

ZNF143 transcription factor mediates cell survival through upregulation of the GPX1 activity in the mitochondrial respiratory dysfunction

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Mitochondrial respiratory dysfunction has intimate relationship with redox regulation. The key mechanism about how the mitochondrial respiration-defective cells survive oxidative stress is still elusive. Here, we report that transcription factor zinc-finger protein 143 (ZNF143) expression and glutathione peroxidase (GPX) activity are markedly increased in the mitochondrial respiratory-defective cells induced by dominant-negative DNA polymerase γ (POLGdn). In this work, investigation of the cellular antioxidant glutathione (GSH) and enzyme GPX activity in the mitochondrial dysfunction revealed the presence of an increased synthesis of GSH through the activation of GCLC (glutamate–cysteine ligase catalytic subunit) and GCLM (glutamate–cysteine ligase regulatory subunit) gene expression, and also a positive upregulation of glutathione peroxidase 1 (GPX1) activity by the transcription factor ZNF143. Significant increase in gene expression of SepSecS, the key enzyme responsible for selenocysteine transfer RNA (tRNA) synthesis, further confirmed the activation of the selenocysteine synthesis pathway. By using both GPX1 and ZNF143 knockdown, we provided insight into the involvement of ZNF143 in promoting GPX1 activity and protecting cells from oxidative damage and cisplatin treatment in the mitochondrial dysfunction. Furthermore, we reported the possible regulation of mitochondrial transcription factor A (TFAM) in the mitochondrial dysfunction. Our findings delineate an important antioxidant survival pathway that allows the mitochondrial-defective cells to survive oxidative stress and cisplatin treatment.

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The homeostasis of intracellular redox status is highly regulated by the mitochondria and is likely to be important for the pathogenesis of various human diseases. The mitochondrial dysfunction observed by mtDNA mutation in cancer has increased reactive oxygen species (ROS) stress.¹ To survive the oxidative stress condition, cancer cells need to adjust their antioxidant system to cope with the toxic effects of the free radicals and initiate adaptation process, but the underline need for regulatory mechanism of the survival pathway to deal with ROS stress in the mitochondrial respiratory dysfunction is elusive. Antioxidant systems are reported to be involved in cancer cell survival and drug resistance.² Thus, insights into understanding the antioxidant regulatory mechanism in the mitochondrial respiratory dysfunction may be important for the design of novel therapeutic agents.

Free radicals can be eliminated by reducing equivalent glutathione (GSH), an abundant cellular thiol and the major determinant of cellular redox equilibrium, and GSH-coupled

antioxidant enzymes such as GSH peroxidases (GPXs) and glutathione reductase to maintain redox homeostasis.³ GSH peroxidase 1 (GPX1) is an abundant and ubiquitously expressed selenoprotein and functions as a major intracellular peroxide-scavenging enzyme.^{4,5} It utilizes GSH as a substrate to catalyze the reduction of H₂O₂ and lipid peroxides.^{6,7} The status of GSH and GPX and their regulatory mechanisms in combat with ROS in the mitochondrial respiratory dysfunction is elusive. Glutamate–cysteine ligase, composed of catalytic subunit (called GCLC) and regulatory subunit (called GCLM), is important for GSH biosynthesis in combating a variety of oxidative stress-related complications, thereby activating the body's own protective response.

Zinc-finger protein 143 (ZNF143) is the human homolog of selenocysteine transfer RNA (tRNA^{Sec}) gene-transcription-activating factor. ZNF143 is required for the transcription activation of tRNA^{Sec}, which mediates the incorporation of Sec into selenoproteins, such as GPX1.⁸ Sec (U) insertion

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Abbreviations: COII, cytochrome *c* oxidase subunit II; ddC, 2',3'-dideoxycytidine; GCLC, glutamate–cysteine ligase catalytic subunit; GCLM, glutamate–cysteine ligase regulatory subunit; GSH, glutathione; GPX, glutathione peroxidase; GSS, glutathione synthetase; POLGdn, dominant-negative form of DNA polymerase γ ; PGC-1 α , peroxisome proliferator-activated receptor γ , coactivator 1 α ; PRC, peroxisome proliferator-activated receptor γ , coactivator-related 1; ROS, reactive oxygen species; TFAM, mitochondrial transcription factor A; ZNF143, zinc-finger protein 143

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into proteins is directed by translational recoding of specific UGA codons located upstream of a stem-loop structure known as Sec insertion sequence element. Selenoproteins with known functions are oxidoreductases containing a single redox-active Sec in their active sites. As a transcription factor, ZNF143 is also involved in RNA-polymerase-II-dependent gene expression. The expression of ZNF143 was reported to be induced by DNA-damaging agents and is enhanced in cisplatin-resistant cell lines.⁹ Recently, ZNF143 was found to be highly expressed in multiple solid tumors and related to the transcriptional regulation of DNA replication and cell-cycle-associated genes.¹⁰

In this study, we adapted the molecular strategy by induction of mitochondrial respiratory dysfunction through tetracycline-controlled expression of dominant-negative DNA polymerase γ (POLGdn),¹¹ and used this experimental model system to investigate the adaptive changes associated with ROS stress, and to identify major redox regulators in the mitochondrial respiratory dysfunction, which reflect the metabolic shift from oxidative phosphorylation to aerobic glycolysis. We discovered that transcription factor ZNF143 protein level is significantly increased in the metabolic change. Through activating tRNASec gene transcription, ZNF143 increases GPX1 protein level and overall GPX activity to combat ROS stress in the mitochondrial respiration dysfunction. Increased gene expression of SepSecS, the key enzyme responsible for tRNASec synthesis, further confirms the activation of the Sec synthesis pathway. Knockdown of ZNF143 leads to decreased GPX activity, increased ROS stress, increased sensitivity to cisplatin and causes massive cell death in the mitochondrial respiratory-defective cells. This observation is further confirmed by GPX1 gene knockdown. Upregulation of GSH content through increased GSH synthesis by GCLC and GCLM gene expression is also observed. Taken together, upregulation of cellular GSH antioxidant system through increasing GPX activation pathway by ZNF143 together with GSH synthesis could provide survival advantages to combat oxidative stress and cisplatin treatment in the mitochondrial respiration dysfunction.

Results

POLGdn expression led to defective oxygen respiration and ROS generation. To investigate the survival mechanism in terms of oxidative stress under mitochondrial respiratory dysfunction, we first adapted an experimental cell model where the mitochondrial respiratory status can be regulated via a tetracycline-controlled dominant-negative form of DNA polymerase γ expression system.¹¹ POLGdn can effectively abolish mtDNA replication leading to respiration defect.^{11–13} Induced expression of POLGdn by adding doxycycline (Tet/on) to the culture medium led to a dramatic decrease of mtDNA-encoded ATPase 6 gene expression when compared with cells without doxycycline-induced POLGdn expression (Tet/on day 0, or Tet/off) (Figure 1a). As complex I deficiency could result in greater production of ROS, we analyzed mtDNA-encoded complex I subunit ND1 gene expression. ND1 gene expression was sharply decreased with POLGdn expression (Figure 1b). To confirm that POLGdn expression in Tet/on situation could affect

mitochondrial respiration, cellular oxygen consumption capacity was measured. We found that oxygen consumption ability was decreased in a time-dependent manner upon POLGdn expression (Figure 1c). Concurrently, western blot analysis detected that mtDNA-encoded cytochrome c oxidase subunit II (COII) protein level was significantly decreased and depleted around day 10 of POLGdn expression (Figure 1c, inset). As mitochondrion is regarded as one of the major sites of ROS generation, the mitochondrial dysfunction induced by POLGdn expression would be predicted to cause profound increase in free radical stress in POLGdn-induced mitochondrial respiratory dysfunction cells. To explore this possibility, we measured intracellular ROS levels by using the hydrogen peroxide-sensitive dye dichlorofluorescein diacetate (DCF-DA). To our surprise, we did not find significant increase on ROS level during the first 6 days of POLGdn induction, even when the mitochondrial respiratory components were largely decreased (Figures 1d and c). In comparison to their Tet/off counterparts, the increase of cellular ROS levels was observed at day 9 and markedly elevated at day 12 of POLGdn expression (Figure 1d).

