Parkin, a p53 target gene, mediates the role of p53 in glucose metabolism and the Warburg effect

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Regulation of energy metabolism is a novel function of p53 in tumor suppression. Parkin (PARK2), a Parkinson disease-associated gene, is a potential tumor suppressor whose expression is frequently diminished in tumors. Here Parkin was identified as a p53 target gene that is an important mediator of p53’s function in regulating energy metabolism. The human and mouse Parkin genes contain functional p53 responsive elements, and p53 increases the transcription of Parkin in both humans and mice. Parkin contributes to the function of p53 in glucose metabolism; Parkin deficiency activates glycolysis and reduces mitochondrial respiration, leading to the Warburg effect. Restoration of Parkin expression reverses the Warburg effect in cells. Thus, Parkin deficiency is a novel mechanism for the Warburg effect in tumors. Parkin also contributes to the function of p53 in antioxidant defense. Furthermore, Parkin deficiency sensitizes mice to γ-irradiation-induced tumorogenesis, which provides further direct evidence to support a role of Parkin in tumor suppression. Our results suggest that as a novel component in the p53 pathway, Parkin contributes to the functions of p53 in regulating energy metabolism, especially the Warburg effect, and antioxidant defense, and thus the function of p53 in tumor suppression.

Metabolic alterations are a hallmark of tumor cells (1, 2). Whereas normal cells use mitochondrial respiration to provide energy, the majority of tumor cells preferentially use aerobic glycolysis, a switch known as the Warburg effect (3). Because glycolysis produces ATP much less efficiently than mitochondrial respiration, tumor cells compensate by having a much higher rate of glucose uptake and utilization than normal cells (1, 2). Recent studies have strongly suggested that the Warburg effect is a key contributor to malignant progression (1, 2), and reversing the Warburg effect inhibits the tumorigenicity of cancer cells (4, 5). However, the underlying mechanisms for the Warburg effect are not well-understood (1, 2).

p53 plays a central role in tumor prevention. As a transcription factor, in response to stress, p53 transcribes its target genes to start various cellular responses, including cell-cycle arrest, apoptosis, and/or senescence, to prevent tumor formation (6, 7). Recent studies have revealed that regulating energy metabolism and the Warburg effect is a novel function of p53 in tumor suppression (2, 8). p53 induces TIGAR (TP53-induced glycolysis and apoptosis regulator) to reduce glycolysis (9), and induces SCO2 (10) and GLS2 (11, 12) to promote mitochondrial respiration. Loss of p53 results in decreased mitochondrial respiration and enhanced glycolysis, leading to the Warburg effect. Furthermore, regulating antioxidant defense has recently been revealed as another novel function for p53 (8, 13). p53 induces several antioxidant genes, including Sestrins (14), TIGAR (9), ALDH4 (15), and GLS2 (11, 12), to reduce the levels of reactive oxygen species (ROS) and DNA damage in cells, which contributes greatly to the role of p53 as a tumor suppressor.

Parkin (PARK2) was first identified as a gene associated with Parkinson disease (PD), a neurodegenerative disease. Mutations of Parkin account for most autosomal recessive forms of juvenile Parkinson disease. Parkin deficiency leads to mitochondrial dysfunction and enhanced oxidative stress in neuronal cells in Drosophila and mice, which are believed to contribute greatly to PD (16, 17). Recently, Parkin has been suggested to be a potential tumor suppressor, and diminished expression and mutations of the Parkin gene have been frequently observed in various tumors (18–22). However, the mechanisms by which Parkin contributes to tumor suppression and the regulation of Parkin are not well-understood.

Here we identified Parkin as a p53 target gene. p53 increases the transcription of Parkin gene both in vitro and in vivo. Parkin contributes to p53’s role in regulating glucose metabolism and the Warburg effect: Parkin deficiency results in the Warburg effect, whereas restoration of Parkin expression reverses the Warburg effect in cells. Parkin also mediates p53’s role in antioxidant defense. These results suggest that the functions of Parkin in regulating energy metabolism and antioxidant defense should contribute greatly to Parkin’s role in tumor suppression and, furthermore, contribute to p53’s role as a tumor suppressor.

Results

Human Parkin Gene Is a p53 Target Gene. As a transcription factor, p53 binds to the p53 responsive elements (REs) in its target genes to transcriptionally regulate their expression in response to stress (23). The p53MH algorithm is a computational program developed for genome-wide scanning for potential p53 targets by identifying putative p53 REs in genes (24), which has been successfully applied to identify new p53 targets (11, 25, 26). Using this algorithm, we identified human Parkin gene as a potential p53 target.

