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Down-regulation of the mitochondrial matrix peptidase ClpP in muscle cells causes mitochondrial dysfunction and decreases cell proliferation

Sathyaseelan S. Deepa^{a,*}, Shylesh Bhaskaran^a, Rojina Ranjit^a, Rizwan Qaisar^a, Binoj C. Nair^b, Yuhong Liu^c, Michael E. Walsh^c, Wilson C. Fok^c, and Holly Van Remmen^{a,d}

^aAging and Metabolism Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, USA

^bThe University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

^cBarshop Institute for Longevity and Aging Studies, University of Texas Health Science Center at San Antonio, San Antonio, TX 78245, USA

^dOklahoma City VA Medical Center, Oklahoma City, OK 73104, USA

Abstract

The caseinolytic peptidase P (ClpP) is the endopeptidase component of the mitochondrial matrix ATP-dependent ClpXP protease. ClpP degrades unfolded proteins to maintain mitochondrial protein homeostasis and is involved in the initiation of the mitochondrial unfolded protein response (UPR^{mt}). Outside of an integral role in the UPR^{mt}, the cellular function of ClpP is not well characterized in mammalian cells. To investigate the role of ClpP in mitochondrial function, we generated C2C12 muscle cells that are deficient in ClpP using siRNA or stable knockdown using lentiviral transduction. Reduction of ClpP levels by ~70% in C2C12 muscle cells resulted in a number of mitochondrial alterations including reduced mitochondrial respiration and reduced oxygen consumption rate in response to electron transport chain (ETC) complex I and II substrates. The reduction in ClpP altered mitochondrial morphology, changed the expression level of mitochondrial fission protein Drp1 and blunted UPR^{mt} induction. In addition, ClpP deficient cells showed increased generation of reactive oxygen species (ROS) and decreased membrane potential. At the cellular level, reduction of ClpP impaired myoblast differentiation, cell proliferation and elevated phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) suggesting an inhibition of translation. Our study is the first to define the effects of ClpP deficiency on mitochondrial function in muscle cells *in vitro*. In addition, we have uncovered novel effects of ClpP on mitochondrial morphology, cell proliferation and protein translation pathways in muscle cells.

*Correspondence to: Aging and Metabolism Research Program, Oklahoma Medical Research Foundation, 825 NE 13th Street, Oklahoma City, OK 73104, USA. Deepa-Sathyaseelan@omrf.org (S.S. Deepa).

Author contribution

S.S.D. contributed to the study design. S.S.D., S.B., R.R., R.Q., Y.L., Y.S., and W.C.F. were responsible for data collection and analysis. S. S.D. wrote the manuscript and H. V. R., B. N. and M. E. W. reviewed/edited the manuscript.

Keywords

ClpP; ClpX; Mitochondrial unfolded protein response; Mitochondrial fission/fusion; Respiration; Reactive oxygen species

1. Introduction

Proper functioning mitochondria are essential for cellular homeostasis. To maintain a healthy mitochondrial population, a quality control (QC) system consisting of mitochondrial proteases and chaperones has evolved and deficiencies in mitochondrial protein QC have been linked to various neurological disorders and aging [1,2]. There are a number of QC proteases located in the mitochondria including the cytosol-localized ubiquitin–proteasome system in the outer mitochondrial membrane, the proteases PARL, YME1L1, AFG3L2 and paraplegin in the inner mitochondrial membrane and the HtrA2 in the intermembrane space. The Lon and ClpXP proteases are located in the mitochondrial matrix. The Lon protease has been studied extensively and has been shown to play an important role in removing oxidized proteins from the mitochondrial matrix [3,4]. ClpP forms complexes with AAA+ chaperone, ClpX to form an active protease ClpXP [5]. Eukaryotic ClpP has been shown to degrade unfolded proteins in the mitochondrial matrix and is induced in response to stress [6–8].

One of the well-characterized functions of ClpP in eukaryotes is its role in the mitochondrial unfolded protein response (UPR^{mt}), a retrograde signaling pathway that maintains mitochondrial protein homeostasis in response to mitochondrial-specific stress. This pathway has been best characterized in *Caenorhabditis elegans* where the UPR^{mt} has been shown to be induced by the presence of unfolded proteins in the mitochondrial matrix and ClpP has been shown to be essential for mediating UPR^{mt} [6]. In mammalian cells, UPR^{mt} is also induced when the stoichiometry of proteins encoded by mitochondria and nucleus is altered, resulting in the up-regulation of both ClpP and heat shock protein 60 (Hsp60), thereby minimizing mitochondrial protein aggregation [6,9]. We previously reported that mice lacking *Surf1*, a mitochondrial electron transport complex IV assembly factor, show impaired processing of Complex IV assembly and upregulation of the UPR^{mt} characterized by induction of ClpP and Hsp60 that may contribute to the lack of deleterious phenotypes in this mouse model [10]. The importance of ClpP is further supported by a number of studies suggesting a role of ClpP in neurodegenerative diseases in humans such as spastic paraplegia, Friedreich's ataxia, and Parkinson's disease, and recessive missense mutations in *CLPP* cause Perrault syndrome, which is characterized by ovarian failure and sensorineural deafness [11–13]. Recently, in a mouse model of ClpP ablation (*ClpP*^{−/−} mice), loss of ClpP is associated with infertility, growth retardation and auditory deficits [14]. Surprisingly, mitochondrial function measured in four-month-old *ClpP*^{−/−} mice in heart and muscle revealed only mild effects on respiration and electron transport complex activities and in brain mitochondria these parameters were unaffected. To define the function of ClpP in mitochondria at cellular level, we have generated C2C12 muscle cells deficient in ClpP. Our findings show that loss of ClpP in C2C12 cells alters mitochondrial morphology, causes mitochondrial dysfunction, elevates oxidative stress, inhibits protein translation, decreases cell proliferation and impair myoblast differentiation.