GSH redox status was altered at an early stage of the mitochondrial dysfunction. To understand if the unaltered ROS level in early stage of the mitochondrial dysfunction is due to the increased antioxidant activity, we investigated GSH antioxidant system, a major cellular antioxidant system in combating ROS stress. Mitochondrial-defective Tet/on cells showed significant increase in cellular total GSH when compared with Tet/off cells (Figure 2a). Tet/on day 6 cells displayed significant higher level of GSH along with Tet/on day 9 and day 12 cells (Figure 2a). To understand the mechanism of GSH level increase in mitochondrial-defective cells, we first analyzed the genes involved in GSH synthesis. Correlate to GSH level increase at day 6 of POLGdn expression, gene expression of GCLC and GCLM, were significantly increased (Figures 2b and c). Although GCLC gene expression continuously increased with POLGdn induction, GCLM gene expression was decreased at day 12 when compared with day 6, which corresponded to the GSH level change. GSH synthetase (GSS) gene expression did not have significant alteration during POLGdn induction process (Figure 2d). Protein levels of the GCLC and GCLM were also increased (Figure 2e). Taken together, these data suggest that GSH synthesis pathway was activated and GSH content was increased in the mitochondrial respiratory-defective cells.

GPXs are the major antioxidant enzymes by using GSH as substrate for the elimination of ROS. We investigated the cellular GPX activity in Tet/on cells as a functional evaluation in comparison to their Tet/off cell counterparts. Significant GPX activity increase was observed even at an early stage of the mitochondrial dysfunction (at day 3 of POLGdn induction), when the increase of ROS level was not observed (Figure 3a). As GPX1 serves as a key antioxidant enzyme, we first analyzed GPX1 gene expression to further investigate GPX activation mechanism. qRT-PCR data showed that GPX1 gene expression did not increase under the duration of POLGdn induction (Figure 3b). These data suggest that GPX

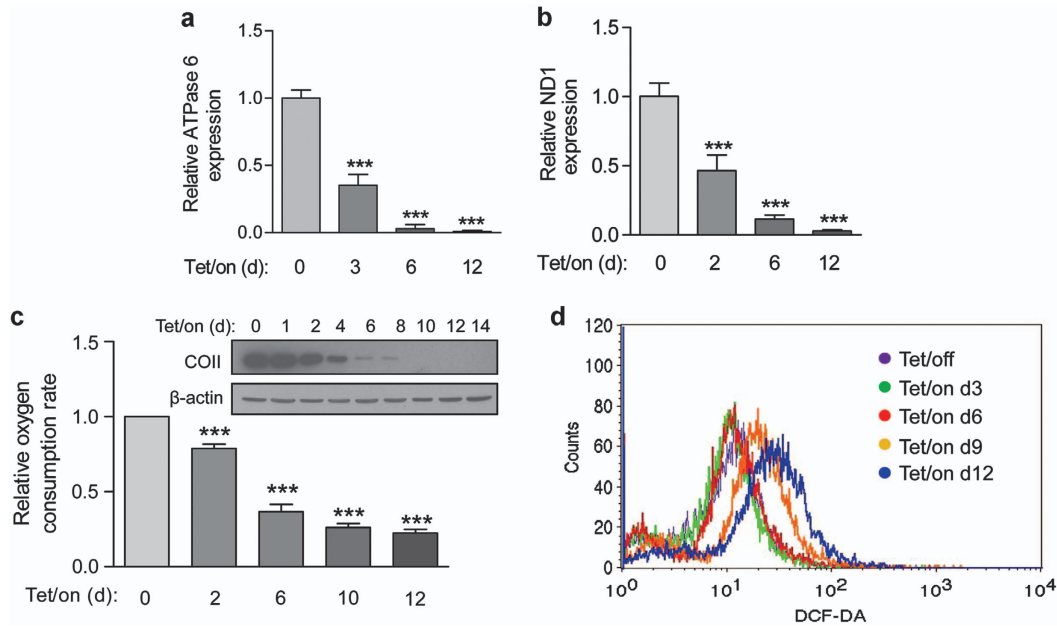


Figure 1 POLGdn expression led to impaired mitochondrial respiration and ROS generation. (a) ATPase 6 mRNA level decreased in a time-dependent manner after doxycycline-induced POLGdn gene expression. Real-time qRT-PCR was used to measure mRNA level of ATPase 6 and β -actin served as control ($n = 3$). Values are shown as mean \pm S.D.; *** $P < 0.001$. (b) ND1 mRNA level decreased in a time-dependent manner after doxycycline-induced POLGdn gene expression. Real-time qRT-PCR was used to measure mRNA level of ND1 and β -actin served as control ($n = 3$). Values are shown as mean \pm S.D.; *** $P < 0.001$. (c) Time-dependent reduction of oxygen consumption rate following POLGdn expression. Tet/off cells were used as control ($n = 3$). Values are shown as mean \pm S.D.; *** $P < 0.001$. (Inset) Time-dependent decrease of mtDNA-encoded COII protein following POLGdn expression. COII protein level was assessed by western blot assay. β -Actin was probed as a loading control. (d) Cellular ROS levels were significantly increased after profound mitochondrial respiration damage. Cellular H_2O_2 was measured by flow cytometry using $4 \mu\text{M}$ DCF-DA as a fluorescent dye

activity increase may due to post-transcriptional regulation of GPX. Thus, GPX1 protein level was measured at indicated days of POLGdn induction by western blot assay. The data showed that GPX1 protein levels were increased constantly after POLGdn expression (Figure 3c). Catalase, another H_2O_2 detoxification enzyme, was stable in the protein level during POLGdn induction (Figure 3d), suggesting that GPX activity increase is a major factor in detoxification of ROS in the mitochondrial respiratory dysfunction.

