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G-protein-coupled Receptor Kinase 5 Phosphorylates p53 and Inhibits DNA Damage-induced Apoptosis*

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Xiaoqing Chen[‡], Huiling Zhu[‡], Man Yuan[‡], Jie Fu[§], Yuqing Zhou[‡], and Lan Ma[‡1]

From the [‡]State Key Laboratory of Medical Neurobiology and Pharmacology Research Center, Shanghai Medical College and Institutes of Brain Science, Fudan University, Shanghai 200032 and the [§]Department of Radiation Oncology, Sixth Hospital of Jiao Tong University, Shanghai 200233, China

G-protein-coupled receptor kinases (GRKs) are an important family of Ser/Thr kinases that specifically phosphorylate and desensitize the activated receptor in response to environmental stimulation. Here we identify p53, a key tumor suppressor, as a novel GRK substrate *in vivo*, revealing a previously unknown function of GRKs in regulation of genome stability. Knockdown GRK5 in osteosarcoma cells inhibits DNA damage-induced apoptosis via a p53-mediated mechanism. Furthermore, GRK5, but not GRK2 or GRK6, phosphorylates p53 at Thr-55, which promotes the degradation of p53, leading to inhibition of p53-dependent apoptotic response to genotoxic damage. Consistently, the increase of p53 and irradiation-induced apoptosis were observed in GRK5-deficient mice. These results demonstrate GRK5 as a novel kinase of p53, as well as a negative regulator of p53-mediated signal transduction.

G-protein-coupled receptor (GPCR)[2] kinases (GRKs) are known as a family of serine/threonine kinases that catalyze phosphorylation of seven-transmembrane-spanning G-protein-coupled receptors. They play important roles in the initiation of homologous desensitization of stimulated receptors and thus act as crucial negative regulators of GPCRs (1). GRKs have been demonstrated to phosphorylate and sequester a variety of GPCRs, including adrenergic receptor, muscarinic receptor, dopamine receptors, opioid receptors, chemokine receptor (2–6), etc.

Regulation of receptors other than GPCR family by GRKs has also been shown recently by others and our laboratory (7–9). GRK2 can phosphorylate tyrosine kinase-coupled receptors, including epidermal growth factor receptor and platelet-derived growth factor receptor (7, 8), and regulate 12-transmembrane receptor Patched 1 (9). Transgenic or knock-out studies have demonstrated that deletion or overexpression of GRK in mice causes dysfunction of cardiovascular, nervous, respiratory, optical, and immune systems or even embryonic lethality (3, 10–13). In addition to their well established roles in the regulation of receptor-mediated signaling, emerging evidence indicates that GRKs are also capable of phosphorylating non-receptor substrates, such as synuclein (14) and β-arrestin 1 (15). However, the physiological significance of these phosphorylation events mediated by GRKs remains unclear.

Seven subtypes of GRKs (GRK1–7) have been identified to date. Members of the GRK family share highly homologous structure and similar function in the regulation of GPCR signaling (10). Most subtypes of GRKs, including GRK2, GRK3, GRK5, and GRK6, are ubiquitously expressed in most tissues (1, 16). Deletion of most individual GRK subtypes, such as GRK1, GRK3, GRK4, GRK5, GRK6, and GRK7, does not result in serious phenotype in mice (10), which indicates a redundancy of GRK subtypes in the regulation of GPCR signaling. In this regard, the functional specificity of an individual GRK subtype remains to be explored. Johnson *et al.* (17) and Yi *et al.* (18) showed recently that GRK5 contains a putative nuclear localization sequence and displays a nuclear distribution in the cardiomyocytes. Martini *et al.* (19) reported that GRK5 phosphorylates histone deacetylase 5 (HDAC5), a repressor of myocyte enhancer factor 2 (MEF2), and thus leads to nuclear export of HDAC5 and allows MEF2-mediated transcription. These observations suggest that GRK5 may have novel functions in the nucleus, distinct from other GRK subtypes.

In the present study, we demonstrate that GRK5 phosphorylates p53, a transcriptional factor and tumor suppressor, and thus down-regulates p53 and inhibits p53-mediated apoptosis in cultured osteosarcoma cells and in mice. Our study reveals a novel substrate of GRK5 and demonstrates that GRK5 plays a role in physiological functions.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Plasmids encoding FLAG-tagged human p53 constructed in pcDNA3 were provided by Dr. G. Pei (Shanghai Institutes of Biological Sciences, Chinese Academy of Science). Plasmids encoding bovine GRK5 were prepared as described previously (20, 21). Plasmids encoding FLAG-, HA-, or GST-GRK5, GRK5 mutants, and p53 mutants were constructed by PCR mutagenesis followed by subcloning into pcDNA3 or PGEX-4T-1 vector.

Cell Culture and Transfection—U2OS and Saos-2 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum. HEK293T cells were cultured in modified Eagle's medium containing 10% fetal bovine

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[1] To whom correspondence should be addressed: State Key Laboratory of Medical Neurobiology and Pharmacology Research Center, Fudan University, 138 Yixueyuan Rd., Shanghai 200032, China. Tel.: 86-21-54237522; Fax: 86-21-54237621; E-mail: lanma@fudan.edu.cn.

[2] The abbreviations used are: GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; HDAC, histone deacetylase; siRNA, short interfering RNA; HA, hemagglutinin; ANOVA, analysis of variance; GST, glutathione *S*-transferase; ATM, ataxia telangiectasia-mutated; FACS, fluorescence-activated cell sorting; KO, knock-out; WT, wild type.

serum. U2OS and Saos-2 cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. HEK293T cells were transfected with the calcium phosphate/DNA coprecipitation method. U2OS cells stably expressing GRK5, K215R, or pcDNA3 were selected and maintained in Dulbecco's modified Eagle's medium containing 600 μg/ml G418.

