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 \(\gamma\)-irradiation-induced tumorigenesis, 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 and mRNA levels at both protein and mRNA levels (Fig. 1A). This induction is p53-dependent because no clear induction of Parkin was observed in H460-p53siRNA cells at both protein and mRNA levels (Fig. 1A and B). 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. 1C 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 mainly 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-
cells treated with Etoposide (10 μM) for different amounts of time. The protein levels were determined by Western blot assays. mRNA levels of Parkin were measured by real-time PCR.

**Fig. 1.** p53 regulates the expression of human Parkin gene. H460-con and H460-p53siRNA cells were treated with Etoposide (Etp, 10 μM) or H2O2 (200 μM) for different amounts of time. The protein levels were determined by Western blot assays. mRNA levels of Parkin were measured by real-time PCR and normalized with actin. Data are presented as mean ± SD (n = 3).

**Human Parkin Gene Contains a Functional p53 Responsive Element.** To investigate whether p53 regulates Parkin expression through its direct binding to the two putative p53 REs in human Parkin promoter region and intron 1 predicted by the p53MH algorithm (Fig. 2A), H460-con and H460-p53siRNA cells were treated with Etoposide to activate p53, and chromatin immunoprecipitation (ChIP) assays were performed. The anti-p53 antibody specifically pulled down the DNA fragment containing the putative p53 RE in Parkin intron 1 in H460-con cells treated with Etoposide, but not in H460-p53siRNA cells (Fig. 2B). These results demonstrate that p53 protein physically interacts with the putative p53 RE in human Parkin intron 1 in vivo.

To further investigate whether these two putative p53 REs 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. 2A). 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 assays 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. 2B). Furthermore, H2O2 clearly induced Parkin expression at both mRNA and protein levels in p53−/−MEF but not in p53−/−MEF cells (Fig. 2C). p53−/−MEF cells also displayed a higher level of basal Parkin expression than p53−/−MEF cells. 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 in p53−/− mice (Fig. 2E 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. 4 A–C, ectopic expression of Parkin in H460-p53siRNA cells significantly decreased glucose uptake, the rate of glycolysis, and lactate production. Furthermore, knockout 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−/− MEF compared with Parkin+/+ MEF cells (Fig. 4 A–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 induces reduced mitochondrial respiration. Parkin knockout in mice was reported to result in the decreased expression of several mitochondrial proteins, including pyruvate dehydrogenase E1α1 (PDH1A1), in the mouse brain as detected by sodium dodecyl sulfate gel electrophoresis 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. 5 A–D clearly show that Parkin knockdown in H460-con cells decreased PDHA1 protein levels, the activity of the PDH complex, and levels of acetyl-CoA, whereas ectopic Parkin expression in H460-p53siRNA cells increased PDHA1 protein levels, the activity of the PDH complex, and levels of acetyl-CoA. Consistently, PDHA1 levels were much higher in Parkin+/+ MEF than Parkin−/− MEF cells (Fig. 5A, B). Furthermore, PDHA1 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 PDHA1. Parkin does not regulate PDHA1 expression at the mRNA level (Fig. 5A, D). Knockdown of Parkin or PDHA1 did not decrease intracellular ATP levels (Fig. 5B), 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. 6 E 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 knockout in MEF cells significantly decreased GSH levels and the GSH/GSSG ratio (Fig. 6D).
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−/− C57BL/6J male mice (28) were subjected to a single dose of 4-Gy IR. As shown in Fig. 7A, 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. 3E 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.

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. S3A–D). Interestingly, in human neuroblastoma SH-SH5Y cells, whereas Parkin represses p53 expression and transcriptional activity (Fig. S3A–D), which is consistent with the previous report (29), p53 does not regulate Parkin expression (Fig. S4A and B). So far, no negative feedback loop between p53 and Parkin was observed in these three cell lines and several
other cell lines we tested. These results strongly suggest that the regulation of Parkin by p53, and the regulation of p53 by Parkin, could be cell type- or tissue-specific.

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 PDHA1, 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 fission (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 (Super-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 PDHA1 by Parkin contributes to the role of Parkin in regulating the Warburg effect in cells. (A) Parkin regulates the expression of PDHA1. (B) PDHA1 knockdown by siRNA in H460-con cells. (C) The levels of Parkin and PDHA1 affect PDH complex activity in H460 cells. (D) Parkin and PDHA1 regulate the intracellular levels of acetyl-coA. (E) PDHA1 knockdown reduces oxygen consumption in H460-con cells. (F) PDHA1 knockdown results in enhanced glucose uptake, rate of glycolysis, and lactate production in H460-con 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), *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.
demonstrates that Parkin shares 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-p53S1RNA 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).

Measurements 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 H2O as described (5, 9). Lactate 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).

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 tumorigenesis assays, 2- month-old wild-type and Parkin−/− C57BL/6J mice (28) were subjected to 4-Gy IR. Mice were examined three times wk/vk 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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