Mikawa et al., http://www.jcb.org/cgi/content/full/jcb.201306149/DC1

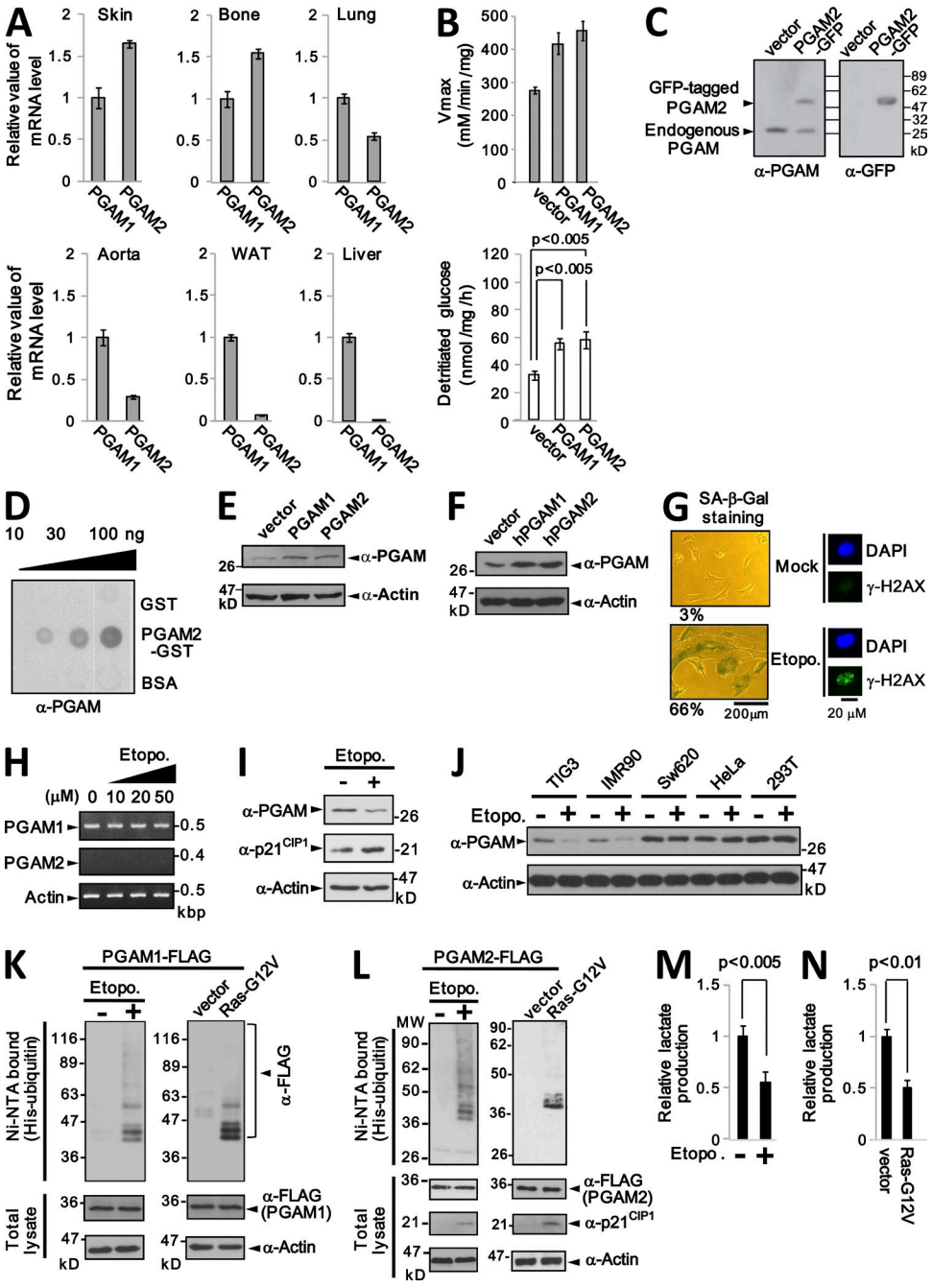


Figure S1. **Detection of PGAM1 and -2 and validation of the anti-PGAM polyclonal antibody.** (A) Total RNA was extracted from the indicated tissues of C57BL6 mice, and the levels of PGAM1 and PGAM2 RNA were compared by qRT-qPCR using the primer sets described in the Materials and methods section. The values were normalized to those of β-actin and are presented as relative expression levels. WAT, white adipose tissue. (B) Measurement of PGAM enzymatic activity (top) and glycolytic flux (bottom) in MEFs expressing either PGAM1 or PGAM2. (C) Cell lysates from MEFs transfected with PGAM2-GFP or empty vector were separated by SDS-PAGE and immunoblotted with affinity-purified anti-PGAM polyclonal antibody (left) and anti-GFP antibody (right). (D) Dot blot showing that the anti-PGAM polyclonal antibody recognized recombinant PGAM2-GST fusion protein, but not GST alone or BSA. (E and F) Primary MEFs were infected with retroviruses encoding mouse PGAM1 or PGAM2 (E) or their human homologues (F). Immunoblotting of cell lysates confirmed that the anti-PGAM polyclonal antibody recognized all of the ectopically expressed isoforms as well as the endogenous proteins. (G) Primary MEFs at passage 2 were exposed to DNA damage, as indicated in Fig. 1 A. 5 d later, cells were stained for SA-β-Gal activity (left) or analyzed for DAPI (middle) and γ-H2AX staining (right) by immunofluorescence. (H) Semiquantitative RT-PCR analysis of *PGAM1*, *PGAM2*, and *β-actin* RNA levels in primary MEFs treated with increasing doses of etoposide, as in Fig. 1 B. (I) Stress-induced reduction of PGAM protein levels in the WI-38 strain of human primary fibroblasts, WI-38, under the conditions described in Fig. 1 A. (J) Stress-induced reduction of PGAM protein levels was detected by Western blotting in primary human fibroblasts (TIG3 and IMR90) but not in immortalized cell lines (HeLa, 293T, and SW620). (K and L) Stress-induced ubiquitination of PGAM1 and PGAM2. MEFs were transfected with vectors encoding His_6-ubiquitin and either PGAM1-FLAG (K) or PGAM2-FLAG (L). The cells were then exposed to etoposide (left) or Ras-G12V expression (right) to induce stress. Ubiquitinated proteins were recovered using Ni-NTA agarose and immunoblotted with FLAG antibody. Samples of total lysate were immunoblotted with the indicated antibodies. (M and N) Measurement of lactate production in primary MEFs exposed to DNA damage (M) or expressing Ras-G12V (N). In A, B, M, and N, error bars indicate SEM (n = 3).

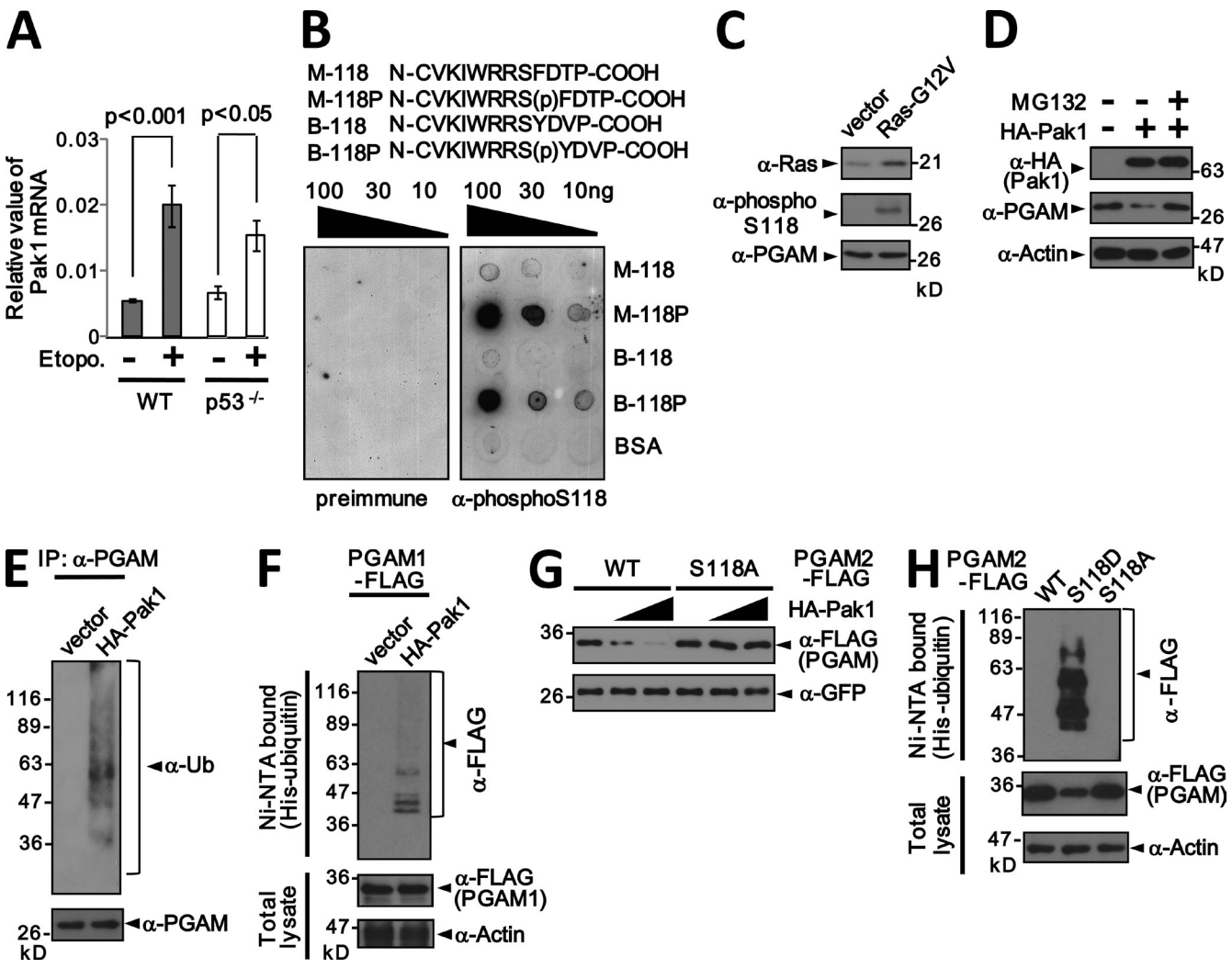


Figure S2. **Pak1 promotes phosphorylation and ubiquitination of PGAM (A) Real-time qRT-PCR analysis of Pak1 mRNA levels in wild-type or *p53*$^{-/-}$ MEFs after etoposide treatment.** The mRNA levels were normalized to those of *RPL13* and are presented as relative values. Error bars indicate SEM (n = 3). (B) Synthetic peptides were prepared representing the sequences surrounding the S118 residue in PGAM1 (B) and PGAM2 (M) and its phosphorylated form (top). The phospho-peptides—B-118P