ZNF143 has survival role through upregulation of the GPX activity in the mitochondrial dysfunction. Tet/on cells displayed higher GPX activity and increased GPX1 protein level, but with unaltered GPX1 gene expression, which suggest that GPX1 activity is regulated at post-transcription level. GPX1 is a selenoprotein and its activity is dependent on the translational incorporation of the amino-acid Sec to the activation site of the protein.¹⁴ Selenoprotein synthesis is dependent on the presence of tRNA^{Sec}, which is regulated by transcription factor ZNF143.¹⁵ We speculated that Tet/on cells may have higher ZNF143 level in comparison to their Tet/off counterparts. ZNF143 mRNA expression was evaluated by qRT-PCR and the data showed that ZNF143 mRNA was significantly increased on day 3 of POLGdn induction, the early stage of mitochondrial respiratory dysfunction, and its expression was further increased after 9 days of POLGdn expression (Figure 4a). Concurrently, ZNF143 protein level was significant increased at day 4 of Tet/on cells and maintained at higher level afterwards (Figure 4b). Sec is the only amino acid that does not have its own tRNA synthetase. Its biosynthesis occurs on its cognate

tRNA. SepSecS is an enzyme that catalyzes the conversion of *O*-phosphoseryl-tRNA(Sec) to selenocysteinyl-tRNA(Sec), one of the major steps for Sec synthesis.¹⁶ To further investigate whether Sec synthesis is activated, we tested SepSecS gene expression using qRT-PCR and found that the gene was significantly increased in a time-dependent manner upon POLGdn induction (Figure 4c). To further identify that Sec synthesis pathway was activated, tRNA^{Sec} level was evaluated by qRT-PCR. The data revealed that tRNA^{Sec} level was significantly upregulated after 3 days of POLGdn expression (Figure 4d). To further prove that ZNF143 is induced by the mitochondrial dysfunction, Tet/off cells were treated with indicated mitochondrial respiratory chain inhibitors. We observed that ZNF143 protein levels were significantly increased with the treatments of these inhibitors (Figure 4e). To ensure that mitochondrial dysfunction-induced ZNF143 activation is not limited to the POLGdn Tet/on system, we measured GPX1 and ZNF143 protein levels in mtDNA-depleted p° cells (also called C6F) in the comparison to control HL60 cells, another model of mitochondrial respiratory dysfunction, and observed increased ZNF143 and GPX1 protein levels in p° cells (Figure 4f). These data strongly suggest that mitochondrial respiratory dysfunction could induce transcription factor ZNF143 gene expression and protein level increase, which further directs increased tRNA^{Sec} level, to activate Sec synthesis contributing to selenoprotein GPX1 activity increase and protein level increase.

To investigate the relationship between ZNF143 and GPX1 and the functional role of ZNF143 in mitochondrial respiratory dysfunction, siRNA of ZNF143 was transfected 2 days after

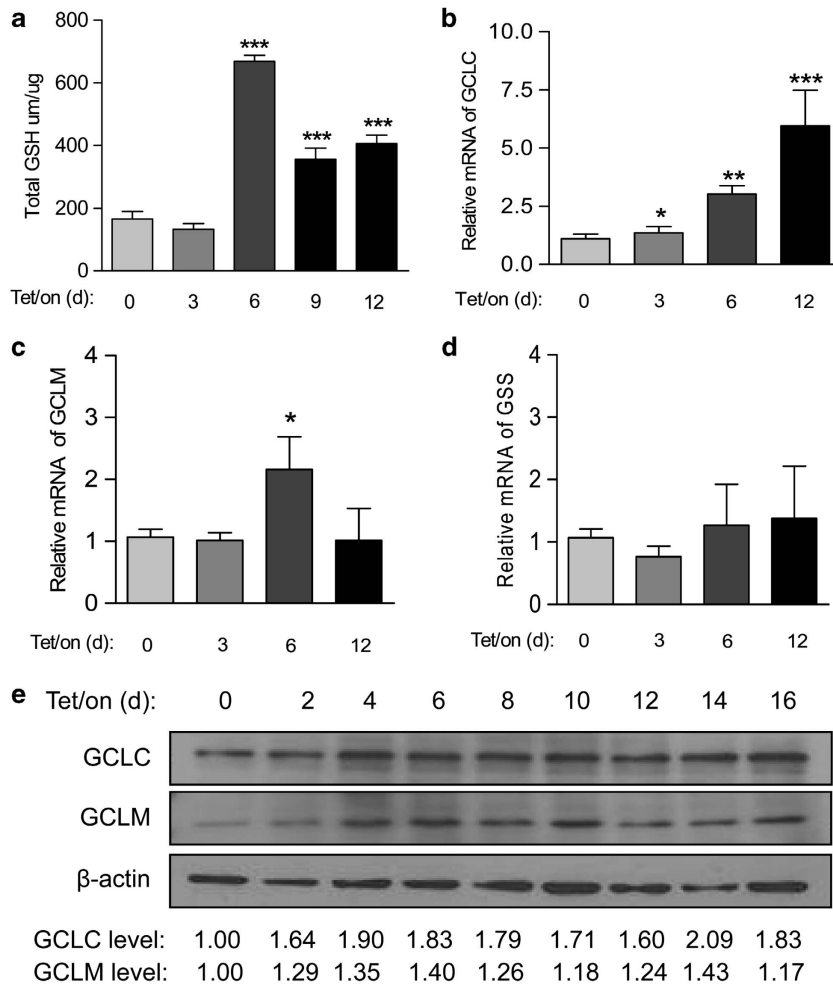


Figure 2 Changes in cellular GSH content and the expression of the genes responsible for GSH synthesis. **(a)** Comparison of cellular glutathione contents in Tet/off and Tet/on cells at the indicated time points using a GSH assay kit from Cayman Chemical Co. ($n = 3$). Values are shown as mean \pm S.D.; *** $P < 0.001$. **(b)** Real-time qRT-PCR analysis of GCLC in Tet/off and Tet/on cells at the indicated time point. β -Actin was used as the sample control ($n = 3$). Values are shown as mean \pm S.D.; * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$. **(c)** Real-time qRT-PCR analysis of GCLM in Tet/off and Tet/on cells at the indicated time points. β -Actin was used as the sample control ($n = 3$). Values are shown as mean \pm S.D.; * $P < 0.05$. **(d)** Real-time qRT-PCR analysis of GSS in Tet/off and Tet/on cells at the indicated time points. β -Actin was used as the sample control ($n = 3$). Values are shown as mean \pm S.D. No significant difference of GSS was found between the control and the POLGdn-expressing samples. **(e)** GCLC and GCLM protein level assessed by western blotting was increased after POLGdn expression. β -Actin was used as a loading control. Scanning and ImageJ software (a public domain Java image processing program developed at the National Institutes of Health) was used for the quantification of western blot results. Results are expressed as integrated optical density. Each sample was normalized to β -actin content

POLGdn induction. The knockdown efficiency was evaluated after 4 days of siRNA transfection, considering that ZNF143 protein level is significantly increased at day 6 of POLGdn induction (Figure 5a). ZNF143 knockdown led to the decrease of GPX1 protein level when compared with scrambled siRNA (scRNA) knockdown (Figure 5a). Concurrently, GPX activity was significantly decreased in the ZNF143 knockdown cells (Figure 5b). As GPX1 is an important ROS scavenger, we speculate that ZNF143 knockdown would cause an increase in ROS generation. We analyzed cellular H_2O_2 level on day 3 of Tet/on, which has either 1 day of ZNF143 siRNA or scRNA transfection. We did not observe ROS alteration in the short time of ZNF143 knockdown (Figure 5c, left). We then analyzed cellular H_2O_2 level on day 5 of Tet/on cells, which have 3 days of ZNF143 siRNA or scRNA transfection. ZNF143 knockdown significantly increased cellular ROS content (Figure 5c, right), which further proved the role of

ZNF143 in the maintenance of cellular redox balance through the upregulation of antioxidant enzyme GPX function in the mitochondrial respiratory dysfunction. Mitochondria have an important role in the regulation of apoptosis, and mitochondrial respiratory-defective cells were reported to have less sensitivity to chemotherapeutic agents commonly used in the clinical treatment of cancer.¹⁷ We examined the effect of mitochondrial defects on the cellular response to anticancer agents, gemcitabine and taxol, through the comparison of the drug sensitivity of Tet/off and respiration-defective Tet/on day 9 cells. We observed that the respiration-defective cells were less sensitive than the Tet/off cells to both anticancer agents (Figure 5d). The transcription factor ZNF143 was reported to be overexpressed in cisplatin-resistant cells.^{9,22} We reasoned that ZNF143 knockdown in respiration-defective cells might increase the cellular sensitivity to cisplatin, an anticancer agent commonly used in the clinic. Tet/on day 6 cells were

