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Research Paper

Ablation of the mitochondrial complex IV assembly protein Surf1 leads to increased expression of the UPR^{MT} and increased resistance to oxidative stress in primary cultures of fibroblasts

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ABSTRACT

Mice deficient in the electron transport chain (ETC) complex IV assembly protein SURF1 have reduced assembly and activity of cytochrome *c* oxidase that is associated with an upregulation of components of the mitochondrial unfolded protein response (UPR^{MT}) and increased mitochondrial number. We hypothesized that the upregulation of proteins associated with the UPR^{MT} in response to reduced cytochrome *c* oxidase activity in *Surf1*$^{-/-}$ mice might contribute to increased stress resistance. To test this hypothesis we asked whether primary cultures of fibroblasts from *Surf1*$^{-/-}$ mice exhibit enhanced resistance to stressors compared to wild-type fibroblasts. Here we show that primary dermal fibroblasts isolated from *Surf1*$^{-/-}$ mice have increased expression of UPR^{MT} components ClpP and Hsp60, and increased expression of Lon protease. Fibroblasts from *Surf1*$^{-/-}$ mice are significantly more resistant to cell death caused by oxidative stress induced by paraquat or tert-Butyl hydroperoxide compared to cells from wild-type mice. In contrast, *Surf1*$^{-/-}$ fibroblasts show no difference in sensitivity to hydrogen peroxide stress. The enhanced cell survival in response to paraquat or tert-Butyl hydroperoxide in *Surf1*$^{-/-}$ fibroblasts compared to wild-type fibroblasts is associated with induced expression of Lon, ClpP, and Hsp60, increased maximal respiration, and increased reserve capacity as measured using the Seahorse Extracellular Flux Analyzer. Overall these data support a protective role for the activation of the UPR^{MT} in cell survival.

1. Introduction

Mitochondrial electron transport chain complex IV (cytochrome *c* oxidase) is composed of 13 protein subunits that are assembled in the mitochondrial inner membrane into the holoenzyme by a regulated series of assembly proteins. Previous studies have shown that mice with a complex IV assembly factor *SURF1* null mutation (*Surf1*$^{-/-}$ mice) have impaired assembly of complex IV resulting in a 50–75% reduction in cytochrome *c* oxidase content and activity [8,35,39]. Surprisingly, despite the significant reduction in cytochrome *c* oxidase activity, the *Surf1*$^{-/-}$ mice exhibit a number of beneficial phenotypic changes including increased insulin sensitivity [7], increased mitochondrial number and activation of the UPR^{MT} in several tissues [35], increased blood flow and memory function in brain [23], increased resistance to kainic acid toxicity, and increased lifespan [8].

Cellular stress resistance is considered an important component of longevity in mammalian organisms [16,27]. For example, primary fibroblasts isolated from long-lived mouse models, such as the Ames dwarf mouse, have been shown to have a significantly elevated LD_{50} compared to wild-type controls in response to exogenous oxidative stressors such as paraquat or hydrogen peroxide [22,38]. Additionally, fibroblasts isolated from species with divergent lifespans indicate that greater survival following various stressors corresponds to the lifespan of the animal species [38]. The increased kainic acid resistance in adult *Surf1*$^{-/-}$ mice and increased longevity in *Surf1*$^{-/-}$ mice led us to hypothesize that cells from the *Surf1*$^{-/-}$ mice would exhibit increased resistance to

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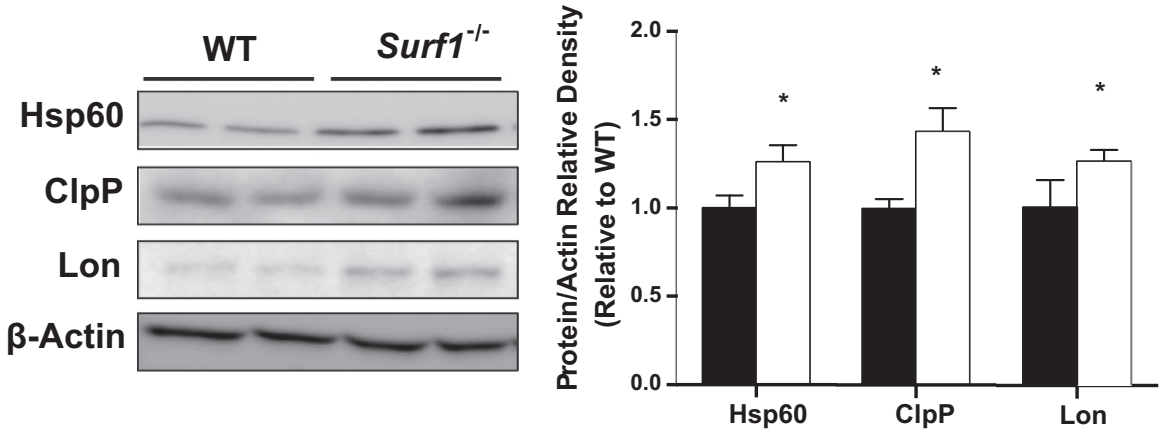

Fig. 1. The UPR^{MT} is elevated in *Surf1*$^{-/-}$ dermal fibroblasts. Primary dermal fibroblast cultures were isolated from 3 to 6 month old wild-type and *Surf1*$^{-/-}$ tails. Following an overnight incubation, cell lysates were prepared and immunoblotted with antibodies to Hsp60, ClpP, and Lon as surrogate for UPR^{MT} activation. Right panel indicates the histogram of the immunoblot quantification. Bars depict the mean ± SEM from 3 independent experiments. Statistical significance was determined by two-tailed Students *t*-test; $^{*}p < 0.05$.

oxidative stress [8]. Consistent with this, we report that primary dermal fibroblasts isolated from *Surf1*$^{-/-}$ mice are more resistant to cell death caused by paraquat (PQ) or tert-Butyl hydroperoxide (*t*-BuOOH), but not to hydrogen peroxide (H_2O_2), compared to cells from wild-type mice. Interestingly, PQ and *t*-BuOOH also result in induction of the UPR^{MT} related proteins Hsp60 and ClpP protease as well as Lon protease. These data suggest that reduced assembly of complex IV holoenzyme associated with reduced cytochrome *c* oxidase activity leads to enhanced resistance to stress that may be associated with enhanced upregulation of the UPR^{MT}.

