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Control of Trx1 redox state modulates protection against methyl methanesulfonate-induced DNA damage via stabilization of p21

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Thioredoxin 1 (Trx1) is known to play an important role in protecting against cell death. However, the mechanism for control of Trx1 in cell death resulting from DNA damage has not been fully investigated. In this study, we used the DNA-damaging agent methyl methanesulfonate (MMS) to investigate the protective effects of Trx1 against DNA damage and cell death in HEK293 cells. We found that MMS application caused dose-dependent changes in the Trx1 redox state determined by redox western blotting. At lower concentrations, both reduced and oxidized Trx1 were observed, whereas the reduced band was fully oxidized at the higher concentration. Trx1 overexpression and small interfering RNA knockdown in cells revealed that reduced Trx1 after exposure to lower doses of MMS attenuated DNA damage, assessed by comet assay, and level of the DNA-damage marker histone γ-H2AX, possibly through scavenging intracellular ROS and an increase in p21 protein level via enhancing its stability. However, oxidized Trx1 lost its protective ability to DNA damage in response to higher concentration of MMS. Corresponding to the redox state control of Trx1, cell death induced by different dose of MMS was also found, by inhibiting phosphorylations of p38 and 4E-BP1. These results indicate that reduced Trx1 plays important protective roles against MMS-induced DNA damage and cell death, suggesting that cell protection is regulated by the intracellular redox state. Control of the redox state of Trx1 and its regulating proteins may offer a novel therapeutic strategy for the control of cancer.

Keywords: cell death/DNA damage/methyl methanesulfonate/reactive oxygen species/thioredoxin 1.

Methyl methanesulfonate (MMS) is a highly toxic DNA-alkylating agent that modifies DNA by adding methyl groups to a number of nucleophilic sites on the DNA bases. MMS also has a misleading reputation as a radiomimetic capable of directly producing DNA-strand breaks (*1*). Studies showed that MMS treatment increased intracellular reactive oxygen species (ROS) in *Saccharomyces cerevisiae* (*2*, *3*), and may further depleted glutathione and induced lipid peroxidation and cell death, which processes were reversed by antioxidant activity (*4*). These data suggested that ROS may be involved in MMS-induced cytotoxicity. The importance of alkylating agents in DNA damage and tumorigenesis means that the mechanisms responsible for MMS cytotoxicity need to be thoroughly investigated.

Trx1 is a 12-kDa protein with a redox-active disulfide/dithiol within the conserved active site (*5*, *6*). During the reduction of target proteins, Trx1 is oxidized to form a disulfide bond between the two cysteine residues at 32 and 35 in its catalytic core. Oxidized Trx1 is then reduced and regenerated by thioredoxin reductase (TrxR) and nicotinamide adenine dinucleotide phosphate (NADPH). Trx1, Trx reductase and NADPH, collectively called the Trx system, operate as a powerful protein-disulfide oxidoreductase system (*5*, *6*). Trx1 interacts directly with various intracellular-signalling molecules, as well as with transcription factors including AP-1 and nuclear factor κB (*7*, *8*), thereby affecting cell growth and cell survival. A recent study showed that overexpression of Trx in Fanconi's anaemia fibroblasts prevented the cytotoxic and DNA-damaging effects of mitomycin C and diepoxybutane (*9*). The cytosolic isoforms of Trx (Trxh1) were shown to be responsible for sensitivity to DNA-damaging agents, including MMS, in the green alga *Chlamydomonas reinhardtii* (*10*). However, the mechanisms of Trx1 responsible for regulating DNA damage are not fully understood.

DNA-damage-induced cellular responses can be regulated by factors such as the cyclin-dependent kinase inhibitor p21Cip1 and the stress-induced kinase p38. p21 plays significant roles in several aspects of the DNA-damage response, including cell cycle arrest, DNA replication (*11*), DNA repair (*12*) and cell apoptosis. However, p21 regulation is complex; while transcriptional regulation by p53-dependent and p53-independent mechanisms is well established, studies have suggested that p21 can also be regulated by proteasomal degradation under oxidative stress. For example, ROS triggered proteasome-dependent degradation of p21 in GM00637 human fibroblast cells and cystic fibrosis lung epithelial cells (*13*, *14*). p38 is activated by ASK1, the N-terminal domain of which binds to reduced Trx1, and p38 can also function in cell survival. 4E-Binding protein 1 (4E-BP1) has

been implicated in certain stress signals. Treatment of cells with DNA-damaging agents led to changes in 4E-BP1 phosphorylation, thus regulating growth and apoptosis (*15*). However, the involvement of p21, p38 and 4E-BP1 in Trx1-regulated DNA damage induced by MMS to control cell growth and death is not known. Clarification of the functions and regulation of these factors will provide further evidence for the mechanisms responsible for DNA-damage-induced cellular responses. This study therefore investigated the effect of Trx1 on MMS-induced damage and explored the related molecular mechanisms, with particular regard to p21.

Materials and Methods

Cell culture and transfection

HEK293 and H1299 cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM) and RPMI 1640 medium, respectively (Biological Industries, Beit Haemek, Israel) supplemented with 10% newborn calf serum. Cells were transfected with pcDNA3.1 and pcDNA3.1-Trx1 plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and with small interfering RNA (siRNA) targeting human Trx1 or scrambled siRNA (Thermo, Waltham, MA) using DharmFECT4 transfection reagent (Thermo), according to the manufacturer's instructions. Plasmids were kindly provided by Dr Dean Jones (Emory University, USA).

Assessment of cell viability

To evaluate the effect of Trx1 on cell viability with or without MMS treatment, 5×10^4 cells were inoculated into 96-well plates after transfection with Trx1 or vector for 24 h and incubated overnight. The following day, freshly-diluted MMS was added at a final concentration of 0.05, 0.1, 0.3 or 0.5 mM. The cells were incubated for a further 24 h and cell viability was estimated by MTT assay.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analysis

For western blot analysis, cells were harvested in sodium dodecyl sulfate (SDS) lysis buffer and homogenized by sonication. Protein concentration was determined using a bicinchoninic acid kit (Pierce, Rockford, IL). Proteins were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 10% skimmed milk, the membranes were probed with primary antibodies overnight at 4°C. Primary antibodies against Trx1, phospho-H2AX (Ser139), phospho-p38 (Thr180/Tyr182), p38, phospho-4E-BP1 (Thr37/46), β-actin and p21 (all from Cell Signaling, Beverly, MA) were diluted 1:1,000 in blocking buffer. Peroxidase-coupled secondary antibodies were diluted 1:3,000 and applied for 1 h at room temperature. Protein was visualized using an enhanced chemiluminescence (ECL) detection kit (Millipore, Billerica, MO). Densitometric analysis of membranes was performed using ImageJ software.