and M-118P—were used to generate polyclonal antibodies, which were then tested for their ability to recognize the phosphorylated peptides, but not the unphosphorylated peptides, by dot-blotting (bottom). (C) Primary MEFs were infected with a vector encoding Ras-G12V, and cell lysates were immunoblotted with antibodies against Ras, PGAM, and the phospho-specific antibody against Ser118 of PGAM. (D) Pak1-transfected primary MEFs were treated with or without 20 μM MG132 for 3 h. Endogenous PGAM protein levels were analyzed by immunoblotting with actin as a loading control. (E) Ubiquitination of endogenous PGAM in MEFs transfected with either HA-Pak1 or empty vector. Cell lysates were immunoprecipitated with a rabbit antibody against PGAM, and ubiquitinated forms of PGAM were detected by immunoblotting with a mouse monoclonal antibody against ubiquitin. (F) Ubiquitination of ectopically express PGAM1-FLAG in MEFs transfected with either HA-Pak1 or empty vector. The cells were cotransfected with His_6-ubiquitin, and His-tagged ubiquitinated proteins were recovered on Ni-NTA agarose and immunoblotted with FLAG antibody. (G) Primary MEFs were cotransfected with vectors encoding GFP, PGAM2-WT-FLAG, or PGAM2-S118A-FLAG and increasing amounts of a plasmid encoding HA-Pak1. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies against FLAG and GFP. (H) MEFs were transfected with His_6-ubiquitin and various mutants of PGAM2-FLAG (WT, S118D, or S118A). His-tagged ubiquitinated proteins were recovered on Ni-NTA agarose and immunoblotted with FLAG antibody.