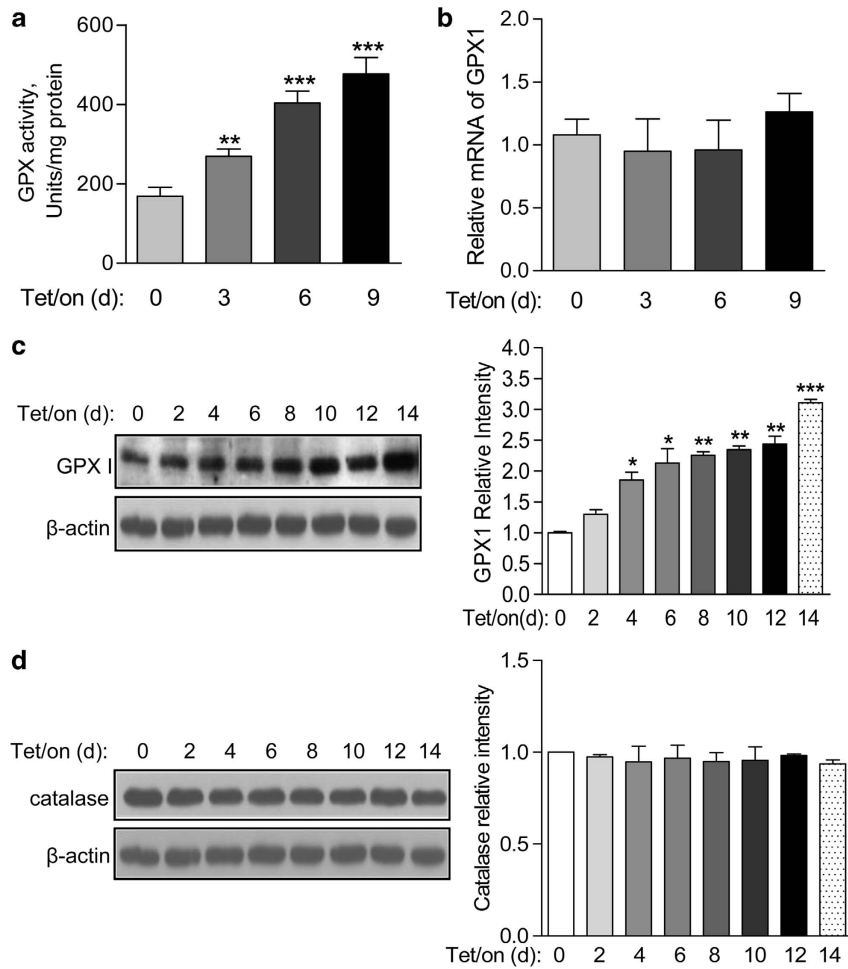


Figure 3 GPX activity and GPX1 protein level were constantly increased in the mitochondrial respiratory dysfunction induced by POLGdn expression. (a) Intracellular GPX activity increased within the duration of POLGdn induction. Intracellular GPX activity was expressed as units/mg protein. Data correspond to means \pm S.D. from at least three independent determinations of GPX. ** $P < 0.01$; *** $P < 0.001$. (b) GPX1 mRNA level was unchanged within the indicated duration of POLGdn induction. Real-time qRT-PCR analysis was used to assess GPX1 gene expression. β -Actin was used as the sample control ($n = 3$). Values are shown as mean \pm S.D. No significant change in gene expression was observed. (c) Time-dependent increase in the protein level of GPX1 following POLGdn expression. Left panels show representative GPX1 protein level with POLGdn induction assessed by western blotting. β -Actin was probed as a loading control. Right panels show quantification of western blot results using scanning and ImageJ software. Results are expressed as integrated optical density. Each sample was normalized to β -actin content. Each bar represents the mean \pm S.E.M. of three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (d) Catalase protein level was unaltered within the indicated duration of POLGdn induction. Left panels show representative catalase protein levels assessed by western blotting. β -Actin was probed as a loading control. Right panels show quantification of western blot results using scanning and ImageJ software. Results are expressed as integrated optical density. Each sample was normalized to β -actin content. Each bar represents the mean \pm S.E.M. of three independent experiments. No significant change was observed

transfected with either scRNA or ZNF143 siRNA for 24 h followed by treatment with cisplatin for 48 h. As illustrated in Figure 5e, cells with ZNF143 knockdown were more sensitive to cisplatin treatment as shown by a substantial increase in the percentage of cells with Annexin V/propidium iodide (PI)-positive staining. There is a stable ZNF143 protein level increase at day 6 of Tet/on (Figure 4b). We decided to knockdown transiently the gene at day 6 of Tet/on by transfecting ZNF143 siRNA at day 2 of Tet/on to investigate the effect of ZNF143 knockdown. We observed that ZNF143 knockdown caused more than 25% cell death compared with cells transfected with scRNA (Figure 5f). Altogether, our data led to the conclusion that ZNF143 is an important survival factor in the mitochondrial respiratory dysfunction through increasing cellular GPX activity to allow mitochondrial-defective

cells to escape from higher oxidative stress. Targeted inhibition of ZNF143 substantially increased cellular sensitivity to cisplatin in respiration-defective Tet/on cells.

To further investigate a functional role of GPX1 activation in mitochondrial dysfunctional cells, we transfected the plasmid expressing nonspecific shRNA (NS-shRNA) and the plasmid expressing GPX1 shRNA (GPX1-shRNA) in Tet/off cells. GPX1 protein was effectively reduced by GPX1-shRNA as determined by western blot analysis (Figure 6a). POLGdn induction caused GPX1 protein level increase in NS-shRNA plasmid-transfected control cells, whereas cells with GPX1 knockdown by GPX1-shRNA plasmid had no detectable GPX1 expression even after POLGdn induction, further confirming the GPX1 knockdown efficiency (Figure 6a). To determine if GPX1 is the major antioxidant for the GPX

