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Redox shuttle of cytosolic Thioredoxin to mitochondria protects against hyperoxia-mediated alteration of mitochondrial structure and dysfunction

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A B S T R A C T

Cytosolic thioredoxin (Trx) is a critical redox protein that converts protein disulfides to thiols via catalytic activity of thioredoxin reductase-1 (TrxR1) and NADPH. Thioredoxin-2 (Trx2) is a mitochondria-localized isoform. It is generally believed that Trx and Trx2 perform similar functions within the cytosol and mitochondria respectively. Here, we demonstrate that cytosolic Trx shuttles into mitochondria in the presence of normal levels of Trx2 in physiological state and higher levels of Trx translocate to mitochondria in oxidative stress conditions such as exposure to high concentrations of oxygen. This shuttle is required to maintain mitochondrial structure and function during physiological and oxidative stress conditions. Further, reduced Trx (Trx-SH) shuttle into mitochondria to protect against the down-regulation of several mitochondrially coded genes and proteins of respiratory chain complexes in oxidative stress. Translocation of Trx occurs only in the reduced state as oxidized or cysteine mutant Trx is unable to translocate to the mitochondria. Accumulation of mitochondrial DNA damage product 8-Oxo-dG in hyperoxia is decreased in the presence of higher levels of cytosolic Trx within the mitochondrion. Collectively, our data demonstrate that shuttling of reduced cytosolic Trx into mitochondria protects against mitochondrial DNA damage, decreased gene and protein expression of respiratory chain complexes and mitochondrial dysfunction resulting in restoration of their native function and cell survival in physiological and oxidative stress conditions.

1. Introduction

Cytosolic thioredoxin (Trx or Trx1; TXN1) is ubiquitously distributed, and was discovered as an electron donor for ribonucleotide reductase [1] for the synthesis of deoxyribonucleotides during DNA replication [2]. In addition, Trx regenerates enzymes and proteins with sulfhydryl groups [3] inactivated by oxidation, via its protein disulfide reductase activity in conjunction with thioredoxin reductase-1 (TrxR1) and NADPH as the electron donor [4,5]. Trx is also an electron donor for peroxiredoxins which enzymatically reduce H_2O_2 [6] to water and molecular oxygen [5,7]. The mammalian Trx has 5 cysteine residues at positions 32, 35, 62, 69 and 73 [8]. The Trx2, a mitochondrial isoform contains the conserved cysteines C32 and C35, but not the regulatory cysteines that are present in Trx [9,10]. Whereas Cys32 and Cys35 perform the direct transfer of electrons to a disulfide, Cys62, Cys69 and Cys73 are involved in regulatory functions in mammalian cells [9], and post-translational modification of these residues such as nitrosylation is known to alter the activity of Trx [9]. The active site of Trx (Trp-Cys--Gly-Pro-Cys) is highly conserved across species [4,10]. Whereas, Trx and Trx2 are functionally similar with respect to their disulfide reductase properties, there is a marked difference in their substrate specificity [11] and they are able to function in opposite manners [12]. TrxR1 efficiently reduces both Trx and Trx2; in contrast, the K_m value of Trx2 for TrxR2 is significantly lower than for Trx [11]. The catalytic efficiency of TrxR2 using Trx2 is 10 times higher than when it uses Trx [11]. Additionally, the catalytic efficiency of TrxR2 is higher at pH 8.0, indicating that mitochondrial environment favors it higher catalytic activity [11]. Thus, Trx2-TrxR2 is not efficient to reduce other disulfide substrates [11]. The limitation of Trx2-related disulfide reduction is due to tight substrate specificity for TrxR2 and lack of regulatory cysteines in the Trx2 protein. Reduction of disulfides by Trx requires a redox-cycling process where reducing equivalents are needed to be transferred to the oxidized substrate via TrxRs. Because Trx or Trx2 is not an enzyme, but a thiol protein they depend on catalytic exchange of electrons by their respective reductases (TrxR1 or TrxR2). Therefore, the function of mitochondrial Trx2 and cytosolic Trx are less overlapping than is generally believed.

Mitochondria are powerhouse of the cell that produce energy in the form of ATP for many energy-dependent processes [13]. Mitochondria are also the organelle that produce significant amount of deleterious superoxide anion ($O_2^{\bullet -}$) due to electrons generated by the electron transport chain [14] and accepted by oxygen [13]. The $O_2^{\bullet -}$ generates additional secondary radicals such as hydroxyl radicals (•OH) and other reactive oxygen species [15], such as cell permeable hydrogen peroxide

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(H_2O_2) [6]. Several antioxidant enzymes are located within the mitochondria, such as mitochondrial superoxide dismutase-2 (Sod2) [16] and various peroxidases of glutathione and thioredoxin systems to continuously remove these radicals, failure of which may not only damage mitochondria, but also could result in death of the organism [17,18]. Additionally, non-enzymatic antioxidant thiols such as proteins of the glutathione system and Trx2 [19] are also present within the mitochondria [18]. However, overproduction of $O_2^{\bullet-}$, H_2O_2 and other secondary reactive species during exposure of cells to oxidative conditions such as hyperoxia overwhelms the antioxidant defense system resulting in mitochondrial dysfunction and cellular damage.

Mitochondrial function is dynamically regulated by continuous trafficking of proteins, as 99 % of all mitochondrial proteins are synthesized in the cytosol and transported to the mitochondrion [20]. The import of mitochondrial proteins synthesized in the cytosol is regulated by their redox state with reduced proteins as import competent [21]. About 1500 proteins that are synthesized in the nucleus are found within the mitochondria [22]. The mitochondrion has its own DNA in mammalian cells, which is a ~16 kb circular double stranded DNA [23]. The mitochondrial DNA codes for 13 proteins all of which are involved in the oxidative phosphorylation and ATP synthesis [22]. Cytosolic signaling regulates mitochondrial protein turnover in physiological as well as pathological conditions [20,21,24]. One of the major functions of the mitochondrion is to produce energy in the form of ATP via passage of electrons in respiratory chain complexes (OXPHOS, complex I, III and IV) that form an electrochemical proton gradient in the mitochondrial inner membrane allowing ATP synthesis from ADP via the H^+ and ATP synthase (complex V). The electron is transferred from complex I (NADH dehydrogenase), complex III (cytochrome *b*), and complex IV (cytochrome oxidase) [25] resulting in extrusion of protons. Proton re-entry via complex V produces ATP. The proton pump of ETC and ATP synthase create a proton circuit across the inner membrane which is central to mitochondrial bioenergetics. These respiratory complex proteins are encoded by both nuclear and mitochondrial genes. Out of 44 total complex I proteins, only 7 are encoded by mitochondrial DNA and 37 are encoded by nuclear genes [26]. Sub-units of complex I that are encoded by mitochondrial DNA that include ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 which are the subunits in the L-shaped dehydrogenase complex I [27]. Complex II protein, succinate dehydrogenase (SDH) is encoded by nuclear gene and complex III protein cytochrome-b and complex IV protein cytochrome *c* oxidase (COX) subunits I, II and III are encoded by mitochondrial genes [22].

8-Oxoguanine glycosylase I (Ogg1) is a base excision repair enzyme that removes 8-oxo deoxyguanosine (8-Oxo-dG) produced due to oxidative guanine base damage [22]. Oxidants such as high concentration of oxygen (hyperoxia) is known to cause increased accumulation of 8-Oxo-dG and overexpression of Ogg1 has been shown to protect against hyperoxia-mediated lung injury by removal of 8-Oxo-dG [28], demonstrating the importance of oxidized base removal in protection against hyperoxic lung injury. The Ogg1 has several isoforms, all containing a mitochondrial-targeting sequence (MTS) [22]. However, the Ogg1α has both, a nuclear localization signal (NLS) and an MTS [22]. In mouse, only Ogg1α has been identified that corresponds to human Ogg1 [22]. Ogg1 is critically important to maintain mitochondrial DNA integrity during mitochondrial oxidative stress conditions. Since mitochondria are potent producers of oxidants such $O_2^{\bullet-}$ and •OH in normoxia and produces these radicals in significantly higher amounts in hyperoxia, maintenance of Ogg1 enzymatic activity during physiological or pathological oxidative load is crucial for mitochondrial function. Ogg1 contains eight cysteine residues, inactivation of which is linked to loss of various activities of the enzyme [29]. Oxidation of these cysteine renders the enzyme inactive including loss of glycosylase activity.

Here, we show that low levels of cytosolic Trx translocate to mitochondria in normoxia in a constitutive manner; however, significantly higher levels of Trx translocate to mitochondria in oxidative stress conditions, such as exposure of cells to hyperoxia. We found that redox active cysteines in Trx are required for this translocation, as mutant Trx (C32S, C35S) and C69S and C73S did not translocate to mitochondria. Additionally, significant increase in Trx levels within the mitochondrion occurs in response to exposure of cells to hyperoxia when cells are treated with human recombinant Trx (rhTrx). We show that rhTrx treatment significantly decreases the level of $O_2^{\bullet-}$ and 8-Oxo-dG and maintains the mitochondrial length in hyperoxia. The level of Trx2 did not change in normoxia or hyperoxia in wildtype mice [30], thioredoxin overexpressing mice (*Trx-Tg*) or mice with decreased expression and activity of Trx (*dnTrx-Tg*) [31–33]. Additionally, mitochondrially coded genes and proteins of complex I such as, ND1, ND2, ND3 and ND4 were significantly decreased in hyperoxia in MLE12 cells, but treatment with rhTrx prevented this decrease. Collectively, our study shows an important and previously unrecognized role of cytosolic Trx shuttle into mitochondria under physiological condition, which increases during oxidative stress in pathological condition in a hyperoxia-mediated lung injury model using high oxygen concentrations (hyperoxia). Translocated cytosolic Trx protects against hyperoxia-mediated downregulation of mitochondrially coded genes of complex I and apoptosis of lung cells by promoting regeneration of Ogg1 activity. Additionally, downregulation of Drp1, TFAM and UCP-1 expression is restored in mitochondria of hyperoxia exposed mice overexpressing cytosolic Trx. Further, decreased oxidative phosphorylation in hyperoxia was improved due to translocation of cytosolic Trx into the mitochondrion.

