formation (Fig. 1A). The Maf group comprises 4 large Maf subfamilies (MafA/L-Maf, MafB/kreisler, c-Maf, and neural retina leucine zipper [NRL]) that contain an acidic transcription-activating domain (TAD) located at their N-terminus and 3 small Maf proteins that contain only the bZIP region (MafF, MafG, and MafK). These factors act as key regulators of terminal differentiation in many tissues such as the bone, brain, kidney, lens, pancreas, retina, and in blood [1]. Moreover, the large Maf proteins have been directly implicated in carcinogenesis, as demonstrated in cell culture, animal models and human cancers [2]. Of these factors, MafB is expressed in a wide variety of tissues [3,4], and gene inactivation studies or mutant analysis of MafB have demonstrated its important roles in the development and differentiation processes of the gonads, hematopoietic system, hindbrain, pancreatic islets, and renal cells [5-11]. Investigation of the molecular mechanisms of gene regulation by MafB is, however, a new undertaking. A recent study reported that MafB was phosphorylated by p38 mitogen-activated protein (MAP) kinase [12] and glycogen synthase kinase 3 (GSK3) [13], and SUMO-1 modification of MafB regulated MafB-driven macrophage differentiation [14]. It was also shown that during osteoclastogenesis, the expression levels of MafB were significantly reduced by the receptor activator of nuclear factor-kB ligand (RANKL). Furtherore, RANKL downregulated MafB expression at least on the transcriptional level via the c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathways [15]. The present study demonstrates that MafB is phosphorylated by JNK and degraded through the ubiquitin-proteasome pathway in COS7 cells.

Materials and Methods

Plasmid construction

Bacterial expression vectors for the synthesis of N-terminal thioredoxin (Trx)·His·S-tagged mouse MafB and human c-Jun (residues 1−79) proteins were obtained by polymerase chain reaction (PCR) and subsequent cloning into the pET32a

vector (Merck). Single and multiple phosphorylation mutants of mouse MafB were generated by PCR-based site-directed mutagenesis [16] using KOD-Plus-DNA polymerase (Toyobo), followed by subcloning and sequencing of the whole reading frame. For mammalian expression vectors for hemagglutinin (HA) epitope-tagged mouse MafB (wild and phosphorylation mutants), a consensus Kozak sequence was introduced at the 5' terminus of MafB using PCR. PCR fragments were cloned into the pcDNA^{TM}3.1/Zeo (-) (Invitrogen) vector. An N-terminal Myc-tagged ubiquitin-encoding construct was generated by PCR-mediated cloning and subcloning into the pcDNA^{TM}3.1/Zeo (-) vector. The plasmids pEF-Flag-MKK7α1-JNK1β1 and pEF-Flag-MKK7α1-JNK1β1 (kinase negative (KN)), which has the Lys434-Lys435 → Met-Met mutation of JNK, were used as the constitutively active form and the kinase-negative form of JNK, respectively [17].

Cell culture and transfections

COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were transfected with the indicated expression plasmids by using the linear 25-kDa polyethylenimine (PEI) "Max" (Polysciences Inc.) as the polycationic vector for nucleic acid delivery, essentially as described by Roseanne et al. (18) in adherent cell condition. For transfection of COS7 cells in a 10-cm plate, 48 μg of PEI and 12 μg of plasmid DNA were used. The cells were harvested after 24-h incubation.

Immunoprecipitation and phosphatase treatment

COS7 cells in a 10-cm plate were transfected with MafB wild or mutant plasmid. Total cell lysates were prepared by lysing cells on ice in 2 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 8.0], 500 mM NaCl, 1% NP-40, 0.5% deoxycholic acid sodium salt, and 0.1% sodium dodecyl sulfate [SDS]) supplemented with protease inhibitors CelLytic^{TM}M (Sigma), phosphatase inhibitors (20 mM sodium *β*-glycerophosphate and 1 mM sodium orthovanadate), and 5 mM *N*-ethylmaleimide followed by brief sonication. Lysates were cleared by centrifugation (20,800 × *g* for 30 min). Because the MafB protein has 2 internal His_6 sequences, MafB proteins were purified by incubating for 2 h at 4℃ with 20 μl of Ni-NTA agarose (Qiagen). The beads were washed 3 times in RIPA buffer and then washed twice in calf intestine alkaline phosphatase (CIAP) buffer (50 mM Tris [pH 8.0]). The beads were resuspended in up to 200 μl of CIAP buffer and 1 mM

phenylmethanesulfonyl fluoride. The reaction was carried out (1 h, 37℃) in a tube containing 10 μl of the beads by the addition of 3 units of CIAP (Toyobo), and it was arrested by the addition of 4× SDS sample buffer. A SDS-polyacrylamide gel electrophoresis (SDS-PAGE) western blot analysis was then performed using horseradish peroxidase (HRP)-conjugated anti-HA rat monoclonal antibody 3F10 (Roche).

In vitro protein kinase assays

According to the manufacture's recommendations, N-terminal thioredoxin (Trx)·His·S-fusion recombinant proteins were purified from *Escherichia coli* BL21 (DE3) extracts by using Ni-NTA agarose. COS7 cells cultured in the 10-cm plate were transiently transfected with pEF-Flag-MKK7α1-JNK1β1 or KN. After a 24-h incubation period following transfection, Flag-tagged kinases were purified from cell extracts as described [19], using the mouse anti-Flag antibody M2 (Sigma) bound to protein G-agarose (Roche). M2 immunoprecipitated Flag-MKK7α1-JNK1β1 or KN was used to phosphorylate 3 μg of Trx·His·S-MafB fusion proteins that carry the indicated substitutions of the putative MAP kinase phosphorylation site. Trx·His·S-c-Jun (1-79) fusion protein was used as a positive control. The kinase reaction was carried out as described [19] at 30℃ for 20 min, and it was arrested by the addition of 4× SDS sample buffer. Samples were analyzed by 8% SDS-PAGE and autoradiography.

Metabolic labeling and pulse-chase experiments

Metabolic labeling and pulse-chase experiments were performed essentially as described in [20]. In brief, 24-h post-transfection cells (24-well plate) were washed and preincubated for 30 min in Met/Cys-free DMEM supplemented with 5% dialyzed FCS, after which they were labeled for 1 h with 2.32 MBq Tran^{35}S-Label No thaw Metabolic Labeling Reagent (MP Biomedical Inc.) in 0.4 ml of the medium per well. The cells were washed and then chased in DMEM supplemented with 10% FCS for 1, 2, or 4 h. They were then homogenized with QIAshredder homogenizer (Qiagen) in RIPA buffer supplemented with protease inhibitors, phosphatase inhibitors, and 5 mM *N*-ethylmaleimide. The lysates were precleared with protein-G agarose (Roche) and incubated overnight at 4℃ with anti-HA mouse monoclonal antibody 12C5 (Roche) and protein-G agarose. The beads were washed 3 times in RIPA buffer. Immunoprecipitates were eluted by boiling in SDS sample buffer. The eluted proteins

were resolved by 7.5% SDS-PAGE, and the gel was dried. The radioactivity in cellular HA-MafB was analyzed using a Fuji BAS1500 imaging analyzer system (Fujifilm).

Proteasomal and lysosomal inhibition

Twenty-four hours after the transfection, the stability of the MafB was analyzed by incubating the cells in a 12-well plate for 6 h in the presence of a proteasome inhibitor (50 μM MG132 [Merck] or 20 μM *clasto*-lactacystin β-lactone (also known as Omuralide) [Merck]) or lysosomal inhibitor (20 mM ammonium chloride [Sigma] or 100 μM chloroquine [Sigma]). After the treatment, the cells were lysed in SDS sample buffer and subjected to 7.5% SDS-PAGE and western blot analysis.