Comet assay

The comet assay was performed as described previously (*16*) with minor modifications. Cells were trypsinized and resuspended in phosphate-buffered saline at a final dilution of 2.5×10^6/ml. A volume of 30 µl of single-cell suspension was mixed carefully with 30 µl of 1.0% low-melting agarose, layered onto microscope slides pre-coated with 100 µl of 0.5% normal-melting agarose, covered with a coverslip and kept at 4°C for 15 min. The coverslips were then removed and cells were lysed for 1 h at 4°C with lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris (pH 10.0), 1% sodium N-lauroylsarcosine and 1% Triton X-100). Slides were placed in a gel-electrophoresis tank filled with cold electrophoresis solution (1 mM Na_2EDTA, 300 mM NaOH, (pH 13)) for 20 min. Electrophoresis was performed in the same buffer for 20 min at 300 mA. After electrophoresis, slides were neutralized with 0.4 M Tris (pH 7.5) for 15 min, stained with Hoechst 33342 (5 µg/ml) and examined under a fluorescence microscope. A total of 100 randomly-selected cells were analyzed per sample. Tail moment (TM; (% DNA in tail × tail length)/100) was used as a measure of DNA damage and computed using Komet version 5.5 software.

Measurement of ROS

ROS generation was measured using dichlorofluorescein diacetate (DCFHDA; Molecular Probes, Eugene, OR) as described previously (*17*). Cells transfected with vector or Trx1 were plated at 7×10^4 per well in a 96-well plate 1 day before the experiment. The following day, the medium was removed and cells were washed with Krebs-Ringer-Hepes (KRH) buffer (129 mM NaCl, 5 mM $NaHCO_2$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 2.8 mM glucose, 10 mM Hepes (pH 7.4)). The cells were then incubated with 100 µM DCFHDA in the loading medium (DMEM with 1% fetal bovine serum) at 37°C for 30 min. After removing DCFHDA, cells were washed with KRH buffer and incubated in KRH buffer with different concentrations of MMS for 1 h at 37°C. Fluorescence emission was measured at 530 nm wavelength following excitation at 485 nm.

Assessment of Trx1 redox status

The redox status of Trx1 was measured by redox western blot. Cells were lysed in collection buffer (6 M guanidine HCl, 50 mM Tris, 3 mM EDTA, 0.5% Triton X-100, pH 8.3). For redox western blot, 50 mM iodoacetic acid (IAA) was added to the lysis buffer and incubated at 37°C for 30 min. Excess IAA was removed by Sephadex chromatography (MicroSpin G-25 columns, Amersham-Pharmacia). Samples were subjected to native electrophoresis (15%) to separate the reduced and oxidized forms of Trx1. Proteins were electrotransferred to PVDF membranes and immunodetected with Trx1 antibody and V5 antibody (Invitrogen). Proteins were visualized using an ECL-detection kit. Densitometric analysis of membranes was performed using ImageJ software. The redox potential (E_h) of Trx1 was calculated based on the ratio of oxidized to reduced form using the Nernst equation, $E_h = -254 + 30 * [\log(\text{oxidized Trx1/reduced Trx1})]$.

Statistical analysis

All values were expressed as mean ± SEM. The significance of differences between controls and samples treated with different concentrations of drug was determined by one-way ANOVA and paired *t*-tests followed by the *post hoc* least significant difference test. Differences were considered significant at $P<0.05$.

Results

Trx1 protected cells against MMS-induced DNA damage and cell death

Previous reports have shown that MMS causes DNA-strand breaks, which induces serine-139 phosphorylation in the C-terminus of H2AX and formation of γ-H2AX. To explore the effect of Trx1 on MMS-induced DNA damage, we detected the levels of γ-H2AX in HEK293 cells transfected with either Trx1 or vector control. MMS exposure increased γ-H2AX in control vector-transfected cells. Overexpression of Trx1 attenuated the increase in DNA damage at lower dose of MMS (0.05 and 0.1 mM) compared with the control, but failed to offer protection against higher MMS concentrations (0.5 mM) (Fig. 1A). Conversely, knock-down of Trx1 expression using siRNA in HEK293 cells aggravated DNA damage at lower dose of MMS (0.05 and 0.1 mM) compared with negative control cells (scrambled-sequence-transfected group), but had no significant effect at higher MMS concentrations (0.5 mM) (Fig. 1B). The comet assay was also used to detect the effect of Trx1 on MMS-induced DNA damage. Consistent with the results in Fig. 1A, MMS exposure caused DNA damage in vector-transfected cells. Transfection of Trx1 alleviated the damage caused by lower-dose MMS, but had little