Animals—GRK5-KO mice were gifts from Drs. Robert J. Lefkowitz and Richard T. Premont (Duke University Medical Center). Heterozygote mice were interbred to obtain the wild-type and homozygote GRK5-KO mice. Genotypes of the mice were determined by PCR amplification using tail tip DNA as described (3). All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Antibodies and Reagents—Goat polyclonal antibody against GRK5 was purchased from R&D systems. Mouse polyclonal antibodies against p53, p21, bax, and phospho-p53 (Thr-55 and Ser-315) and rabbit polyclonal antibodies against GRK5 were purchased from Santa Cruz Biotechnology, and rabbit polyclonal antibodies against phospho-p53 (Ser-9, Ser-6, and Ser-15) were purchased from Cell Signaling. Rabbit monoclonal antibodies against actin, FLAG epitope, and HA epitope were purchased from Sigma. Purified recombinant full-length human GRK5 kinase (supplied as a GST fusion protein) was purchased from Cell Signaling. [γ-^{32}P]Adenosine 5′-triphosphate was purchased from PerkinElmer Life Sciences. Cisplatin, camptothecin, cyclohexmide, nutlin, heparin, MG132, and propidium iodide were purchased from Sigma. GRK5 siRNA-1 (5′-AGU AGA AGU CGU CGU CUG UGU GGU C), GRK5 siRNA-2 (5′-AUU AGG UCC AAA CAC GUU CAG CUC C-3′), GRK2 short interfering RNA (siRNA) (5′-GUU CCA GAA AUU CAU UGA GAG CGA U-3′), and control siRNA were purchased from Invitrogen.

Immunoprecipitation—To detect the interaction between p53 and MDM2 or GRK5, cells were treated with MG132 for 4 h before harvesting. Cells were washed with ice-cold phosphate-buffered saline and lysed in solubilization buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 20 mM NaF, 0.5% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride) for 1.5 h. The lysate was centrifuged, and the supernatant was incubated with 1 μg of antibody and 15 μl of 50% slurry of protein A-Sepharose beads (Sigma) at 4 °C for 4 h. For immunoprecipitation of FLAG-tagged proteins, cell lysates were precipitated with M2-conjugated Sepharose (Sigma). The Sepharose was subsequently washed, and the proteins bound to the Sepharose were eluted in SDS-PAGE sample buffer and separated by SDS-PAGE. The samples were detected in Western procedures with the corresponding antibody.

Cell Proliferation and Apoptosis Analyses—To generate proliferation curves, cells were transfected with plasmids or siRNA as indicated, seeded in dishes in triplicate, and counted at 0, 1, 2, 3, 4 and 5 days after transfection. For apoptosis analysis, U2OS or Saos-2 cells were transfected with plasmids or siRNA as indicated for 48 h, treated with cisplatin for the indicated time or not treated, collected, fixed in 70% ethanol, and then stained with propidium iodide and analyzed using flow cytometry assay for apoptotic cells (sub-G_1).

Isolation of Thymocytes—To analyze apoptosis of thymocytes, wild-type and GRK5$^{-/-}$ mice (6 weeks old) were given whole-body irradiation (5 grays) and sacrificed 24 h afterward. Mouse thymocytes were isolated as described (22). In brief, thymus was removed and passed through a nylon cell strainer (BD Biosciences) in RPMI 1640 (Invitrogen) at 4 °C to generate a single-cell suspension. After being washed with phosphate-buffered saline and medium, cells were fixed in 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry for apoptotic cells.

Preparation of Purified Proteins—HEK293T cells overexpressing FLAG-GRK5, FLAG-GRK2, FLAG-GRK6, or mutant proteins were lysed in lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 1% Triton, 10% glycerol, and 2 mM EDTA, plus 10 μg/ml aprotinin, 10 μg/ml benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride). After centrifugation, proteins were immunoprecipitated with M2-conjugated Sepharose (Sigma). After extensive wash in lysis buffer, beads were rinsed with Tris-buffered saline twice. Bound proteins were eluted by 3×FLAG peptide (Sigma). 3×FLAG peptide and salt were removed by centrifugation in Amicon Ultra-4 (30-kDa cutoff size, Millipore). Protein was concentrated and adjusted to a final concentration of 3 μg/ml in buffer containing 5 mM Tris-Cl, pH 8.0, 0.2 mM ATP, 0.5 mM dithiothreitol, and 0.2 mM $CaCl_2$. Protein is >85% pure as determined by 10% SDS-PAGE and Coomassie Blue staining.

GST fusion proteins were expressed in *Escherichia coli* strain BL21. Cultures were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 6 h. Cells were collected, resuspended in phosphate-buffered saline with 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride, sonicated, and centrifuged. The supernatant was incubated with 1 ml of glutathione agarose matrix (Sigma) for 4 h at 4 °C. After being washed with 1×phosphate-buffered saline, bound proteins were eluted by 100 mM glutathione; glutathione and salt were removed by centrifugation in an Amicon Ultra-4.

In Vitro Kinase Assay—Phosphorylation of GST-p53 was assessed by incubating 5 μg of purified GST or GST-p53 with 50 or 100 ng of the indicated kinase in 20 μl of Buffer A (20 mM Tris-HCl, pH 8.0, 7.5 mM $MgCl_2$, 2 mM EDTA) with 0.1 mM (1 μCi) [γ-^{32}P]ATP for 30 min at 30 °C or for the indicated times. As the molecular weight of autophosphorylated FLAG-GRK5 is close to that of GST-p53, FLAG-tagged GRK5 was removed by M2-conjugated Sepharose after the reaction. Samples were electrophoresed on a 10% SDS-polyacrylamide gel. Phosphorylation of protein was detected by autoradiography. For Western blotting, purified GST-p53 or GST-T55A was incubated with the indicated kinase in 20 μl of Buffer A containing 0.1 mM ATP at 30 °C for 30 min.