2. Methods

2.1. Animals

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Health Science Center at San Antonio (UTHSCSA) and the Oklahoma Medical Research Foundation (OMRF). The *Surf1*$^{-/-}$ mice were generated as previously described [8]. The *Surf1*$^{-/-}$ colony was maintained by breeding male and female heterozygous *Surf1*$^{+/-}$ mice on a B6D2F1/J (C57/Bl6JxDBA2) mixed background. All wild-type mice were littermate controls of the *Surf1*$^{-/-}$ animals. The mice were maintained under specific pathogen-free barrier conditions with access to water and food ad libitum.

2.2. Primary fibroblast isolation

Primary fibroblasts were isolated from tail snips collected from *Surf1*$^{-/-}$ and wild-type mice. Tail snips taken from young, 3- to 6-month old mice were washed in 70% ethanol and rinsed in DMEM (Gibco, 4.5 g/l glucose, Glutamax) supplemented with 1% Penicillin/Streptomycin (P/S) antimicrobial solution (Gibco). The tail snip was added to fresh DMEM (4.5 g/l glucose with 1%P/S) and minced using a sterile scalpel. 1 mg of Liberase DL (Roche) was added to the minced tail and samples were incubated overnight in a cell culture incubator at 37 °C, 5% CO_2, and 21% O_2. The next day, the tail fragments were pipetted 7–10 times in complete DMEM (DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% P/S) and centrifuged at 200 × *g* for 5 min. The supernatant was aspirated and the cell pellet resuspended in complete DMEM and transferred to a 25 ml cell culture flask for subsequent culturing and expansion.

2.3. Cell survival assay

Primary fibroblasts were seeded 2.5×10^4 per well in a 96-well plate in complete DMEM. Following an overnight incubation, complete DMEM was replaced with DMEM+2% Bovine Serum Albumin (BSA) for 18 h prior to addition of the stressors in serum-free DMEM. Fibroblasts were treated with increasing concentrations of PQ, *t*-BuOOH, or H_2O_2 in triplicate for 6 h. Following stress, the media was aspirated and the cells were washed with dPBS and cultured in DMEM+2% BSA for 18 h. Cell viability was then measured after a three-hour incubation with the extracellular tetrazolium dye WST-1. The LD_{50} was calculated using non-linear regression (curve fit) analysis for wild-type and *Surf1*$^{-/-}$ fibroblasts.

2.4. XF24 Seahorse Flux Analyzer

Cells were seeded into Seahorse Flux Analyzer plate (30,000 cells/well) and incubated overnight at 37 °C, 5% CO_2 in complete DMEM. Following overnight incubation, cells were treated with vehicle (MEM), 1 mM PQ, 100 μM *t*-BuOOH, or 200 μM H_2O_2 for 30 min. These concentrations were chosen based on the LD_{50} measurements for each stress. We used a concentration that would give a stress that did not result in significant loss of cell viability. Following stress, the culture medium was changed to DMEM, pH 7.4 supplemented with 4 mM glutamine and placed in a dry incubator (non-CO_2) for 1 h at 37 °C. Mitochondrial function was then assessed by monitoring changes in oxygen consumption rate (OCR) as previously described ([30] (3T3 fibroblast); [31] (C2C12 cells); [6] (C2C12 cells)). Briefly, at the time of the assay run, three measurements of the basal level of oxygen consumption were recorded. Subsequently, oligomycin (1 μM), a complex V inhibitor, was injected, mixed, and two measurements were recorded to determine ATP-linked oxygen consumption and proton leak. Following oligomycin, FCCP (1 μM), a proton uncoupler, was injected, mixed, and another two measurements were recorded to determine maximal respiration capacity. Finally, antimycin A (1 μM), a complex III inhibitor, was injected, mixed, and two measurements were recorded to determine non-mitochondrial oxygen consumption. The oxygen consumption rate of each well was then normalized to total protein.

2.5. Cell culture, treatment and preparation of extracts

Primary fibroblasts from *Surf1*$^{-/-}$ and wild-type mice were seeded at 1.5×10^5 in a 6-well plate with 3 ml complete DMEM