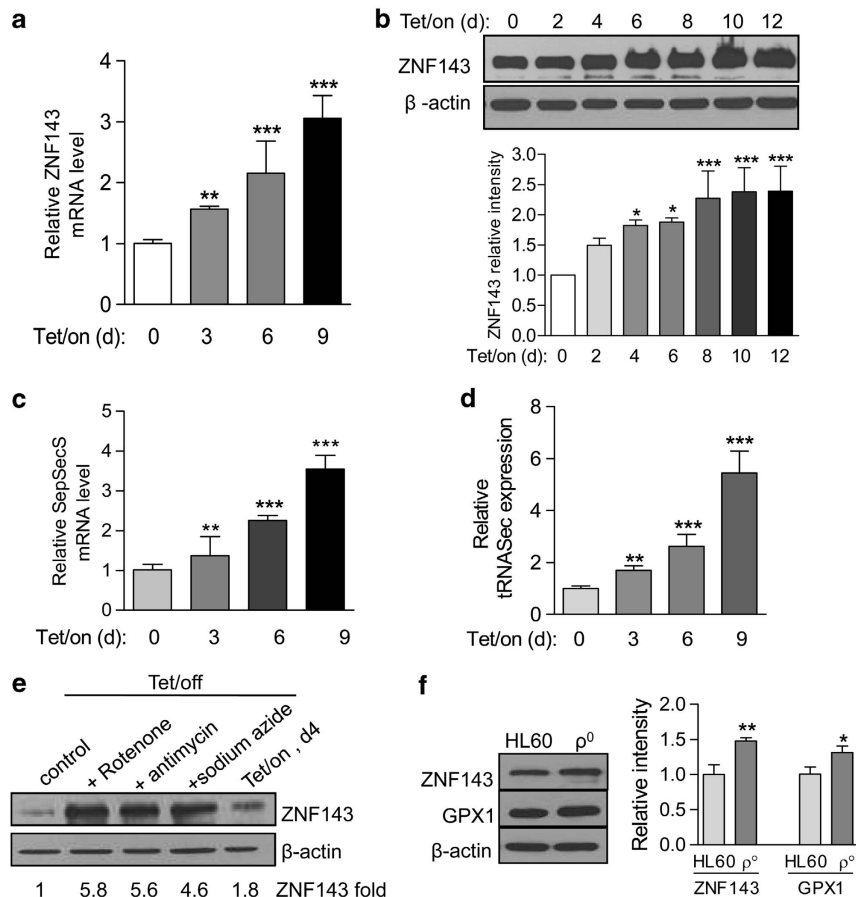


Figure 4 ZNF143 gene expression and protein level were significantly increased in the cells with mitochondrial respiratory dysfunction. (a) ZNF143 mRNA level, as determined by real-time qRT-PCR, was significantly increased after POLGdn induction by doxycycline. β -Actin was used as the sample control ($n=3$). Values are shown as mean \pm S.D.; ** $P<0.01$; *** $P<0.001$. (b) ZNF143 protein level detected by western blot was increased upon POLGdn expression at indicated time points. β -Actin was probed as a loading control. Upper panels show representative ZNF143 protein levels assessed by western blot. Lower panels show quantification of western blot results using scanning and ImageJ software. Results are expressed as integrated optical density. Each sample was normalized to β -actin content. Each bar represents the mean \pm S.E.M. of three independent experiments. * $P<0.05$; *** $P<0.001$. (c) SepSecS gene expression, as determined by qRT-PCR, was significantly increased after 3 days of POLGdn induction by doxycycline. β -Actin was used as the sample control ($n=3$). Values are shown as mean \pm S.D.; ** $P<0.01$; *** $P<0.001$. (d) tRNA^{Sec} expression, as determined by qRT-PCR, was significantly increased after 3 days of POLGdn induction. β -Actin was used as the sample control ($n=3$). Values are shown as mean \pm S.D.; ** $P<0.01$; *** $P<0.001$. (e) Western blot analysis of ZNF143 protein level in Tet/off cells were treated with mitochondrial respiratory chain inhibition agents, rotenone (100 nM), antimycin (5 μ M) or sodium azide (500 μ M), for 24 h, respectively. β -Actin was probed as a loading control. ZNF143 fold change was quantified by scanning and ImageJ software. Results are expressed as integrated optical density. Each sample was normalized to β -actin content. (f) ZNF143 and GPX1 protein levels were increased in mitochondrial respiration-defective p⁰ cells. Left panels show representative ZNF143 and GPX1 protein levels in mitochondrial respiration-defective p⁰ (C6F) cells as compared with their parental HL60 cells using western blot assay. β -Actin was probed as a loading control. Right panels show quantification of western blot results using scanning and ImageJ software. Results are expressed as integrated optical density. Each sample was normalized to β -actin content. Each bar represents the mean \pm S.E.M. of three independent experiments. * $P<0.05$; ** $P<0.01$

activation in the mitochondrial dysfunction, GPX activity was measured. Cells with NS-shRNA plasmid showed increased GPX activity after POLGdn induction, whereas cells with GPX1 knockdown did not have any GPX activity increase after POLGdn induction and even lower than the Tet/off control cells (Figure 6b). These data further confirm that GPX activity increase mainly comes from GPX1. To test the effect of GPX1 knockdown in response to cisplatin treatment in the respiration-defective cells, we induced POLGdn expression for 7 days in either NS-shRNA or GPX1-shRNA stably transfected POLGdn Tet/on models followed by treatment of cisplatin for 48 h. As illustrated in Figure 6c, Tet/on day 9 cells with GPX1 knockdown showed approximately 25% more cell death compared with same stage of Tet/on cells with scramble

vector transfection under cisplatin treatment. To examine the effect of GPX1 knockdown on cell death of long time Tet/on cells, Annexin V/PI double staining assay was used. Figure 6d indicates that Tet/on day 12 cells with GPX1 knockdown resulted in approximately 20% more cell death than same stage of Tet/on cells with scramble vector transfection.

There was a significant increase in cellular GSH and an upregulation of GPX activity, which mainly came from GPX1 activation. These data together suggest that expression of POLGdn induced mitochondrial dysfunction, which in turn led to an increase in cellular GSH content and an increase in ZNF143-mediated GPX antioxidant activity in protecting mitochondrial respiration-defective cells from oxidative damage and cisplatin treatment.

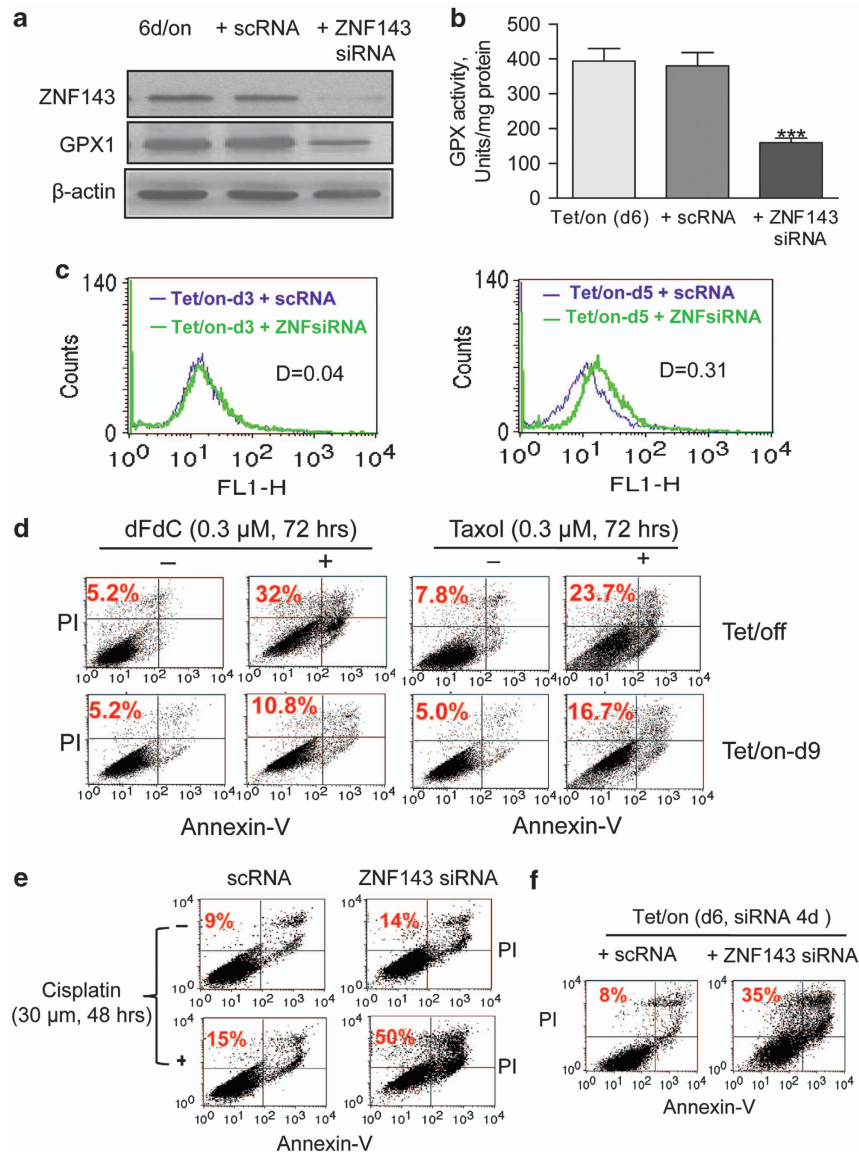
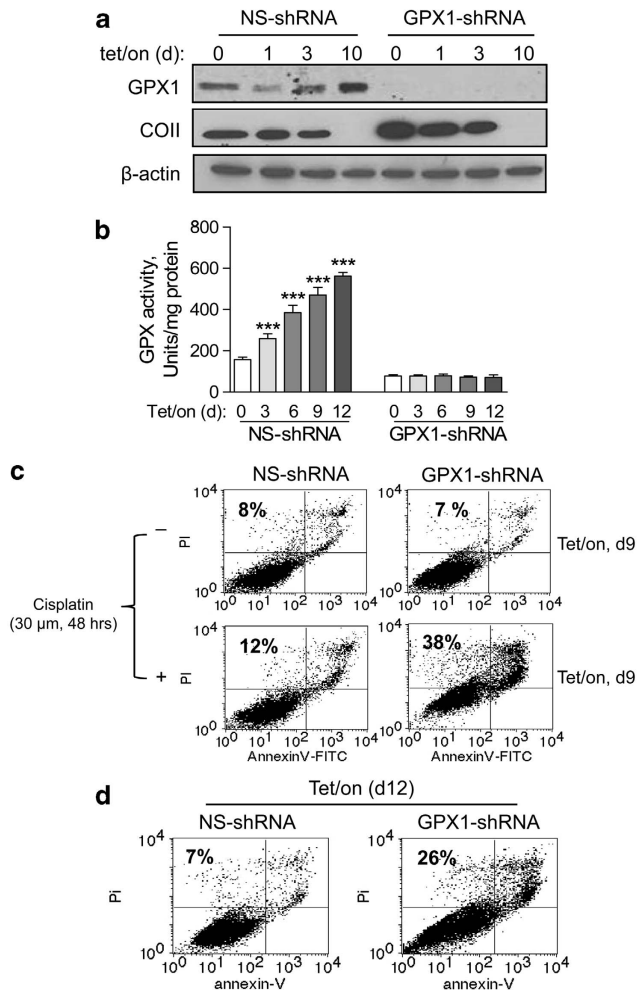