Ubiquitination assay

HA-MafB wild or mutant expression vector was cotransfected into COS7 cells in a 10-cm plate with a Myc-ubiquitin expression vector and kinase expression vectors as indicated in Fig. 6. Cells were lysed 24 h later with RIPA buffer supplemented with protease inhibitors, phosphatase inhibitors, and 5 mM *N*-ethylmaleimide and briefly sonicated. Insoluble materials were removed by centrifugation. The HA-tagged proteins were immunoprecipitated with 3F10-conjugated agarose beads (Roche) for 4 h at 4℃. The beads were washed 3 times with RIPA buffer, boiled in SDS sample buffer, and then subjected to western blot analysis. Myc-tagged ubiquitin conjugates were detected using an HRP-conjugated rabbit anti-Myc antibody (Sigma).

Results and Discussion

MafB is phosphorylated by JNK

Exogenously expressed HA-MafB was detected as a band of approximately 50-kDa by western blot analysis. When the purified HA-MafB protein was treated with CIAP, the mobility of the band shifted to more rapidly migrating forms, indicating that the overexpressed MafB protein was phosphorylated in COS7 cells (Fig. 1B). The MafB protein has 10 putative MAP kinase phosphorylation sites (Ser/Thr-Pro) (Fig. 1A). To examine whether MafB acts as a substrate for JNK and to identify the residue(s) that could be phosphorylated by JNK, 1 of the 10 putative MAP kinase phosphorylation sites was chosen, and 12 MafB mutants carrying substitutions of Ser and Thr residues (conforming to the MAP kinase consensus sites) for Ala were constructed. Trx·His·S-MafB (wild and mutants) fusion recombinant proteins were prepared, and an in vitro protein kinase assay was performed. For the constitutively active form and the

kinase-negative form of JNK, pEF-Flag-MKK7α1-JNK1β1 and KN plasmids, respectively, were used. MafB was phosphorylated by Flag-MKK7α1-JNK1β1 but not KN at the phosphoacceptor site Thr62 (Fig. 1C). To investigate whether MafB is indeed phosphorylated directly by JNK in living cells, the MafB 10A mutant carrying substitutions of Ser and Thr residues of all Ser/Thr-Pro sites for Ala and the 9AT62 mutant (the 10A mutant but carrying Thr62 site) were expressed in COS7 cells together with Flag-MKK7α1-JNK1β1. Western blot analysis indicated that the purified 9AT62 mutant was more immunoreactive for phosphothreonine antibody than the 10A mutant (Fig. 1. D and E). These results indicate that Thr62 site of the MafB protein is phosphorylated by JNK in COS7 cells. In addition, the sequences surrounding mouse MafB Thr62 are strikingly conserved among large Maf proteins (Fig. 2).

Activation of JNK promoted the degradation of MafB in COS7 cells

To examine the effects of JNK activation on MafB in COS7 cells, transfections were performed with HA-MafB and increasing amounts of Flag-MKK7α1-JNK1β1 plasmids. Interestingly, the concentration of HA-MafB decreased owing to the activation of JNK in a dose-dependent manner (Fig. 3. A and B). However, in transient transfection, co-overexpression may alter the expression levels from the different cotransfected plasmid. Therefore, we measured the half-life of HA-MafB to assess whether HA-MafB is modified by a constitutively active form or a kinase-negative form of JNK. Metabolic labeling and pulse-chase experiments showed that ^{35}S-labeled HA-MafB disappeared more rapidly in cells cotransfected with the kinase-active form than in cells cotransfected with the kinase-negative form (Fig. 3. C and D). These results indicate that the rate of MafB protein degradation is regulated by the activation of JNK.

Mutation in the JNK target site on MafB protects the instability of MafB

The effects of mutating the phosphorylation site (conforming to the MAP kinase consensus site) on stability were investigated. HA-MafB wild, HA-MafB carrying a T62A substitution, the 4A mutant (HA-MafB with T58A, T62A, S70A, and S74A substitutions), the 6A mutant (HA-MafB with S14A, T58A, T62A, S70A, S74A, and T103A substitutions), and the 10A mutant were introduced into COS7 cells and subjected to western blot analysis. Fig. 4 shows that the introduction of a mutation (T62A) into the JNK target site on the MafB protein reduced the instability of MafB resulting from the activation of JNK; the introduction of the four-fold substitutions

almost protected the instability, which was similar to the effects of the six-fold and the ten-fold substitutions. These results suggest that the phosphorylation status of the Thr62 site is associated with stability. However, additional phosphorylation status of the Thr58, Ser70, and Ser74 sites may be also needed for enhancing stability.

MafB is degraded by ubiquitin-dependent pathway

Eukaryotic cells contain 2 major systems for protein degradation: the proteasome, an ATP-dependent proteolytic complex that mostly degrades ubiquitinated proteins, and the lysosomal apparatus, a membrane-enclosed vacuole containing multiple acid proteases. To determine whether the degradation of MafB is due to proteasomal or lysosomal activity, we analyzed the effect of 2 proteasome inhibitors (MG132 or *clasto*-lactacystin β-lactone [also known as Omuralide]) and 2 lysosomal inhibitors (ammonium chloride or chloroquine) on the degradation of exogenously expressed HA-MafB protein (Fig. 5. A and B). Both MG132 and Omuralide induced a marked increase in the HA-MafB protein levels in the cells transfected with both a constitutively active and a kinase-negative form of JNK. Neither ammonium chloride nor chloroquine exerted any effect. As a positive control for lysosomal degradation, we assessed the protein levels of beta-site amyloid precursor protein cleaving enzyme (BACE), a protein known to be degraded by the lysosome [21]. Treatment with lysosomal inhibitors resulted in an increase in BACE protein levels (data not shown). Under these conditions, the amount of actin was constant. These results suggest that MafB is degraded by the proteasome. To examine the relationship between exogenously expressed HA-MafB protein stability and phosphorylation status in COS7 cells, COS7 cells were transfected with plasmids expressing HA-MafB wild or T62A mutant and Flag-MKK7α1-JNK1β1 or KN and treated with or without MG132. Western blot analysis indicates that MG132 slightly reduced the instability of the T62A mutant owing to the activation of JNK, but not statistically significant. These results suggest that the phosphorylation status of the Thr62 site is associated with the degradation caused by the proteasome.

An important component of proteasome-mediated degradation is the proper targeting of the protein to be degraded by the ubiquitin conjugation complex. This process results in the attachment of multiple ubiquitin chains to the target protein. To determine whether MafB was ubiquitinated, HA-MafB was expressed in COS7 cells with Myc-ubiquitin and Flag-MKK7α1-JNK1β1 and purified from the

HA-MafB-containing cell extracts using anti-HA-agarose. A high-molecular-weight smear, characteristic of polyubiquitination, was observed in lysates from cells in which HA-MafB, Myc-ubiquitin, and Flag-MKK7α1-JNK1β1 were co-expressed, demonstrating that MafB is degraded by the ubiquitin-proteasome pathway (Fig. 6). A high-molecular-weight smear was also observed in lysates from cells in which the T62A mutant, Myc-ubiquitin and Flag-MKK7α1-JNK1β1 were co-expressed. However, this smear was weaker than that of HA-MafB wild, and the input of the T62A mutant had approximately 4 times more HA-MafB protein than that of HA-MafB wild (compared input lanes 3 with 5). Thus, the overexpressed T62A mutant is less sensitive to proteasomal degradation owing to the activation of JNK.