To investigate whether p53 transcriptionally regulates the Parkin gene, Parkin expression was examined in various cell lines exposed to different stress signals. A pair of isogenic p53 wild-type and p53-deficient human lung cell lines, H460-con and H460-p53siRNA, which stably express a control vector and a p53 shRNA vector in p53 wild-type H460 cells, respectively, were used. H460-p53siRNA cells displayed greatly decreased p53 protein levels and decreased induction of p53 target MDM2 in response to stress (27), clearly induced Parkin expression in a p53-dependent manner in H460 cells (Fig. 1 C and D). This induction is p53-dependent because no clear induction of Parkin was observed in H460-p53siRNA cells. Furthermore, p53 increased the basal levels of Parkin under nonstressed conditions. In addition to these stress signals, Nutlin-3a, a nongenotoxic small molecule that activates p53 through disruption of p53-MDM2 interaction (27), clearly induced Parkin expression in a p53-dependent manner in H460 cells (Fig. 1 C and D). The regulation of Parkin by p53 activation was also confirmed by immunofluorescence (IF) staining (Fig. 1E). Results in Fig. 1E further show that Parkin is primarily localized in mitochondria and cytoplasm, and not in nuclei. Similar results were observed in p53 wild-type HCT116-con and p53-deficient HCT116-p53siRNA (Fig. S1). These results to-
and mRNA (C and B) confer p53-dependent transcriptional activities, the DNA fragments containing one copy of these two putative p53 REs were inserted into the promoter of PGL2 luciferase reporter vector. p53 null human lung H1299 cells were cotransfected with the reporter vectors and a vector expressing either wild-type or R273H mutant p53. Compared with mutant p53, the expression of wild-type p53 greatly enhanced luciferase activities of the reporter vector containing the putative p53 RE in the Parkin intron 1 (by >20-fold), but not in the promoter region (by less than twofold) (Fig. 2C). Taken together, our data demonstrate that human Parkin gene is a p53 target gene; p53 binds to the p53 RE in Parkin intron 1 and increases Parkin transcription in cells.

Mouse Parkin Gene Is a p53 Target Gene. The p53MH algorithm also showed that mouse Parkin gene contains three putative p53 REs in its promoter region, including RE A and two overlapped REs, B and C (Fig. 4A). This suggested that mouse Parkin gene was a potential p53 target. Two luciferase reporter vectors were constructed that contained a copy of RE A and REs B+C. Luciferase reporter assay showed that the expression of wild-type p53 in p53 null H1299 cells and mouse embryonic fibroblasts (p53−/−MEF) clearly enhanced the luciferase activities of both reporter vectors by approximately four- to sixfold compared with mutant p53 (Fig. 3B). Furthermore, H2O2 clearly induced Parkin expression at both mRNA and protein levels in p53−/−MEF but not mutant p53−/−MEF cells (Fig. 3C and D). p53−/−MEF cells also displayed a higher level of basal Parkin expression than p53−/−MEF. These results demonstrate that mouse Parkin is a p53 target gene; p53 increases Parkin transcription through the regulation of p53 REs in Parkin promoter region.

To investigate whether p53 activation induces Parkin expression in vivo, p53−/− and p53−/−C57BL/6j mice were subjected to whole-body γ-irradiation (IR) (4 Gy). Parkin was clearly induced at both mRNA and protein levels (by approximately four- to sixfold at 20 h after IR) in the spleen and thymus, two highly radiosensitive tissues that display the clearest p53 responses to IR, in p53−/− but not p53−/− mice (Fig. 3E and F). The p53-dependent induction of Parkin by IR appears to be tissue-specific because Parkin was not induced in the cortex of brain, liver, or kidney. Together, these results demonstrate that the regulation of Parkin by p53 is evolutionarily conserved from mice to humans.