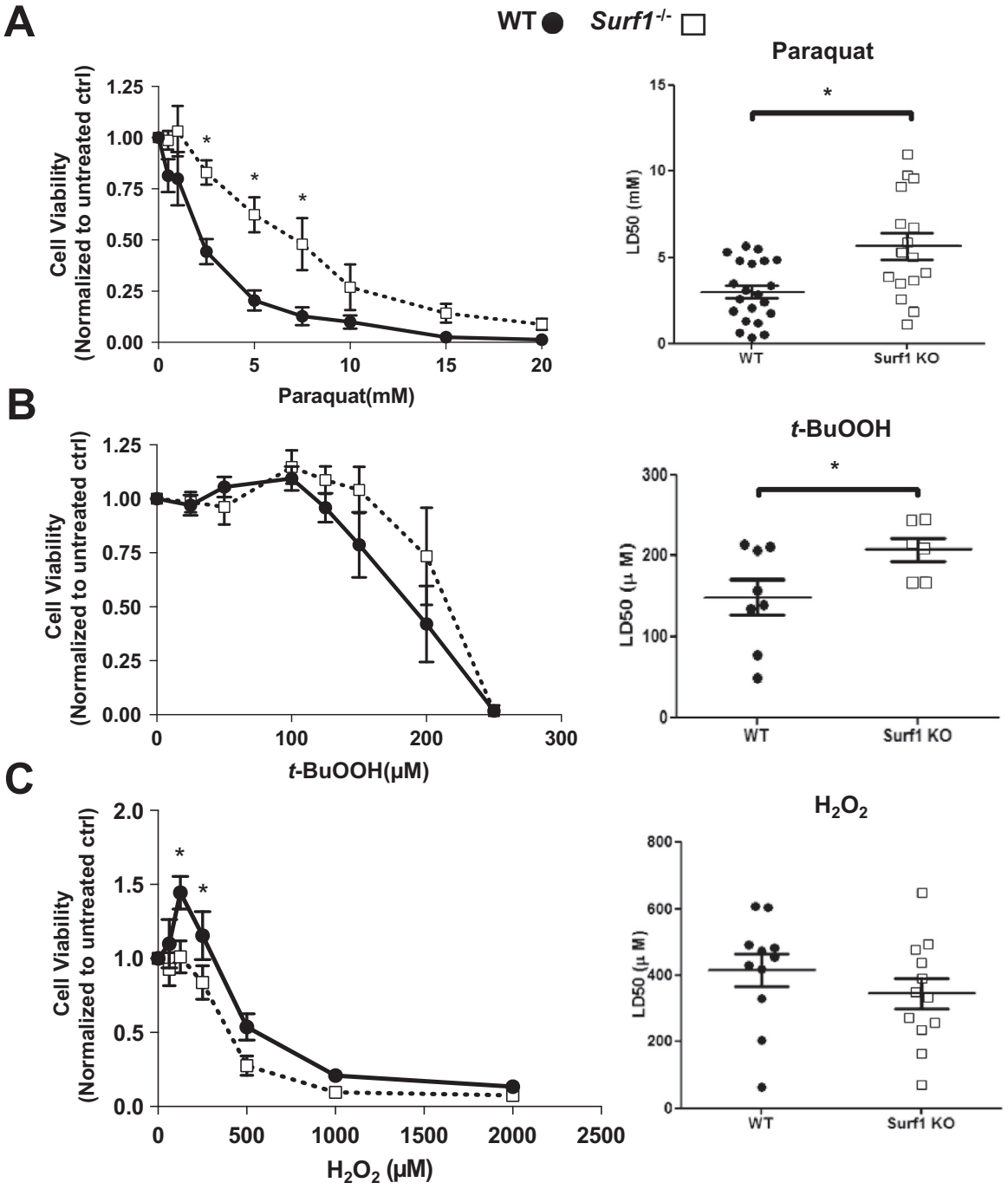

Fig. 2. *Surf1*$^{-/-}$ fibroblasts are resistant to PQ and *t*-BuOOH. Cell survival in the presence of increasing doses of (A) PQ, (B) *t*-BuOOH, and (C) H_2O_2 was determined with WST-1. In the right panels, each point represents the individual LD50 of a primary fibroblast line from either WT or Surf1$^{-/-}$ mice; LD50 was calculated using non-linear regression (curve fit) analysis. Bars depict means ± SEM (n=6–16); statistical significance was determined by two-way ANOVA. *$p < 0.05$ for comparing wild type vs. Surf1$^{-/-}$ at same treatment (Sidak's post-hoc test).

(4.5 g/l glucose, 10% FBS, 1% P/S) and incubated overnight at 37 °C, 5% CO_2, and 21% O_2. After 24 h, cells were incubated with PQ (1 mM), *t*-BuOOH (100 μM) or H_2O_2 (200 μM). Cells were harvested at 1, 6, and 24 h of treatment for each group, and the addition of the stressors was timed such that all the cell culture plates were harvested simultaneously. At the end of the stress time points, the media was aspirated, the plate was washed with cold dPBS, and 150 μl of RIPA buffer with 1x protease inhibitor cocktail (Set III, Calbiochem) and 1 mM PMSF was added to each well. The plates were immediately put at −20 °C overnight. Cells were thawed at 4 °C on a tilt-shaker at 9 rpm for 30 min, then the wells were scraped, and the lysate collected. The lysate was spun at 16,000 × *g* for 20 min at 4 °C, and the supernatant was collected. Protein concentrations were measured using the Bradford method and diluted to equal concentration. Samples were then diluted in 6x Laemmli Sample Buffer and heated at 95 °C for 10 min.

2.6. Western blot analysis

Western blot analysis was carried out as previously described [35]. Briefly, equal amounts of protein samples were run on 10% gel (casting system from Bio-Rad) with 1x Tris/Glycine/SDS buffer.

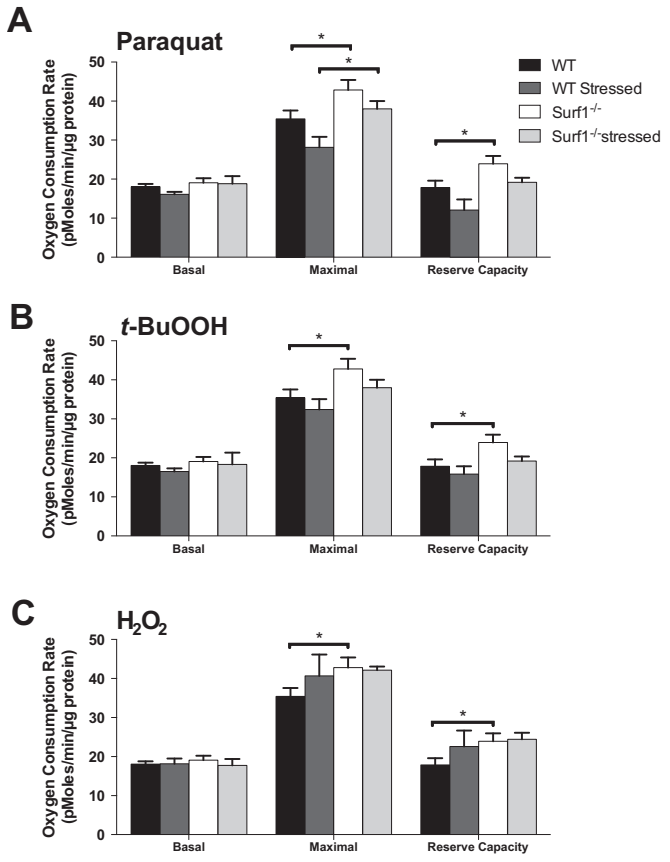

Fig. 3. *Surf1*$^{-/-}$ fibroblasts have increased maximal respiration and reserve capacity. Cells were treated with either (**A**) 1 mM PQ, (**B**) 100 μM *t*-BuOOH, or (**C**) 200 μM H_2O_2 for 30 min followed by a one hour recovery period in seahorse running media. Oxygen consumption rate (OCR) was determined with the Seahorse XF-24 Flux Analyzer. Basal respiration was determined by the third OCR reading prior to the addition of substrates and inhibitors. Maximal respiration was determined as the highest OCR value following the addition of FCCP (mitochondrial uncoupler). Reserve capacity was calculated as the difference between basal and maximal respiration. Data are representative of 4 independent experiments (cell lines, n=4–5) and expressed as mean ± SEM. Statistical significance was determined by two-way ANOVA; $^{*}p < 0.05$.