2. Results

2.1. Increased levels of cytosolic Trx in cells or mice with overexpression of cytosolic Trx (Trx-Tg) inhibit 8-oxo-dG accumulation, $O_2^{\bullet-}$ generation, mitochondrial fragmentation, Drp1 phosphorylation and promotes translocation of cytosolic Trx into mitochondria

Hyperoxia is known to cause mitochondrial DNA damage and impairs mitochondrial function in the lung [31,34–36]. We have previously shown that hyperoxia-induced lung injury is ameliorated in *Trx-Tg* mice but not *dnTrx-Tg* mice. Further, mitochondrial aconitase and NADH dehydrogenase activities are decreased in *dnTrx-Tg* mice, but not *Trx-Tg* mice in hyperoxia [31]. Murine epithelial cells exposed to hyperoxia showed decreased ATP production along with increased expression of p53 and p21; and increased apoptotic and inflammatory markers in *dnTrx-Tg* mice, but not *Trx-Tg* mice [31]. Therefore, we sought to determine whether cytosolic Trx plays a role in hyperoxia-mediated mitochondrial dysfunction. Here, we determined whether increased levels of cytosolic Trx would prevent mitochondrial DNA damage in hyperoxia. As shown in Fig. 1a and b, hyperoxia significantly increased the level of 8-Oxo-dG, an oxidative DNA damage product within the mitochondria compared to normoxic cells, suggesting mitochondrial DNA damage. However, the level of 8-Oxo-dG was significantly decreased in the presence of human recombinant Trx (rhTrx) (Fig. 1a and b), suggesting that externally added rhTrx protects against mitochondrial DNA damage, even if Trx2 was present within the mitochondria. The enlarged figure presented in Fig. S1 was acquired by high resolution LSM880 microscope [37] that shows the localization of 8-Oxo-dG (green) within mitochondrial membrane (red, Tom20, Fig. S1). As shown in Fig. 1a-c, exposure to hyperoxia caused significant fragmentation of mitochondria as quantitated by mitochondrial length. However, cells treated with rhTrx showed decreased mitochondrial fragmentation and restored normal mitochondrial length in hyperoxia (Fig. 1a–c). Since hyperoxia is known to produce $O_2^{\bullet-}$ [37,38] in the mitochondria we determined mitochondrial $O_2^{\bullet-}$ levels by EPR using mitoTEMPO and found significant increase in $O_2^{\bullet-}$ levels in hyperoxia as expected, but rhTrx decreased the level of $O_2^{\bullet-}$ (Fig. 1d and e). Dynamin-related protein-1(Drp-1) is a mitochondrial fission protein which promotes mitochondrial fragmentation when phosphorylated. Since we found increased mitochondrial fragmentation, we evaluated the level of phosphorylated Drp-1 (pDrp-1) in the mitochondria from

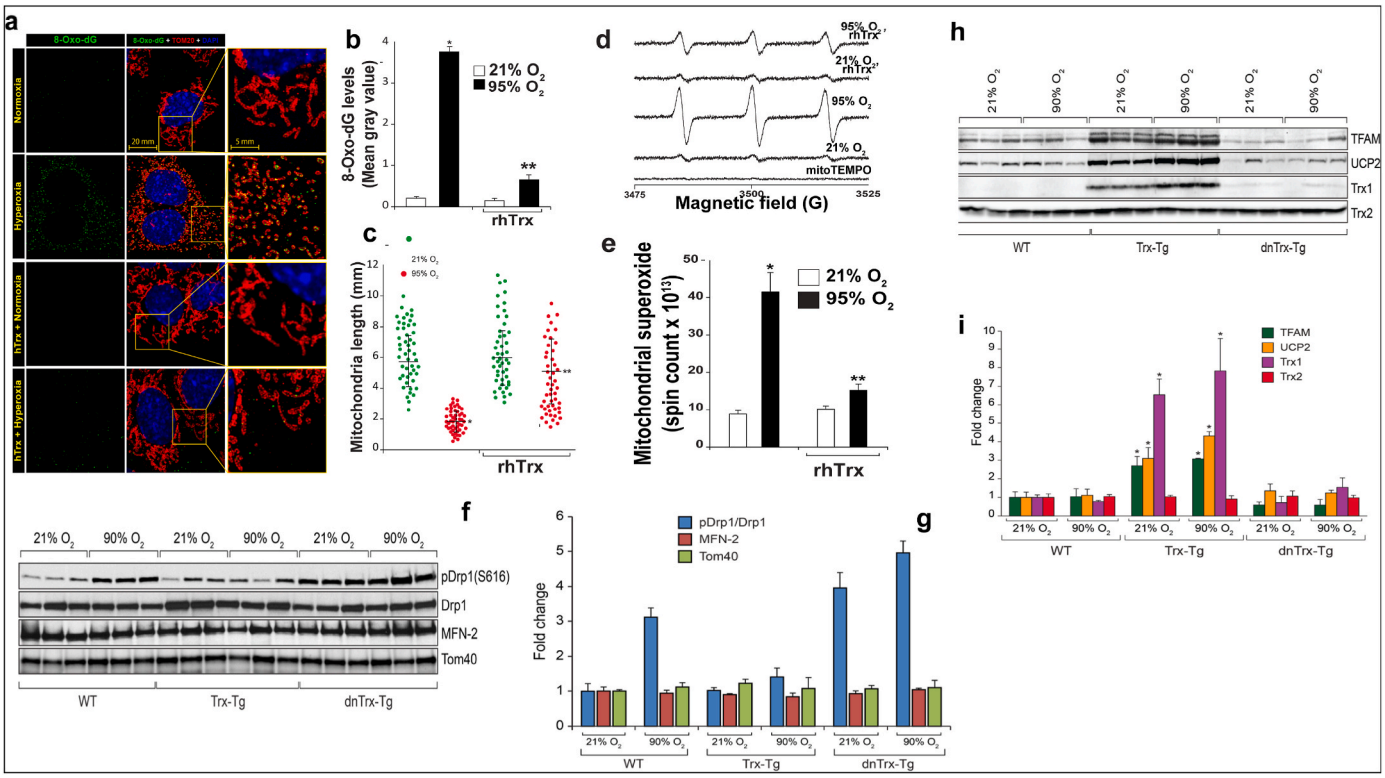

Fig. 1. Trx decreases 8-Oxox-dG levels, restores mitochondrial length, decreases $O_2^{\bullet-}$ generation, reduces Drp1 phosphorylation and translocates to the mitochondria in hyperoxia: (a) Trx blocks accumulation of 8-Oxo-dG in mitochondria. MLE12 cells were exposed to 21 % O_2 or 95 % O_2 with or without rhTrx (2ug/mL) for 18 h, following which cells were fixed and probed for 8-Oxo-dG and Tom20; **(b)** 8-Oxo-dG levels (mean gray value); *p < 0.01 versus normoxia (21 % O_2); **p < 0.01 versus hyperoxia (95 % O_2); n = 3,*t*-test; **(c) Trx restores mitochondrial length shortened due to hyperoxia** (n = 3,*t*-test, **P < 0.05); **(d) Trx decreases O_2•− generation in hyperoxia**, MLE-12 cells, untreated or treated with rhTrx (2ug/mL) were exposed to normoxia or hyperoxia, $O_2^{\bullet-}$ was detected by EPR using CMH as spin probe as mentioned in methods; **(e),** Absolute spin counts determined by Bruker Xenon software and plotted as bar graph, n = 3,*t*-test; **(f), Trx inhibits Drp1 phosphorylation:** Mitochondria were isolated from lungs of *WT*, *Trx-Tg* or *dnTrx-Tg* mice exposed to normoxia or hyperoxia and mitochondrial lysates were subjected to western analysis of Drp/pDrp(Ser^{616}), MFN and Tom40; **(g)** densitometry of Drp1/pDrp1, *P < 0.05 vs. 21 %O_2 *WT*, **P < 0.05 vs. 21 %O_2 and 21 %O_2 or 90 % O_2 *Trx-Tg* mice; n = 3,*t*-test **(h)** Western analysis of TFAM, UCP2, Trx, and Trx2, **(i)** Densitometry of TFAM, UCP2, Trx and Trx2 in *Trx-Tg* mice, *P < 0.05 vs. *WT* or *dnTrx-Tg* mice; densitometry of TFAM and UCP2, n = 3,*t*-test (P < 0.05 *Trx-Tg* vs. *WT* or *dnTrx-Tg* mice, normoxia and hyperoxia).