Some regulatory proteins have been reported to be ubiquitinated depending on their phosphorylation status [22,23]. Several previous studies have reported post-translational processes in the regulation of MafA, which is a member of the large Maf family of proteins. For example, quail and chicken MafA were shown to be phosphorylated by the extracellular signal-regulated kinase (ERK), and the phosphorylation status of MafA influenced the transactivation potential, and the phosphorylation also induced the degradation of MafA by the proteasome [24,25]. In addition, p38 MAP kinase was shown to be a major regulator of mouse MafA protein stability under both basal and high-glucose conditions as well as during oxidative stress [26]. Another study showed quail and mouse MafA to be constitutively phosphorylated by GSK3, and the phosphorylation induced ubiquitination and degradation of MafA [13,27]. Furthermore, SUMO-1 and SUMO-2 modifications of MafA have been shown to reduce the transcriptional activity of MafA in pancreatic β-cells [28].

In the present study, it was found that MafB was phosphorylated by JNK and a phosphorylation site, Thr62, was identified in TAD. Activation of JNK promoted the degradation of MafB in COS7 cells, while the introduction of a mutation into the JNK target site on MafB significantly reduced the instability of MafB. This degradation occurred via the ubiquitin-proteasome pathway. However we cannot exclude indirect effects of JNK-mediated MafB degradation such as destabilizing factors induced or activated by JNK. Short motifs that mediate phosphorylation-dependent recognition by an E3 ubiquitin ligase are known as phosphodegrons. The degron is generally found within the TAD of transcription factors [29]. This is in accordance with our result. Because introduction of a mutation into the Thr62 site on the MafB protein partially

protected the instability of MafB and the phosphorylation status of the Thr58, Ser70, and Ser74 sites was also associated with the stability, E3 ubiquitin ligase(s) induced or activated by JNK may recognize these phosphodegrons.

We attempted to observe the effect of Itch in one of the E3 ubiquitin ligases activated by JNK [30] on the stability of MafB. Exogenously expressed Itch did not enhance the destabilization of MafB by the activation of JNK (data not shown). We also investigated whether MafB phosphorylation affects its transcriptional activity. However, because MafB was degraded by the activation of JNK, it was not apparent whether MafB phosphorylation affects its transcriptional activity. Expression levels of MafB are significantly reduced by RANKL during osteoclastogenesis at the mRNA and protein levels, and RANKL downregulates MafB expression via the JNK and p38 MAP kinase pathways [16]. During osteoclastogenesis, RANKL may regulate MafB expression via the ubiquitin-proteasome pathway.

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References

[1] Y. Yang, A. Cvekl, Large Maf Transcription Factors: Cousins of AP-1 Proteins and Important Regulators of Cellular Differentiation, Einstein J. Biol. Med. 23 (2007) 2-11.

[2] A. Eychene, N. Rocques, C. Pouponnot, A new MAFia in cancer, Nature Rev. Cancer 8 (2008) 683-693.

[3] K. Kataoka, K.T. Fujiwara, M. Noda, M. Nishizawa, MafB, a new Maf family transcription activator that can associate with Maf and Fos but not with Jun, Mol. Cell. Biol. 14 (1994) 7581-7591.

[4] P.W. Wang, J.D. Eisenbart, S.P. Cordes, G.S. Barsh, M. Stoffel, M.M. Le Beau, Human KRML (MAFB): cDNA cloning, genomic structure, and evaluation as a

candidate tumor suppressor gene in myeloid leukemias, Genomics 59 (1999) 275-281.
[5] M.A. Li, J.D. Alls, R.M. Avancini, K. Koo, D. Godt, The large Maf factor Traffic Jam controls gonad morphogenesis in Drosophila, Nat. Cell Biol. 5 (2003) 994-1000.
[6] B. Blanchi, L.M. Kelly, J.C. Viemari, I. Lafon, H. Burnet, M. Bévengut, S. Tillmanns, L. Daniel, T. Graf, G. Hilaire, M.H. Sieweke, MafB deficiency causes defective respiratory rhythmogenesis and fatal central apnea at birth, Nat. Neurosci. 6 (2003) 1091-1100.
[7] S.P. Cordes, G.S. Barsh, The mouse segmentation gene kr encodes a novel basic domain-leucine zipper transcription factor, Cell 79 (1994) 1025-1034.
[8] V. Sadl, F. Jin, J. Yu, S. Cui, D. Holmyard, S. Quaggin, G. Barsh, S. Cordes, The mouse Kreisler (Krml1/MafB) segmentation gene is required for differentiation of glomerular visceral epithelial cells, Dev. Biol. 249 (2002) 16-29.
[9] T. Moriguchi, M. Hamada, N. Morito, T. Terunuma, K. Hasegawa, C. Zhang, T. Yokomizu, R. Esaki, E. Kuroda, K. Yoh, T. Kudo, M. Nagata, D.R. Greaves, J.D. Engel, M. Yamamoto, S. Takahashi, MafB is essential for renal development and F4/80 expression in macrophages, Mol. Cell. Biol. 26 (2006) 5715-5727.
[10] A. Aziz, L. Vanhille, P. Mohideen, L.M. Kelly, C. Otto, Y. Bakri, N. Mossadegh, S. Sarrazin, M.H. Sieweke, Development of macrophages with altered actin organization in the absence of MafB, Mol. Cell. Biol. 26 (2006) 6808-6818.
[11] I. Artner, B. Blanchi, J.C. Raum, M. Guo, T. Kaneko, S. Cordes, M. Sieweke, R. Stein, MafB is required for islet beta cell maturation, Proc. Natl. Acad. Sci. USA 104 (2007) 1043853-3858.
[12] K. Sii-Felice, C. Pouponnot, S. Gillet, L. Lecoin, J.-A. Girault, A. Eychene, M.-P. Felder-Schmittbuhl, MafA transcription factor is phosphorylated by p38 MAP kinase, FEBS lett. 579 (2005) 3547-3554.
[13] N. Rocques, N.A. Zeid, K. Sii-Felice, L. Lecoin, M.-P. Felder-Schmittbuhl, A. Eychene, C. Pouponnot, GSK-3-mediated phosphorylation enhances Maf-transforming activity, Mol. Cell 28 (2007) 584-597.
[14] S. Tillmanns, C. Otto, E. Jaffray, C. Du Roure, Y. Bakri, L. Vanhille, S. Sarrazin, R.T. Hay, M.H. Sieweke, SUMO modification regulates MafB-driven macrophage differentiation by enabling Myb-dependent transcriptional repression, Mol. Cell. Biol. 27 (2007) 5554-5564.
[15] K. Kim, J.H. Kim, J. Lee, H.M. Jin, H. Kook, K.K. Kim, S.Y. Lee, N. Kim, MafB