Gels were transferred to PVDF membrane using wet transfer system (Bio-Rad) overnight at 16 V in Tris/Glycine/SDS buffer containing 20% methanol then blocked for one hour in 1% bovine serum albumin (BSA) in TBS buffer containing 0.05% Tween-20 (TBS-T). The membranes were incubated at RT for 2 h with 1:2000 Lon protease rabbit polyclonal antibody (gift from Luke Szweda), 1:2000 Hsp60 mouse monoclonal antibody (Enzo ADI-SPA-806), 1:2000 mouse monoclonal ClpP protease antibody (Sigma WH0008192M1), or 1:2000 β-Actin rabbit polyclonal antibody (Cell Signaling 4967 S). The blots were washed 3 times with TBS-T and then incubated with 1:10,000 HRP-conjugated secondary antibodies (Vector Laboratories) for 2 h. The blots were washed 3 more times with TBS-T, detected using ECL, and imaged using a G: BOX imaging system (Syngene). The image was then quantified using GeneTools software (Syngene).

2.7. Statistical analyses

Two-tailed student's *t*-test or two-way ANOVA with Dunnett's or Sidak's post-hoc tests were performed for comparison of groups. Data were analyzed using GraphPad Prism 6 and expressed as mean ± SEM for each experiment. *P* values of less than 0.05 were accepted as statistically significant.

3. Results

3.1. Primary fibroblasts from *Surf1*$^{-/-}$ mice have an increase in the UPR^{MT} compared to WT mice

Our previous studies revealed that tissues of *Surf1*$^{-/-}$ mice have elevated expression of a number of UPR^{MT} associated proteins [35] suggesting that the *Surf1*$^{-/-}$ deletion might confer an enhanced stress resistance. We measured protein levels of Hsp60, ClpP, and Lon protease in primary fibroblasts isolated from *Surf1*$^{-/-}$ mice using western blot analysis. The data in Fig. 1 confirm that the UPR^{MT} is upregulated in a primary cell culture model from *Surf1*$^{-/-}$ mice.

3.2. Primary cultures of fibroblasts from *Surf1*$^{-/-}$ mice have an increased resistance to PQ and t-BuOOH treatment, but not to H_2O_2 treatment

To test whether fibroblasts from *Surf1*$^{-/-}$ mice are more resistant to exogenous stress than cells from wild-type mice, we treated primary cultures of fibroblasts with the following to induce oxidative stress: PQ (a superoxide anion generator), *t*-BuOOH (an organic hydroperoxide analog that induces lipid peroxidation), and H_2O_2. Cell viability and LD_{50} (lethal median dose) in response to each stressor were determined using the tetrazolium salt, WST-1, that is enzymatically cleaved to formazan by mitochondrial dehydrogenase enzymes in viable cells and detected using a spectrophotometer. The formation of dark yellow formazan is directly correlated to viable cells. A dose response curve showed that fibroblasts from *Surf1*$^{-/-}$ mice are significantly more resistant to cell death caused by PQ treatment at 2, 5, and 7.5 M (Fig. 2a). The LD_{50} for both PQ and *t*-BuOOH is also increased (Fig. 2a and b) compared to wild type. However, fibroblasts from *Surf1*$^{-/-}$ mice have reduced cell viability when treated with 125 μM or 250 μM H_2O_2 and no change in LD_{50} compared to wild-type cells (Fig. 2c).

3.3. Primary cultures of fibroblasts from *Surf1*$^{-/-}$ mice exhibit a significant increase in maximal oxygen consumption and reserve capacity

Mitochondrial function assays in isolated mitochondria from tissues of *Surf1*$^{-/-}$ mice show a mild decline in respiration in a tissue specific manner [8,23,35]. Here we measured oxygen consumption rate (OCR) in primary fibroblasts isolated from young adult *Surf1*$^{-/-}$ and wild-type mice using an XF24 analyzer (Seahorse Biosciences). Basal rates of oxygen consumption are not different between primary fibroblasts from *Surf1*$^{-/-}$ and wild-type mice (Fig. 3). ATP-linked OCR, and proton leak are also not significantly different between primary fibroblasts from *Surf1*$^{-/-}$ and wild-type mice (data not shown). Interestingly, the maximal OCR and the reserve capacity are significantly increased in *Surf1*$^{-/-}$ fibroblasts (Fig. 3).

3.4. Mitochondrial function is not altered in primary fibroblasts from *Surf1*$^{-/-}$ mice in response to low doses of oxidative stressors

Previous studies have shown that PQ and *t*-BuOOH generate oxidative stress targeted to the mitochondria [21,3–5,9]. Our data show that fibroblasts from *Surf1*$^{-/-}$ mice are resistant to cell death caused by these mitochondrial stressors, i. e., PQ and *t*-BuOOH. Thus, we hypothesized that *Surf1*$^{-/-}$ fibroblasts may have improved mitochondrial function in response to treatment with exogenous oxidative stressors compared to wild type. To test this hypothesis, mitochondrial function, as assessed by OCR, was measured in primary fibroblasts from *Surf1*$^{-/-}$ and wild-type mice