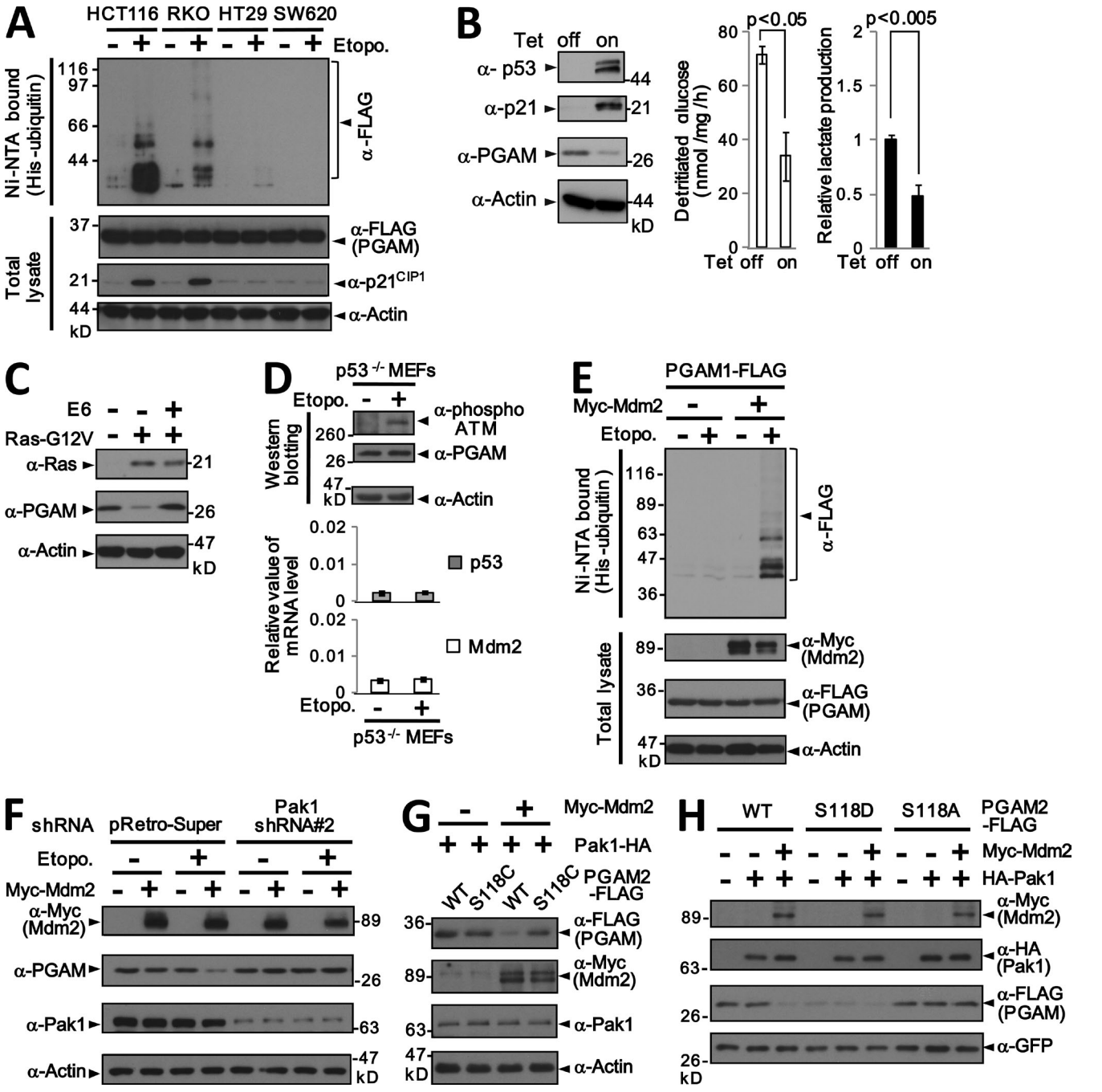
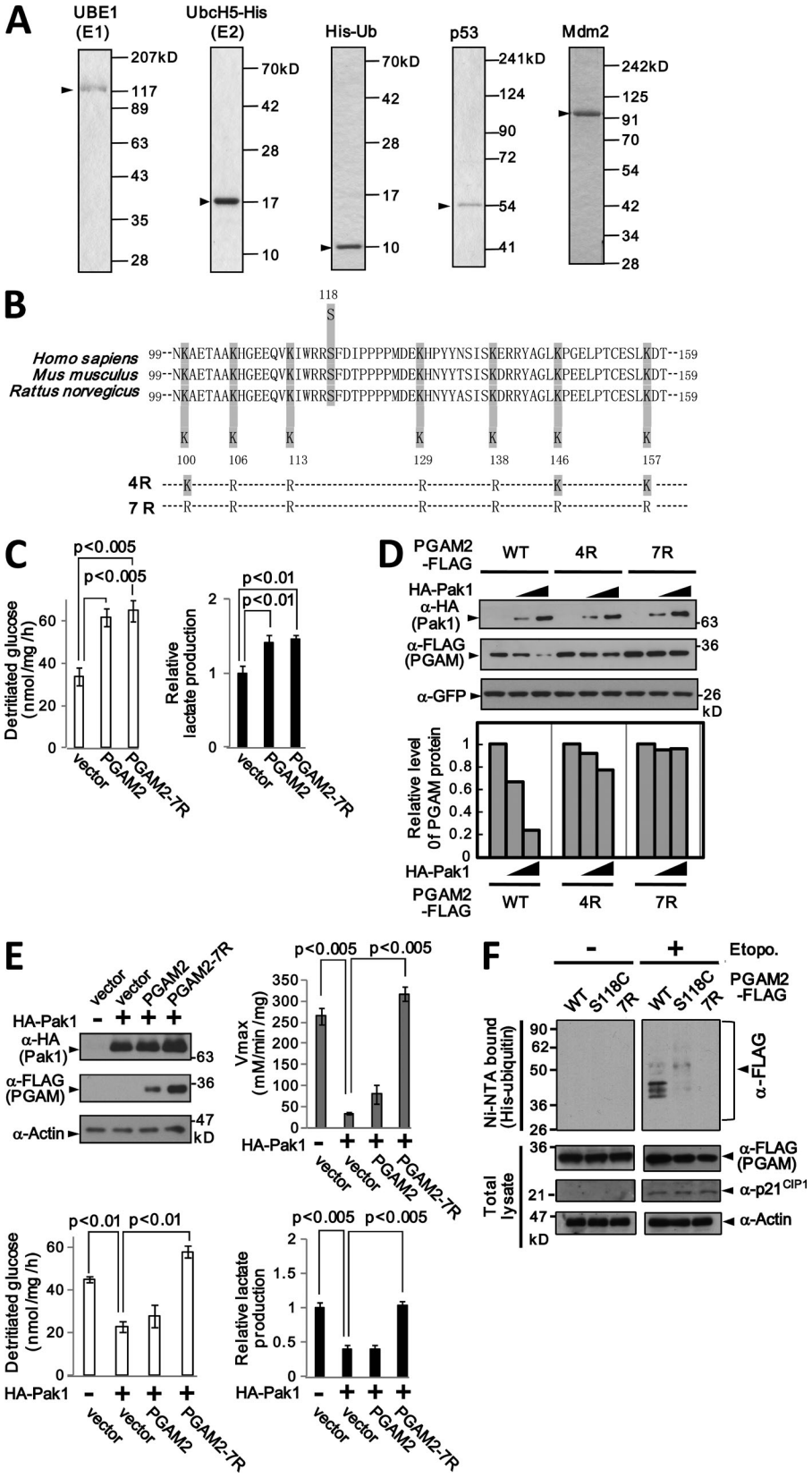


Figure S3. **Effects of p53 and Mdm2 on PGAM protein levels.** (A) The human colon cancer cell lines HCT116, RKO, HT29, and SW620 were cotransfected with His_6-ubiquitin and PGAM2-WT-FLAG and then treated with or without etoposide to generate DNA damage. His-tagged ubiquitinated proteins were recovered on Ni-NTA agarose, analyzed by SDS-PAGE, and immunoblotted with FLAG antibody. As controls, samples of total cell lysate were immunoblotted for PGAM (FLAG), $p21^{CIP1}$, and actin. Note that p53 function is intact in HCT116 and RKO cells but impaired in HT29 and SW620 cells. (B) Reduction of endogenous PGAM levels and activity after overexpression of p53. The left panel confirms that tetracycline-induced expression of a p53-GFP fusion protein in the TGP53-4 mouse cell line results in down-regulation of PGAM protein levels, as previously reported (Kondoh et al., 2005). Induction of p53 also caused a reduction in glycolytic flux (middle) and lactate production (right) in these cells. (C) Primary MEFs were coinfected with retroviral vectors encoding Ras-G12V and the HPV E6 protein. Immunoblotting of the cell lysates showed that while Ras-G12V caused down-regulation of PGAM, the protein levels were restored in cells that coexpress HPV E6. (D) DNA damage has no effect on PGAM levels in *p53*$^{-/-}$ MEFs. The top panels show the results of immunoblotting for phospho-ATM, PGAM, and actin in cells treated with and without etoposide; the bottom panels show real-time qRT-PCR for the indicated target genes. The mRNA levels were normalized to those of *RPL13* and are presented as relative values. These data are related to Fig. 4 A. (E) Myc-Mdm2 restores DNA damage–dependent ubiquitination of PGAM1-FLAG in *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs. Cells cotransfected with His_6-ubiquitin and the indicated vectors were treated with or without etoposide, and the ubiquitination of PGAM-FLAG was assessed as described in the Materials and methods. (F) Pak1 knockdown impairs Mdm2-mediated turnover of PGAM after DNA damage. *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs infected with Pak1 shRNA No. 2 or control retroviruses were transfected with Myc-Mdm2 and treated with etoposide as indicated. The protein level of endogenous PGAM was assessed by immunoblotting. (G and H) Effects of Pak1 and Mdm2 on the expression of wild-type and mutant forms of PGAM. *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs expressing either wild-type PGAM2 or the S118C (G), S118D, and S118A variants (H) were transfected with vectors encoding Myc-Mdm2 and HA-Pak1 as indicated. Cell lysates were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. In B and D, error bars indicate SEM (n = 3).