negatively regulates RANKL-mediated osteoclast differentiation, Blood 109 (2007) 3253-3259.
[16] M.P. Weiner, G.L. Costa, W. Schoettlin, J. Cline, E. Mathur, J.C. Bauer, Site-directed mutagenesis of double-stranded DNA by the polymerase chain reaction, Gene 151 (1994) 119-123.
[17] F. Tsuruta, J. Sunayama, Y. Mori, S. Hattori, S. Shimizu, Y. Tsujimoto, K. Yoshioka, N. Masuyama, Y. Gotoh, JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins, EMBO J. 23 (2004) 1889-1899.
[18] T. Roseanne, L. Bisson, D. Yves, Transfection of HEK293-EBNA1 cells in suspension with linear PEI for production of recombinant proteins, Cold Spring Harb. Protoc., 2008, doi:10.1101/pdb.prot4977.
[19] B. Derijard, M. Hibi, I.-H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, R.J. Davis, JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain, Cell 76 (1994) 1025-1037.
[20] H. Tanahashi, T. Tabira, Three novel alternatively spliced isoforms of the human beta-site amyloid precursor protein cleaving enzyme (BACE) and their effect on amyloid beta-peptide production, Neurosci. lett. 307 (2001) 9-12.
[21] Y.H. Koh, C.A.F. von Arnim, B.T. Hyman, R.E. Tanzi, G. Tesco, BACE is degraded via the lysosomal pathway, J. Biol. Chem. 280 (2005) 32499-32504.
[22] M. Gao, M. Karin, Regulating the regulators: control of protein ubiquitination and ubiquitin-like modifications by extracellular stimuli, Mol. Cell 19 (2005) 581-593.
[23] T. Hunter, The age of crosstalk: Phosphorylation, Ubiquitination, and Beyond, Mol. Cell 28 (2007) 730-738.
[24] S. Benkhelifa, S. Provot, E. Nabais, A. Eychene, G. Calothy, M.-P. Felder-Schmittbuhl, Phosphorylation of MafA is essential for its transcriptional and biological properties, Mol. Cell. Biol. 21 (2001) 4441-4452.
[25] H. Ochi, H. Ogino, Y. Kageyama, K. Yasuda, the stability of the lens-specific Maf protein is regulated by fibroblast growth factor (FGF)/ERK signaling in lens fiber differentiation, J. Biol. Chem. 278 (2003) 537-544.
[26] T. Kondo, I.El Khattabi, W. Nishimura, D.R. Laybutt, P. Geraldes, S. Shah, G. King, S. Bonner-Weir, G. Weir, A. Sharma, p38 MAPK is a major regulator of MafA protein stability under oxidative stress, Mol. Endocrinol. 23 (2009) 1281-1290.
[27] S.-i. Han, S. Aramata, K. Yasuda, K. Kataoka, MafA stability in pancreatic β cells

is regulated by glucose and is dependent on its constitutive phosphorylation at multiple sites by glycogen synthase kinase 3, Mol. Cell. Biol. 27 (2007) 6593-6605.
[28] C. Shao, M.H. Cobb, Sumoylation regulates the transcriptional activity of MafA in pancreatic β cells, J. Biol. Chem. 284 (2009) 3117-3124.
[29] M. Muratani, W.P. Tansey, How the ubiquitin-proteasome system controls transcription, Nat. Rev. Mol. Cell Biol., 4 (2003) 192-201.
[30] M. Gao, T. Labuda, Y. Xia, E. Gallagher, D. Fang, Y.-C. Liu. M. Karin, Jun turnover is controlled through JNK-dependent phosphorylation of the E3 ligase Itch, Science, 306 (2004) 271-275.
[31] K. Igarashi, K. Kataoka, N. Itoh, N. Hayashi, M. Nishizawa, M. Yamamoto, Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins, Nature 367 (1994) 568-572.

Legends to Figures.

Fig. 1. MafB Thr62 is phosphorylated by JNK. (A) Schematic representation of mouse MafB. The putative MAP kinase phosphorylation sites (S/T-P) are indicated on MafB structure (TAD: transcription activation domain, H: histidine repeat [His_8, His_6]). (B) Phosphatase treatment. Purified HA-MafB wild or the 10A mutant (HA-MafB with S14A, T58A, T62A, S70A, S74A, T103A, S170A, S178A, S315A, and S318A substitutions) were treated with or without CIAP and subjected to SDS-PAGE and western blot analysis using HRP-conjugated anti-HA antibody 3F10. (C) In vitro protein kinase assays. Either Flag-MKK7α1-JNK1β1 (a constitutively active form) or Flag-MKK7α1-JNK1β1 (KN) (a kinase-negative form) was used to phosphorylate Trx·His·S-MafB fusion proteins carrying the indicated substitutions of the putative MAP kinase phosphorylation site. Trx·His·S-c-Jun (1-79) fusion protein was used as a positive control. Samples were analyzed by SDS-PAGE and autoradiography. Arrow: Trx·His·S-MafB fusion protein. Arrow head: Trx·His·S-c-Jun (1-79) fusion protein. (D) Phosphorylation of the MafB T62 site in COS7 cells. The 9AT62 mutant (HA-MafB with S14A, T58A, S70A, S74A, T103A, S170A, S178A, S315A, and S318A substitutions) or the 10A mutant was co-expressed with Flag-MKK7α1-JNK1β1 or KN in COS7 cells. Twenty-four hours after the transfection, the cells were incubated for 6 h in the presence of 50 μM MG132 to protect HA-MafB from proteasomal degradation. Each HA-MafB mutant was purified by Ni-NTA agarose and subjected to SDS-PAGE

and western blot analysis using anti-phospho-Thr mouse monoclonal antibody PTR-8 (BioMakor). Inputs were also examined by western blot analysis using HRP-conjugated 3F10, anti-phospho-c-Jun (Ser63) II (Cell Signaling Technology), and anti-actin (Sigma) antibodies. (E) The amounts of phospho-Thr HA-MafB. Western blots were visualized and quantitatively analyzed using the Densitograph AE-6930 Lumino CCD and Lane analyzer 10H (ATTO). The values of HA-MafB with phospho-Thr were normalized to HA-MafB. The value of the KN-transfected cells in each independent experiment couple was set as 100%. The results are expressed as mean ± S.D. of 3 independent experiments. *$P < 0.05$; NS, nonsignificant ($P > 0.05$) compared with the KN-transfected cells (paired *t*-test).

Fig. 2. Sequence comparison of the TAD of large Maf proteins surrounding Thr62. Asterisks represent amino acid residues that are conserved with respect to mouse MafB protein. S/T-P sites are underlined. Both chicken and quail MafA are the same as mouse MafA except for P47A, T75G, and G76Q substitutions.

Fig. 3. Activation of JNK-promoted degradation of MafB. (A) COS7 cells in a 12-well plate were transfected with pcDNA-HA-MafB plasmids (0.25 μg) and increasing amounts of pEF-Flag-MKK7α1-JNK1β1 plasmids (0, 0.03, 0.08, 0.25 and 0.75 μg). Empty pEF-Flag vector was used to equalize the total amount of transfected DNA (1 μg). Western blot analysis was performed using HRP-conjugated 3F10, anti-phospho-c-Jun, and anti-actin antibodies. (B) The amounts of HA-MafB. Western blots were visualized and quantitatively analyzed. The values were normalized to the level of actin in the same sample. The value of the pEF-Flag-MKK7α1-JNK1β1 (0 μg)-transfected cells in each independent experiment was set as 100%. The results are expressed as mean ± S.D. of 3 independent experiments. (C) Turnover of HA-MafB. COS7 cells transiently expressing HA-MafB with Flag-MKK7α1-JNK1β1 or KN were metabolically labeled for 1 h and chased for the indicated time points. Cells were then lysed and immunoprecipitated with anti-HA mouse monoclonal antibody 12C5. (D) The radioactivity of HA-MafB. The results are expressed as percentages (± S.D., $n = 2$) of HA-MafB radioactivity at the 0-h time point.