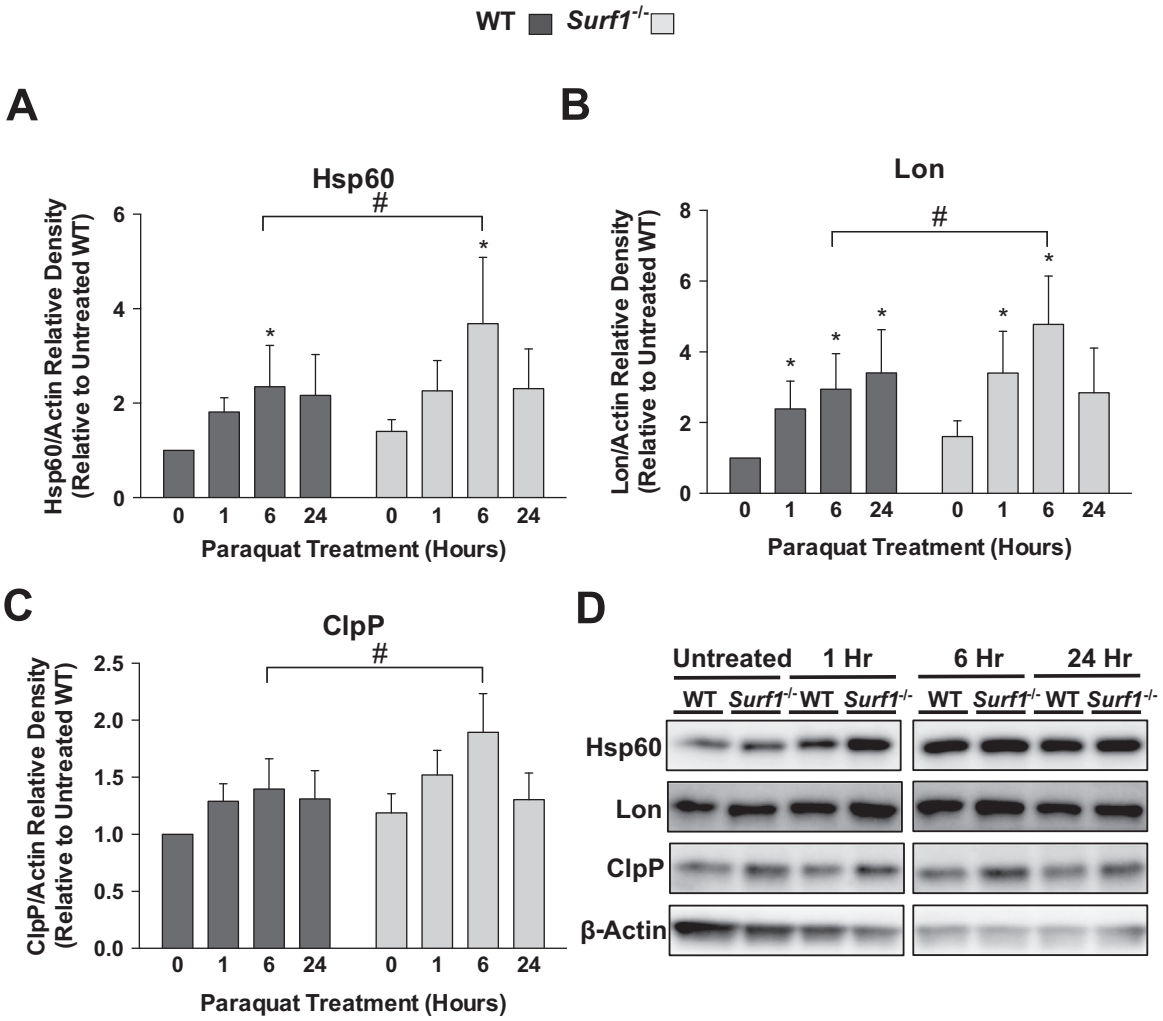

Fig. 4. Effect of PQ on UPR^{MT} induction. Fibroblasts from wild-type and *Surf1*$^{-/-}$ mice were incubated with 1 mM PQ and lysed at the designated time points. Cell lysates were processed for western blot analysis of (**A**) Hsp60, (**B**) Lon and (**C**) ClpP. (**D**) A representative blot with β-actin as the loading control. Bars depict means ± SEM at each time point (n=6). Significance was determined by two-way ANOVA. $^{*}p < 0.05$ for comparing treated vs. untreated of same line (Dunnett's post-hoc test). $^{\#}p < 0.05$ for comparing WT vs. *Surf1*$^{-/-}$ at same time point (Sidak's post-hoc test).

treated with 1 mM PQ, 100 μM *t*-BuOOH, or 200 μM H_2O_2 for 30 min. Fibroblasts treated with sub-lethal doses of PQ, *t*-BuOOH, or H_2O_2 do not show a significant immediate change in basal, maximal, or reserve capacity compared to untreated controls for both wild-type and *Surf1*$^{-/-}$ fibroblasts in response to stress (Fig. 3A, B, and C).

3.5. The UPR^{MT} response to oxidative stress in fibroblasts

3.5.1. PQ treatment induces the UPR^{MT} response in fibroblasts

We have previously shown that UPR^{MT} associated mitochondrial specific chaperones (Hsp60) and proteases (ClpP and Lon) that aid in maintaining mitochondrial proteostasis are significantly elevated in skeletal muscle, brain, liver, and adipose tissue from young adult *Surf1*$^{-/-}$ mice compared to wild-type mice [35]. Because fibroblasts isolated from *Surf1*$^{-/-}$ mice show increased cell survival in response to mitochondrial specific oxidative stressors, we hypothesized that the UPR^{MT} may contribute to the increased survival following treatment with PQ and *t*-BuOOH. To test this, we treated primary fibroblasts with 1 mM PQ, 100 μM *t*-BuOOH, or 200 μM H_2O_2 for one, six, or 24 h. As shown in Fig. 2, fibroblasts from *Surf1*$^{-/-}$ mice have enhanced survival to PQ treatment. 1 mM PQ treatment significantly induced Hsp60, Lon, and ClpP expression in *Surf1*$^{-/-}$ fibroblasts at six hours compared to untreated controls (Fig. 4A, B, and C). Fibroblasts showed significantly increased Hsp60 expression compared to untreated control cells at six hours for both *Surf1*$^{-/-}$ and wild-type cells (Fig. 4A). Lon expression is significantly increased at one, six, and 24 h for wild type and one and six hours for *Surf1*$^{-/-}$ compared to untreated controls (Fig. 4B). PQ treatment did not increase ClpP expression relative to untreated controls (Fig. 4C). Taken together, these data indicate that prolonged PQ treatment of primary mammalian fibroblasts can significantly induce markers of the UPR^{MT}.

3.5.2. t-BuOOH differentially regulates UPR^{MT} in wild-type and *Surf1*$^{-/-}$ fibroblasts

t-BuOOH treatment for one and six hours significantly induces Hsp60 expression in *Surf1*$^{-/-}$ fibroblasts compared to untreated controls but not in wild-type cells (Fig. 5A). Lon protease expression is significantly increased one and 24 h after treatment in wild-type cells, is trending towards increased at six hours in wild-type cells, and is significantly increased at six hours of treatment in *Surf1*$^{-/-}$ fibroblasts compared to untreated controls (Fig. 5B). ClpP expression is significantly induced at 24 h compared to untreated controls for wild type (Fig. 5C). After one hour of *t*-BuOOH treatment, Hsp60 is significantly increased in *Surf1*$^{-/-}$ fibroblasts compared to wild-type controls (Fig. 5A).