WT, *Trx-Tg* and *dnTrx-Tg* mice [31–33] exposed to hyperoxia. As shown in Fig. 1f and g, significant increase in the level of pDrp-1 was observed in isolated mitochondria from *WT* mice exposed to hyperoxia. However, the level of pDrp-1 did not increase in mitochondria from *Trx-Tg* mice exposed to hyperoxia (Fig. 1f and g). In contrast, the phosphorylation of Drp-1 was increased in *dnTrx-Tg mice* exposed to normoxia or hyperoxia. Further, the expression of mitochondrial fusion protein, mitofusion-2 (MFN-2) or the mitochondrial Tom-40 expression did not change in normoxia or hyperoxia. We determined the level of mitochondrial transcription factor A (TFAM), mitochondrial inner membrane uncoupler-2 (UCP2), Trx and Trx2 expression in *WT*, *Trx-Tg* or *dnTrx-Tg* mice exposed to normoxia or hyperoxia. Although we found no change in the levels of Trx2 in response to hyperoxia (Fig. 1h and i), there was significant increase in cytosolic Trx in the mitochondrial lysates in *Trx-Tg* mice, which was further increased in hyperoxia. In contrast, *dnTrx-Tg* mice did not show any increase in the level of Trx in the mitochondrial lysates (Fig. 1h and i), suggesting that cytosolic Trx is able to translocate to mitochondria in hyperoxia. In addition, we found that the expression of TFAM or UCP2 is increased in *Trx-Tg* mice lungs in normoxia or hyperoxia in contrast to *dnTrx-Tg* mice (Fig. 1h and i). These studies show that cytosolic Trx translocates to mitochondria and has profound impact on mitochondrial structure, DNA damage and mitochondrial protein expression. Intriguingly, our data show that Trx2 level did not change in response to hyperoxia, but Trx levels increased within mitochondria in hyperoxia.

2.2. Reduced Trx, but not oxidized or mutant Trx translocates to the mitochondria

Since we found significantly higher levels of Trx in mitochondrial lysates in *Trx-Tg* mice exposed to hyperoxia, we determined whether externally added rhTrx would translocate to mitochondria in oxidative stress conditions such as hyperoxia. As shown in Fig. 2a and b, high levels of rhTrx translocated to mitochondria in MLE12 cells when treated with rhTrx and exposed to hyperoxia. However, although significant, lesser amount of rhTrx translocated to mitochondria in normoxia compared to hyperoxia. To further confirm the translocation of cytosolic Trx into mitochondria and its effect on mitochondrial morphology, we performed immunofluorescence analysis and found that the elongated mitochondrial structure was changed to rounded form in hyperoxia, but MLE-12 cells supplemented with rhTrx retained higher number of elongated mitochondria (Fig. 2c and d) in hyperoxia exposed cells. Further, we noted that Trx co-localizes with Mito Tracker during hyperoxia and to a lesser extent in normoxia (Fig. 2d). As shown in Fig. 2e–g, Trx was found to co-localize with TOM-40 (translocase of outer mitochondrial membrane), glucose regulated protein-75 (Grp75), which is associated with mitochondrial membrane-associated membrane (MAM) and cytochrome oxidase-4 (COX4), demonstrating that Trx translocates into mitochondria and localizes to the outer and inner membranes, and mitochondrial matrix. We eliminated the possibility of cross reactivity with mitochondrial Trx2 by performing experiment with histidine-tagged rhTrx and using anti-His antibodies (Fig. S2). To further confirm the translocation of Trx into the mitochondria we performed

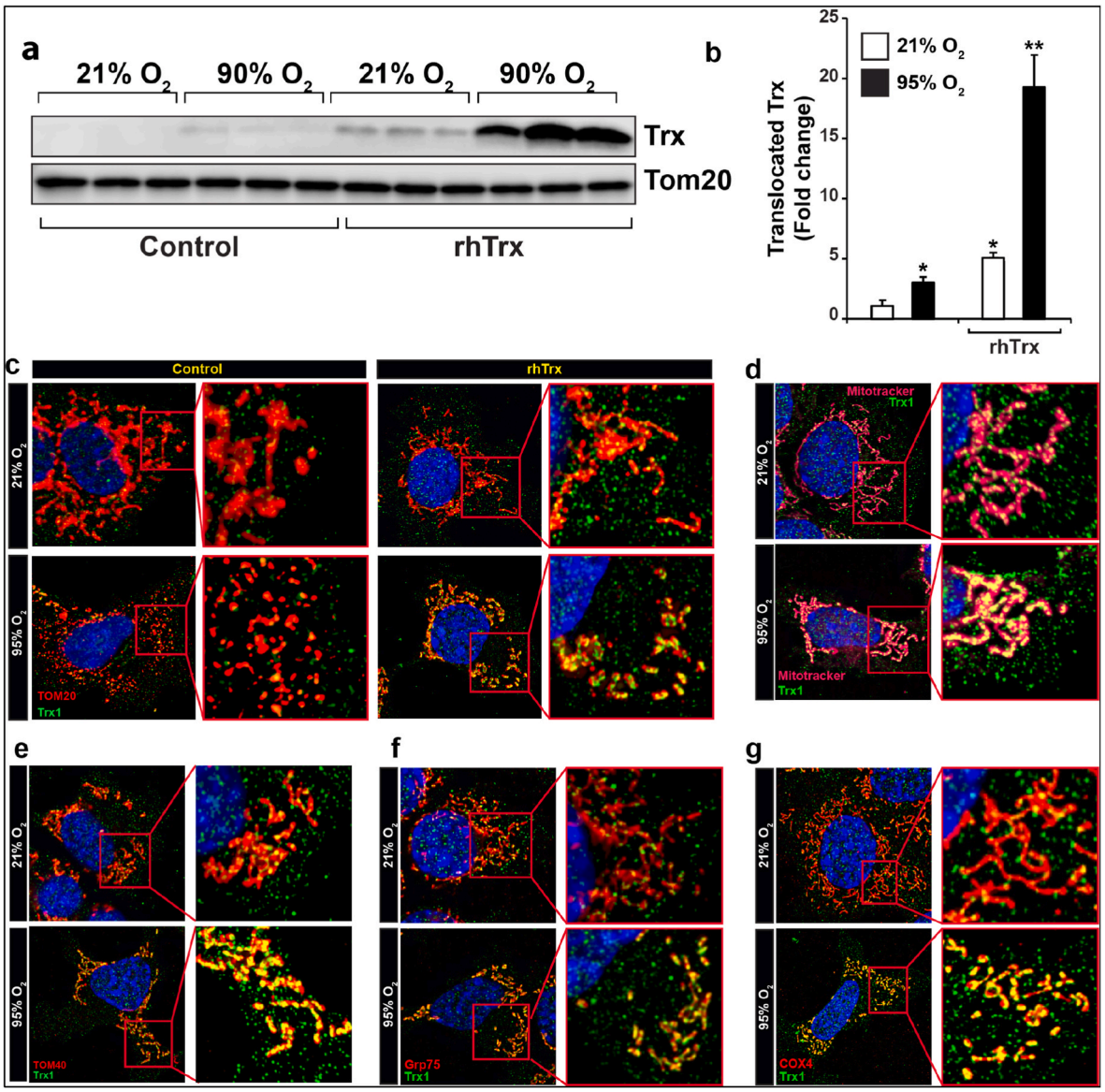

Fig. 2. Cytosolic Trx translocates to mitochondria in normoxia and hyperoxia: (a) MLE12 cells were incubated with rhTrx (2ug/mL) for 16- hours, followed by isolation of mitochondria and western blotting of mitochondrial lysates for Trx and Tom20; **(b)** Densitometry of Western blot for Trx and Tom20. *P < 0,0.05 vs normoxia control; **P < 0.05 vs. cells treated with rhTrx and exposed to hyperoxia; n = 3, *t*-test, **(c) Immuno-fluorescence analysis of rhTrx translocation to mitochondria:** MLE12 cells were cultured on glass cover slips, incubated with or without recombinant human Trx (2 μg/mL) and exposed to 21 % or 95 % O_2 for 16 h. Then cells were stained with MitoTracker Deep Red, fixed, permeabilized, blocked and probed with anti-Trx antibodies alone or in combination. Control reaction without addition of hTrx; detected with Trx and TOM20 antibody; **(d)** only anti-Trx with mito-tracker dye; **(e)** anti-Trx and anti-TOM20; **(f)** anti-Trx and anti-Grp75; **(g)** anti-Trx and anti-Cox4 antibodies followed by Alexa Fluor 488 and Alexa Fluor 568 conjugated secondary antibodies. Cellular nuclei were stained with Hoechst 33342. 20 to 25 fluorescent images along the *z*-axis with 80 nm interval (optical sections) were obtained using Zeiss Axio Imager Z2 upright fluorescent microscope via a 100x/1.40 NA objective and deconvolved using AxioVision 4.9 software.