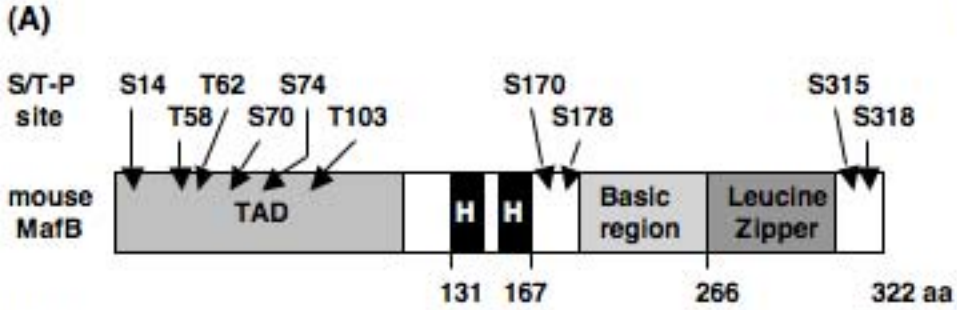
Fig. 4. Effect of mutation in the phosphorylation site on the stability of MafB. (A) COS7 cells in a 12-well plate were transfected with the plasmid for expressing HA-MafB wild or mutants carrying the indicated substitution(s) (0.4 μg), and either Flag-MKK7α1-JNK1β1 or KN (0.6 μg). Western blot analysis was performed using

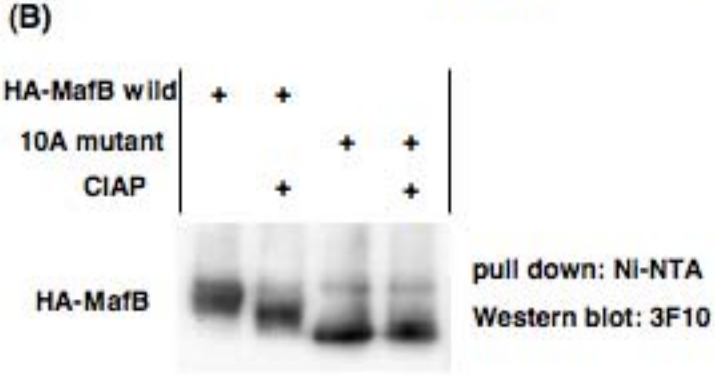
HRP-conjugated 3F10, anti-phospho-c-Jun, and anti-actin antibodies. T62A (HA-MafB with T62A substitution), 4A mutant (HA-MafB with T58A, T62A, S70A, and S74A substitutions), 6A mutant (HA-MafB with S14A, T58A, T62A, S70A, S74A, and T103A substitutions), 10A mutant (HA-MafB with S14A, T58A, T62A, S70A, S74A, T103A, S170A, S178A, S315A, and S318A substitutions) (B) The amounts of HA-MafB. Western blots were visualized and quantitatively analyzed. The values were normalized to the level of actin in the same sample. The value of the KN-transfected cells in each independent experiment couple was set as 100%. The results are expressed as mean ± S.D. of 3 independent experiments *$P < 0.05$; NS, nonsignificant ($P > 0.05$) compared with the KN-transfected cells (paired *t*-test).

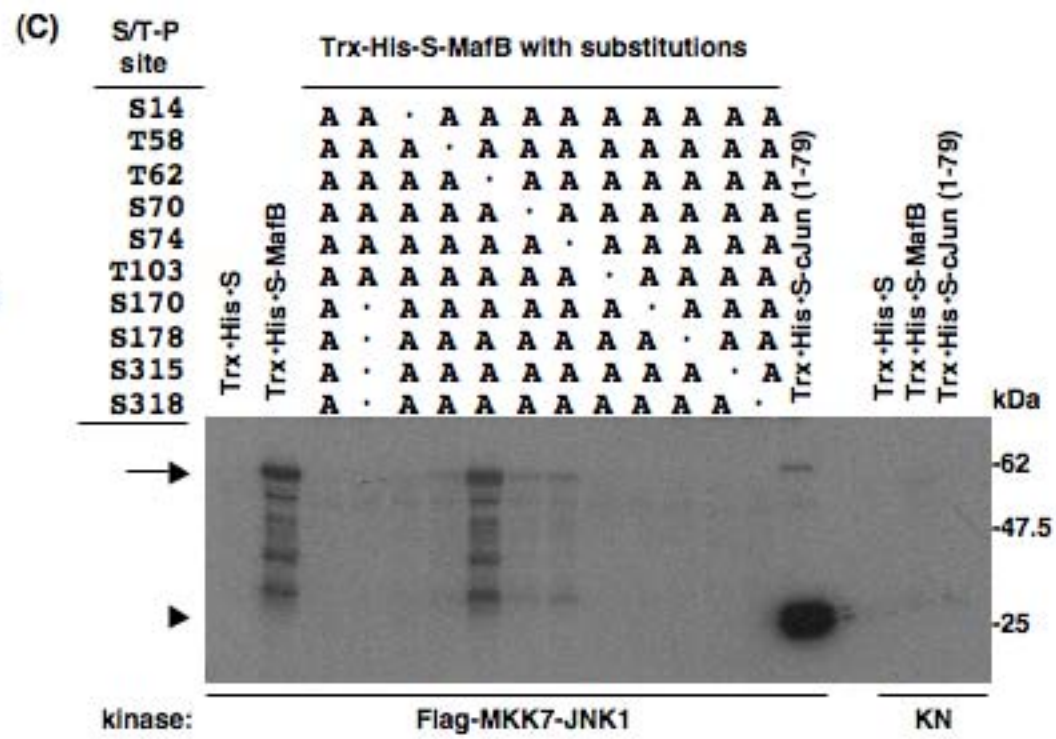
Fig. 5. MafB is degraded by the ubiquitin-proteasome pathway. (A) Treatment of proteasome and lysosomal degradation inhibitors. COS7 cells in a 12-well plate were transfected with pcDNA-HA-MafB (0.4 μg) and/or either pEF-Flag-MKK7α1-JNK1β1 or KN (each 0.6 μg). Twenty-four hours after the transfection, the cells were incubated for 6 h in the presence of a proteasome inhibitor (MG132 or Omuralide), or a lysosomal inhibitor (ammonium chloride or chloroquine). Western blot analysis was performed using HRP-conjugated 3F10, anti-phospho-c-Jun, and anti-actin antibodies. (B) The amounts of HA-MafB. Western blots were visualized and quantitatively analyzed. The values were normalized to the level of actin in the same sample. The value of the vehicle (DMSO) only-treated cells in each independent experiment couple was set as 100%. The results are expressed as mean ± S.D. of 3 independent experiments *$P < 0.05$; NS, nonsignificant ($P > 0.05$) compared with the DMSO only-treated cells (paired *t*-test). (C) Effect of MG132 on T62A mutant. COS7 cells were transfected with plasmids expressing HA-MafB wild or T62A mutant and either Flag-MKK7α1-JNK1β1 or KN. Twenty-four hours after the transfection, the cells were incubated for 6 h in the presence or absence of MG132. Western blot analysis was performed using HRP-conjugated 3F10, anti-phospho-c-Jun, and anti-actin antibodies. (D) The amounts of HA-MafB. Western blots were visualized and quantitatively analyzed. The values were normalized to the level of actin in the same sample. The value of the DMSO only-treated cells in each independent experiment couple was set as 100%. The results are expressed as mean ± S.D. of 3 independent experiments *$P < 0.05$; NS, nonsignificant ($P > 0.05$) compared with the DMSO only-treated cells (paired *t*-test).