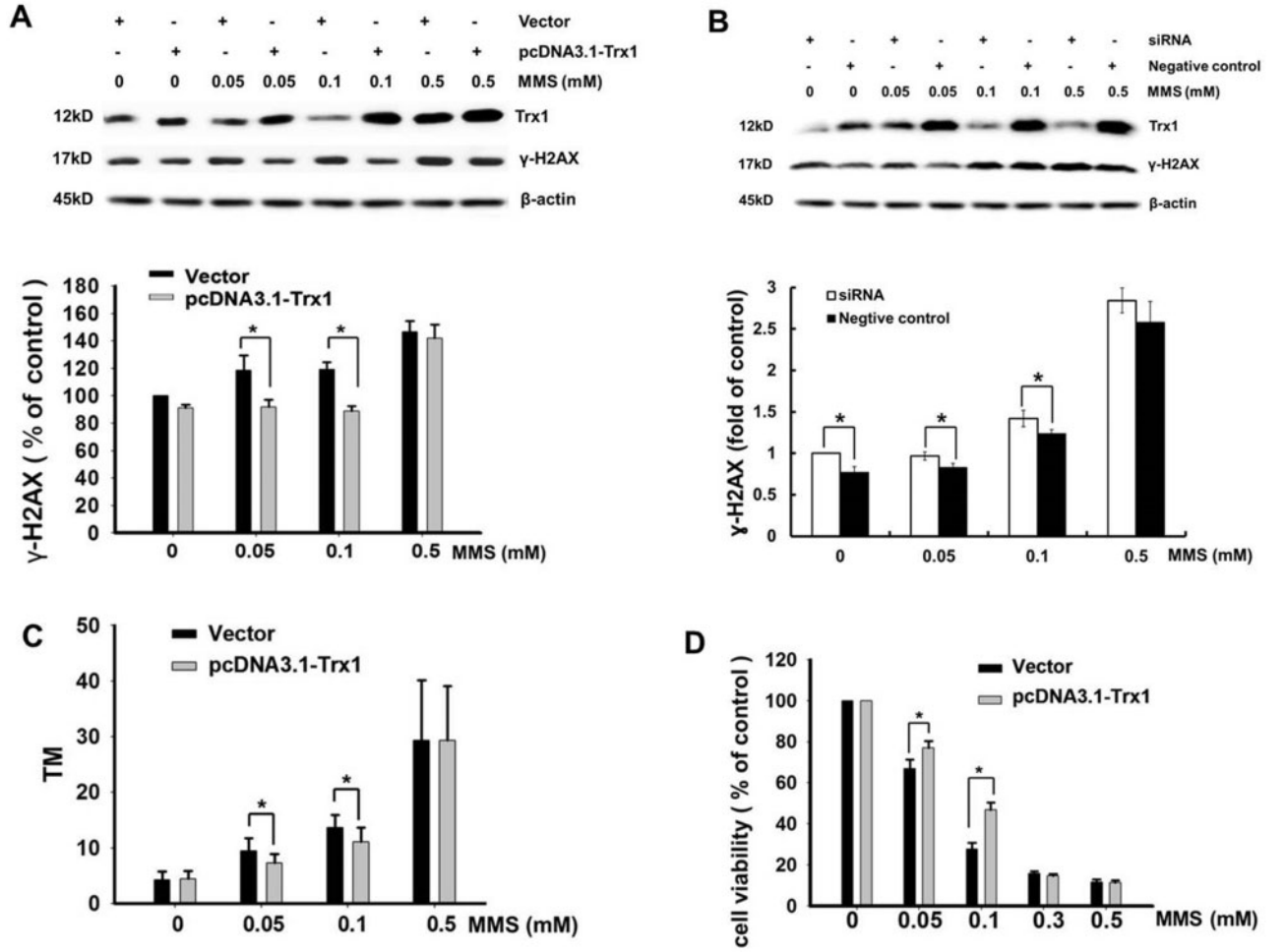

Fig. 1 Trx1 protected against MMS-induced DNA damage and cell death. Effect of Trx1 on MMS-induced DNA damage. **(A)** HEK293 cells were transiently transfected with vector or Trx1 plasmid for 48 h. **(B)** Knockdown of Trx1 using siRNA. HEK293 cells were transfected with Trx1 siRNA for 72 h. Cells were treated with 0.05, 0.1 or 0.5 mM MMS for 1 h. Levels of Trx1 and γ-H2AX were detected by western blot. Levels of γ-H2AX were normalized to β-actin and presented as percentage of control. Representative immunoblots are shown above. **(C)** TM in comet assay, as described in Materials and methods, was used as a measure of DNA damage. **(D)** Trx1 protected cells against MMS-induced death. Cell viability was measured by MTT assay. Vector-transfected and negative control cells without MMS treatment were used as controls. $*P<0.05$ versus correspondingly-treated vector-transfected cells or negative control cells in *t*-tests. $N = 3$–6.

effect on DNA damage caused by higher doses of MMS (Fig. 1C).

MMS is a highly toxic DNA-alkylating agent that induces cell death, while Trx1 is involved in cytoprotection (*18*). To examine the protective effects of Trx1 against MMS-induced cell death in more detail, we transiently transfected HEK293 cells with vector or Trx1. Transfected cells were then treated with MMS at 0.05, 0.1, 0.3 or 0.5 mM for 24 h and cell viability was evaluated by MTT assay. MMS exposure reduced cell viability in a dose-dependent manner in vector-transfected cells. However, the viability of MMS-treated (0.05 or 0.1 mM) cells was increased in Trx1-transfected cells, compared with vector-transfected cells. The protective effect was not observed at higher concentrations of MMS (0.3 or 0.5 mM) (Fig. 1D). These results indicate that Trx1 can protect HEK293 cells against cell death induced by relatively low doses of MMS.

Taken together, the above results suggest that Trx1 is able to protect HEK293 cells against MMS-induced DNA damage or cell death, though its protective effect seems to be limited to lower doses of DNA-damaging agents.

Trx1 protected against MMS by scavenging ROS via alteration of the redox state

Trx1 inhibits oxidative-stress-induced cell death (*19*) by scavenging ROS (*20*). MMS can induce ROS production in *S. cerevisiae*. We therefore investigated the role of Trx1 in scavenging MMS-induced ROS during cell damage in HEK293 cells. Intracellular ROS increased significantly and peaked with the application of 0.3 mM MMS in vector-transfected cells, but remained low in Trx1-transfected cells throughout the incubation period (Fig. 2A). These results suggest that MMS caused ROS production in HEK293 cells, and that overexpression of Trx1 prevented the production of ROS in response to MMS treatment.

Trx1 acts as an intracellular reductase to scavenge ROS by converting two-SH groups to its disulfide bonds. We therefore determined if MMS treatment resulted in a redox change of Trx1. The intracellular Trx1 redox state was detected by redox western blot