Parkin Contributes to the Function of p53 in Regulating Glucose Metabolism and the Warburg Effect. p53 has been reported to reduce glycolysis and promote mitochondrial respiration in cells.
p53 deficiency leads to the Warburg effect in tumors, which is characterized by higher glucose uptake, a higher rate of glycolysis, and higher lactate production in tumor cells than normal cells (9, 10). We found that, as a downstream target of p53, contributes to the role of p53 in regulating glucose metabolism and the Warburg effect in cells. As shown in Fig. 4A–C, ectopic expression of Parkin in H460-p53siRNA cells significantly decreased glucose uptake, the rate of glycolysis, and lactate production. Furthermore, knockdown of endogenous Parkin in H460-con cells significantly enhanced glucose uptake, the rate of glycolysis, and lactate production. Similar effects of Parkin on the Warburg effect were also observed in Parkin+/− MEFs compared with Parkin+/+ MEFs (Fig. 4A–C, Right). These results demonstrate that Parkin deficiency results in the Warburg effect, whereas restoration of Parkin expression reverses the Warburg effect in cells. Furthermore, Parkin mediates the role of p53 in glucose metabolism. As shown in Fig. 4D, Parkin knockdown significantly enhanced glucose uptake, the rate of glycolysis, and lactate production in H460 cells. Simultaneous knockdown of p53 and Parkin results in higher glucose uptake, rate of glycolysis, and lactate production compared with individual knockdown of p53 or Parkin in cells, but the effects are less than additive effects. These results suggest that Parkin is one of the important mediators for p53’s role in glucose metabolism.

Parkin deficiency leads to mitochondrial dysfunction in neuronal cells, which contributes to the development of PD (16). Therefore, the role of Parkin in preventing the Warburg effect could be mainly due to its function in maintaining mitochondrial respiration. Consistent with the role of p53 in enhancing mitochondrial respiration (Fig. 4E, first panel), ectopic expression of Parkin in H460-p53siRNA cells enhanced oxygen consumption (Fig. 4E, second panel). Furthermore, Parkin knockdown in H460-con and Parkin knockout in MEF cells (Parkin+/− MEF) decreased oxygen consumption (Fig. 4E, third and fourth panels), which indicates reduced mitochondrial respiration. Parkin knockout in mice was reported to result in the decreased expression of several mitochondrial proteins, including pyruvate dehydrogenase E1α (PDH1A1), in the mouse brain as detected by Western blot and mass spectrometry analysis (16). PDH1A1 is a critical component of the pyruvate dehydrogenase (PDH) complex, which catalyzes the conversion of pyruvate into acetyl-CoA and serves as a critical link between glycolysis and mitochondrial respiration. It is unknown whether Parkin can regulate the expression of PDH1A1 in human cells, which may in turn affect the activity of the PDH complex and therefore the levels of acetyl-CoA and glucose metabolism in cells. Results in Fig. 5A–D clearly show that Parkin knockdown in H460-con cells decreased PDH1A1 protein levels, the activity of the PDH complex, and levels of acetyl-CoA, whereas ectopic Parkin expression in H460-p53siRNA cells increased PDH1A1 protein levels, the activity of the PDH complex, and levels of acetyl-CoA. Consistently, PDH1A1 levels were much higher in Parkin+/+ MEF than Parkin−/− MEF cells (Fig. 5A). Furthermore, PDH1A1 knockdown in H460-con cells significantly reduced mitochondrial respiration (Fig. 5E), which in turn increased glucose uptake, the rate of glycolysis, and lactate production, leading to the Warburg effect (Fig. 5F). It is still unclear how Parkin regulates PDH1A1. Parkin does not regulate PDH1A1 expression at the mRNA level (Fig. 5A). Knockdown of Parkin or PDH1A1 did not decrease intracellular ATP levels (Fig. 5A), which suggests that enhanced glycolysis compensates the decreased mitochondrial respiration for ATP generation in cells.

Parkin Contributes to the Role of p53 in Regulating Antioxidant Defense. The antioxidant function is a novel mechanism for p53 in tumor suppression (8, 13). Parkin regulates antioxidant function in neuronal cells (16). To investigate whether the induction of Parkin by p53 also contributes to the role of p53 in antioxidant defense, Parkin was overexpressed or knocked down in cells. Ectopic Parkin expression significantly reduced ROS levels in H460-p53siRNA cells (Fig. 6A) treated with or without H2O2. Parkin knockdown in H460-con (Fig. 6B) and Parkin knockout in MEF cells (Fig. 6C) significantly increased ROS levels. Furthermore, Parkin mediates the role of p53 in ROS regulation. As shown in Fig. 6D, simultaneous knockdown of p53 and Parkin results in higher intracellular ROS levels than individual knockdown of p53 or Parkin, but the effect is less than additive. These results suggest that Parkin is one of the important mediators for p53’s role in ROS regulation in cells.