Figure S4. **Ubiquitination of PGAM2 in vitro and mutation of relevant lysine residues.** (A) Coomassie brilliant blue staining of the recombinant proteins used for in vitro ubiquitination assays. (B) Conservation of lysine residues surrounding Ser118 in the PGAM2 homologues in *Homo sapiens*, *Mus musculus*, and *Rattus norvegicus*. In the PGAM2-4R mutant, residues K106, K123, K129, and K138 were substituted with arginine, whereas in the PGAM2-7R mutant, residues, K100, K146, and K157 were also mutated. (C) Measurement of glycolytic flux (left) and lactate production (right) in MEFs expressing either PGAM2-WT-FLAG or PGAM2-7R-FLAG. (D) Primary MEFs were cotransfected with vectors encoding GFP, HA-Pak1, and the WT, 4R, or 7R variants of PGAM2-FLAG. Whole cell extracts were analyzed by SDS-PAGE and immunoblotted with antibodies against FLAG, HA, and GFP (top). The bottom panel shows quantitation of the PGAM signal normalized to that of GFP. (E) Primary MEFs were infected with vectors encoding HA-Pak1 and either PGAM2-WT-FLAG or PGAM2-7R-FLAG, as indicated. The levels of PGAM protein were assessed by immunoblotting (top left), and samples of cell extract were used to measure PGAM enzymatic activity (top right), glycolytic flux (bottom left), and lactate production (bottom right). (F) The S118C and 7R mutations impair the ubiquitination of PGAM2 in response to DNA damage. MEFs were cotransfected with His_6-ubiquitin and the WT, S118C, or 7R variants of PGAM2-FLAG. After treatment with or without 20 μM etoposide, cell lysates were either analyzed immediately or after purification of ubiquitinated proteins on Ni-NTA agarose. In C and E, error bars indicate SEM (n = 3).



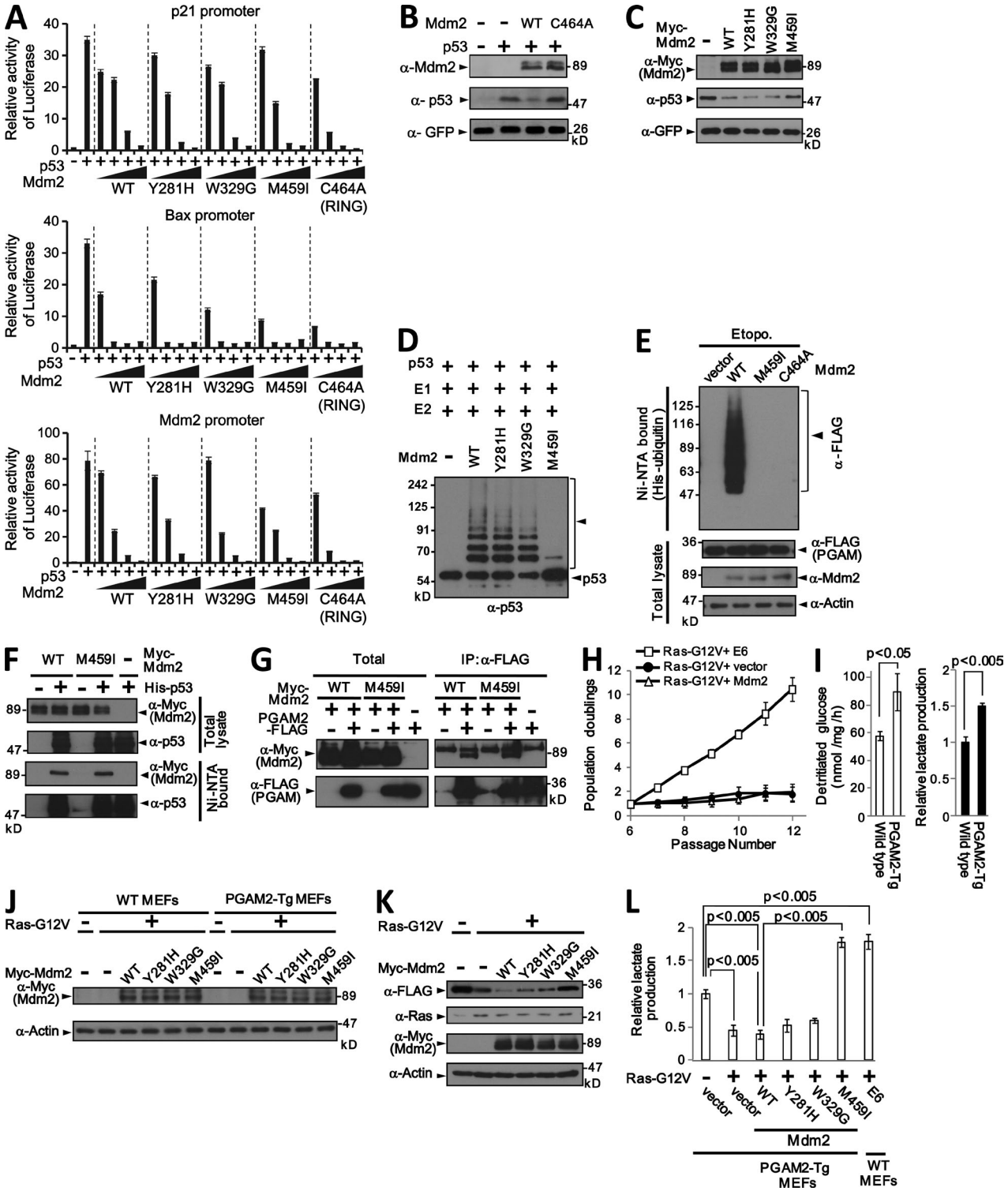


Figure S5. **Mdm2-M459I affects ubiquitination of both PGAM and p53.