RESULTS

GRK5 Regulates Apoptosis of Osteosarcoma Cells—To explore the potential role of GRK5 in the regulation of cell proliferation and apoptosis, U2OS, a human osteosarcoma cell line derived from the bone tissue of an osteosarcoma patient, was transfected with control or GRK5 siRNA. As shown in Fig. 1*A*, transfection of siRNAs targeting to two distinct GRK5 sequences significantly reduced proliferation of U2OS cells. Given that cell proliferation is tightly regulated by cell cycle progression

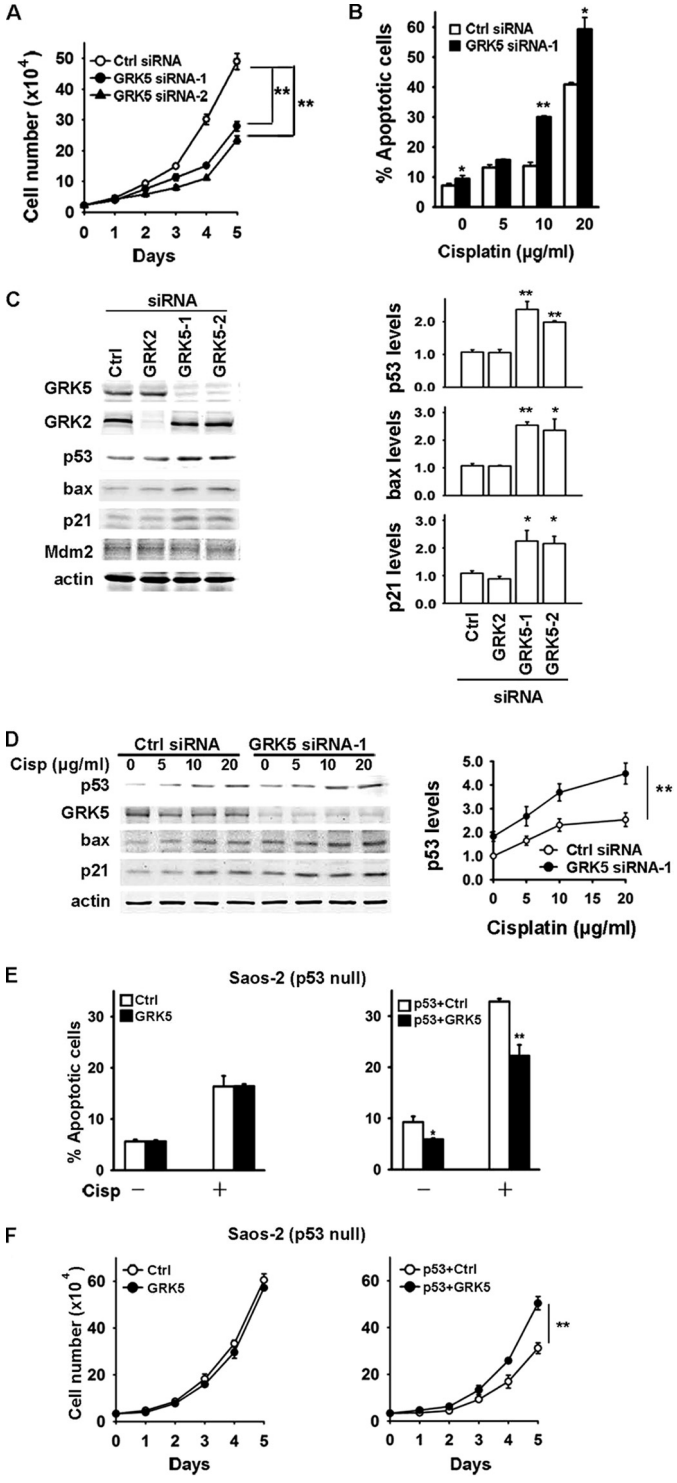

FIGURE 1. **GRK5 inhibits apoptosis in osteosarcoma cells through a p53-dependent mechanism.** *A*, U2OS cells were transfected with control or GRK5 siRNA and counted on the indicated days after transfection. Data are mean ± S.D. ($n = 3$). **, $p < 0.01$, two-way ANOVA. *B*, 48 h after transfection with control or GRK5 siRNA, U2OS cells were treated with the indicated concentrations of cisplatin for 24 h, and the number of apoptotic cells (sub-G_1) was analyzed by FACS. Data are mean ± S.D. ($n = 3$). **, $p < 0.01$, *, $p < 0.05$ *versus* control siRNA (*Ctrl siRNA*), Student's *t* test. *C*, Western blots of lysates from U2OS cells transfected with GRK2, GRK5, or control siRNA. *Right*, quantification of Western results. Data are mean ± S.D. ($n = 3$). **, $p < 0.01$, *, $p < 0.05$ *versus* control siRNA Student's *t* test. *D*, Western blots of lysates from U2OS cells transfected with siRNA and treated with different concentrations of cisplatin (*Cisp*). *Right*, quantification of Western results. Data are mean ± S.D. ($n = 3$). **, $p < 0.01$, two-way ANOVA. *E*, Saos-2 cells coexpressing pcDNA3 (*Ctrl*) or GRK5 plasmid, with or without p53 as indicated, were incubated in the presence or absence of 10 μg/μl cisplatin for 24 h, and the number of apoptotic cells was analyzed by FACS. Data are mean ± S.D. ($n = 3$). **, $p < 0.01$, *, $p < 0.05$, Student's *t* test. *F*, Saos-2 cells were transfected as in *E* and counted on the indicated days after transfection. Data are mean ± S.D. ($n = 3$). **, $p < 0.01$, two-way ANOVA.

and apoptosis, the effect of GRK5 on these two processes was then examined. Transfection of GRK5 siRNA induced a slight increase of G_1/S ratio, which represents a G_1 phase arrest (data not shown). Moreover, a 40% increase of apoptosis was observed in cells treated with GRK5 siRNA (Fig. 1*B*). Furthermore, treatment with GRK5 siRNA increased apoptosis of U2OS cells by 120 and 30%, respectively, in cells treated with 10 and 20 μg/ml cisplatin (Fig. 1*B*).

GRK5 Inhibits Apoptosis through a p53-dependent Mechanism—The tumor suppressor p53 is a critical mediator of cell apoptosis and proliferation (23, 24). Therefore, the effect of GRK5 knockdown on p53 pathway was examined next. As shown in Fig. 1*C*, knockdown of endogenous GRK5, but not GRK2, in U2OS cells with siRNA resulted in an increase of p53, as well as p21 and bax, two transcriptional targets of p53, whereas the level of MDM2, a p53 regulator and transcriptional target (25, 26), remained unchanged. In addition, knockdown GRK5 also increased p53, bax, and p21 in cells treated with cisplatin (Fig. 1*D*), consistent with the result of apoptosis (Fig. 1*C*). To verify whether p53 mediates the GRK5-regulated apoptosis, the effect of GRK5 on DNA damage-induced apoptotic response was tested in Saos-2, a p53-deficient human osteosarcoma cell line. Overexpression of GRK5 in Saos-2 cells had no effect on cisplatin-induced apoptosis (Fig. 1*E*), whereas coexpression of GRK5 with p53 in Saos-2 cells did result in inhibition of apoptosis in the presence or absence of cisplatin treatment. Cotransfection of p53 with GRK5, but not pcDNA3, enhanced cell proliferation, whereas transfection of GRK5 alone failed to cause any change in Saos-2 proliferation (Fig. 1*F*). The above results suggest that GRK5 regulates apoptosis through a p53-dependent mechanism.