Reduced glutathione (GSH) is an important antioxidant molecule and a scavenger for ROS. GSH/GSSG (oxidized glutathione) balance reflects the redox state of cells. p53 has been reported to up-regulate GSH levels and the GSH/GSSG ratio in cells (11, 12). As shown in Fig. 6E and F, H460-con displayed significantly higher GSH levels and GSH/GSSG ratio than H460-p53siRNA cells. Ectopic Parkin expression significantly increased GSH levels and GSH/GSSG ratio in H460-p53siRNA cells. Furthermore, Parkin knockdown in H460-con and Parkin knockdown in MEF cells significantly decreased GSH levels and the GSH/GSSG ratio (Fig. 6F).
Fig. 4. Parkin regulates the Warburg effect in cells. (A) Parkin reduces glucose uptake in H460 and MEF cells. (B) Parkin reduces the rate of glycolysis in H460 and MEF cells. (C) Parkin reduces the lactate production of H460 and MEF cells. (D) Parkin contributes to the role of p53 in glucose metabolism. Simultaneous knockdown of p53 and Parkin in H460 cells results in higher glucose uptake, rate of glycolysis, and lactate production than individual knockdown of p53 or Parkin, but the effects are less than the additive effects. (E) Parkin increases oxygen consumption of H460 and MEF cells. First panel: p53 deficiency reduces oxygen consumption in cells. For A–C and E, H460-p53siRNA cells were transfected with Parkin expression vector, and H460-con cells were transfected with siRNA oligos against Parkin 24 h before assays. For D, H460 cells were transfected with siRNA oligos against p53 and/or Parkin 24 h before assays. Three different siRNA oligos were used, and similar results were obtained as the represented one. Data are presented as mean ± SD (n = 3). *P < 0.05; **P < 0.01.

Discussion
Parkin has recently been suggested to be a potential tumor suppressor (18–22). Here we demonstrate that Parkin deficiency sensitizes mice to IR-induced tumorigenesis, providing further direct evidence to support a role of Parkin in tumor suppression. Diminished expression of Parkin has frequently been observed in various tumors, but the mechanisms are not well-understood. Parkin mutations do not account for all of the decreased Parkin expression in tumors (18–22). Our finding that p53 regulates Parkin expression not only provides a mechanism for the regulation of Parkin but also suggests that loss of p53, a common event in tumors, is an important mechanism contributing to the frequently decreased expression of Parkin in tumors.

Recently, Parkin was reported to transcriptionally repress p53 (29). Our results show that Parkin does not repress p53 expression in H460 or HCT116 cells in which p53 induces Parkin expression (Fig. S3 A–D). Interestingly, in human neuroblastoma SH-SY5Y cells, whereas Parkin represses p53 expression and transcriptional activity (Fig. S3 A–D), which is consistent with the previous report (29), p53 does not regulate Parkin expression (Fig. S4 A and B). So far, no negative feedback loop between p53 and Parkin was observed in these three cell lines and several

6 E and F). These results strongly suggest that as a target of p53, Parkin contributes greatly to the role of p53 in antioxidant defense.

Parkin Deficiency Sensitizes Mice to IR-Induced Tumorigenesis. Recent studies have suggested that Parkin is a potential tumor suppressor (18–22). It has been well-established that IR induces tumorigenesis in mice. To study the impact of Parkin deficiency upon tumorigenesis, 2-mo-old Parkin+/+ and Parkin−/− C57BL6/J male mice (28) were subjected to a single dose of 4-Gy IR. As shown in Fig. 7,4, Parkin knockout did not enhance the rate of spontaneous tumors but sensitized mice to IR-induced tumorigenesis; Parkin−/− mice displayed a shorter tumor latency induced by IR compared with wild-type mice (P < 0.01). The IR-induced tumor spectrum is similar between Parkin−/− and wild-type mice; IR mainly induced lymphomas in the spleen in both mice (Fig. 7B). Interestingly, our results have shown that IR specifically induced Parkin expression in a p53-dependent manner in mouse spleen (Fig. 3 E and F), which suggests that the induction of Parkin by p53 in response to IR may contribute to the role of Parkin in preventing IR-induced lymphomas in the spleen. These results provide further direct evidence to support the role of Parkin as a potential tumor suppressor.
Our results demonstrate that as a newly identified important component of the p53 signaling pathway, Parkin contributes to the functions of p53 in both energy metabolism and antioxidant defense. Parkin deficiency results in the Warburg effect, whereas ectopic expression of Parkin reverses the Warburg effect in cells. These results indicate that decreased expression of Parkin, which has been frequently observed in tumors, should be an important mechanism for the Warburg effect in tumors. The reduced mitochondrial respiration resulting from Parkin deficiency could be an important mechanism that contributes to enhanced glycolysis and the Warburg effect in tumor cells. The decreased expression of mitochondrial proteins resulting from Parkin deficiency, such as PDHΔ1, contributes to reduced mitochondrial respiration, which in turn promotes the Warburg effect. Recently, several additional mechanisms by which Parkin regulates mitochondrial function have been proposed, including regulating autophagy to clear damaged mitochondria (30), promoting mitochondrial fusion (31), and maintaining mitochondrial genome integrity (32), all of which may contribute to the role of Parkin in regulation of the Warburg effect. p53 has also been reported to play similar roles in some of these processes, such as autophagy and maintaining mitochondrial genome integrity (6). It is possible that Parkin also contributes to the role of p53 in the regulation of these processes. Thus, maintaining the homeostasis of energy metabolism and preventing the Warburg effect could be an important mechanism contributing to the tumor-suppressive function of Parkin. Furthermore, Parkin enhances GSH levels and decreases ROS levels. Considering the important role of ROS in tumorigenesis, the antioxidant function of Parkin should also contribute greatly to its role in tumor suppression. Thus, as a direct p53 target, Parkin contributes to the functions of p53 in tumor suppression through the regulation of energy metabolism, especially the Warburg effect, and antioxidant defense.