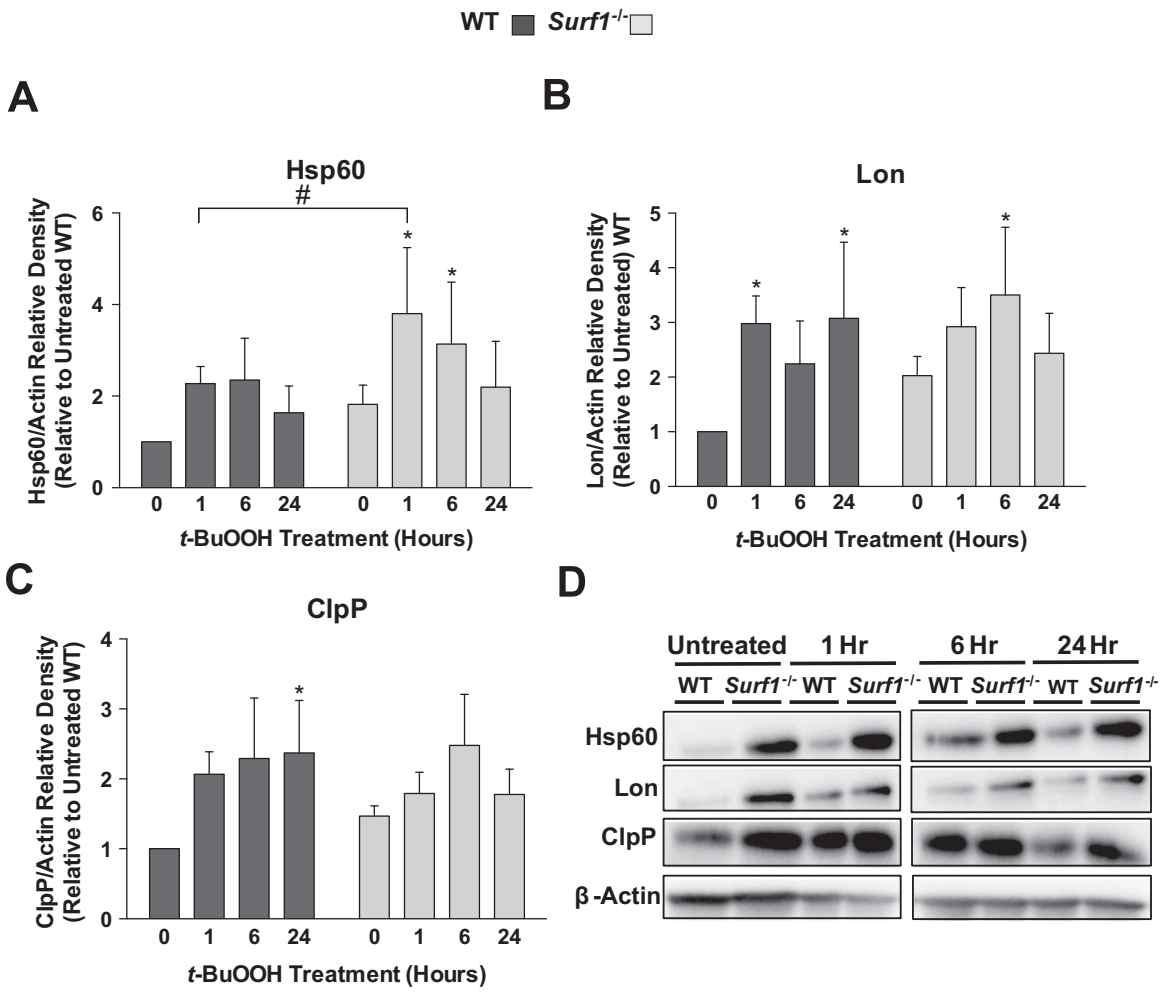

Fig. 5. Effect of *t*-BuOOH on UPR ^{MT} induction. Fibroblasts from wild-type and *Surf1*$^{-/-}$ mice were incubated with 100 μM *t*-BuOOH and lysed at the designated time points. Cell lysates were processed for western blot analysis of (**A**) Hsp60, (**B**) Lon and (**C**) ClpP. (**D**) A representative blot with β-actin as the loading control. Bars depict means ± SEM at each time point (n=6). Significance was determined by two-way ANOVA. $^{*}p < 0.05$ for comparing treated vs. untreated of same line (Dunnett's post-hoc test). $^{\#}p < 0.05$ for comparing WT vs. *Surf1*$^{-/-}$ at same time point (Sidak's post-hoc test).

3.5.3. H_2O_2 does not induce the UPR^{MT} in fibroblasts from Surf1$^{-/-}$ mice

Cell viability and mitochondrial function are not different in fibroblasts isolated from wild-type and *Surf1*$^{-/-}$ mice following exposure to 200 μM H_2O_2 (Fig. 2c). Furthermore, there is no significant difference in the expression levels of Hsp60, ClpP, or Lon in fibroblasts from *Surf1*$^{-/-}$ mice treated with H_2O_2 compared to wild-type fibroblasts (Fig. 6). Hsp60 expression is significantly increased at one and six hours of H_2O_2 treatment in wild-type cells, while *Surf1*$^{-/-}$ fibroblasts had reduced expression at 24 h (Fig. 6(A)). Lon protease expression is significantly increased in wild-type cells, but remains unchanged in *Surf1*$^{-/-}$ fibroblasts (Fig. 6B). ClpP protease expression is not different between wild-type and *Surf1*$^{-/-}$ fibroblasts. However, *Surf1*$^{-/-}$ fibroblasts have significantly reduced ClpP expression following H_2O_2 treatment compared to untreated controls (Fig. 6C).

4. Discussion

The goal of this study was to determine whether cells from *Surf1*$^{-/-}$ mice exhibit increased resistance to stress in culture. Previous studies from our laboratory and others have shown that null mutation of the complex IV assembly protein *SURF1* in mice is unexpectedly associated with a number of positive phenotypes despite reduced activity of cytochrome *c* oxidase, including an elevated level of UPR^{MT} proteins and increased mitochondrial number in several tissues [7,8,35]. Together these findings suggest that the impaired complex IV assembly or reduced cytochrome *c* oxidase activity might induce compensatory protective pathways in the cell. Our current findings demonstrate upregulation of UPR^{MT} related proteins in *Surf1*$^{-/-}$ fibroblasts associated with increased resistance to paraquat (PQ) and tert-Butyl hydroperoxide (*t*-BuOOH) induced cell death compared to wild-type cells. In contrast, the response to hydrogen peroxide (H_2O_2) was not different between wild-type and *Surf1*$^{-/-}$ cells. These data suggest that the upregulation of UPR^{MT} may play a role in cell stress resistance to some types of oxidative stress.