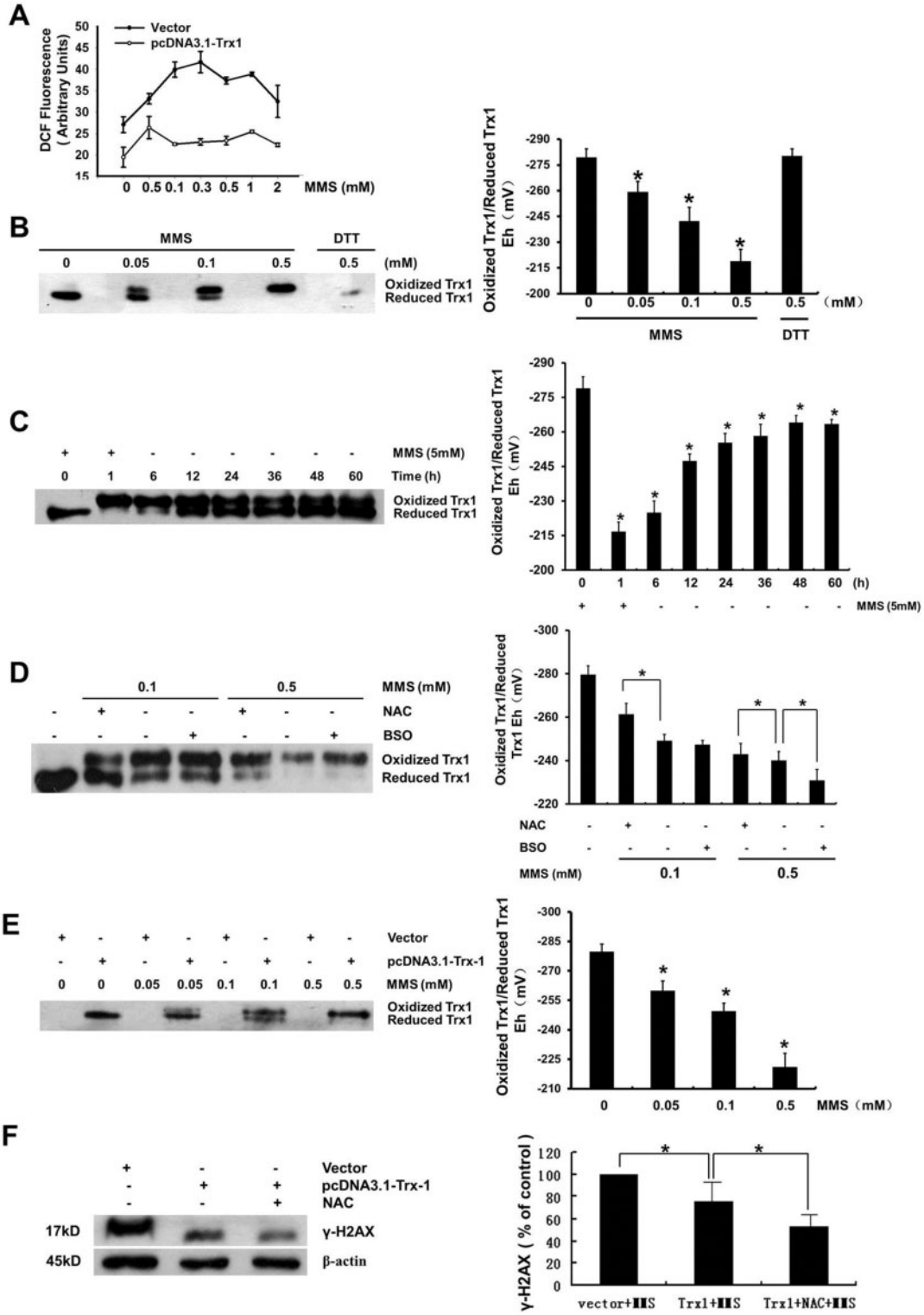

Fig. 2 Trx1 exerted its protective effect by scavenging ROS via alteration of its redox status. (A) DCFDA assay to detect intracellular ROS after MMS exposure. HEK293 cells were transiently transfected with vector or pcDNA.3.1-Trx1 plasmids. After 48 h, cells were treated with the indicated concentration of MMS for a further 24 h. (B, E) Redox western blot showing the change in redox state of intracellular Trx1 or transfected Trx1. After MMS treatment, anti-Trx1 antibody was used to detect the change in intracellular Trx1 **(B)**, and anti-V5 antibody against the V5 tag in pCDNA3.1 plasmids was used to detect the redox state of transfected Trx1 (E). $^*P < 0.05$ versus cells without MMS treatment. **(C)** Redox state of Trx1 after washing off MMS. Cells were treated with 0.5 mM MMS for 1 h, and the medium was then replaced with normal culture medium after washing off MMS. The redox state of intracellular Trx1 was evaluated at different time points by redox western blot. Representative immunoblots are shown to the left of the quantitative data in all panels $^*P < 0.05$ versus cells treated with MMS for 1 h. **(D)** Redox state of Trx1 pretreated with NAC or BSO prior to MMS. HEK293 cells were pretreated with 5 mM NAC for 1 h or 5 mM BSO for 12 h, followed by 0.1 or 0.5 mM MMS for a further 1 h. Trx1 redox status was determined by redox western blot. $^*P < 0.05$ versus cells without NAC or BSO treatment. **(F)** Levels of γ-H2AX after pretreatment with NAC prior to MMS. Transfected cells with or without NAC (5 mM for 1 h) pretreatment were exposed to 0.05 mM MMS for 1 h. Levels of γ-H2AX were normalized to β-actin and presented as ratios compared with the control (vector + MMS). Statistical significance was assessed by Anova. $^*P < 0.05$ versus MMS + vector or Trx1 + MMS ($N = 4$).

after exposure to different concentrations of MMS for 1 h. Trx1 generated its reduced form ($E_h = -279 \pm 5$ mV) under basal conditions, whereas the redox status of Trx1 changed gradually from the reduced ($E_h = -259 \pm 6$ mV) to the oxidized form ($E_h = -219 \pm 7$ mV) after treatment with MMS, in a concentration-dependent manner. Trx1 existed in both reduced and oxidized forms at lower doses of MMS (0.05 and 0.1 mM), and in a fully oxidized form at the higher concentration of MMS (0.5 mM) (Fig. 2B). We also investigated the change in Trx1 redox state during depletion of MMS. Cells were treated with 0.5 mM MMS for 1 h and the medium was then replaced with normal medium after washing off the MMS. The redox state of intracellular Trx1 was evaluated at different incubation times. The reduced form of Trx1 was gradually rescued with incubation in normal culture medium (Fig. 2C), where Trx1 was reduced from 1 h ($E_h = -217 \pm 4$ mV) to 60 h ($E_h = -263 \pm 2$ mV), further implying that a redox-state change in Trx1 was caused by MMS treatment. To confirm the Trx1 redox change in response to MMS treatment, we introduced an antioxidant, N-acetylcysteine (NAC), and an inhibitor of glutathione (GSH) synthesis, buthionine sulfoximine (BSO), into the system. HEK293 cells were pretreated with 5 mM NAC for 1 h or 5 mM BSO for 12 h, and exposed to MMS for a further 1 h. As shown in Figure 2D, oxidation of Trx1 was prevented by NAC and exacerbated by BSO, indicating that Trx1 was involved in scavenging MMS-induced ROS via alteration of its redox status. We also examined the redox state of Trx1 in transfected cells and found a similar redox alteration of transfected Trx1 in response to MMS (Fig. 2E), suggesting that the effects of overexpressed Trx1 on MMS-induced ROS were also dependent on redox regulation, possibly as a result of absolute increases in the reduced and oxidized forms, rather than a change in the reduced to oxidized ratio. To confirm that the Trx1 redox change was involved in its protection against DNA damage, we investigated the levels of γ-H2AX in Trx1-transfected or vector-transfected cells with or without NAC after MMS treatment. NAC pretreatment decreased γ-H2AX in the Trx1-transfected group, compared with the Trx1-transfected group with MMS treatment alone (Fig. 2F). These results indicate that MMS-induced ROS results in a change in Trx1 redox status, and reduced Trx1 protects cells against MMS-induced DNA damage by scavenging ROS.