Materials and Methods

Cell Culture. p53-deficient H460-p53siRNA cells were established by stable transduction of a p53 shRNA retroviral vector (pSuper-puro-si-p53) in p53 wild-type H460 cells. H460-con cells are H460 cells with stable transduction of a control retroviral vector. p53 wild-type HCT116-con and p53-deficient HCT116-p53siRNA cells were generous gifts from M. Oren (Weizmann Institute of Science, Rehovot, Israel) (33). MEF Parkin+/− and Parkin−/− cells were established from wild-type and Parkin−/− C57BL6/J mice (The Jackson Laboratory) (28).

Fig. 5. The regulation of PDHΔ1 by Parkin contributes to the role of Parkin in regulating the Warburg effect in cells. (A) Parkin regulates the expression of PDHΔ1. (B) PDHΔ1 knockdown by siRNA in H460-con cells. (C) The levels of Parkin and PDHΔ1 affect PDH complex activity in H460 cells. (D) Parkin and PDHΔ1 regulate the intracellular levels of acetyl-coA. (E) PDHΔ1 knockdown reduces oxygen consumption in H460-con cells. (F) PDHΔ1 knockdown results in enhanced glucose uptake, rate of glycolysis, and lactate production in H460-con cells. Three different siRNA oligos against Parkin or PDHΔ1 were used for all assays, and similar results were observed. Data are presented as mean ± SD (n = 3). *P < 0.01.

Fig. 6. Parkin reduces ROS levels and increases GSH levels in cells. (A and B) Parkin reduces ROS levels in cells. H460-p53siRNA cells were transfected with Parkin expression vectors (A), and H460-con cells were transfected with siRNA oligos against Parkin (B) for 24 h. The cells were then treated with H2O2 (200 μM) for 6 h before assays. (C) Parkin knockout increases ROS levels in MEF cells. (D) Parkin contributes to the role of p53 in ROS regulation. Simultaneous knockdown of p53 and Parkin in H460 cells results in higher ROS levels than individual knockdown of p53 or Parkin, but the effect is less than additive. (E and F) Parkin regulates GSH levels (E) and the GSH/GSSG ratio (F) in H460 and MEF cells. The levels of GSH and GSSG were measured in cells at 24 h after transfection. Three different siRNA oligos against Parkin were used for all assays, and similar results were observed. Data are presented as mean ± SD (n = 3). #P < 0.05; *P < 0.01.
Fig. 7. Loss of Parkin sensitizes mice to IR-induced tumorigenesis. Two-month-old wild-type and Parkin−/− (C7BL6/J) male mice were subjected to a single dose of 4-Gy IR and monitored for survival. (A) Kaplan–Meier curve demonstrates that Parkin−/− mice had a significantly shorter tumor latency induced by IR compared with wild-type mice (P < 0.01). (B) The similar IR-induced tumor spectrum between Parkin−/− and wild-type mice.

ChiP and Luciferase Activity Assays. ChiP assays were performed in H460-con and H460-p53siRNA cells treated with Etoposide (10 μM for 16 h) to activate p53 as described (11, 25). The pGL2 firefly luciferase reporter containing putative p53 REs in human and mouse Parkin genes was constructed, and luciferase activity assays were performed as described (11, 25).