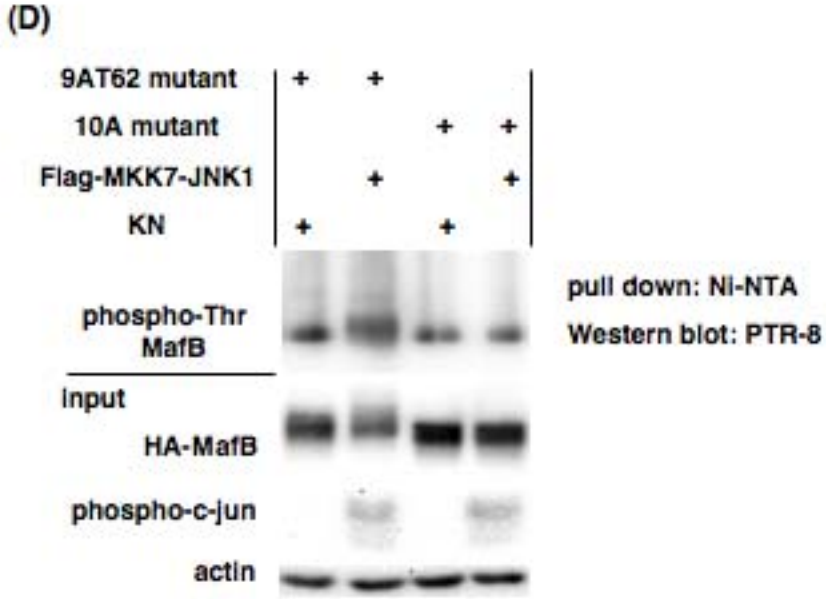
Fig. 6. Ubiquitination assay. COS7 cells in a 10-cm plate were transfected with plasmid

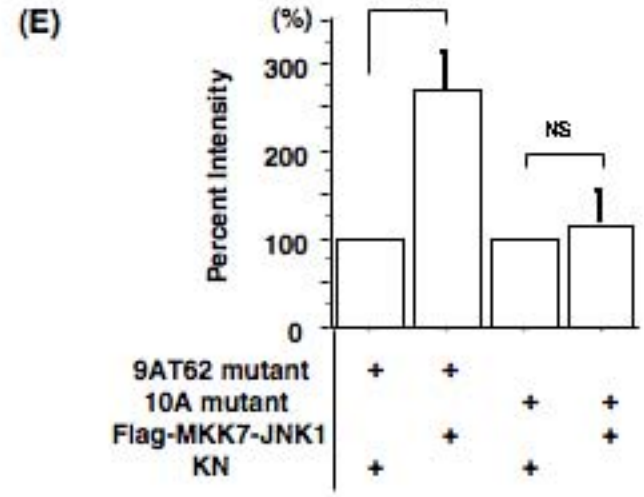
for expressing HA-MafB wild or the mutant (each 3 μg), either Flag-MKK7α1-JNK1β1 or KN (each 4.5 μg), and/or Myc-ubiquitin (each 4.5 μg). Empty pcDNA™3.1/Zeo (-) vector was used to equalize the total amount of transfected DNA (12 μg). Cell lysates were immunoprecipitated with the 3F10 conjugated agarose beads. The beads were washed and subjected to western blot analysis. Myc-tagged ubiquitin conjugates were detected using an HRP-conjugated anti-Myc antibody. Cell lysates were also analyzed by western blot analysis using HRP-conjugated 3F10, anti-phospho-c-Jun, and anti-actin antibodies. A bracket indicates the polyubiquitinated HA-MafB.

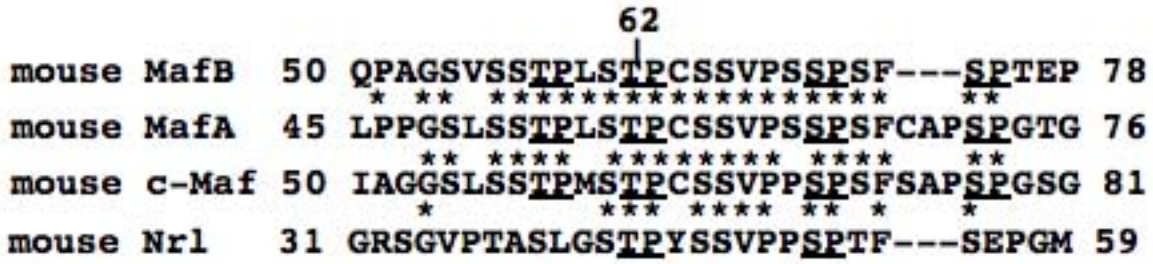

(A)
S/T-P site
S14
T58
T62
S70
S74
T103
S170
S178
S315
S318
mouse MafB
TAD
H
H
Basic region
Leucine Zipper
131
167
266
322 aa