Trx1 increased p21 protein levels in response to MMS

p21 is as an important regulator of the cell cycle checkpoint and other pathways, and plays significant roles in the DNA-damage response. We therefore investigated the involvement of p21 in the protective effect of Trx1 against MMS treatment. As shown in Figure 3A, p21 protein levels decreased in response to MMS treatment. Furthermore, the levels of p21 were increased in Trx1-transfected cells (Fig. 3B) and decreased in Trx1-knock-down cells (Fig. 3C). Consistent with the results in Figure 1, the alteration in p21 protein associated with Trx1 was also found following treatment with lower doses of MMS. We also examined whether involvement of p53-regulated p21 protein level by Trx1 in response to MMS treatment and found that treatment of MMS for 1 h did not significantly change the protein level of p53 in the presence or absence of Trx1 (Fig. 3B). In order to further investigate the involvement of p53 in the protection of Trx1-mediated p21 protein level against MMS treatment, Trx1 was transfected in p53 null cell line H1299 and p21 protein level was detected. The result showed that the levels of p21 also decreased in Trx1 untransfected cells and increased in Trx1 transfected cells with lower doses of MMS (Fig. 3D), further indicating that involvement of p21 in the protective effect of Trx1 against MMS treatment is independent of p53.

To explore the mechanism whereby Trx1 increased p21 protein in response to MMS treatment, we investigated if Trx1 altered p21 protein levels by modulating post-translational protein degradation. After treatment with MMS, protein translation was inhibited by adding cycloheximide (CHX) and the remaining p21 was detected at different time points. As shown in Figure 3E and F, Trx1 overexpression or knock-down correspondingly altered the stability of p21 protein with MMS treatment. In addition, the proteasome and calpain inhibitor MG132 inhibited MMS-induced p21 protein degradation (Fig. 3G). These results suggest that Trx1 is involved in the protection of p21 protein stability at the post-translational level.

p38 phosphorylation may be involved in Trx1-mediated protection against cell death in response to MMS treatment

Trx1 can act as a redox-active protein to regulate cell death by modulating ASK1 protein, resulting in p38 phosphorylation (*21*). To determine if Trx1 protected against MMS-induced damage by inhibiting p38 activity, we transfected HEK293 cells with either vector control or Trx1. Treatment with MMS led to phosphorylation of p38 in vector-transfected cells, indicating that the p38 signalling pathway was activated in response to MMS, consistent with a previous report (*22*). Trx1 suppressed p38 phosphorylation induced by lower doses of MMS (0.05 and 0.1 mM), but not that induced by higher doses (0.5 mM) (Fig. 4A). Knock-down of Trx1 expression also showed that the level of p38 phosphorylation was higher in Trx1-knock-down cells at lower doses of MMS (0.05 and 0.1 mM) compared with negative control cells. No difference was observed at higher doses (0.5 mM) of MMS (Fig. 4B). We further investigated the protective role of Trx1 by examining the possibility that Trx1 might regulate p38 and thus affect the phosphorylation of 4E-BP1, which is involved in the regulation of protein translation and therefore in cell growth. Overexpression of Trx1 decreased 4E-BP1 phosphorylation at lower MMS doses (Fig. 4C). To confirm this effect, we compared the levels of 4E-BP1 phosphorylation between cells transfected with siRNA to Trx1 and scrambled-sequence siRNA. 4E-BP1 phosphorylation in the Trx-siRNA-transfected group was higher than in the control group at lower MMS doses, but not at higher MMS doses (Fig. 4D). These results suggest