The UPR^{MT} is a molecular pathway that results in the induction of the chaperone Hsp60 and the protease ClpP to maintain mitochondrial proteostasis in response to mitochondrial dysfunction or misfolded mitochondrial proteins [15,25,44]. A number of studies in *Caenorhabditis elegans* have shown that perturbations in mitochondrial protein folding result in the translocation of the transcription factors UBL-5, DVE-1, and ATFS-1 to the nucleus and the upregulation of genes associated with the UPR^{MT} including mitochondrial chaperones, antioxidants, and glycolysis genes [14,28,29]. While several studies have focused on the UPR^{MT} response in *C. elegans* and in *Saccharomyces cerevisiae* [1,13,14,20,26], studies in mammalian systems are relatively few,

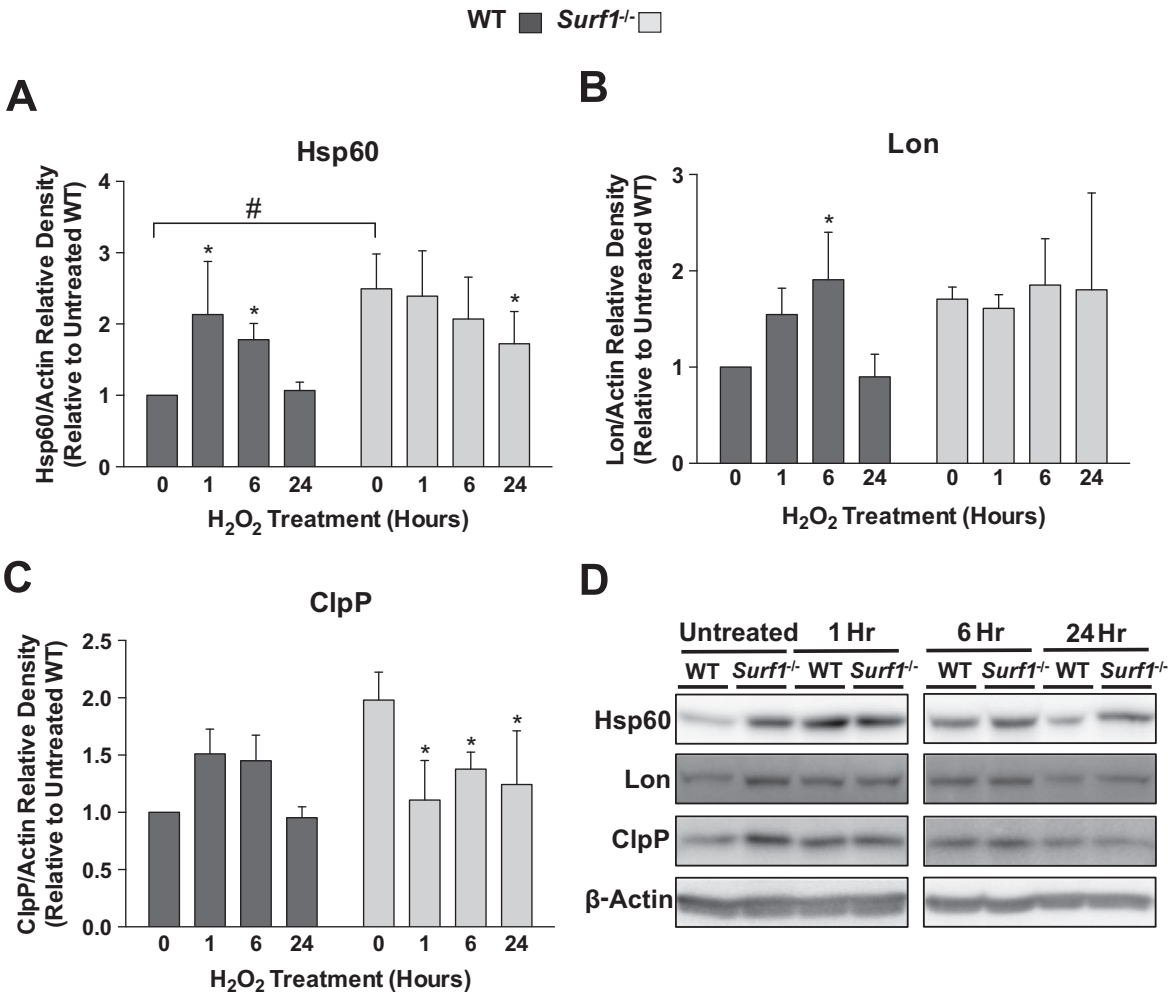

Fig. 6. Effect of H_2O_2 on UPR^{MT} induction. Fibroblasts from wild-type and *Surf1*$^{-/-}$ mice were incubated with 200 μM H_2O_2 and lysed at the designated time points. Cell lysates were processed for western blot analysis of (**A**) Hsp60, (**B**) Lon and (**C**) ClpP. (**D**) A representative blot with β-actin as the loading control. Bars depict means ± SEM at each time point (n=4). Significance was determined by two-way ANOVA. $^{*}p < 0.05$ for comparing treated vs. untreated of same line (Dunnett's post-hoc test). $^{\#}p < 0.05$ for comparing WT vs. *Surf1*$^{-/-}$ at same time point (Sidak's post-hoc test).

and the mammalian molecular pathways involved and the specific role of oxidative stress in the induction of the UPR^{MT} has not been defined.