Figure 5 ZNF143 protein level increase is important for the increase of GPX1 activity in the mitochondrial-defective cells to survive oxidative stress. (a) GPX1 protein level was decreased after ZNF143 siRNA knockdown on day 6 of Tet/on cells. At 2 days after POLGdn expression, cells were transiently transfected with nonspecific scramble siRNA (scRNA) or ZNF143 siRNA for 4 days in a doxycycline medium. Western blotting was conducted to confirm ZNF143 knockdown. β -Actin served as a loading control. (b) GPX activity significantly decreased after ZNF143 gene knockdown. Tet/on day 6 cells or Tet/on day 6 cells with scRNA transient transfection were used as control ($n = 3$). Values are shown as mean \pm S.D.; *** $P < 0.001$. (c) ZNF143 gene knockdown at day 3 of Tet/on cells (1 day after ZNF143 siRNA transfection) has same H_2O_2 level as its counterpart with scRNA knockdown (left). ZNF143 gene knockdown at day 5 of Tet/on cells (3 days after ZNF143 siRNA transfection) has higher H_2O_2 level than its counterpart with scRNA knockdown (right). DCF-DA was used as a probe and detected by flow cytometry. (d) Comparison of drug sensitivity in Tet/off and Tet/on day 9 cells. Cells were incubated with the indicated concentrations of gemcitabine (dFdC) and taxol for 72 h, and cell death was analyzed by flow cytometry after double staining with Annexin V and PI. (e) Tet/on cells with ZNF143 knockdown were sensitive to cisplatin treatment. Tet/on day 6 cells were transfected with ZNF143 siRNA or scramble siRNA (scRNA) for 24 h followed by treatment with cisplatin at the indicated concentration for 48 h. Cell death was measured by flow cytometry after double staining with Annexin V and PI. (f) ZNF143 gene knockdown caused massive cell death on day 6 of Tet/on cells (4 days after ZNF143 siRNA transfection) when compared with its counterpart with scRNA knockdown. Cell death was detected by Annexin V/PI double stain and detected by flow cytometry

ZNF143 expression could not protect TFAM from degradation in mitochondrial dysfunction. ZNF143 was previously reported as one of the several transcription factors responsible for maintaining the normal transcription levels of the mammalian mitochondrial transcription factor A (TFAM), a nuclear-encoded mitochondrial transcription factor that is indispensable for the expression of key mitochondrial-encoded genes and for the replication of mitochondrial

DNA.¹⁸ To investigate the effect of ZNF143 on TFAM regulation in the mitochondrial respiratory dysfunction, TFAM gene expression was evaluated with qRT-PCR and did not have corresponding increase during POLGdn induction (Figure 7a). The data showed that the expression of TFAM transcription factor (nuclear respiratory factor-1 (NRF-1)) and activators such as peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α) and PGC-1-related coactivator



(PRC) did not have significant alteration even after 6 days of POLGdn induction (Figure 7a). TFAM protein was significantly decreased on day 2 and became undetectable by day 4 after POLGdn induction (Figure 7b). To further confirm that TFAM protein might be destabilized as a consequence of reduced mtDNA content, we used 2',3'-dideoxycytidine (ddC), a nucleoside analog known to incorporate efficiently into mtDNA and terminate its replication, as another method to examine its effect on TFAM protein levels. Treatment of

Tet/off cells with ddC led to a time-dependent decrease of TFAM protein (Figure 7c). These findings suggest that lower mtDNA was associated with decreased TFAM protein level, and are consistent with the report that p° cells have significant decrease of TFAM protein level.¹⁹ Interestingly, the proteasome inhibitor MG132 partially prevented the disappearance of TFAM protein in Tet/on cells, whereas the caspase inhibitor Z-VAD did not suppress TFAM degradation (Figure 7d), suggesting that TFAM was degraded mainly by proteasome or proteasome-like structure when mtDNA replication was inhibited by POLGdn induction. These data suggest that TFAM is mainly regulated at post-transcription level, but not transcriptional level in the mitochondrial respiration dysfunction induced by POLGdn.