Real-Time PCR, Western Blot Analysis, and IF Staining. TaqMan real-time PCR was performed as described (11, 25). Western blot analysis and IF staining were performed as previously described (11).

Measurement of Glucose Uptake, Glycolysis Rate, Lactate Production, and Oxygen Consumption. Glucose uptake was measured by determining the uptake of 2-[3H]deoxyglucose (American Radiolabeled Chemicals) by cells as previously described (34). Glycolysis rate was measured by monitoring the conversion of 5-[3H]glucose to 3H2O as described (5, 9). Latent levels in the culture media of cells were determined by using a Lactate Assay Kit (Biovision). Oxygen consumption in cells was measured by using the BD Oxygen Biosensor System (BD Biosciences) as described (11).

Measurements of Activity of PDH and Levels of Acetyl-CoA, ATP, ROS, and GSH. The activity of PDH was measured by using a PDH Enzyme Activity Assay Kit (MitoSciences). The intracellular levels of acetyl-CoA were measured by using an ATP Bioluminescence Assay Kit (Roche) as described (11). ROS levels were measured by flow cytometry as described (9, 11). The levels of GSH and GSSG were measured by using a glutathione detection kit (Biovision) as described (9, 11).

γ-Irradiation and IR-Induced Tumorigenesis of Mice. Two-month-old p53+/+ and p53−/− male mice were treated with IR (4 Gy). Mice were killed at different times after IR (n = 6 for each time point), and different tissues were collected to determine Parkin expression. For tumorgenesis assays, 2-mo-old wild-type and Parkin−/− (C7BL6/J) mice (28) (The Jackson Laboratory) were subjected to 4-Gy IR. Mice were examined three times/wk until moribund. The statistical differences in tumor latency were analyzed by Kaplan–Meier analysis. See SI Materials and Methods for details.

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Supporting Information

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SI Materials and Methods
Reagents, Plasmids, and Cell Culture. Human H460 (p53 wild-type) and H1299 (p53 null) cells were obtained from the American Type Culture Collection. p53-deficient H460-p53siRNA cells were established by stable expression of a p53 shRNA vector (pSuper-puro-si-p53 vector; a generous gift from R. Agami, The Netherlands Cancer Institute, Amsterdam, The Netherlands) to knock down p53 in H460 cells. p53 wild-type H460-con cells are H460 cells stably transfected with a control vector. p53 wild-type HCT116-con and p53-deficient HCT116-p53siRNA cells were generous gifts from M. Oren (Weizmann Institute of Science, Rehovot, Israel) (1). HCT116-p53siRNA cells were established by stable transduction of pSuper-puro-si-p53 vector to knock down p53 in HCT116 cells. Parkin+/− and Parkin−/− mouse embryonic fibroblasts (MEF) were established from wild-type and Parkin−/− C57BL/6j mice (established in J. Shen’s laboratory at Harvard Medical School, Boston, MA) purchased from The Jackson Laboratory (2). siRNA oligos against Parkin or pyruvate dehydrogenase E1α1 (PDHA1) (Ambion) were transfected into cells using Oligofectamine (Invitrogen). To avoid off-target effects, three different siRNA oligos against Parkin or PDHA1 were used. The human Parkin expression vector pRK5-myc-Parkin (a generous gift from T. Dawson, Johns Hopkins University, Baltimore, MD) was transfected into cells using Lipofectamine 2000 (Invitrogen). pLPCX-Parkin retroviral vector was constructed by inserting the myc-Parkin DNA fragment from pRK-myc-Parkin into pLPCX vector (Clontech). For γ-irradiation (IR) treatment, a 137Cs irradiator (CIS Bio International) was used to deliver 4 Gy to cells.

γ-Irradiation of Mice. Two-month-old p53+/+ and p53−/− male C57BL6 mice (provided by T. Jacks, Massachusetts Institute of Technology, Cambridge, MA) were treated with whole-body IR (4 Gy) with a 137Cs-source irradiator. Mice were killed at different times after IR (6, 20, and 24 h; n = 6 for each time point), and different tissues were collected for assays.