In a previous study, adult *Surf1*$^{-/-}$ mice were protected from neurodegeneration and apoptosis following kainic acid injection [8]. Kainic acid mimics glutamate excitotoxicity that results in cell stress through generation of reactive oxygen species (ROS) [36]. *Surf1*$^{-/-}$ neurons also exhibit decreased cell death and cytosolic calcium when challenged with a high concentration of glutamate [8]. Here we measured the resistance of fibroblasts from *Surf1*$^{-/-}$ mice to three compounds that induce oxidative stress, specifically PQ, *t*-BuOOH, and H_2O_2. We determined the LD_{50} to each of the stressors in wild-type and *Surf1*$^{-/-}$ cells and subsequently used a dose that did not cause loss of viability in tests of stress resistance. PQ is a redox cycler that generates superoxide at mitochondrial NADH dehydrogenase (complex I) and ubiquinol: cytochrome *c* oxidoreductase (complex III) in the mitochondrial electron transport chain [3,5,11]. PQ has previously been shown to decrease cell viability and mitochondrial membrane potential, increase the generation of ROS [19,41], inhibit mitochondrial protein import [40], increase oxidative modification of mitochondrial proteins, and induce mitochondrial dysfunction [4]. Transgenic overexpression of the mitochondrial-specific antioxidant peroxiredoxin 3 is capable of preventing PQ induced mitochondrial dysfunction in mice, supporting a role for mitochondrial oxidative stress induced by PQ superoxide anion generation [4]. In *C. elegans*, PQ can induce the UPR^{MT} [37]. *t*-BuOOH is an organic hydroperoxide that inhibits mitochondrial respiratory-chain enzymes and forms hydroxyl radicals that damage lipids and membranes, depolarizes mitochondria, and causes apoptosis by mitochondrial permeability transition and cytochrome *c* release [9,21,43]. In hepatocytes, *t*-BuOOH inhibits mitochondrial ATP generation at low concentrations (25–50 μM) and causes mitochondrial uncoupling at concentrations greater than or equal to 100 μM *t*-BuOOH. Glycolysis was strongly inhibited at 1 mM *t*-BuOOH [17]. In neuronal cell lines, *t*-BuOOH-induced ROS production, mitochondrial depolarization, and cell death was suppressed using the mitochondrial specific antioxidant SS-31 [43]. H_2O_2 is an oxidant and important redox signaling molecule that can induce cell proliferation at relative low doses and cause damage and cell death at higher doses [2,32]. At the relatively low doses we tested, we found that the fibroblasts from *Surf1*$^{-/-}$ animals are more resistant to treatment with PQ and *t*-BuOOH but not H_2O_2 over a range of doses for each stressor. The differential response to PQ and *t*-BuOOH versus H_2O_2 in the cells from wild-type and *Surf1*$^{-/-}$ mice could be due to the mitochondrial specific effects of PQ and *t*-BuOOH. In addition to the increased response to PQ and *t*-BuOOH in *Surf1*$^{-/-}$ fibroblasts, the time course of the UPR^{MT} may be shorter in the *Surf1*$^{-/-}$ cells. For *Surf1*$^{-/-}$ cells treated with PQ or *t*-BuOOH, the relative expression of Hsp60, Lon, and ClpP is

decreased from six to 24 h, while it remains elevated for the wild-type cells in the same parameters except for Hsp60 expression after *t*-BuOOH treatment (Figs. 4 and 5). This suggests that the induction response of the UPR^{MT} is completing at 24 h for *Surf1*$^{-/-}$ fibroblasts but not for wild-type cells. Thus it is possible that cells from the *Surf1*$^{-/-}$ mice in which there is an upregulated UPR^{MT} response might be better able to withstand these stressors.

The most robust induction of UPR^{MT} occurred after PQ treatment. Compared to *t*-BuOOH, PQ specifically damages mitochondrial proteins [4] and inhibits mitochondrial protein import, resulting in accumulation of precursor proteins in the cytosol [40]. Direct damage to mitochondrial proteins causes induction of the UPR^{MT} through ClpP-mediated protein degradation [13]. In *C. elegans*, impairment of ATFS-1 import into the mitochondria causes accumulation of ATFS-1 in the cytosol where it is then imported into the nucleus to function as a transcription factor upregulating UPR^{MT} genes [29]. No mammalian homolog of ATFS-1 has been discovered, although the transcription factor CHOP may be a mammalian ortholog [33].

Our study also revealed that cultured fibroblasts from the *Surf1*$^{-/-}$ mice have no change in basal oxygen consumption rate (OCR) but have an elevated maximal respiration and elevated reserve capacity as measured using the Seahorse Extracellular Flux Analyzer. In contrast, a previous study in fibroblasts from Leigh Syndrome patients that is characterized by a mutation in *SURF1* showed a significant decline in OCR compared to controls [18]. However, the level of complex IV in cells from Leigh syndrome patients is reduced to a much greater extent [42] than we see in the cells from the *Surf1*$^{-/-}$ mice. To test the effect of low level oxidative stress on mitochondrial function in the *Surf1*$^{-/-}$ cells, we measured OCR in primary fibroblasts treated with PQ, *t*-BuOOH and H_2O_2 for 30 min. It has previously been shown that incubation of cells with PQ for over 4 h can induce a mild basal oxygen consumption increase in N27 neural cells at 100, 250, and 500 μM [10]. In 661 W cells, 500 μM of PQ suppressed maximal mitochondrial respiration after 24 h, while 30 min of exposure to 50 μM *t*-BuOOH showed loss of mitochondrial reserve capacity without affecting the basal rate [34]. In the current study, wild-type fibroblasts showed a trend towards decrease in the maximal OCR within 30 min of 1 mM PQ treatment which was preserved in the *Surf1*$^{-/-}$ fibroblast. *t*-BuOOH and H_2O_2 did not result in any changes in basal OCR, maximal respiration, or reserve capacity. HepG2 cells and human dermal fibroblasts showed a marked decrease in mitochondrial function upon treatment with H_2O_2 [12,24]. These studies used a higher dose of H_2O_2 between 1 and 5 mM while we used 200 μM H_2O_2. Thus our data suggest the dose of the stressors, duration of treatment, and cell type are determinants in deciding the cell response/resistance to stress.

In summary, we have shown that cells with reduced complex IV holoenzyme assembly have upregulation of mitochondrial chaperones and proteases that may confer increased resistance to stress despite significantly reduced levels of cytochrome *c* oxidase activity. The increased stress resistance may be related to the other beneficial phenotypes that have been previously reported in these mice including increased lifespan, increased insulin sensitivity, and increased resistance to kainic acid toxicity in the brain. Further studies on the role of the UPR^{MT} in resistance to various types of stress in mammalian models are needed to confirm the role of the UPR^{MT} in cellular stress resistance.

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