GRK5 Interacts with p53—The observation that GRK5 regulates p53 and p53-dependent apoptosis promoted investigation into the possible interaction between GRK5 and p53. As indicated by the coimmunoprecipitation data shown in Fig. 2*A*, association of endogenous p53 and GRK5 was detected in U2OS and HEK293T cells, whereas in Saos-2 cells (p53-null), no p53 was detected in the GRK5 immune complex. The results from coimmunoprecipitation of GRK5 and p53 or their truncated mutants indicate that the interaction between p53 and GRK5 is mainly through the kinase domain of GRK5 (Fig. 2, *B* and *C*) and the central DNA binding domain of p53 (Fig. 2, *D* and *E*).

GRK5 Phosphorylates p53—Because GRK5 has been identified as a G-protein-coupled receptor kinase, whether it could catalyze phosphorylation of p53 was determined via a kinase assay. GRK5 purified from HEK293T cells transfected with FLAG-GRK5 plasmid was incubated with GST-p53 purified from a recombinant *E. coli* strain. As shown in Fig. 3*A*, phosphorylation of GST-p53, but not GST, by GRK5 was detected. Furthermore, the *in vitro* phosphorylation of p53 by GRK5 could be blocked by heparin, an inhibitor of GRK (16) (Fig. 3*B*).

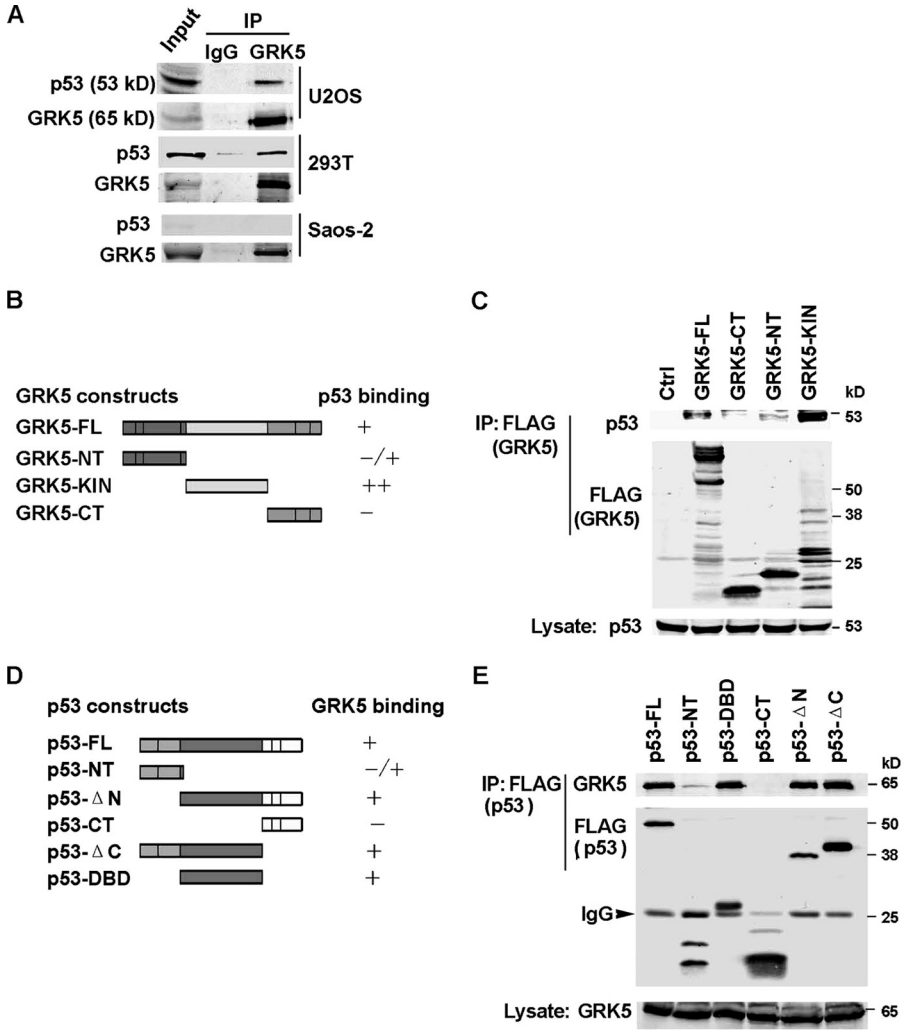

FIGURE 2. **GRK5 interacts with p53.** *A*, coimmunoprecipitation (*IP*) of endogenous p53 and GRK5 with IgG (control) or GRK5 antibody in HEK293T, U2OS, and Saos-2 cells treated with MG132. *B*, schematic diagrams of GRK5 constructs. *GRK5-FL*, full-length; *GRK5-CT*, C-terminal domain (residues 431–590); *GRK5-NT*, N-terminal domain (residues 1–186); *GRK5-KIN*, kinase domain (residues 186–431). *C*, U2OS cells were transfected with FLAG-tagged GRK5 constructs, and immunoprecipitated with M2-Sepharose after MG132 treatment. *Ctrl*, control. *D*, schematic diagrams of p53 truncations. *p53-FL*, full-length; *p53-NT*, N-terminal domain (residues 1–112); *p53-CT*, C-terminal domain (residues 291–393); *p53-DBD*, DNA binding domain (residues 93–291); *p53*-Δ*N*, N terminus-deleted domain (residues 93–393); *p53*-Δ*C*, C terminus-deleted domain (residues 1–291). *E*, U2OS cells were transfected with HA-GRK5 and FLAG-tagged p53 constructs immunoprecipitated with M2-Sepharose.

The above results indicate that p53 serves as a substrate for GRK5.