** (A) Effects of Mdm2 variants on p53-mediated transactivation of the *p21*, *Bax*, and *Mdm2* promoters in $p53^{-/-}Mdm2^{-/-}$ MEFs. Note that the catalytically inactive Mdm2-C464A mutant could suppress p53 activity, as previously reported (Poyurovsky et al., 2003). (B) The C464A RING finger mutation in Mdm2 abolishes its ability to promote turnover of p53. (C and D) The M459I RING finger mutation in Mdm2 does not promote turnover (C) or in vitro ubiquitination (D) of p53. (E) $p53^{-/-}Mdm2^{-/-}$ MEFs were cotransfected with His_6-ubiquitin, PGAM2-FLAG, and the indicated Mdm2 variants (WT, M459I, or C464A). The cells were treated with or without etoposide and cell extracts were analyzed by immunoblotting, either directly (bottom) or after recovery of His-tagged ubiquitinated proteins on Ni-NTA agarose (top). (F) The M459I variant of Mdm2 binds to p53 as efficiently as WT. Primary MEFs were cotransfected with plasmids encoding His_6-p53 and the Myc-tagged WT or M459I variant of Mdm2. Cell lysates were immunoblotted directly (top) or after recovery of His-tagged proteins on Ni-NTA agarose (bottom). (G) The M459I variant of Mdm2 binds to PGAM2 as efficiently as WT. Primary MEFs were cotransfected with PGAM2-FLAG and the Myc-tagged WT or M459I variant of Mdm2. Cell lysates were immunoblotted immediately (left) or after immunoprecipitation with a FLAG antibody (right). (H) Ectopic expression of Mdm2 failed to bypass Ras-G12V–induced senescence in primary MEFs cultured under a 3T3 protocol. HPV E6 served as a positive control. (I) Comparison of glycolytic flux (left) and lactate production (right) in wild-type and *PGAM2*-Tg MEFs. (J) Expression of Myc-Mdm2 variants in Ras-G12V–expressing wild-type or *PGAM2*-Tg MEFs was evaluated by immunoblotting. (K) *PGAM2*-Tg MEFs expressing the indicated combinations of Myc-Mdm2 variants and Ras-G12V were analyzed by immunoblotting with antibodies that detect PGAM (FLAG), Mdm2 (Myc), Ras, and actin. (L) Measurement of lactate production in primary *PGAM2*-Tg MEFs expressing the indicated combinations of Ras-G12V and Mdm2 variants (wild type [WT], Y281H, W329G, or M459I). Wild-type MEFs expressing HPV E6 were used as a control. In A, H, I, and L, error bars indicate SEM ($n = 3$).

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