Discussion

We propose that oxygen consumption by respiration represents a fundamentally important function of mitochondria, which is to keep the essential redox homeostasis. By using a Tet/on-inducible system to regulate POLGdn expression, we have created a pair of respiring and non-respiring human cell lines to investigate possible mechanisms of antioxidant status to the oxidative stress in the mitochondrial respiratory dysfunction, which also reflect the metabolic switch from mitochondrial oxidative phosphorylation to aerobic glycolysis. POLGdn expression in Tet/on cells caused defective mitochondria with depletion of mtDNA-encoded gene expression and protein expression responsible for functional mitochondrial respiratory chain, decreases of mitochondrial respiration. Mitochondrial dysfunction induced by POLGdn has a profound impact on the balance between the ROS and antioxidant homeostasis. From the study, we have established that ZNF143 is responsive to genetic or pharmacological disruption of mitochondrial respiration and that ZNF143 can regulate GPX1 level and GPX activity, an event that promotes the antioxidant defense of the compromised respiration with oxidative stress. This study revealed that increase of GPX1 protein level and GPX activity by ZNF143 may be an important adaptive mechanism for cell survival and drug resistance in the mitochondrial respiratory dysfunction, as knockdown of these genes in the mitochondrial dysfunction increased sensitivity to cisplatin treatment and caused massive cell death. Upregulated GPX activity is orchestrated via increased cellular GSH level in the mitochondrial-defective cells, which further emphasize the role of antioxidants in the defense against the cellular oxidative stress with mitochondrial dysfunction (Figure 8). Thus, ZNF143 and GPX1 together with increased GSH synthesis may have key roles in the control of metabolic network that regulates cell survival and oxidative defense in the mitochondrial respiratory dysfunction. Cancer cells often display different degrees of mitochondrial respiratory dysfunction with resistance to the common chemotherapeutic drug intervention; thus, the identification of pathways that promote cell survival under the mitochondrial respiratory-defective metabolic status may have therapeutic implications.

Mitochondrial dysfunction is an important abnormality in cancer.^{20,21} Mitochondrial-defective p° cells were reported to be resistant to many anticancer agents.¹⁷ The drug sensitivity

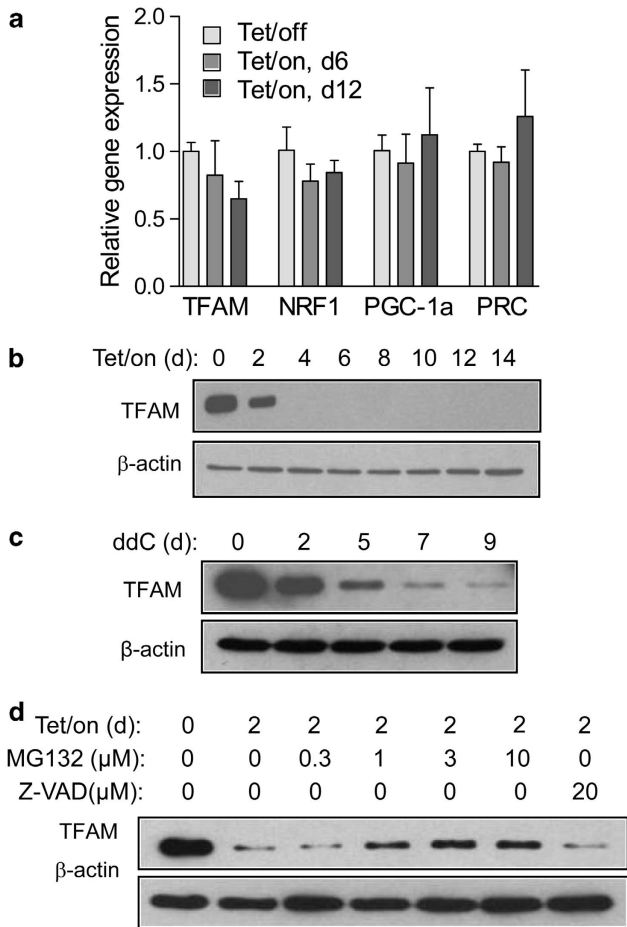


Figure 7 ZNF143 expression could not protect TFAM from degradation in mitochondrial dysfunction. (a) Increased ZNF143 expression did not upregulate mRNA expression of TFAM as measured by qRT-PCR. TFAM gene expression did not change even after 6 days of POLGdn induction. TFAM transcriptional regulators, such as NRF-1, PGC-1 α and PRC gene expression, did not have significant alteration after mitochondrial dysfunction. β -Actin was used as internal control. Data are shown as mean \pm S.D. of triplicate samples from three independent experiments. (b) Disappearance of TFAM protein following inhibition of mtDNA synthesis by POLGdn expression as determined by western blot assay. β -Actin was probed as a loading control. (c) Dramatic decrease of TFAM protein following inhibition of mtDNA synthesis by 0.2 μ M ddC as determined by western blot assay. β -Actin was probed as a loading control. (d) Suppression of TFAM protein degradation by the proteasome inhibitor MG132, but not by pancaspase inhibitor Z-VAD. After doxycycline-induced POLGdn expression for 24 h, cells were treated with the indicated inhibitors for additional 24 h. Protein lysates were subject to western blot analysis for TFAM protein and β -actin was probed as a loading control

of cancer cells is governed by the complex pathways involved in the pathogenesis of cancer, and understand the fact that the survival pathway under the metabolic status may become a primary theme of molecular therapies. The transcription factor ZNF143 is stress induced and overexpressed in cisplatin-resistant cells.^{9,22} The development of cisplatin resistance by tumor cells is a major clinical limitation in cancer chemotherapy. One of the several possible mechanisms of resistance is the increased activity of intracellular pathways of thiol production, such as GSH, which can contribute to the detoxification of cisplatin.²³ GCLC, a key rate-limiting enzyme

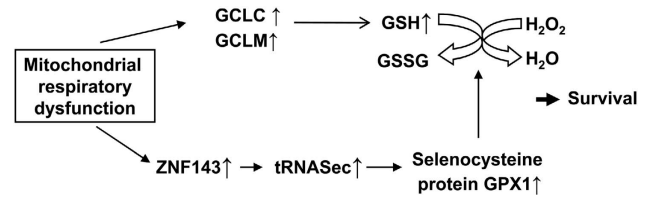


Figure 8 Schematic illustration of ZNF143 function in mitochondrial dysfunction. Disruption of mitochondrial respiration by either genetic or pharmacological approaches led to the prompt increase in the function of transcription factor ZNF143. By increasing the expression of tRNASec, elevated ZNF143 upregulates GPX activity. Increased GPX activity is orchestrated via increased cellular glutathione synthesis by transcriptional upregulation of glutathione synthesis enzyme GCLC and GCLM, which together serve as antioxidants in the defense against the cellular oxidative stress and resistant to cisplatin treatment in the mitochondrial respiratory dysfunction

involved in GSH biosynthesis, was also shown to be associated with cisplatin resistance.²⁴ ZNF143 knockdown in prostate cancer caused increased sensitivity for cisplatin.⁹ Our data have revealed that in orchestra with the increased activity of GCLC and GSH synthesis, ZNF143 upregulates cellular GPX activity to protect cells from oxidative stress induced by mitochondrial dysfunction. Targeted inhibition of ZNF143 substantially increased cellular sensitivity to cisplatin in respiration-defective Tet/on cells. The signal pathway could serve as a potential target for therapeutic intervention as cisplatin-resistant cells also display mitochondrial defects.²⁵

TFAM, one of the key factors for the mtDNA replication and transcription, was reported as a downstream target of ZNF143.¹⁸ POLGdn expression upon doxycycline induction did not induce TFAM gene upregulation even though higher ZNF143 protein level was observed. Instead, TFAM protein was depleted through a pathway that may be similar to the proteasome activation mechanism. To our surprise, other TFAM transcription regulators such as PGC-1 α , PRC and NRF-1 did not respond to the early stage of mitochondrial dysfunction, and their mRNA levels remained stable even after 6 days of POLGdn induction. These data imply that PGC-1 α , a master regulator of mitochondrial function, is not the early response gene in the mitochondrial respiratory dysfunction. This is in contrast to the prompt expression of ZNF143 and GPX1 genes in the mitochondrial respiratory dysfunction.