proximity ligation assay (PLA) [39,40]. We used anti-TOM-20, anti--TOM-40, and anti-aconitase-2 (Aco2) with anti-Trx and found proximity signals that were evident in normoxic MLE-12 cells, which was increased significantly when the cells were exposed to 95 %O_2 (Fig. 3a–d). In addition, we tested whether mouse Trx would translocate to mitochondria in MLE12 cells. As shown in Fig. 3e and f, mouse Trx was translocated to mitochondria in MLE12 cells. We further confirmed the translocation of Trx into the mitochondria by immunoelectron microscopy. As shown in Fig. 4a, we detected Trx within mitochondria in rhTrx treated MLE-12 cells exposed to hyperoxia. Since, we did not find any translocation of mutant Trx from *dnTrx-Tg* mice to the mitochondria (Fig. 1h and i), we determined whether redox-active cysteines within Trx are required for its translocation to the mitochondria. We found that reduced Trx is specifically transported to mitochondria, as mutant Trx (C32A-C35A) or Trx (C69S–C73S) did not translocate to the mitochondrion (Fig. 4b). These studies established that increased levels of reduced cytosolic Trx translocate to mitochondria in normoxia, which is further increased in oxidative stress conditions such as hyperoxia. In addition, overexpression of Trx in *Trx-Tg* mice or rhTrx treatment of MLE12 cell increased the translocation of Trx to the mitochondria in normoxia. We determined whether Trx translocates to mitochondria in other cells in response to oxidative stress. We treated H9c2 (embryonic rat cardiomyocyte) and HCAEC (human coronary artery endothelial cells) with or without rhTrx and exposed these cells to hypoxia/reoxygenation (H/R) followed by immunofluorescence of Trx and Tom-20 (mitochondrial marker). As shown in Fig. S3, rhTrx colocalized with Tom-20 in these cells, suggesting translocation of Trx to mitochondria in these cells in oxidative stress conditions. Taken together, these data

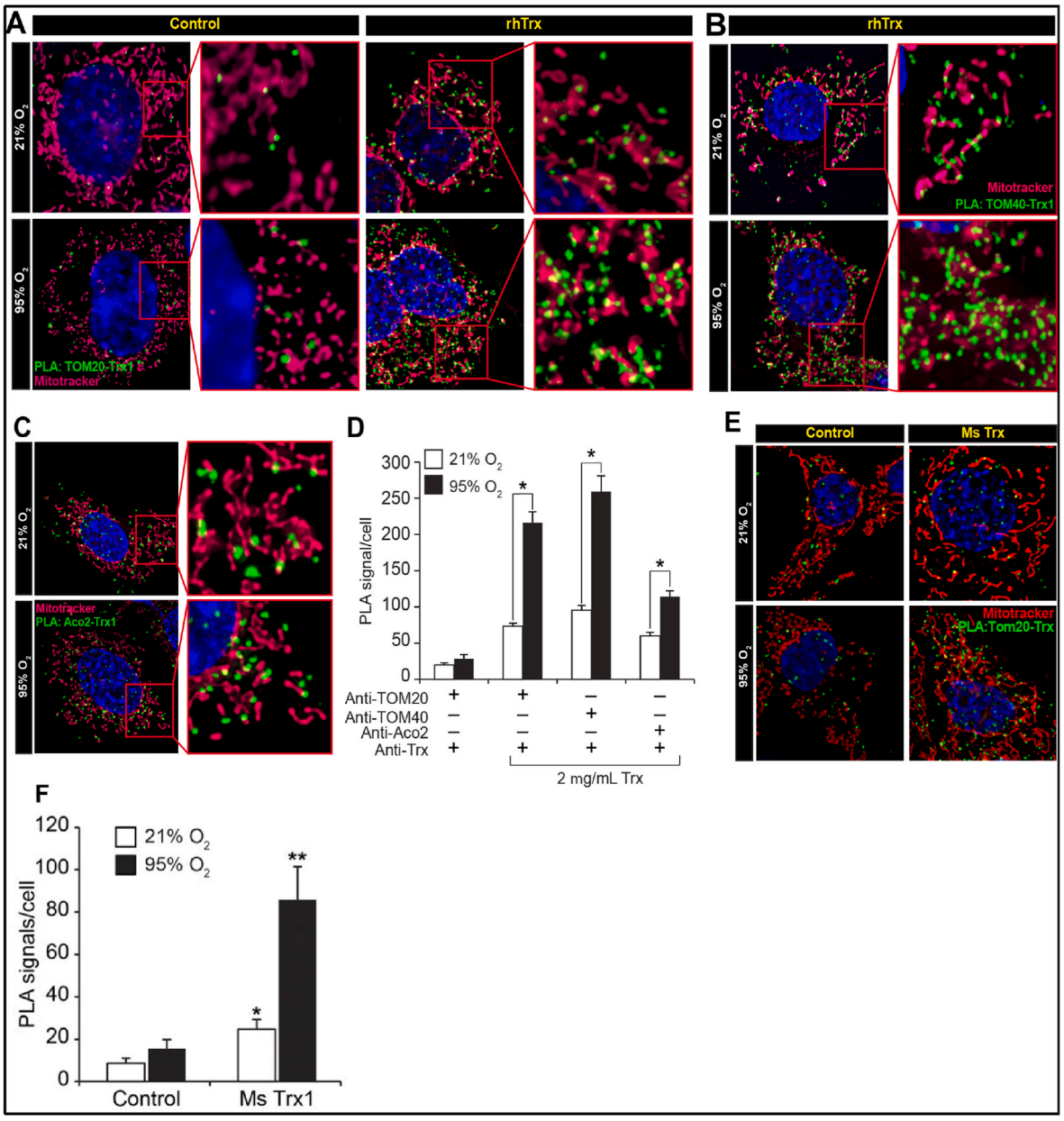

Fig. 3. Proximity Ligation Assay (PLA) demonstrating translocation of rhTrx to mitochondria and its interaction with TOM20, aconitase and TOM40: MLE12 cells were cultured on glass cover slips, incubated with rhTrx (2 μg/mL), and exposed to 21 % or 95 % O_2 for 16 h. Then the cells were stained with MitoTracker Deep Red, fixed, permeabilized and PLA was performed using anti-hTrx antibodies in combination with anti-TOM20 **(a)**, anti-TOM40 **(b)** or anti-Aco2 **(c)** antibodies. Cellular nuclei were stained with Hoechst 33342. 20 to 25 fluorescent images along the *z*-axis with 80 nm interval (optical sections) were obtained using Zeiss Axio Imager Z2 upright fluorescent microscope via a 100x/1.40 NA objective and deconvolved using AxioVision 4.9 software. PLA reaction products appear as green dots which originates from the location of Trx interaction with its partners located in/on mitochondria; **(d)** Quantitation of PLA, n = 3,*t*-test*p < 0.05; **(e)** MLE-12 cells were incubated with mouse Trx (Ms-Trx) for 24 h followed by exposure to 95 %O_2 for 24 h. Following exposure PLA was performed with anti-TOM-20 and anti-Ms-Trx antibody; **(f)** quantitation of PLA signal; n = 3, *t*-test, p < 0.05; **p < 0.05).

established that cytosolic reduced Trx translocate to mitochondria in normal physiological state which is significantly increased in oxidative stress condition such as hyperoxia and is a phenomenon that may occur in all cells including epithelial, endothelial cells and cardiomyocytes that were tested here.

2.3. Loss of function of Trx in dnTrx-Tg mice, promotes mitochondrial dysfunction in hyperoxia

Given that increased levels of $O_2^{\bullet -}$ is produced in hyperoxia in the mitochondrion concomitant with decreased energy production, we hypothesized that *Trx-Tg* mice would be protected against mitochondrial dysfunction in hyperoxia due to translocation of cytosolic Trx. We determined oxygen consumption rate (OCR) in mitochondria isolated from lungs of normoxia or hyperoxia exposed mice. As shown in Fig. 5a–c, the basal OCR (state 2) was significantly decreased in mitochondria isolated from lungs of *WT* or *dnTrx-Tg* mice exposed to hyperoxia, but this rate was increased in *Trx-Tg* mice. Further, the OCR was significantly increased in *Trx-Tg* mice compared to *WT* or *dnTrx-Tg* in the presence of ADP (state 3) in hyperoxia, indicating a higher rate of ATP production in the lungs of these mice (Fig. 5b, Fig. S6A). State 3_{ADP} is regulated by substrate oxidation, substrate uptake, processing of enzymes, as well as ATP turnover and therefore any of these processes would reduce the state 3 [25]. As shown in Fig. S6B, the respiratory