GRK5-catalyzed serine/threonine phosphorylation of GPCR is crucial for the desensitization of cellular signals mediated by these receptors, and many studies have shown that phosphorylation of p53 at N terminus by kinases such as ATM (27), ATM and Rad3-related (28), and CHK2 (29) plays an important role in regulation of p53 functions. In this regard, phosphorylation at Ser-6, Ser-9, Ser-15, Ser-37, Thr-55, and Ser-315 of p53 were examined, and Thr-55 was identified as a GRK5 phosphorylation site (Fig. 3, *C* and *D*). Furthermore, heparin-sensitive phosphorylation of p53 at Thr-55 by wild-type human GRK5 from a commercial source was observed, whereas K215R, a GRK5 kinase-dead mutant, failed to phosphorylate p53 (Fig. 3*D*). These results indicate that the phosphorylation is not due to contamination of other kinases and that the catalytic activity concerning p53 is most likely a conserved function of GRK5. In addition, GRK2 and GRK6 failed to catalyze Thr-55 phosphorylation of p53 under the same conditions (Fig. 3*E*), suggesting that phosphorylation of p53 is GRK subtype-specific.

It has been reported that phosphorylation at Thr-55 contributes to degradation of p53 (30); consistent with this, decrease of endogenous p53 induced by GRK5 was abolished in the presence of proteasome inhibitor MG132, which blocks p53 degradation (Fig. 3*F*). Phosphorylation of endogenous p53 was detected in U2OS cells treated with MG132. Transient overexpression of GRK5, but not K215R, resulted in a significant increase in p53 phosphorylation at Thr-55. Furthermore, knockdown of GRK5 in U2OS cells decreased p53 phosphorylation at Thr-55 (Fig. 3*F*). Moreover, cotransfection of GRK5 and p53 in Saos-2 cells notably increased Thr-55 phosphorylation of wild-type p53 (Fig. 3*G*). These results demonstrate that GRK5 is capable of phosphorylating p53 at Thr-55 *in vivo*.

The effect of GRK5 on MDM2-mediated p53 degradation was then investigated. As shown in Fig. 3*H*, in Saos-2 cells treated with MG132, overexpression of GRK5, but not K215R, increased association of MDM2 with p53 and ubiquitination of p53, whereas such effects were not observed with T55A. Furthermore, overexpression of K215R failed to decrease the p53 level (Fig. 3*F*). These results, taken together, indicate that GRK5-mediated p53 phosphorylation facilitates association of MDM2 with p53 and thus promotes degradation of p53.

Phosphorylation of p53 by GRK5 Inhibits Apoptosis of Osteosarcoma Cells—The effect of GRK5-catalyzed p53 phosphorylation was then examined. Both apoptosis and elevation of p53 induced by cisplatin or camptothecin were significantly reduced in U2OS cells expressing GRK5, but not K215R (Fig. 4, *A*–*C*). Overexpression of GRK2 or GRK6, which is incapable of phosphorylating p53, had no influence on the apoptotic response to cisplatin (Fig. 4*D*). Moreover, coexpression of K215R with p53 or of GRK5 with T55A, in which Thr-55 was substituted to alanine, in Saos-2 cells failed to induce any change in apoptosis or cell proliferation assays (Fig. 4, *E* and *F*), suggesting that phosphorylation of p53 by GRK5 is important for DNA damage-induced apoptosis.