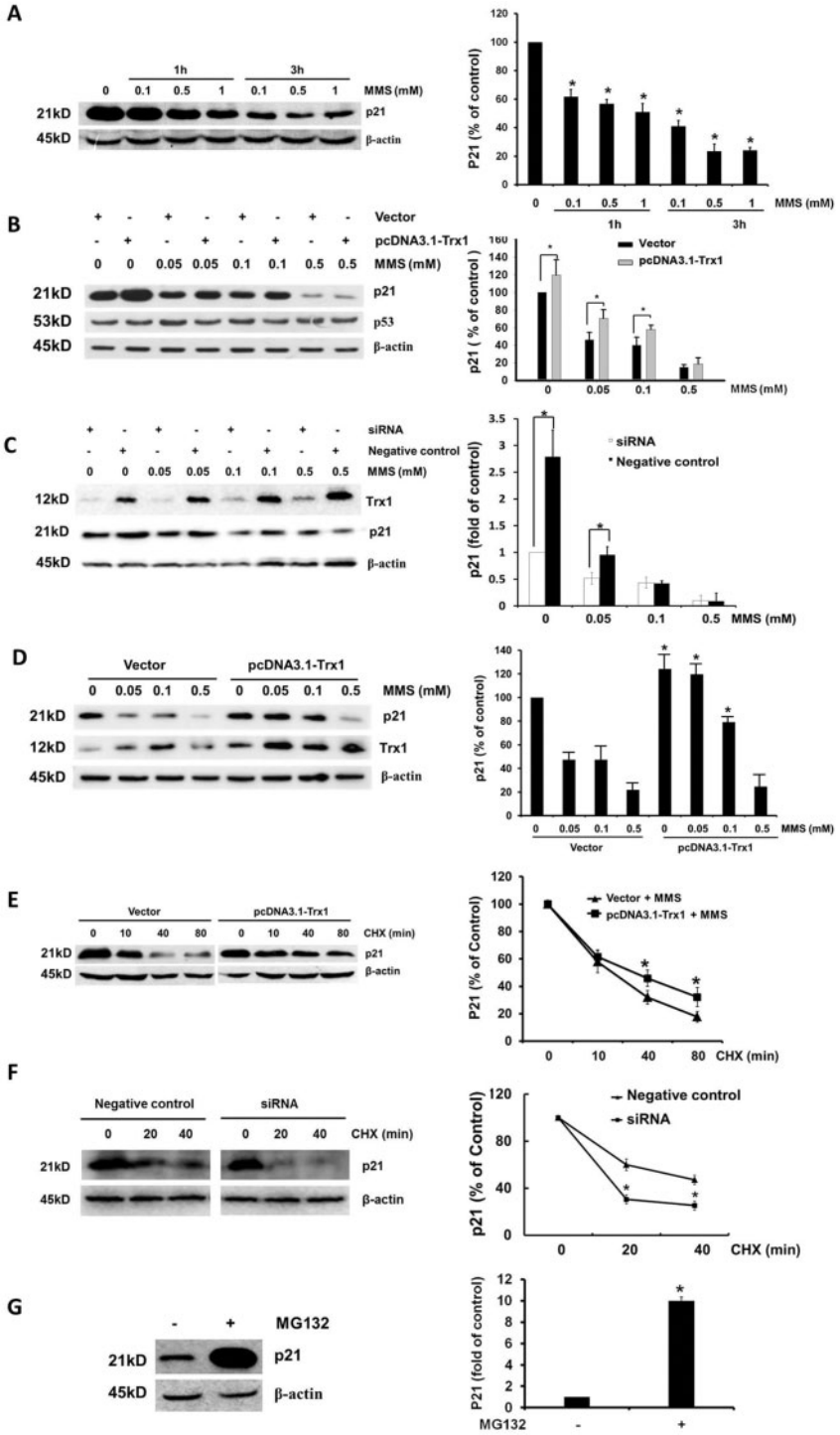

Fig. 3 Trx1 increased p21 levels by regulating its protein stability. (A) Western blot showing the effects of MMS treatment on p21 protein levels. HEK293 cells were treated with MMS (0.1, 0.5 or 1 mM) for 1 or 3 h, and p21 protein levels were examined by anti-p21 antibody staining. p21 levels were normalized to β-actin and presented as ratios compared with control (cells without MMS treatment). $^*P < 0.05$ versus control. **(B, C)** p21 and p53 protein levels were detected after Trx1 overexpression or knock-down. Cells were transiently transfected with Trx1 plasmids **(B)** or Trx1 siRNA **(C)**. After transfection, cells were treated with 0.05, 0.1 or 0.5 mM MMS for 1 h and collected for western blot. Vector-transfected cells and negative control cells without MMS treatment were used as controls. p21 levels were normalized to β-actin and presented as ratios compared with control. $^*P < 0.05$ versus corresponding treated cells transfected with vector or scrambled sequence. **(D)** p21 protein levels were detected in p53 null cell line H1299 after Trx1 overexpression. After transfection, cells were treated with 0.05, 0.1 or 0.5 mM MMS for 1 h and collected for western blot. Vector-transfected cells without MMS treatment were used as controls. p21 levels were normalized to β-actin and presented as ratios compared with control. $^*P < 0.05$ versus corresponding treated cells transfected with vector. Effects of Trx1 on MMS-induced p21 protein degradation. After transfection with Trx1 plasmids **(E)** or siRNA **(F)**, cells were treated with 0.05 mM MMS for 1 h. CHX (100 μM) was then introduced to the system and p21 protein levels were evaluated at the indicated time points. p21 levels were normalized to β-actin and presented as ratios compared with control (cells without CHX treatment). $^*P < 0.05$ versus corresponding treated cells transfected with vector or scrambled sequence. (G) MG132 blocked MMS-induced p21 degradation. HEK293 cells were transfected with Trx1. At 48 h post-transfection, cells were pretreated with 20 μM MG132 for 12 h, followed by MMS for 1 h. p21 levels were normalized to β-actin and presented as fold of the sample with non-MG132 treatment. Representative immunoblots are shown to the left of the quantitative data in all panels. Statistical significance was assessed by Anova. $^*P < 0.05$ versus untreated samples ($N = 3$–5).