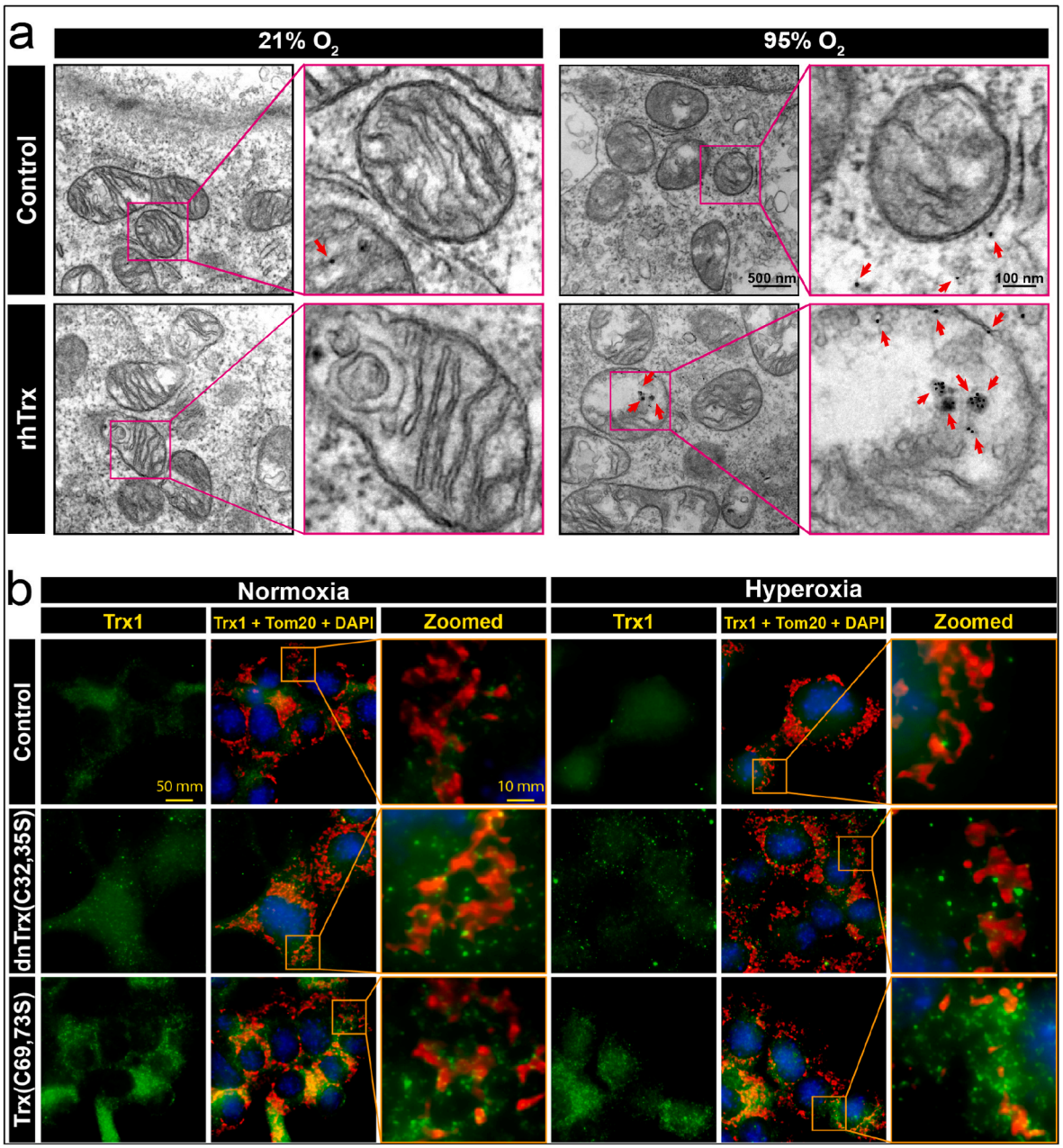

Fig. 4. (a) Immunoelectron microscopy of rhTrx within the mitochondria: MLE-12 cells were incubated with rhTrx (2 μg/ml) and exposed to normoxia or hyperoxia for 18 h. Following exposure cells were processed for immunoelectron microscopy as detailed in the experimental procedure: (**b) Mutant Trx, (C32,35S) and Trx (C69,73S) do not translocate to mitochondria:** MLE12 cells were incubated with rhTrx (C32,35S) and Trx1(C69,73S) mutant proteins (2 μg/mL) and then exposed to 21 % O_2 or 95 % O_2 for 18 h. Then cells were fixed and probed for Trx and Tom20 as described in the experimental procedure.

control ratio (RCR) for *WT* or *dnTrx-Tg* mice exposed to hyperoxia was decreased compared to normoxia exposed animals. However, there was no significant change in RCR for *Trx-Tg* mice exposed to normoxia or hyperoxia. Next, we determined whether hyperoxia impaired the flow of electrons via respiratory chain complexes. The electron flow experiment was performed using pyruvate and malate as substrates, which are oxidized via complex I [25,34]. Exposure to hyperoxia decreased the basal OCR demonstrating complex I malfunction in the isolated lung mitochondria in *WT* mice, but not in *Trx-Tg* mice (Fig. 5d and f). Because the substrate was provided to mitochondria isolated from lungs of hyperoxia or normoxia exposed mice, the data show specific dysfunction of complex I to utilize the substrate, but not the substrate oxidation resulting in decreased OCR. The OCR was significantly increased in normoxia-exposed mitochondria in response to injection of succinate, which is oxidized via complex II, demonstrating a complex II driven respiration (Fig. 5e). However, hyperoxia significantly decreased the OCR suggestive of complex II malfunction as succinate was supplied *in vitro* to the mitochondria. Inhibition of complex III by antimycin A remained unchanged for mitochondria isolated from normoxic or hyperoxic lung. We used ascorbate/TMPD, which is a direct electron donor for complex IV to determine whether complex IV function was compromised in hyperoxia. To our surprise the OCR following ascorbate/TMPD injection was unchanged in hyperoxia in lung mitochondria demonstrating that the complex IV function is not affected in hyperoxia (Fig. 5d–f). However, complex I and II functions were preserved in *Trx-Tg* mice, but not in *WT* or *dnTrx-Tg* mice. Since complex I and II functions are decreased in *WT* mice but not in *Trx-Tg* mice, we determined whether expression of complex I proteins are affected due to