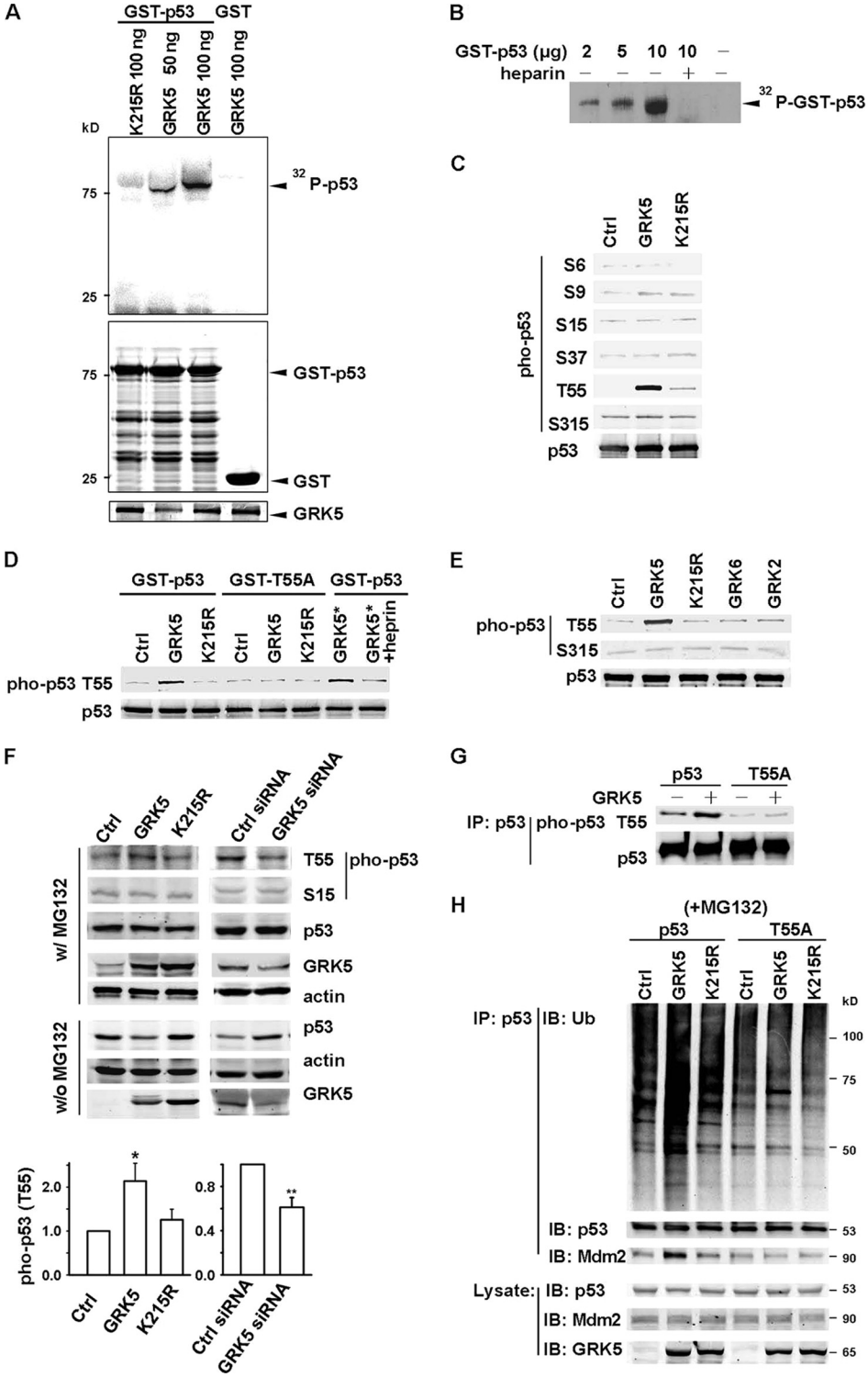

FIGURE 3. **GRK5 phosphorylates p53 at Thr-55.** *A*, FLAG-GRK5 or FLAG-K215R purified from HEK293T cells was incubated with GST-p53 or GST purified from *E. coli* in the presence of [γ-^{32}P]ATP. Isolated GST proteins were resolved by SDS-PAGE and visualized by autoradiography (*top panel*) and Coomassie Blue staining (*middle panel*), and FLAG-GRK5 input was shown (*lower panel*, Coomassie Blue staining). *B*, *in vitro* kinase assay was carried out in the presence or absence of 20 μM heparin. *C–E*, GST-p53 or GST-T55A incubated with GRK purified from HEK293T cells or purified GRK5 obtained from commercial source (GRK5*), subjected to Western analysis with the indicated antibodies. *Ctrl*, control; *pho-p53*, phosphorylated p53. *F*, Western analysis of lysates from U2OS cells transfected as indicated and treated with MG132 or not. The relative ratio of p53 phosphorylated at Thr-55 to total p53 was quantified (*lower panel*). Data are mean ± S.D. ($n = 3$). *, $p < 0.05$, **, $p < 0.01$, Student's *t* test. *G*, FLAG-p53 or FLAG-T55A was cotransfected with vector or GRK5 in Saos-2 cells. Cell lysates were immunoprecipitated (*IP*) with M2-Sepharose after MG132 treatment. *H*, Saos-2 cells coexpressing FLAG-p53 or FLAG-T55A, GRK5 or K215R, or HA-ubiquitin and MDM2 were treated with MG132 and subjected to immunoprecipitation with M2-Sepharose. *IB*, immunoblot; *Ub*, ubiquitin.

GRK5 Serves as a Critical p53 Regulator in Vivo—To further establish the role of GRK5 as a p53 regulator *in vivo*, levels of p53 and phosphorylation of p53 at Thr-55 in heart, lung, spleen, kidney, liver, and thymus of GRK5 knock-out mice were determined. Notably, aberrant up-regulation of p53 was observed in all tissues examined (Fig. 5, *A* and *C*), accompanied by up-regulation of bax. Coincidentally, the percentage of p53 phosphorylated at Thr-55 was decreased in these tissues in GRK5 knock-out mice (Fig. 5*B*). Moreover, a higher percentage of apoptosis induced by whole-body γ-irradiation was observed in the thymocytes of GRK5-deficient mice (Fig. 5*D*). These observations support an essential role of GRK5 as a negative regulator of p53 *in vivo*.

DISCUSSION

GRKs are recognized as critical regulators of GPCR signaling, based on their roles on phosphorylating and sequestering these receptors demonstrated by numerous studies (1, 10, 31). As the most widely expressed and best characterized member of the GRK4 subfamily, GRK5 plays important roles in GPCR-mediated physiological processes, especially in the central nervous and cardiovascular systems. Transgenic mice with cardiomyocyte-specific GRK5 overexpression exhibit marked β-adrenergic receptor desensitization (32) and exaggerated hypertrophic response (19). Deletion of GRK5 in mice results in enhanced cholinergic responses mediated by muscarinic M2 receptor, such as hypothermia, hypoactivity, tremor, and salivation (3). Aged GRK5 knock-out mice show an Alzheimer-like pathology and working memory impairment (33). In addition, recent evidence indicates that GRK5 is capable of phosphorylating non-receptor substrates. Barthet *et al.* (15) reported phosphorylation of β-arrestin 1 by GRK5 *in vitro* and in COS-1 cells. Martini *et al.* (19) demonstrated that GRK5 catalyzes histone deacetylase 5 *in vitro* and in cardiac fibroblasts. These findings