Chromatin Immunoprecipitation Analysis. Chromatin immunoprecipitation (ChIP) assays were performed using the Upstate ChIP Assay Kit as previously described (3, 4). H460-con and H460-p53siRNA cells were treated with Etoposide (10 μM for 16 h) to activate p53 before ChIP assays. DO-1 anti-p53 antibody and mouse IgG (Sigma) were used for the assays. The following primer sets were designed to encompass the potential p53 responsive elements (REs) in human Parkin gene. For intron 1, forward primer: 5′-TGGGCCCCAAGAACAGAATG-3′; reverse primer: 5′-AACCACAGGGAAAGAAAACAAAT-3′. For promoter, forward primer: 5′-ATTCCTCTAAACTTTGAGCATATA-3′; reverse primer: 5′-AAGTCTGTGTTGAGTGTTGATAG-3′. The PCR fragments were cloned into the promoter region of pGL2 luciferase reporter vector (Promega) at KpnI and XhoI sites, respectively. The PCR fragments were cloned into the promoter region of pGL2 luciferase reporter vector (Promega) at KpnI and XhoI sites. Luciferase activity assays were performed as previously described (3, 4). In brief, the reporter vectors were transfected into p53 null H1299 and MEF p53−/− cells along with pRC-p53 (wild-type p53 expression plasmid) or pRC-R273H (mutant R273H p53 expression plasmid). pRL-SV40 vector expressing renilla luciferase was cotransfected as an internal control to normalize transfection efficiency. For H1299 cells, transfection was performed by using Lipofectamine 2000 (Invitrogen). For MEF p53−/− cells, transfection was performed by electroporation in a Gene Pulser Xcell Electroporation System (Bio-Rad). Luciferase activities were measured 24 h after transfection. The relative luciferase activity was calculated as luciferase activity of reporter vectors in cells cotransfected with wild-type p53 compared with that in cells cotransfected with mutant p53.

Quantitative Real-Time PCR. Total RNA was prepared with the RNeasy Kit (Qiagen). cDNA was prepared using a TaqMan reverse transcription kit, and real-time PCR was performed with TaqMan PCR mixture (Applied Biosystems) as previously described (3, 4). The expression of genes was normalized to the actin gene.

Western Blot Analysis. The following antibodies were used for assays: anti-Parkin (PRK8; Santa Cruz Biotechnology); anti-p53 (DO-1 for human p53, and FL393 for mouse p53; Santa Cruz Biotechnology); anti-MDM2 (2A10, Calbiochem); anti-PDHA1 (C54G1; Cell Signaling Technology); and anti-actin (A5441; Sigma). Western blot analysis was performed as previously described (4).

Immunofluorescence Staining. Immunofluorescence staining was performed as previously described (4). PRK8 antibody was used for Parkin staining. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma). For MitoTracker (Molecular Probes) staining, cells were incubated in 50 nm MitoTracker for 30 min before fixation.

Measurement of Glucose Uptake. Glucose uptake was measured as previously described (5, 6). In brief, cells (1 × 105) were seeded onto 12-well plates and cultured for 12 h. Cells were then washed with PBS twice and preincubated in glucose-free media for 30 min before 2-[3H]deoxyglucose (1 μCi per well; American Radiolabeled Chemicals) was added to the cells and incubated for 30 min at 37 °C. Uptake of 2-[3H]deoxyglucose was terminated by a rapid removal of medium, followed by washing with PBS three times. The cells were then lysed in 300 μL of 1% SDS, and the radioactivity of collected cell lysates was determined in a Beckman LS 6000 SC liquid scintillation counter (Beckman Coulter). Glucose uptake was normalized to the protein concentrations of the cell lysates.

Measurement of the Rate of Glycolysis. The rate was determined by monitoring the conversion of 5-[3H]glucose to 3H2O as described (7, 8). In brief, cells (1 × 105) were collected and washed in PBS once and then resuspended in 1 mL of Krebs buffer without glucose for 30 min at 37 °C. Cells were then collected and resuspended in 0.5 mL of Krebs buffer containing 10 mM glucose and 5 μCi of 5-[3H]glucose (American Radiolabeled Chemicals) for 1 h at 37 °C. Triplicate 100-μL aliquots were transferred to uncapped PCR tubes containing 100 μL of 3H2O.
0.2 N HCl, and a tube was transferred to a scintillation vial containing 0.5 mL of H₂O. The scintillation vials were sealed and left for 48 h to allow diffusion to occur. The amounts of diffused and undiffused ³H were determined in a Beckman LS 6000 SC liquid scintillation counter (Beckman Coulter). The rate of glycolysis was calculated as described (7, 8).

**Measurement of Lactate Production.** Cells (1 × 10⁶) were seeded onto 12-well plates for 12 h and then replenished with fresh phenol red-free medium (Sigma). Cells were then incubated for 16–24 h and the culture medium was collected for measurement of lactate concentrations. Lactate levels were determined by using a Lactate Assay Kit (Biovision) and normalized with cell number.