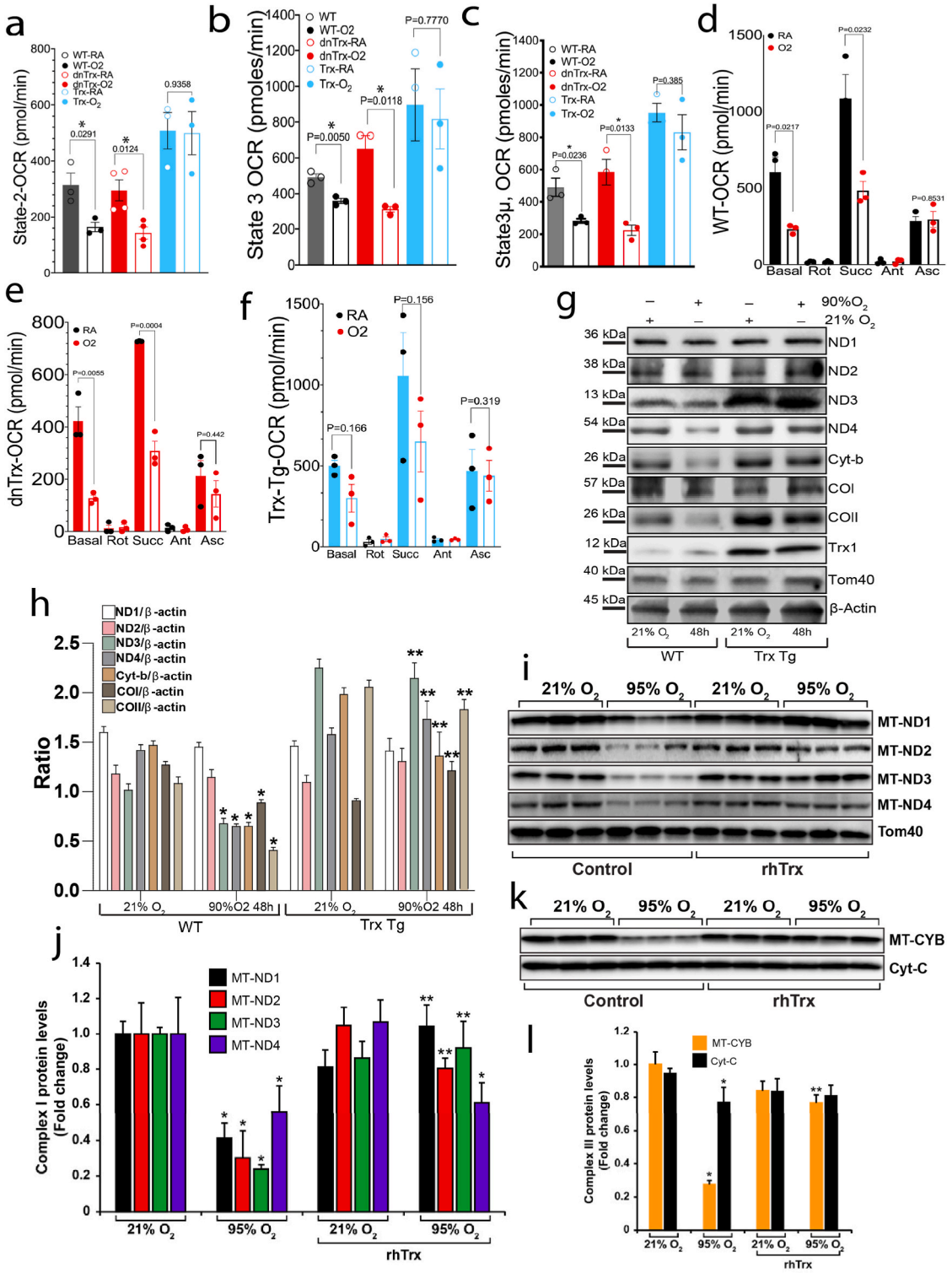

Fig. 5. Decreased oxygen consumption in mitochondria isolated from lungs of *dnTrx-Tg* mice, but not *Trx-Tg* mice: *WT*, *dnTrx-Tg*, and *Trx-Tg* mice were exposed to room air (RA) or 90 % oxygen in animal exposure chambers for 24 h. Mitochondria from lungs of *WT*, *Trx-Tg and dnTrx-Tg* mice were isolated as described in the methods and the OCR was measured in isolated mitochondria (10μg/well) in the presence of ADP, Oligomycin, FCCP and Antimycin A using the XF24 analyzer. **(a)** Basal OCR (succinate) in normoxia or hyperoxia, state 2; **(b)** OCR in response to ADP (state 3); **(c)** OCR in response to FCCP (state 3μ); **(d)** *WT* mitochondria basal OCR in response to pyruvate/malate, rotenone, succinate, AA and ascorbate/TMPD **(e)** *dnTrx-Tg* mitochondria basal OCR in response to pyruvate/malate, rotenone, succinate, AA and ascorbate/TMPD **(f)** *Trx-Tg* isolated mitochondria basal OCR in response to pyruvate/malate, rotenone, succinate, AA and ascorbate/TMPD. Data are presented as means ± SEM, $*P < 0.05$, $**P < 0.0001$ by ANOVA and Tukey-Kramer post-test. OCR measurements taken in 5 replicate wells and data representative of two to three independent experiments. **(g)** Hyperoxia-mediated decreased mitochondrial proteins from *WT* mice (90 % $_{O2}$ exposure for 24 h) are rescued in *Trx-Tg* mice, mitochondrial lysates from *WT* or *Trx-Tg* mice exposed to RA or 90 %O_2 were subjected to western analysis for ND1, ND2, ND3, ND4 and Tom-40 expression; **(h)** densitometry of complex I proteins in Fig. 5g (ANOVA, $P < 0.05$), $N = 3$)**; (i–j); MLE12 cells show loss of ND1-ND4 in hyperoxia:** MLE12 cells were treated with rhTrx (2.5 μg/ml) and exposed to 95 % O_2 for 16–24 h; (**k-l**) **rhTrx treatment of MLE12 cells rescues loss of Cyt. b in hyperoxia:** western analysis of mito-cyt. b and quantitation.

hyperoxia exposure. As shown in Fig. 5g and h, the expression of mitochondrially coded ND3 and ND4 proteins were decreased in hyperoxia in *WT* mice, but not in *Trx-Tg* mice exposed to hyperoxia. However, the expression of ND1 and ND2 did not decrease in *WT* mice or *Trx-Tg* mice in hyperoxia (Fig. 5g and h). In contrast, exposure of MLE12 cells to hyperoxia resulted in decreased ND1-ND4 proteins, but rhTrx treatment prevented this downregulation (Fig. 5i and j). Additionally, we found that the expression of mitochondrial Cyt *b* (Mt-Cyt b) was significantly decreased in *WT* mice, but not in *Trx-Tg* mice (Fig. 5g and h). Additionally, Mt-Cyt-b expression was also decreased in MLE12 cells exposed to hyperoxia; however, treatment with rhTrx rescued Mt-Cyt b expression. Further, the expression of complex IV proteins such as mitochondrial cytochrome oxidase-1(Mt-CO1) and Mt-CO2 were decreased in hyperoxia in *WT* mice, but not in *Trx-Tg* mice (Fig. 5g and h). These data show that loss of complex I and complex III proteins may account for the loss of function of these complexes in hyperoxia, and overall decreased ATP production.

2.4. Modulation of kinetics of $O_2^{\bullet-}$ generation by complex I and complex III in hyperoxia by Trx

To understand the specific mechanism by which complex I and complex III become dysfunctional in hyperoxia, we studied the kinetics of $O_2^{\bullet-}$ generation in isolated mitochondria from MLE12 cells using EPR spectrometry. As shown in Fig. 6a, normoxia or hyperoxia, both generated increased levels of $O_2^{\bullet-}$ with significantly higher levels of $O_2^{\bullet-}$ in hyperoxia compared to normoxia. Treatment of cells with rhTrx decreased the rate of $O_2^{\bullet-}$ generation in hyperoxia. Rotenone is known to inhibit complex I function by blocking the electron transfer from ND2 iron-sulfur cluster to ubisemiquinone [41,42]. This causes the accumulated electrons to react with oxygen resulting in the generation of $O_2^{\bullet-}$. Thus, mitochondria isolated from normoxic cells generated $O_2^{\bullet-}$ in the presence of rotenone (Fig. 6b). Since complex I is already dysfunctional in hyperoxia (Fig. 5h) addition of rotenone did not result in acute increase of $O_2^{\bullet-}$ production. However, diphenyl iodonium (DPI) inhibited superoxide generation in normoxia as well as hyperoxia in mitochondria as it blocks electron transfer from NADH2 to FMN site (Fig. 6b and c). Thus, our data show that complex I is dysfunctional in hyperoxia. We further evaluated the rate of $O_2^{\bullet-}$ generation by complex III in isolated mitochondria. As shown in Fig. 6d–f, $O_2^{\bullet-}$ was produced at an increased rate in hyperoxia compared to normoxia, and rhTrx significantly inhibited the rate of $O_2^{\bullet-}$ generation. We used antimycin A (AA) that is known to induce $O_2^{\bullet-}$ production from complex III [43]. AA induced significant level of $O_2^{\bullet-}$ in normoxia as expected (Fig. 6e and f). Since hyperoxia was already producing significant $O_2^{\bullet-}$ from complex III, AA treatment caused marginal increase the rate of $O_2^{\bullet-}$ compared to hyperoxia alone (Fig. 6e and f). Since myxothiazol inhibits complex III (site III_{Q0}) and therefore, decreases $O_2^{\bullet-}$ production, we further tested whether myxothiazol would inhibit hyperoxia-mediated complex III $O_2^{\bullet-}$ generation. As shown in Fig. 6e and f, myxothiazole decreased rate of $O_2^{\bullet-}$ generation in normoxia or hyperoxia, indicating complex III driven $O_2^{\bullet-}$ generation. Thus, complex I and complex III functions are significantly impaired by hyperoxia, which is rescued by rhTrx.

2.5. Cytosolic Trx interacts with Ogg1 and rescued loss of Ogg1 activity in hyperoxia

Since we found significant decrease in the expression of mitochondrially coded genes and proteins of complex I and complex III (Fig. 5), we speculated that hyperoxia might have caused significant damage to the mitochondrially coded genes as mitochondrial genome is CG rich which is more susceptible for $O_2^{\bullet-}$ -mediated damage. In addition, the protective proteins such as histones are not present in mitochondrial genome [22]. Mitochondrial Ogg1 is known to repair oxidative base modification within the mitochondrion, such as 8-Oxo-dG [22]. Therefore, we determined whether hyperoxia would modulate the expression of Ogg1. As shown in Fig. 7a and b, we found significant increase in Ogg1 expression in hyperoxia. Additionally, treatment of cells with rhTrx further increased Ogg1 expression in hyperoxia. We also found significant apoptosis of MLE12 cells exposed to hyperoxia which was decreased in the presence of rhTrx (Fig. 7c and Fig S4). To evaluate specific role of Ogg1 in apoptosis in hyperoxia, we depleted Ogg1 by siRNA and found increased apoptosis of MLE12 cells in both, normoxia and hyperoxia even in the presence of rhTrx. These data suggest that rhTrx does not have a direct role in protection against apoptosis, but could do so via Ogg1. Therefore, loss of Ogg1 promotes cell apoptosis in normoxia or hyperoxia irrespective of presence of rhTrx (Fig. 7c). Additionally, increased expression of Ogg1 in normoxia or hyperoxia did not provide protection against apoptosis of cells. Therefore, we speculated that rhTrx treatment could regenerate hyperoxia-mediated inactivation of Ogg1. Therefore, we determined whether Ogg1 and rhTrx interact to maintain the redox-active cysteines of Ogg1 that promotes Ogg1 activity during oxidative stress such as hyperoxia. As shown in Fig. 7d, Trx interacted with Ogg1 in hyperoxia as determined by immunoprecipitation of Ogg1 and immunoblotting for Trx (Fig. 7d and e). Thus, we speculated that functional Ogg1 is required to repair mitochondrial DNA damage caused by exposure to hyperoxia. We determined Ogg1 activity and found significant loss of Ogg1 activity in hyperoxia, which was rescued by treatment with rhTrx (Fig. 7f and g). Taken together, these studies suggest that restoration of Ogg1 activity due to translocation of cytosolic Trx into the mitochondria is a major mechanism of protection of mitochondrial function during oxidative stress. Thus, shuttle of cytosolic Trx into mitochondria in oxidative stress conditions such as hyperoxia is a major mechanism mitochondrial DNA repair and cell survival.

3. Discussions

Hyperoxia-mediated pulmonary oxygen toxicity is a major mechanism of pulmonary diseases such as bronchopulmonary dysplasia (BPD) in the neonates and adult respiratory distress syndrome (ARDS) in the elderly. Increased production of $O_2^{\bullet-}$ has been implicated in the loss of alveolar growth and differentiation [44], pulmonary cell death due to necrosis or apoptosis [45,46], and the onset of inflammation of the lung [47,48]. Although mitochondria have several antioxidant proteins to protect its energy producing machinery in normoxic conditions, overwhelming oxidant load in exposure to hyperoxia or other form of oxidative stress would irreparably damage the energy producing function resulting onset of various diseases or death of the organism. Since oxidatively inactivated mitochondrial proteins could not be regenerated by mitochondrially located proteins/enzymes, cytosolic Trx translocates to the mitochondria to regenerate oxidized –SH groups in enzymes (such as Ogg1) or other proteins by its disulfide reductase activity. Oxidized Trx would return to the cytosol where it is reduced by TrxR1 and NADPH and Trx-SH goes back to mitochondrial to further regenerate oxidatively inactivated proteins (Fig. S1).