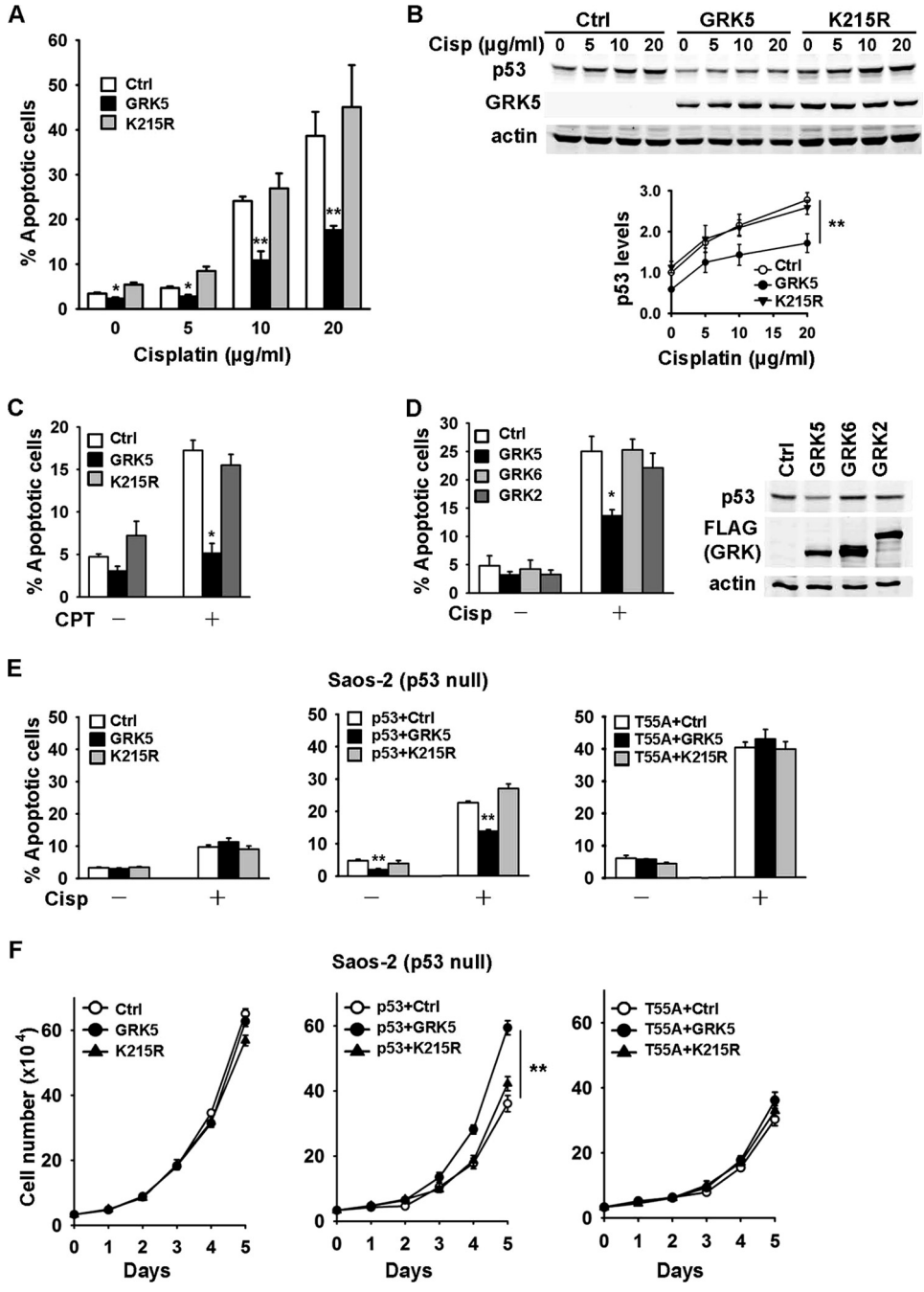

FIGURE 4. **Phosphorylation of p53 by GRK5 inhibits apoptosis of osteosarcoma cells.** *A*, U2OS cells stably overexpressing pcDNA3 (*Ctrl*), FLAG-GRK5, or FLAG-K215R were treated with cisplatin and analyzed by FACS. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$, **, $p < 0.01$ *versus* control group; Student's *t* test. *B*, Western blots of lysates from U2OS cells stably overexpressing control or GRK5 construct treated with different concentration of cisplatin. *Lower panel*, quantification of Western results. Data are mean ± S.D. ($n = 3$). **, $p < 0.01$, two-way ANOVA. *C* and *D*, U2OS cells stably overexpressing control or GRK construct were treated with camptothecin (*CPT*) (*C*) or cisplatin (*Cisp*) (*D*) and analyzed by FACS. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$, Student's *t* test. Western blots of lysates from U2OS cells stably overexpressing control or GRK construct are shown (*D*, *right*). *E*, Saos-2 cells were transfected as indicated and incubated in the presence or absence of 10 μg/μl cisplatin for 24 h, and the number of apoptotic cells was analyzed by FACS. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$, Student's *t* test. *F*, Saos-2 cells were transfected as in *E* and counted on the indicated days after transfection. Data are mean ± S.D. ($n = 3$). **, $p < 0.01$, two-way ANOVA.

elevated p53 level in multiple tissue types and an enhanced sensitivity to irradiation. Our results suggest that GRK5 may play an essential role in the regulation of genome integrity and tumorigenesis.

The tumor suppressor p53, serving as a cell fate determinant, induces cell cycle arrest or apoptosis in response to diverse stress (23, 24). The function of p53 is regulated primarily at the level of protein stability. Posttranslational modifications of p53, especially phosphorylation and acetylation, have been shown to tightly regulate p53 stabilization and activation (26, 34). GRK5 has been shown to phosphorylate HDAC5 and regulate HDAC5-mediated transcriptional activation (19). Unchanged acetylation of p53 was observed in cells overexpressing or deficient in GRK5 (data not shown), suggesting that the effect of p53 by GRK5 is unlikely via the regulation of the p53 acetylation level mediated by HDAC. Phosphorylation within the p53 N terminus, catalyzed by ATM (27), ATM- and Rad3-related (28), DNA-dependent protein kinase (35), or CHK2 (29), enhances p53 stability by preventing MDM2 binding. In the present study, we show that GRK5, a well known kinase of GPCRs, phosphorylates p53 and promotes p53 degradation. Thus, we identified GRK5 as a new p53 kinase, and our results suggest that GRK5 may serve as an indispensable member of the complex regulatory network of p53 (Fig. 6). How GRK5 coordinates with other p53 regulators in response to various genotoxic stresses to maintain genomic stability remains to be further explored.

Sharing conserved structure and regulation pattern, members of GRK family have a common role in phosphorylating GPCRs; however, their selectivity of non-GPCR substrates appears to be specific (14, 19, 36). Hall *et al.* (36) show that GRK6, but not GRK2, is capable of phosphorylating Na^+/H^+ exchanger regulatory factor. GRK5 has been demonstrated to catalyze the phosphorylation of HDAC5 *in vitro* (19), whereas purified GRK2 does not. Our results also show that GRK5 subtype specifically regulates the function of p53, whereas GRK2 indicate that GRK5 may have broader non-receptor substrate selectivity and more functions than assumed. In the current study, we reveal a previously unknown function of GRK5 to phosphorylate p53, a crucial tumor suppressor, and inhibit p53-mediated apoptosis. Moreover, GRK5 knock-out mice showed an