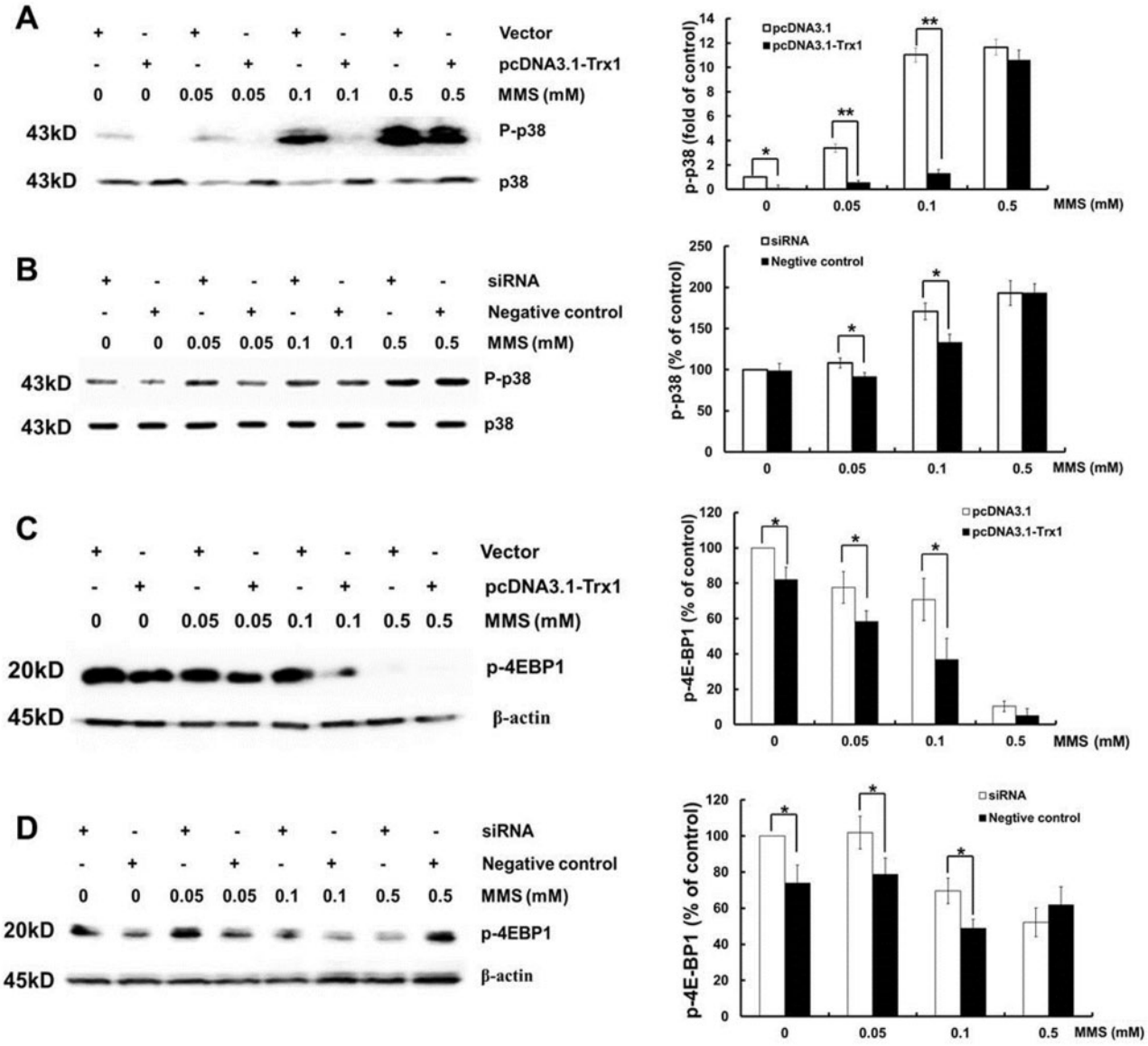

Fig. 4 Trx1 exerted its protective effect against MMS by inhibiting the phosphorylation of p38 and 4E-BP1. (A, B) Effects of Trx1 on p38 phosphorylation. **(C, D)** Effects of Trx1 on 4E-BP1 phosphorylation. HEK293 cells were transiently transfected with vector and Trx1 plasmids for 48 h (A, C), or Trx1 siRNA and scrambled sequence for 72 h (B, D). After transfection, cells were treated with 0.05, 0.1 or 0.5 mM MMS for a further 1 h. Levels of phosphorylated p38 and 4E-BP1 were determined by western blot. Phosphorylated p38 was normalized to total p38 and phosphorylated 4E-BP1 was normalized to β-actin. The results are presented as ratios compared with control (vector-transfected cells or negative control cells without MMS treatment). Representative immunoblots are shown to the left of the quantitative data in both panels. $*P<0.05$, $**P<0.01$ versus corresponding treated cells transfected with vector or scrambled sequence in *t*-tests ($N = 3$–6).

that reduced Trx1 may protect against MMS-induced damage via regulation of p38 activity and inhibition of the translational process, possibly through cell cycle arrest and allowing more time for DNA repair, thus eventually reducing MMS-induced cell death and enhancing cell survival.

Discussion

ether Trx1 is involved in We used MMS as DNA-damage-agent to investigate the DNA-damage-response pathways. MMS induces DNA damage by adding methyl groups to nucleophilic sites on the DNA bases, and by ROS generation. This study tested the possibility that Trx1 may be involved in DNA damage caused by MMS-induced ROS. The results demonstrated that Trx1 was able to protect HEK293 cells against MMS-induced DNA damage and cell death through scavenging ROS. ROS represents a large family of reactive molecules containing oxygen, including superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (•OH), and hydrogen peroxide (H_2O_2), and so on. MMS treatment may react with DNA producing superoxide ($O_2^{\bullet-}$) and entries into a number of secondary reactions leading to other ROS such as H_2O_2 (*23*). Peroxiredoxin (Prx) converted MMS-induced H_2O_2 into H_2O, accompanied with the oxidation of reduced Trx. Oxidized Trx is then reduced by TrxR and NADPH (*23*), suggesting that MMS might also be an inhibitor of TrxR.

Interestingly, Trx1 protection was only effective at lower MMS doses (0.05 and 0.1 mM). Trx1 occurred in

both reduced and oxidized forms at lower concentrations of MMS, but was fully oxidized at higher doses, suggesting that MMS-induced ROS regulates the redox state of Trx1, and reduced Trx1 subsequently participates in protection against MMS-induced cell damage, while oxidized Trx1 fails to exert a protective effect. Indeed, reduced but not oxidized Trx has previously been shown to bind to ASK1 to inhibit its activity in p38 phosphorylation (*22*). Oxidation of Trx leads to its dissociation from ASK1 and subsequent activation of p38 (*22*). In this study, Trx1 decreased p38 phosphorylation at lower doses of MMS, suggesting that inhibition of p38 phosphorylation in response to MMS might also occur through binding of reduced Trx1 to ASK1, to affect cell death. Our results indicate that reduced Trx1 protects from cell death in response to MMS, in accordance with a previous report indicating that oxidized Trx1 participates in oxidative stress-induced cell death (*24*). Although Trx1 plays its anti-apoptosis effect on cells mainly through the change of oxidation, some studies showed that Trx1 still had effects after mutation of Trx1-C32/35S, the conserved active site (*25*, *26*). These studies suggest that the mechanism other than the change of Trx1 oxidation may be involved in Trx1 anti-apoptosis process. It will be interesting to investigate other regions of Trx1 in regulating apoptosis by using Trx1 reducing activity dead mutant in the future. In addition, H_2O_2 can also be neutralized to H_2O through other antioxidants such as the action of the glutathione peroxidase/glutathione reductase cycle at the expense of reducing equivalents (NADPH) (*23*). It will be interesting to examine whether glutathione system is as well involved in the protection against MMS-induced DNA damage.