The mitochondrion is the primary producer of $O_2^{\bullet-}$ during normal respiration in physiological state; however, increased levels of this radical is produced when cells are exposed to higher concentrations of oxygen [37,38,49,50]. The excessive $O_2^{\bullet-}$ production overwhelms the enzymatic and non-enzymatic antioxidant defense system in the mitochondria, resulting in extensive damage to the mitochondrial energy producing machinery triggering the cell death process [37,51]. Mitochondria have their own DNA that codes for 13 proteins that plays critical role oxidative phosphorylation and ATP production. Since some of these proteins are of high GC content and are not associated with histones or other protective proteins, these proteins are extremely susceptible to damage by chronic oxidative stress due to aging or overt oxidant stress cause by chemicals or high oxygen concentrations in the lung.

In this study, we discovered that cytosolic Trx translocates to mitochondria in normoxia and increased levels of Trx translocate to

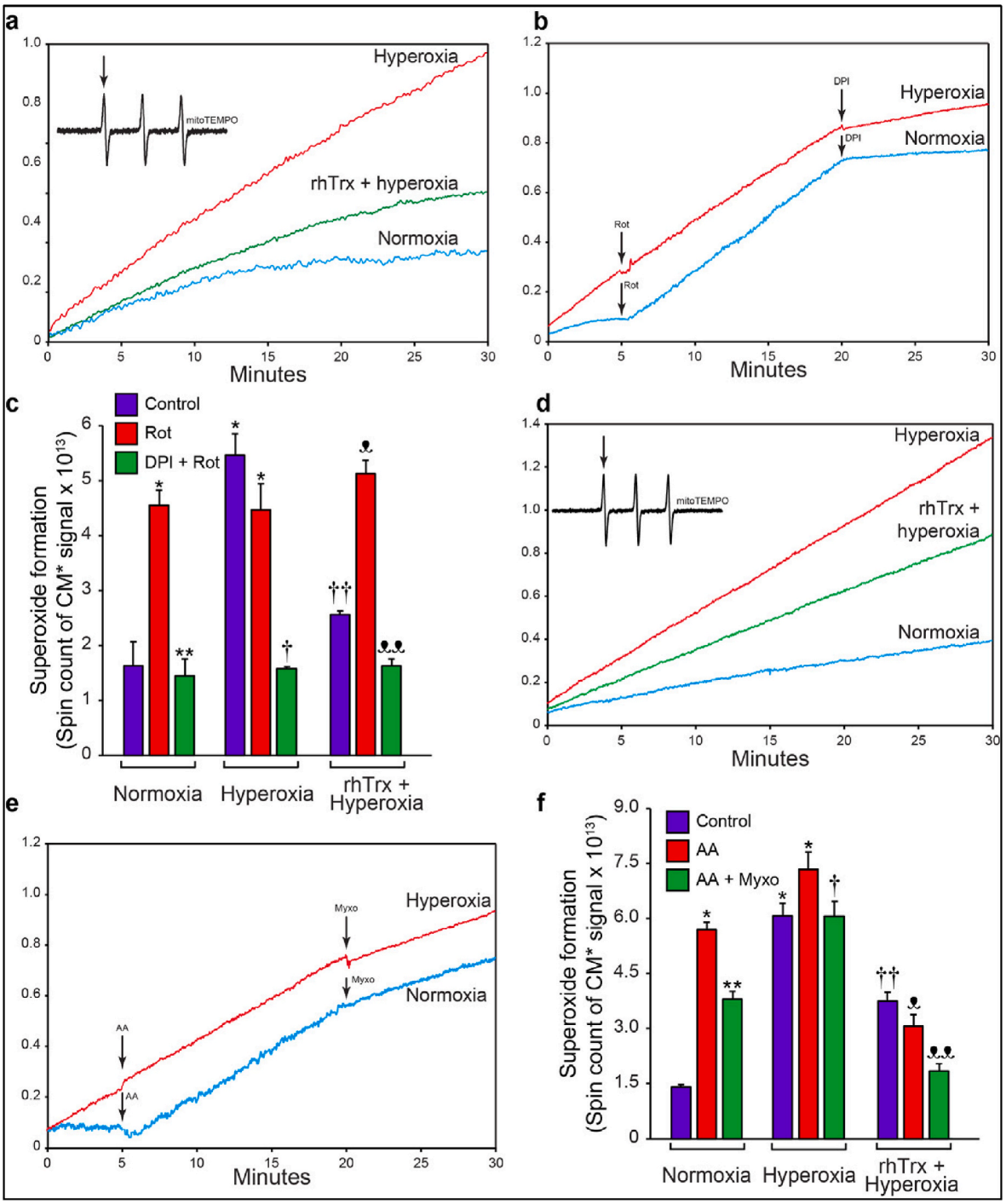

Fig. 6. Mitochondria-translocated Trx attenuates hyperoxia-induced complex I dysfunction: MLE12 cells were incubated with rhTrx (2 μg/mL) and exposed to hyperoxia for 16 h. At the end of treatment, mitochondria were isolated from cells and superoxide formation was determined by EPR using mitoTEMPO as described in methods. (**a**) effect of hyperoxia, rhTrx + hyperoxia and normoxia in time scan of nitroxide accumulation by 10 μg isolated mitochondria; **(b)** Effect of rotenone and DPI on generation of mitochondrial $O_2^{\bullet -}$ in normoxia or hyperoxia. Mitochondria were treated with 2 μM rotenone at 5 min and 0.5 mM DPI at 20 min of incubation and time scan data was collected.; **(c)** $O_2^{\bullet -}$ formation from mitochondria (10 μg) in the presence of rotenone or rotenone + DPI was detected by mitoTEMPO and quantified as absolute spin counts of nitroxide and plotted as a bar graph. *, $p < 0.01$ versus normoxia control; **, $p < 0.01$ versus normoxia + rotenone; †, $p < 0.01$ versus hyperoxia + rotenone; ††, $p < 0.01$ versus hyperoxia control; ♟, $p < 0.01$ versus rhTrx-hyperoxia control; ♟♟, $p < 0.01$ versus rhTrx-hyperoxia + rotenone; **(d)**10 μg mitochondria respiring in MAS buffer containing 10 mM pyruvate, 2 mM malate, and 4 mM ADP. The inset shows EPR spectrum of the nitroxide formed from mitoTEMPO; **(e)** Mitochondria were treated with 2 μM antimycin A (AA) at 5 min and 0.5 mM myxothiazole (Myxo) at 20 min of incubation and time scan data was collected; **(f)** $O_2^{\bullet -}$ formation from mitochondria (10 μg) in the presence of AA or Myxo was detected by mitoTEMPO and quantified as absolute spin counts of nitroxide and plotted as a bar graph. *, $p < 0.01$ versus normoxia control; **, $p < 0.01$ versus normoxia + AA; †, $p < 0.01$ versus hyperoxia + AA; ††, $p < 0.01$ versus hyperoxia control; ♟, $p < 0.01$ versus rhTrx-hyperoxia control; ♟♟, $p < 0.01$ versus rhTrx-hyperoxia + rotenone.