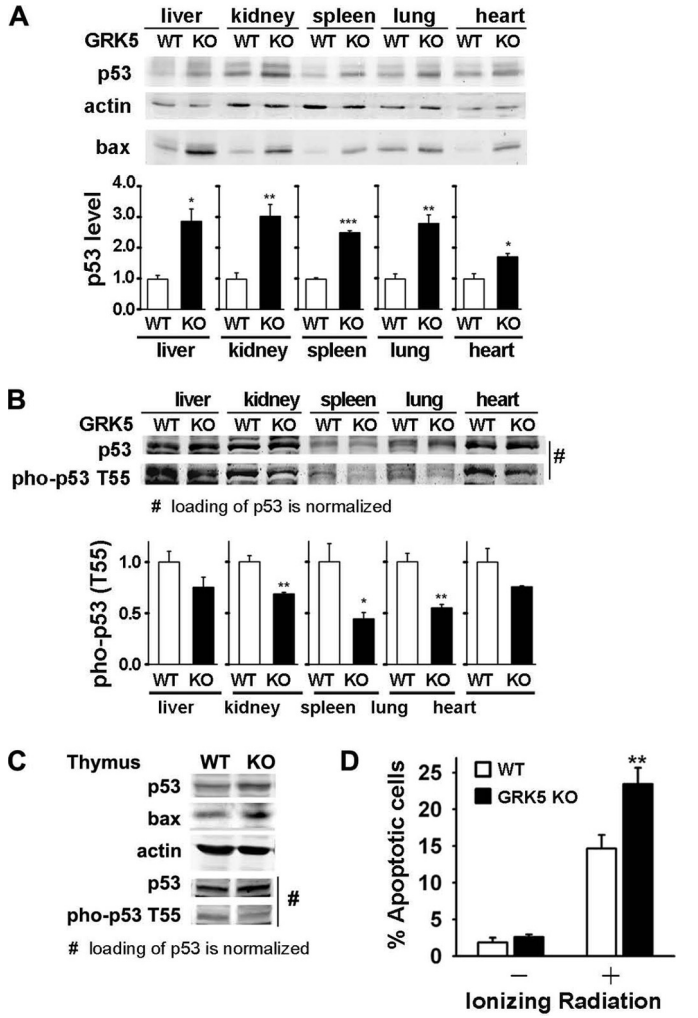

FIGURE 5. **GRK5 serves as a critical p53 regulator *in vivo*.** *A*, Western analysis of p53 and bax level in different tissues of wild-type (*WT*) or GRK5-KO (*KO*) mice. *Lower panel*, quantification of Western results. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ *versus* WT, Student's *t* test. *B*, Western analysis of Thr-55 phosphorylation of p53 (*pho-p53*) in different tissues of wild-type or GRK5-KO mice. #, sample loading was adjusted (input from KO sample was reduced to obtain an equal amount of total p53 loading). *Lower panel*, quantification of Western results. Data are mean ± S.D. ($n = 3$). *, $p < 0.05$, **, $p < 0.01$ *versus* WT, Student's *t* test. *C*, Western analysis of levels p53, bax, and Thr-55 phosphorylation of p53 in the thymus of wild-type or GRK5-KO mice. *D*, wild-type and GRK5-KO mice were subjected to 0 or 5 grays of ionizing radiation, and the number of apoptotic thymocytes was analyzed by FACS. Data are mean ± S.D. ($n = 3$). **, $p < 0.01$ *versus* WT, Student's *t* test.

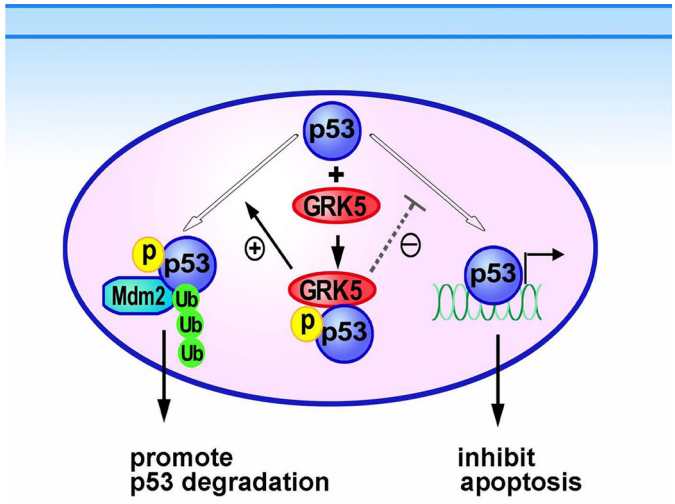

FIGURE 6. **A model for the regulation of p53 by GRK5.** GRK5 phosphorylates p53 (*P*) and enhances MDM2 binding and ubiquitination of p53 (*Ub*), promotes p53 degradation, and thus inhibits p53-mediated apoptosis.

and GRK6 have no catalytic activity toward p53 nor any effect on p53-mediated apoptotic response. Unlike GRK5, GRK2 mainly distributes in cytoplasm. However, our *in vitro* binding assays indicated that both GRK2 and GRK6 can interact with p53 (data not shown); thus, the specificity of GRK5 to catalyze p53 phosphorylation may be attributed to its structural characters rather than its distribution abundance in the nucleus. The variability in GRK structure among different subtypes mainly exists in their C-terminal domain. Unlike other subtypes, The C-terminal domain of GRK5 is rich in basic residues and contains unique autophosphorylation sites, conserved in human, bovine, and mouse GRK5 (37), which is associated with the regulation of GRK5 by calmodulin (38, 39), phospholipids (40), and protein kinase C (41). In our study, both bovine and human GRK5 can phosphorylate p53, and the regulation of p53 by GRK5 was also found in mice, indicating that the role of GRK5 on p53 is conserved across species.

Cellular response to genotoxic stress, especially apoptosis and cell cycle arrest, is pivotal for protection of genomic integrity and avoiding oncogenic mutations. It has been well established that the misregulation of p53 level or activity compromises cellular apoptotic response and contributes to tumorigenesis. Chao *et al.* (42) reported that mutation of phosphorylation sites in the N terminus of p53 impairs apoptotic response to DNA damage and alters susceptibility to spontaneous tumorigenesis. Johnson *et al.* (43) demonstrated that the $p53^{QS}$ mutant, which cannot bind MDM2 and has transactivation deficiency, induces embryonic lethality in mice. In our study, animals deficient in GRK5 show abnormal p53 level. Furthermore, enhanced susceptibility in response to irradiation was found in GRK5 knock-out mice. These findings support an essential role for GRK5 in restricting of p53 and protecting genomic stability under physiological conditions.

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