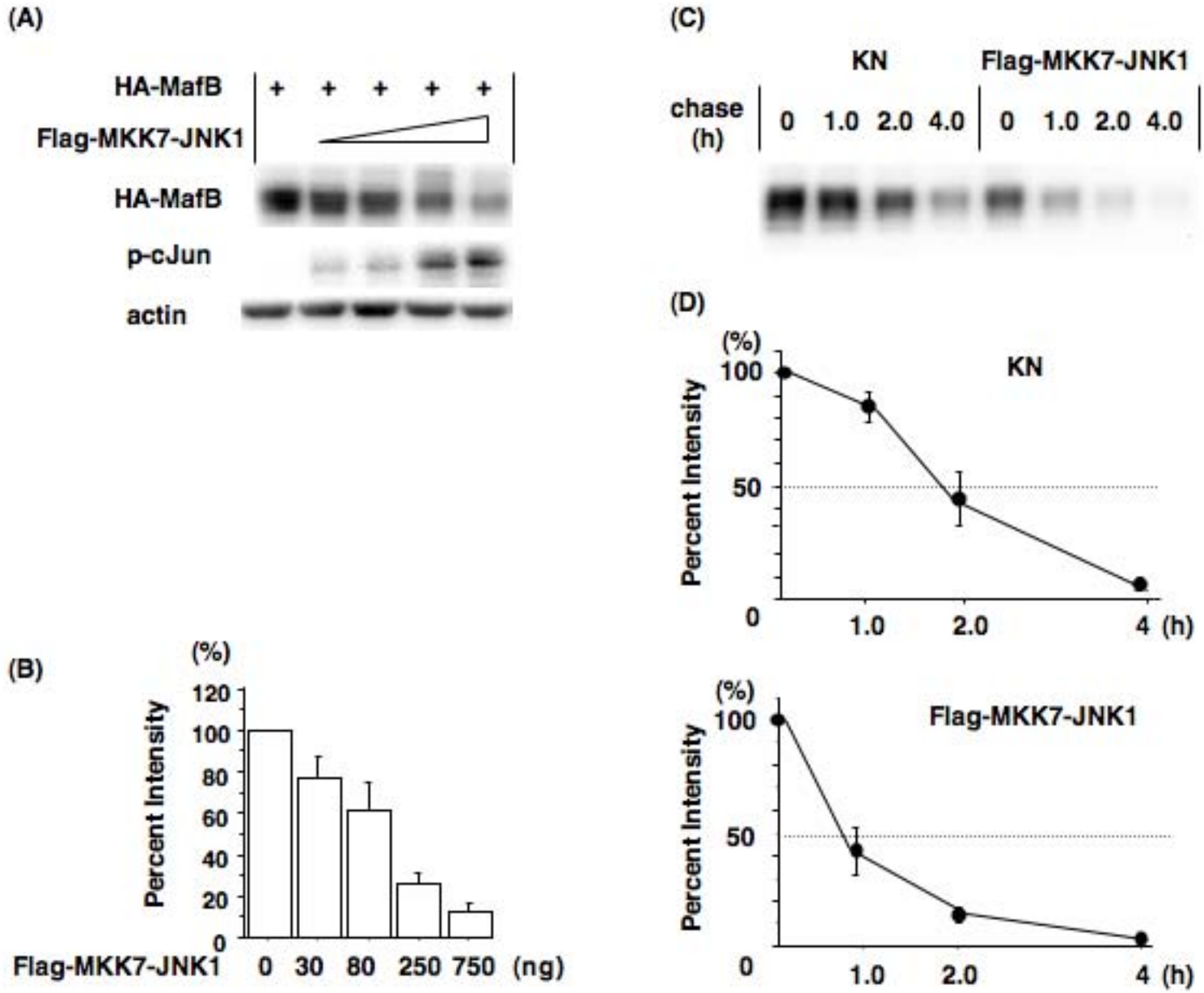

(B)
HA-MafB wild
10A mutant
CIAP
HA-MafB
pull down: Ni-NTA
Western blot: 3F10

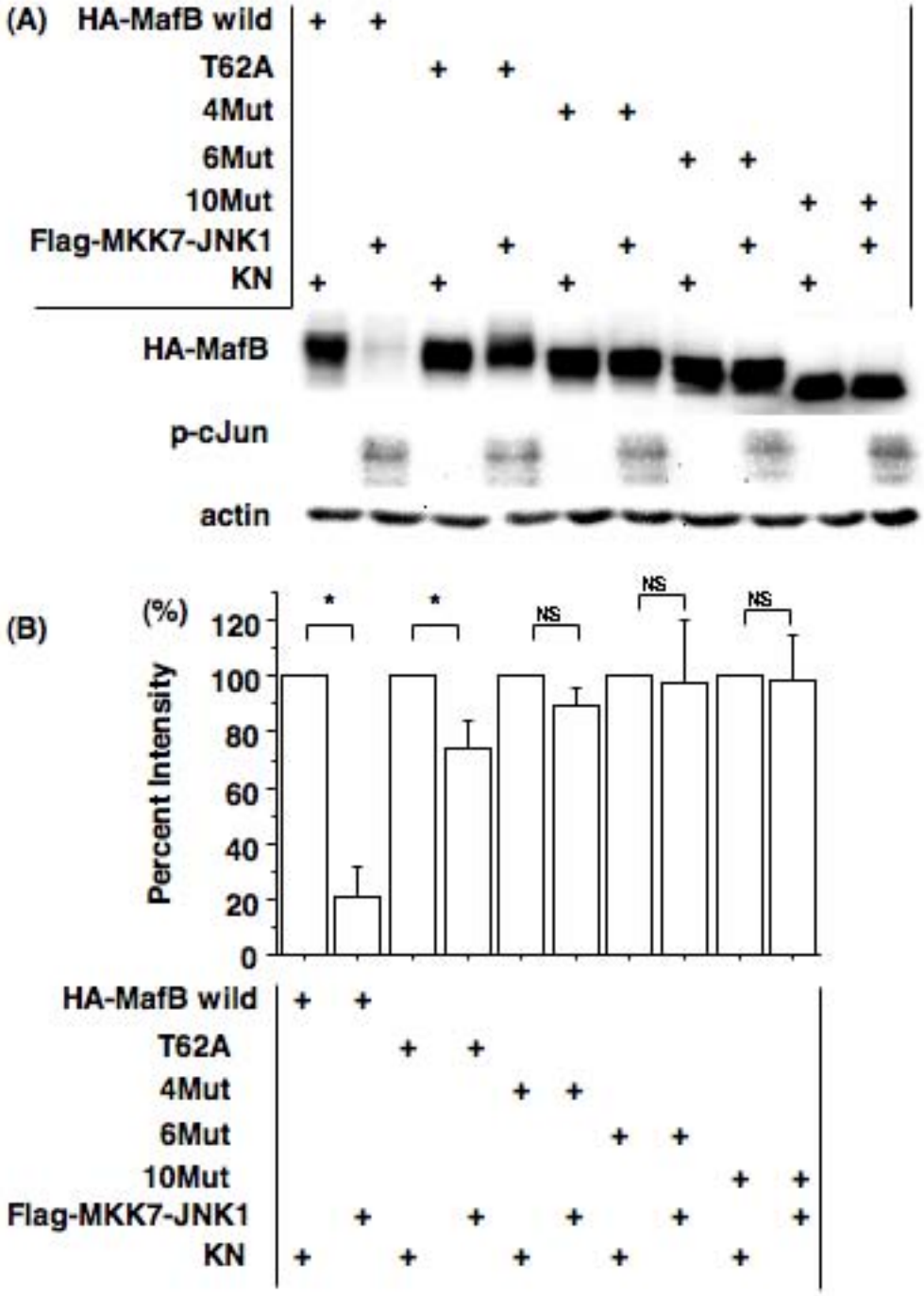

(C)
S/T-P site
Trx-His-S-MafB with substitutions
S14
T58
T62
S70
S74
T103
S170
S178
S315
S318
Trx-His-S
Trx-His-S-MafB
Trx-His-S-cJun (1-79)
Trx-His-S
Trx-His-S-MafB
Trx-His-S-cJun (1-79)
kDa
62
47.5
25
kinase:
Flag-MKK7-JNK1
KN