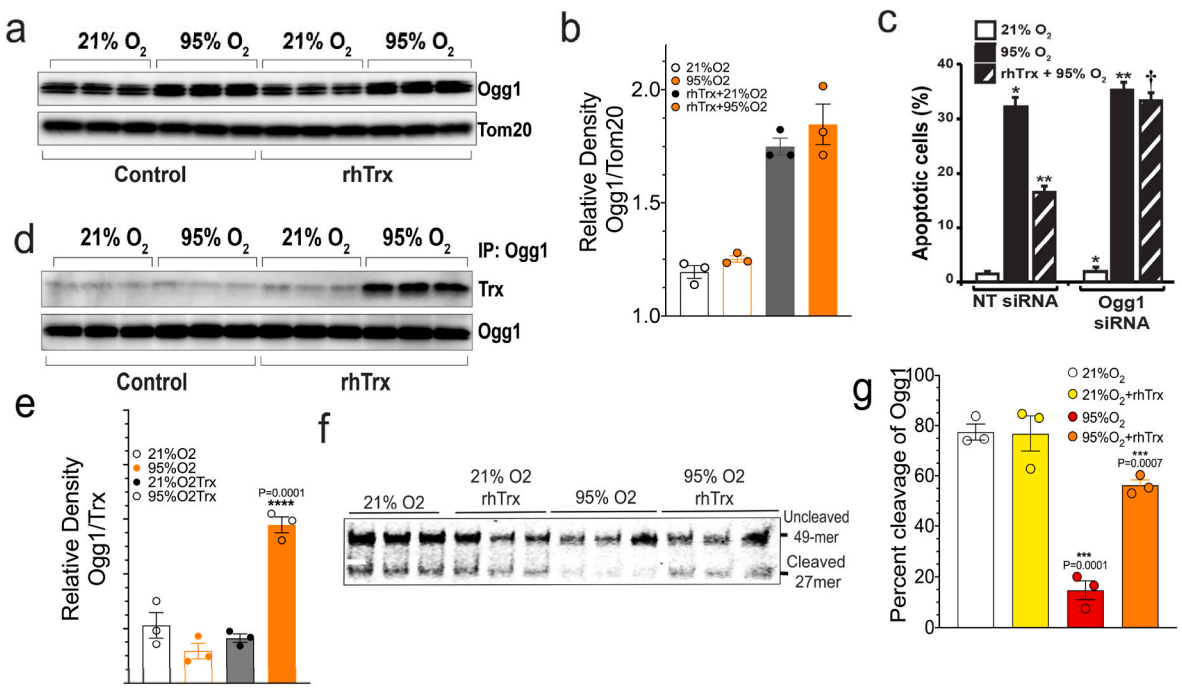

Fig. 7. Trx interacts with Ogg1 and rescue its activity during hyperoxia: MLE12 cells were treated with rhTrx (2.5 μg/mL) and exposed to normoxia or hyperoxia for 24 h, cell lysate was prepared and the expression of Ogg1 was analyzed by western blotting; (**a-b**), the expression of Ogg1 was increased in response to treatment with rhTrx, both in normoxia or hyperoxia; **(c), Ogg1 is required for protection against apoptosis of MLE12 cells by rhTrx:** MLE12 cells were transfected with NT or Ogg1 siRNA, treated or untreated with rhTrx (2.5ug/mL) and exposed to hyperoxia or normoxia. Apoptosis of cells was measured via FACS (Fig. S4) and quantitated. (**d-e**), **Trx interacts with Ogg1 in hyperoxia**: cell lysates were immuno-precipitated (IP) with anti-Ogg1 and blotted for Trx and densitometric data presented in "e"; **(f–g)** MLE12 cells treated with rhTrx (2.5 μg/ml) and treated with or without hyperoxia and were used for 8-oxoG DNA glycosylase activity assay as detailed in methods. TBE-Urea gel-based separation showed 49-mer oligonucleotide substrate (100 fmole) as in upper band and a 27-mer cleaved product in lower band. (**f**); Bar graph showing percentage cleavage of substrate at respective conditions. Ogg1 activity assay was performed using three independent *in vitro* culture samples two times. Data are represented as mean ± SEM. Statistical analyses were performed by one-way ANOVA with post hoc multiple comparisons test and significance level indicated as adjusted P value.

mitochondria in hyperoxia even if mitochondrial Trx2 is present within the mitochondria. We found that the rate of state 3_{ADP}, did not change significantly in mitochondria from *Trx-Tg* mice exposed to normoxia or hyperoxia, suggesting that the oxidative and enzymatic process remained functional in mitochondria form *Trx-Tg* mice in hyperoxia compared to either *WT* or *dnTrx-Tg* mice (Fig. 5b). Therefore, translocated Trx was able to protect mitochondrial substrate oxidation and ATP production in hyperoxia compared to *WT* or *dnTrx-Tg* mice. Further, our study shows that mRNA and protein expression of several mitochondrially encoded genes are decreased in hyperoxia. Lack of nucleosomes, histones, the other DNA binding proteins, and the non-coding DNA repeats in mitochondria, as well as proximal production of superoxide, predisposes it for enhanced DNA damage. In hyperoxia, we found decreased expression of complex I proteins such as ND1, ND2, ND3, ND4; complex III protein such as Cyt B and complex IV protein MT-COX2, all of which are very rich in GC. For example, ND4 has the highest GC content among them and is severely depleted hyperoxia. Additionally, ND1-4 are core proteins of complex one transmembrane and hydrophobic domain of complex I. Their genetic deficiency and single nucleotide polymorphism (SNP) results in cognitive impairment and muscle wasting [26,52]. Furthermore, defects in complex I also promotes increased $O_2^{\bullet -}$ production along with altered membrane potential, changes in calcium homeostasis and decreased NADH enzyme activities [26]. Both are the result of dysfunctional mitochondrial metabolism. If they were depleted in hyperoxia, it would affect electron transport from NADH to CoQ via complex I. Consistent with the loss of these proteins we found significant decrease in the rate of oxygen consumption and mitochondrial complex I and III dysfunction, as measured by their oxygen utilization. We found that MLE12 cells exposed to 95 % oxygen showed significant downregulation of ND1 and ND2 proteins in contrast to *WT* mice. We believe that 95 % O_2 exposure of MLE12 cells to hyperoxia could have been a very strong oxidative impact compared to *WT* mice exposed to 90 % oxygen. Therefore, 95 % O_2 could have decreased ND1/ND2 expression due to significantly higher load of oxidative stress compared to 90 % O_2 in mice. Therefore, endogenous mitochondrial antioxidant defense systems, such as Sod2, Trx2 and/or other mitochondria-specific antioxidant enzymes and small molecules do not protect against ROS-mediated loss of mitochondrial genes and protein in hyperoxia, although they are effective to protect against physiological ROS levels in normoxia.

Exposure to hyperoxia caused significant accumulation of 8-Oxo-dG, indicating failure of oxidative DNA base excision repair by Ogg1 that is correlated with decreased expression of several mitochondrially coded genes. Ogg1 is very prone to oxidation of its cysteine residues which impair its glycosylase activity in oxidative stress conditions [29]. Consistent with this notion we found that decreased Ogg1 activity in hyperoxia was rescued due to rhTrx treatment concomitant with decreased 8-Oxo-dG levels. It has been shown previously that forced overexpression of Ogg1 increases resistance to hyperoxia-mediated lung injury [28]. Ogg1 is known to be redox regulated, where oxidation by diamide completely abolished Ogg1 activity, but treatment with thiol reducing agents such as DTT restored Ogg1 activity [53]. Our data show that Trx directly interacts with Ogg1 in hyperoxia and increased its catalytic activity resulting in decreased apoptosis of pulmonary cells (Fig. 7f and g), suggesting that translocated Trx is critically required to protect the cells against oxidative damage that promotes cell survival. Our study shows that cells treated with rhTrx or mice overexpressing cytosolic Trx are protected against the loss of mitochondrial encoded genes and proteins. Although, mitochondria have their own Trx2, the expression of genes and proteins encoded by mitochondria are not

protected against high $O_2^{\bullet-}$ mediated decrease. The level of Trx2 did not change due to hyperoxia, but increased translocation of Trx occurred in normoxia as well hyperoxia, suggesting that cytosolic reduced Trx shuttle into mitochondria to reduce critical proteins, which are oxidized due to significantly higher $O_2^{\bullet-}$ generation in the mitochondria due to hyperoxia. Mitochondria imports several nuclear coded proteins for its basal activity, and import competent proteins should be in reduced state, as oxidized proteins are not imported to the mitochondrion [20,24]. As shown in our study, only Trx in its full reduce form is specifically translocated to the mitochondrion, as cysteine mutant Trx failed to translocate to the mitochondrion in hyperoxia or normoxia. Mitochondria utilizes several redox-sensitive enzymes to carryout oxidative phosphorylation. Additionally, mitochondrial electron transport system has several iron-sulfur (Fees) clustered enzymes that are reduced by electrons generated during the process. Cytosolic Trx-SH can maintain the function of enzymes by contributing electrons required for generation of ATP and maintenance of redox enzymes in functional state within an oxidizing milieu within the mitochondria. In contrast, Trx2 is not efficient in these processes compared to Trx. The levels of Trx2 did not change within the mitochondria in hyperoxia. Mitochondrial generation of $O_2^{\bullet-}$ is also implicated many other diseases such as age-mediated neurodegenerative diseases, cardiovascular diseases, cancer and metabolic disorders such as diabetes. We demonstrated that translocation of Trx to mitochondria is not limited to pulmonary cells, but also occurs in other cell types such as rat embryonic cardiomyocytes and human coronary endothelial cells (Fig. S2).

Overall, our study demonstrated that cytosolic Trx protects mitochondrial structure and function in oxidative stress conditions such as, hyperoxia by regenerating/or maintaining several –SH containing proteins by its translocation to mitochondria. For example, it regenerates Ogg1, a DNA repair enzyme that is inactivated in hyperoxia due to –SH oxidation. Similarly, NADH dehydrogenase, a redox-enzyme of complex I coded by mitochondrial genes is regenerated by Trx by promoting mitochondrial DNA repair due to restoration of Ogg1, and its activity is regenerated by reducing the catalytic center of NADH dehydrogenase [31]. Likewise, translocated Trx regenerates Fe–S cluster enzyme aconitase by it reducing function [31]. These disulfide exchange reactions could not be performed by Trx2, therefore, cytosolic Trx translocation is required to keep the mitochondria functioning during or after overt oxidant conditions that occur in pathological conditions, such as BPD, ARDS in the lung, ischemia-reperfusion injury in heart failure, neurodegenerative diseases and may be in other diseases where oxidative mitochondrial dysfunction is implicated in disease pathology.

CRediT authorship contribution statement

Venkatesh Kundumani-Sridharan: Supervision, Methodology, Formal analysis, Data curation. **Somasundaram Raghavan:** Methodology, Formal analysis, Data curation. **Sudhir Kumar:** Methodology, Formal analysis, Data curation. **Kumuda C. Das:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kumuda Das reports financial support was provided by National Institutes of Health. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2025.103678.

Data availability

Data will be made available on request.

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