**Measurement of Oxygen Consumption.** Oxygen consumption in cells was measured by using the BD Oxygen Biosensor System (BD Biosciences) as previously described (4). This system is an oxygen-sensitive fluorescent compound embedded in a gas-permeable and hydrophobic matrix attached to the bottom of a multiwell plate. The amount of fluorescence correlates directly with the rate of oxygen consumption in the well. In brief, cells were seeded onto a 96-well plate covered with microcarrier beads (Cytoxex-3; Sigma) for cells to attach better. Oxygen consumption was measured by a fluorescence plate reader using Ex/Em = 485/630 nm at different times after cell seeding.

**Measurement of the Activity of the Pyruvate Dehydrogenase Complex and Intracellular Levels of Acetyl-CoA and ATP.** Pyruvate dehydrogenase (PDH) activity was measured by using a PDH Enzyme Activity Microplate Assay Kit (MitoSciences) according to the manufacturer’s instructions. The intracellular levels of acetyl-CoA were measured by using a PicoProbe Acetyl-CoA Assay Kit (Biovision) according to the manufacturer’s instructions. The intracellular levels of ATP were measured by using an ATP Bioluminescence Assay Kit (Roche) as previously described (4).

**Measurement of Levels of ROS and Reduced Glutathione.** ROS levels were measured by flow cytometry as described (4, 7). In brief, cells (1 × 10⁶) were incubated in PBS containing 5 mM dihydroethidium (DHE, Molecular Probes) or 10 μM 7-di chlorodihydrofluorescein diacetate (H₂DCF-DA) (Molecular Probes) for 30 min at 37 °C. Cells were washed in PBS, trypsinized, resuspended in PBS, and applied on a flow cytometer to measure ROS levels. The levels of reduced glutathione and oxidized glutathione were measured by using a glutathione detection kit (Biovision) as described (4, 7).

**IR-Induced Tumorigenesis in Mice.** Wild-type and Parkin⁻⁻/⁻ C57BL6/J mice (established in J. Shen’s laboratory at Harvard Medical School) (2) were purchased from The Jackson Laboratory. Two-month-old wild-type and Parkin⁻⁻/⁻ C57BL6/J mice were subjected to 4-Gy whole-body IR with a ¹³⁷Cs-source irradiator. After IR, mice were examined three times per week until moribund. Routine histopathological analysis was performed after the mice were killed.

**Statistical Analysis.** The statistical differences in tumor latency in IR-induced tumorigenesis assays were determined by Kaplan–Meier analysis using GraphPad Prism software. All other P values were obtained using a t test.


Fig. S1. p53 regulates the expression of human Parkin gene in HCT116 cells. p53 wild-type HCT116-con cells and p53-deficient HCT116-p53sirRNA cells were treated with (A) H₂O₂ (200 μM) and (B) Etoposide (Etp; 10 μM), respectively. Parkin protein levels at different time points after treatment were measured by Western blot assay.
Fig. S2. Regulation of PDHA1 and intracellular ATP levels by Parkin in cells. (A) Parkin does not regulate PDHA1 at the mRNA level in H460 and MEF cells. (B) The altered expression of Parkin or PDHA1 does not affect intracellular ATP levels in H460 cells. Three different siRNA oligos against Parkin or PDHA1 were used for all assays, and similar results were observed. Data are presented as mean ± SD (n = 3).
Fig. S3. Parkin negatively regulates p53 in a cell type-specific manner. (A and B) Parkin overexpression represses p53 expression in SH-SH5Y cells but not in HCT116 or H460 cells. Parkin was overexpressed in p53 wild-type human neuroblastoma SH-SH5Y, H460-con, and HCT116-con cells by transduction with pLPCX-Parkin retroviral vector expressing human Parkin. The levels of p53 and its targets (MDM2 and p21) were measured by Western blot (A) and TaqMan real-time PCR (B) assays, respectively. mRNA expression levels were normalized with actin. Parkin mRNA levels in cells are presented in B (Right). Parkin levels in control cells transduced with control pLPCX vector were designated as 1. (C and D) Knockdown of endogenous Parkin induces p53 expression in SH-SH5Y cells but not in HCT116 or H460 cells. Cells were transfected with siRNA oligos against Parkin to knock down Parkin 24 h before assays. The levels of p53 and its targets (MDM2 and p21) were measured by Western blot (C) and TaqMan real-time PCR (D) assays, respectively. mRNA expression levels were normalized with actin. Parkin mRNA levels in cells are presented in D (Right). Three different siRNA oligos against Parkin were used, and similar results were observed.

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