Several studies have indicated that p21 regulation is sensitive to redox status (*27*, *28*), but the underlying mechanism is not clear. Mild oxidative stimulation was shown to cause rapid nuclear export and a transient decrease in p21 protein levels (*27*), while p21 protein levels increased in response to sublethal doses of H_2O_2 (*29*, *30*), inducing G2/M-phase and multi-phase cell cycle arrest in human lung cancer cells (*29*). This study further found that MMS treatment reduced p21 protein levels, while Trx1 enhanced the stability of p21 protein. The Trx1-mediated intracellular redox status might be involved in the underlying mechanism. MMS caused ROS production and altered the Trx1 redox state, possibly protecting against p21 protein degradation. In response to extracellular stimuli, the stability of p21 is regulated by ubiquitylation and proteasome-mediated degradation (*31*), in which the free 20S proteasome (20SPT) activity is controlled by post-translational regulatory mechanism partially due to the S-glutathiolation of specific Cys residues (*32*). Trx1 and GSH are two major thiol-based antioxidative cellular systems (*33*). Expression of the Trx1 protein increases and colocalizes with the 20S proteasome during DNA synthesis (*34*). We therefore speculate that Trx1 may modulate p21 degradation through ubiquitin-dependent proteasome pathway. Modification of the Trx1 redox state by NAC reduced γ-H2AX, implying that the Trx1-mediated intracellular redox status might control p21 protein levels in response to MMS,

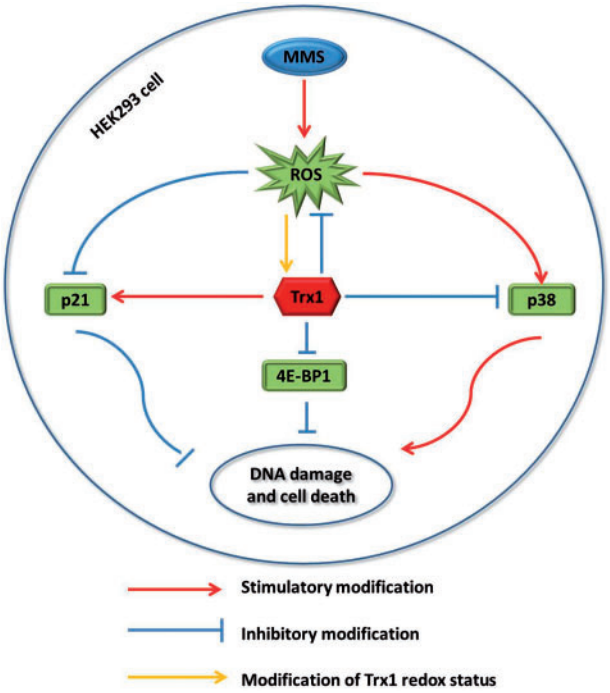

Fig. 5 Schematic representation of reduced Trx1 protecting cells against MMS-induced DNA damage. Trx1 protected cells against MMS-induced DNA damage and cell death by scavenging ROS via alteration of the redox state, increased p21 protein levels by enhancing its stability. In addition, Trx1 inhibit p38 and 4E-BP1 phosphorylation in response to MMS treatment. These results indicate that reduced Trx1 plays important protective roles against MMS-induced DNA damage and cell death.

subsequently protecting against DNA damage. Thioredoxin-dependent redox state was previously shown to regulate p53-mediated p21 activation (*35–37*). However, we found that the protein level of p53 in the presence or absence of Trx1 did not significantly change after treatment of MMS for 1 h, suggesting that short-term treatment of MMS decrease p21 protein stability is independent of p53 and Trx1. We also found that p21 protein level decreased with Trx1 untransfection and increased with Trx1 transfection both in p53 null cell line, further indicating that involvement of p21 in the protective effect of Trx1 against MMS treatment is independent of p53. Although the relationships among the above processes require further investigation, the current study revealed the novel finding that Trx1 regulated p21 expression in response to MMS at the post-translational level. Another important finding in this study was the regulatory effect of Trx1 on the phosphorylation of 4E-BP1, which is involved in the regulation of protein translation, and therefore in cell growth. Our results showed that Trx1 reduced the phosphorylation of 4E-BP1 in response to MMS. Furthermore, Trx caused a decrease in ROS without MMS treatment, possibly as a result of scavenging of the basal ROS levels in cells, and subsequently regulating the expression of p21, and phosphorylation of p38 and 4E-BP-1. However, DNA damage below basal levels was not found in overexpressing cells. Trx1 may therefore also play a protective role in avoiding DNA damage, as indicated in Trx1

knock-out cells. Collectively, it is possible that Trx1 may exert a protective role against DNA damage through inhibiting translation by regulating phosphorylation of 4E-BP1 to save energy, possibly through cell cycle arrest by affecting p21 stability and allowing more time for DNA repair, thus eventually reducing MMS-induced cell death and enhancing cell survival. Indeed, protection of Trx1 against MMS-induced DNA damage could have been involved in DNA repair as redox signalling has been shown to affect APE1/Ref1 function, an apurinic/apyrimidinic endonuclease in the DNA base excision repair pathway (*38*, *39*), and APE1/Ref1 was shown to regulate the p53/p21 system (*37*, *40*). In addition, it was reported that the heat-stable cytosolic factor that promotes glucocorticoid receptor binding to DNA after MMS treatment is neither thioredoxin nor ribonuclease (*41*), suggesting that Trx1-mediated protection against MMS-induced DNA damage might be specific through certain cellular factors.

In conclusion, the results of the present study suggest that MMS alters Trx1 redox status, and reduced Trx1 is then able to protect against MMS-induced DNA damage and cell death at low dose. Increased expression of Trx1 was suggested to be associated with decreased patient survival in human colorectal cancer (*42*). Our findings further suggest that the reduced Trx1 may prevent early cancer via protection against DNA damage and possibly lose its reduced capacity resulting in facilitating the development at late stage of cancer. The responsible mechanism may involve attenuation of intracellular ROS, and modulation of p21 protein stability, phosphorylation of p38 and 4E-BP1 (Fig. 5), though the exact role of reduced Trx1 in the process requires further investigation. These findings imply that Trx1 is a promising protein for protecting cells against MMS-induced damage, and that control of the redox state of Trx1 and its regulating proteins possibly further contribute to understanding of the pathogenesis of cancer.

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Conflict of Interest

None declared.

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