Materials and Methods

Cell lines and reagents. Tet/off T-Rex 293 cells were cultured in the DMEM culture medium supplemented with 10% tetracycline-free FBS in 5% CO₂ at 37 °C in the presence of 100 μ g/ml zeocin and 5 μ g/ml blasticidin as the selection agents. Tet/on cells were maintained in DMEM supplemented with 10% FBS, 1 mmol/l sodium pyruvate, 50 mmol/l uridine, 0.47% glucose and 100 μ g/ml zeocin, 5 μ g/ml blasticidin and 1 μ g/ml doxycycline.¹¹ HL60 and its derived C6F (p⁻) cells were maintained as described.¹⁷ MG132, PI, rotenone, sodium azide and antimycin A were obtained from Sigma (St Louis, MO, USA). 2,7-DCF-DA was obtained from Invitrogen (Carlsbad, CA, USA). Z-VAD-FMK was purchased from Promega (Fitchburg, WI, USA) and FITC Annexin V was from BD Biosciences (Franklin Lakes, NJ, USA).

RNA preparation and real-time qRT-PCR. Total RNA isolation and cDNA synthesis were carried out as described previously.²⁶ The mRNA expression of ZNF143, GPX1, GCLC, GCLM, GSS, SepSecS, ATPase 6, tRNASec, NRF-1, PGC-1 α , PRC and TFAM were analyzed by qRT-PCR using primers as described²⁷ or otherwise as listed below: ZNF143 forward,

5'AGACTTGGCAGCATTCCATAC3' and reverse, 5'CCATTGGATGTTGCTAC TAAGGT3'; GPX1 forward, 5'CTCTTCGAGAAGTGCAGGTT3' and reverse, 5'GATGTCAGGCTCGATGTCAA3'; GCLC forward, 5'GGCACAAGGACGTTTCAAGT3' and reverse, 5'CAAAGGGTAGGATGGTTTGGG3'; GCLM forward, 5'TGTGTGATGCCACCAGATTGG3' and reverse, 5'CGTGCCTTGAATGTCAGG3'; GSS forward, 5'CTCTACGGCTACCCAATGC3' and reverse, 5'TCGTCGGATCACATGGATGTT3'; SepSecS forward, 5'ATTGGACGATCCGGTGATATTTTC3' and reverse, 5'AAAGCAGTTGGCTACTGTATGG3'; PGC-1 α forward, 5'-ACTCAGCAGCAGTTGACCCT3' and reverse, 5'GGACTAGGAGAAGCAGGGCT3'; ATPase 6 forward, 5'CTGTTGCTTCATTTCATGCCC3' and reverse, 5'GTGGCGCTTCCAATTAGGTG3'; tRNasec forward, 5'CGGATGATCCTCAGTGGTCT3' and reverse, 5'CGGGCAACAAGCAAAATAAG3'; ND1 forward, 5'AACA TACCCATGGCCAACCT3' and reverse, 5'GGCAGGAGTAATCAGAGGTC3'; β -actin forward, 5'GCATCGTACCAACTGGGAC3' and reverse, 5'ACCTGG CCGTCAGGCAGCTC3' was used as internal control. For tRNA expression, the reverse primer of tRNasec was annealed to RNA and reverse transcribed (Fermentas, Burlington, ON, Canada). The forward primer of tRNasec was later on added for the amplification of the cDNA.²⁸ Real-time qRT-PCR was performed and normalized by measuring average cycle threshold (Ct) ratios between the gene of interest and β -actin gene using a ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Western blot analysis. Proteins from whole cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed with the following primary antibodies: anti-GCLC (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-GCLM (Novus Biologicals, Littleton, CO, USA), anti-TFAM (Aviva Systems Biology, San Diego, CA, USA), anti-COII (Invitrogen), anticalcanealase (Calbiochem, Darmstadt, Germany), anti- β -actin (Sigma), anti-GPX 1 (Lab Frontier, Seoul, Korea) and anti-ZNF143 (Novus). The bound primary antibodies were then probed with their respective secondary antibodies conjugated to horseradish peroxidase. Immunolabeled proteins were detected by using a SuperSignal enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) and autoradiography. For sequential blotting, the membranes were stripped by using stripping buffer (Pierce) and reprobed with other antibodies or β -actin-specific antibody to provide a loading control.

Determination of cellular ROS and oxygen consumption. Measurement of cellular ROS and oxygen consumption were performed as described.^{11,17,29} Briefly, cellular ROS of Tet/off and Tet/on cells were measured with 4 μ M DCF-DA using a BD Biosciences FACSCalibur flow cytometer and analyzed using the CellQuest software (Becton Dickinson, San Jose, CA, USA). DCF-DA was detected as FL1 (525 \pm 20 nm). Oxygen consumption was measured with a Clark-type oxygen electrode system (Oxytherm, Hansatech Instrument, Cambridge, UK).^{11,17}

Assays for cellular GSH and GPX enzyme activity. Cellular GSH was measured using a GSH assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's recommendation. Briefly, cell extracts were prepared by sonication and deproteination and cellular total GSH was detected by measuring the product of glutathionylated DTNB by UV spectrophotometer at 405 nm. The standard curve was also generated to calculate cellular GSH contents.

GPX enzyme activity in the cell lysates was determined by coupled chemical reactions using GSH reductase and the GPX assay kit (Cayman Chemical Co.). The reaction mixture contained 50 mM sodium phosphate, 0.4 mM EDTA buffer (pH 7.0), 2 mM GSH, 0.2 mg/ml NADPH, 0.1 U of GSH reductase and a certain volume of cell extract; 0.001% H₂O₂ was added to initiate the reaction. The kinetics of NADPH oxidation was monitored for 5 min at 340 nm. Cellular GPX activity was calculated based on equal concentrations of protein extracts determined by BCA protein assay kit (Pierce) with BSA as a standard. Results were expressed as units/mg protein.

Knockdown analysis using siRNA/shRNA methods. Double-strained siRNA of ZNF143 was generated from Stealth Select RNAi (Invitrogen; HSS111600) with 25 bp oligonucleotides: 5'-AUAAGCUGUGUACCAUCUUC CAGC-3' and 5'-GCUGGAAGAUGGUACCAAGCAGCUUUAU-3'. Scramble control siRNA was purchased along with siRNA of ZNF143. SureSilencing shRNA plasmids for human GPX1 were purchased from SABiosciences (Frederick, MD, USA) and transfected into Tet/on T-Rex 293 cell model bearing inducible

POLGdn gene expression.¹¹ Transfection by using Lipofectamine 2000 and colony selection by using puromycin were performed according to the manufacturer's instruction. Nonspecific shRNA plasmid sequencing is 5'-TAGAGAAGTCCCGCCT CATAA-3'.

Determination of cell death. Apoptosis was determined by flow cytometry using the Annexin V and PI double staining method. Briefly, after drug treatment, cells were collected and washed twice with cold PBS and then stained with Annexin V for 15 min at room temperature in dark followed by staining with PI. The levels of apoptosis in Annexin V/PI assay were determined by measuring the fluorescence of the cells using a FACSCalibur flow cytometer equipped with CellQuest Pro software. A total of 10 000 cells were analyzed per sample.

Statistical analyses. The Kolmogorov-Smirnov test (CellQuest Pro software; Becton Dickinson) was used to evaluate the significant difference between control and test samples in flow cytometry analysis. For comparison of the statistical differences of more than two groups, one-way ANOVA and Newman-Keul's multiple comparison test was used. All other statistical significant difference analyses were evaluated using Student's *t*-test (Prism GraphPad, San Diego, CA, USA). A *P*-value < 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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