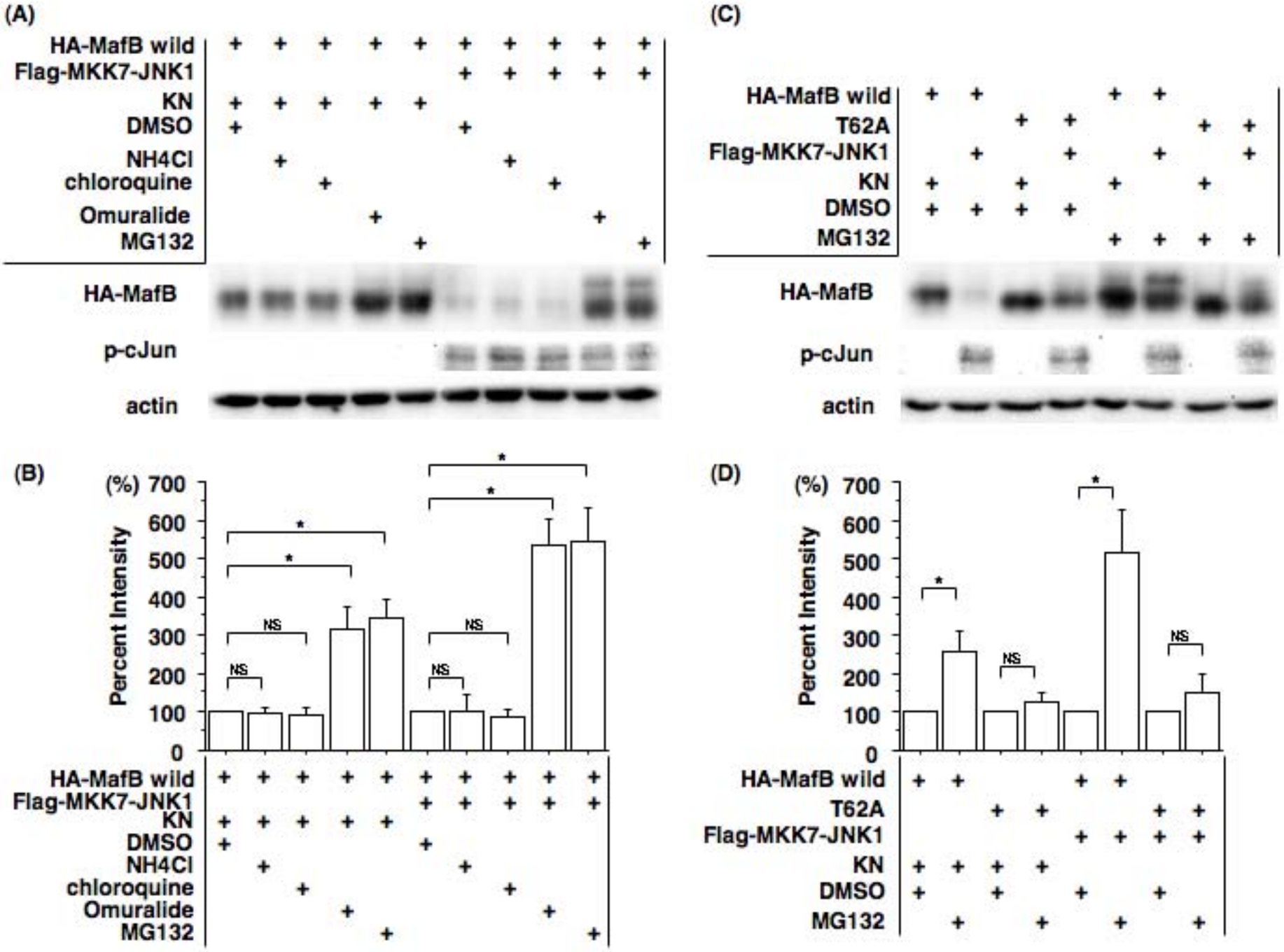

(D)
9AT62 mutant
10A mutant
Flag-MKK7-JNK1
KN
phospho-Thr MafB
pull down: Ni-NTA
Western blot: PTR-8
input
HA-MafB
phospho-c-jun
actin

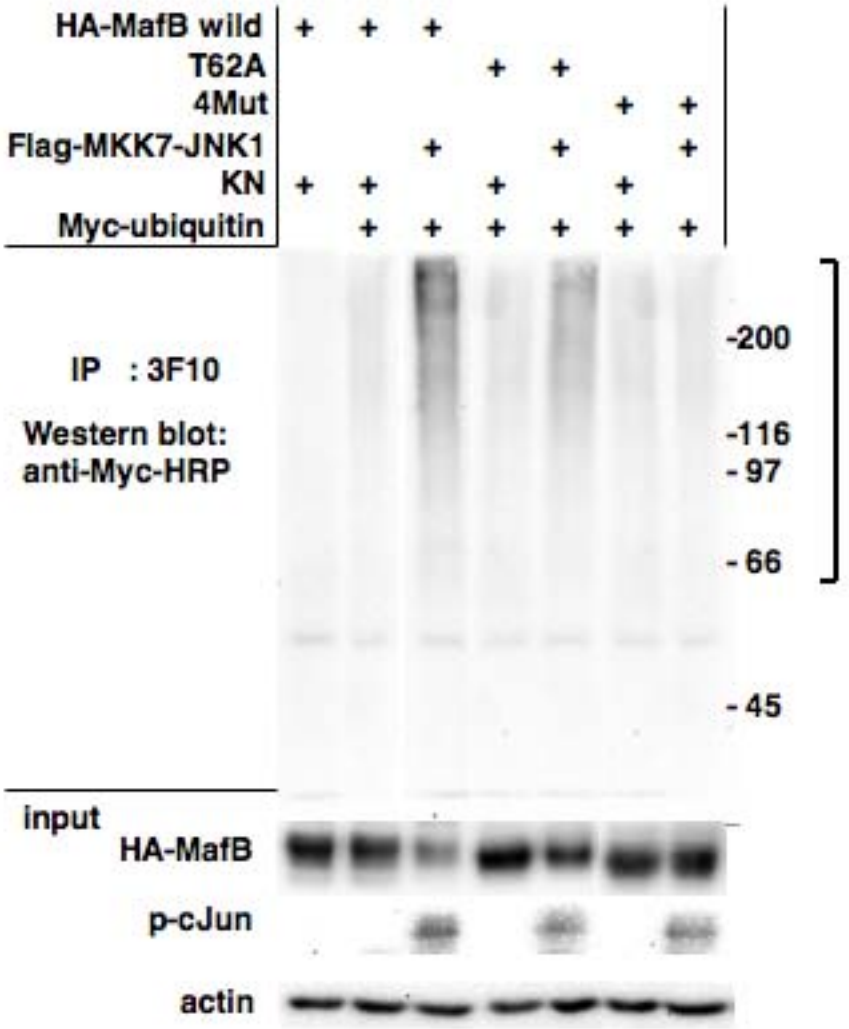

(E)
Percent Intensity
(%)
300
200
100
0
*
NS
9AT62 mutant
10A mutant
Flag-MKK7-JNK1
KN


62
mouse MafB 50 QPAGSVSSTPLSTPCSSVPSSPSF---SPTEP 78
mouse MafA 45 LPPGSLSSTPLSTPCSSVPSSPSFCAPSPGTG 76
mouse c-Maf 50 IAGGSLSSTPMSTPCSSVPPSPSFSAPSPGSG 81
mouse Nrl 31 GRSGVPTASLGSTPYSSVPPSPTF---SEPGM 59


(A)
HA-MafB
Flag-MKK7-JNK1
+ + + + +
HA-MafB
p-cJun
actin
(B)
(%)
Percent Intensity
120
100
80
60
40
20
0
Flag-MKK7-JNK1
0 30 80 250 750 (ng)
(C)
KN
Flag-MKK7-JNK1
chase (h)
0 1.0 2.0 4.0 0 1.0 2.0 4.0
(D)
(%)
100
50
0
Percent Intensity
KN
1.0 2.0 4 (h)
(%)
100
50
0
Percent Intensity
Flag-MKK7-JNK1
1.0 2.0 4 (h)


(A)
HA-MafB wild
T62A
4Mut
6Mut
10Mut
Flag-MKK7-JNK1
KN
HA-MafB
p-cJun
actin
(B)
(%)
Percent Intensity
120
100
80
60
40
20
0
*
*
NS
NS
NS
HA-MafB wild
T62A
4Mut
6Mut
10Mut
Flag-MKK7-JNK1
KN


(A)
HA-MafB wild
Flag-MKK7-JNK1
KN
DMSO
NH4Cl
chloroquine
Omuralide
MG132
HA-MafB
p-cJun
actin
(C)
HA-MafB wild
T62A
Flag-MKK7-JNK1
KN
DMSO
MG132
HA-MafB
p-cJun
actin
(B)
(%)
Percent Intensity
700
600
500
400
300
200
100
0
NS
*
HA-MafB wild
Flag-MKK7-JNK1
KN
DMSO
NH4Cl
chloroquine
Omuralide
MG132
(D)
(%)
Percent Intensity
700
600
500
400
300
200
100
0
NS
*
HA-MafB wild
T62A
Flag-MKK7-JNK1
KN
DMSO
MG132


HA-MafB wild + + +
T62A + +
4Mut + +
Flag-MKK7-JNK1 + +
KN + + + + +
Myc-ubiquitin + + + + + +
IP : 3F10
Western blot:
anti-Myc-HRP
-200
-116
- 97
- 66
- 45
input
HA-MafB
p-cJun
actin