2. Material and methods

2.1. Cell culture, ClpP siRNA and generation of a stable ClpP deficient cell line

The mouse myoblast cell line C2C12 (ATCC) was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptavidin and maintained at 37 °C in 5% humidified incubator. For transient knockdown of ClpP, C2C12 cells were transfected with control or ClpP siRNA (Sigma, St. Louis, MO). For generation of the stable cell line, C2C12 myoblasts were infected with mission shRNA lentiviral transduction particles for ClpP (Sigma, St. Louis, MO) or shRNA control transduction particles and transduced cells were obtained by puromycin selection. C2C12Myoblasts were differentiated to myotubes by growing them in DMEM containing 2% horse serum and 1% penicillin–streptavidin for four days.

2.2. Measurement of mitochondrial respiration and glycolysis

Mitochondrial respiration and glycolysis in intact cells were measured using a Seahorse Bioscience XF24 Extracellular Flux Analyzer (North Billerica, MA). To measure respiration, control and ClpP KD myoblasts were seeded at a density of 20,000 cells/well kept at 37 °C incubator with 5% CO₂ for 24 h. After incubation, cells were changed to assay media [XF base medium (Cat#102353-100, Seahorse Biosciences) containing 25 mM glucose, 1 mM sodium pyruvate and 4 mM L-glutamine, pH 7.4] and kept at 37 °C incubator without CO₂ for 60 min. Oxygen consumption rate (OCR) was recorded when cells were metabolically perturbed by the sequential injections of oligomycin, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and antimycin A (1 mM, final concentration) [15]. From the obtained OCR values calculations were made as follows: basal respiration (third basal measurement), ATP-linked (difference between basal respiration rate and oligomycin-induced respiration), proton leak (difference between oligomycin-induced and antimycin A-induced respirations), maximal respiration (maximum rate after FCCP injection), reserve capacity (maximal respiration–basal respiration) and non-mitochondrial.

To measure glycolysis, control and ClpP KD myoblasts were seeded at a density of 20,000 cells/well kept at 37 °C incubator with 5% CO₂ for 24 h. After incubation, cells were changed to assay media [Bicarbonate-free, low phosphate DMEM (Cat#D5030, Sigma, St. Louis, MO), 143 mM NaCl, 3 mg/L phenol red, 10 mM glucose and 2 mM L-glutamine, pH 7.4] and kept at 37 °C incubator without CO₂ for 60 min. Extracellular acidification rate (ECAR) was recorded when cells were metabolically perturbed by the sequential injections of glucose (10 mM, final concentration), oligomycin (1 mM, final concentration) and 2-deoxyglucose (100 mM, final concentration). From the obtained ECAR values calculations were made as follows: basal ECAR (3rd basal measurement), glycolysis (difference between glucose-induced ECAR and basal ECAR), glycolytic capacity (difference between oligomycin-induced ECAR and basal ECAR), and glycolytic capacity (difference between oligomycin-induced and glucose-induced ECAR).

2.3. Mitochondrial electron transport chain (ETC) complex activity

Complex I and II activities were measured in control and ClpP KD cells using a Seahorse Bioscience XF24 Extracellular Flux Analyzer (North Billerica, MA) in substrate-free KHB

buffer as previously described [16]. Increasing concentrations of glutamate/malate or succinate were sequentially injected to cells in substrate-free KHB buffer to a final concentration of 10 mM. At the end of the experiments, total protein in each well was quantified using Bio-Rad protein assay dye reagent (Bio-Rad, Hercules, CA, USA) and OCR values were normalized with the protein concentration.

2.4. Measurement of H₂O₂ release

H₂O₂ generation in cells was measured using Amplex Red-horseradish peroxidase (Molecular Probes, Eugene, OR). 1×10^6 cells were incubated with digitonin (40 μ M, final concentration) in reaction buffer (10 mM HEPES, 125 mM KCl, 5 mM MgCl₂, 2 mM K₂HPO₄, pH 7.44) for 10 min at 4 °C. It is known that at this concentration digitonin do not affect mitochondrial function [17,18]. The reaction was initiated by adding Amplex Red (80 mM) and fluorescence was measured. The slope of the increase in fluorescence is converted to the rate of H₂O₂ production with a standard curve [19]. For the H₂O₂ inhibition experiments, catalase (~4 units) (Sigma, St. Louis, MO) was added to the reaction mix prior to the addition of substrates/inhibitors.

2.5. Aconitase activity assay

While aconitase is present in both mitochondria and cytosol in most tissues, muscle has only mitochondrial aconitase. Hence we used total cell homogenate of C2C12 muscle cells for aconitase activity assay [20]. Buffer containing 25 mM MOPS and 0.05% Triton X-100 (pH 7.4) was used for performing the assay. Aconitase activity was measured as the rate of NADP⁺ reduction by isocitrate dehydrogenase. The reaction was initiated by the addition of 1 mM sodium citrate, 0.6 mM MnCl₂, 0.2 mM NADP⁺, 1 unit/mL isocitrate dehydrogenase and protein extracts containing equal amounts of protein [21].

2.6. Mitochondrial membrane potential assay

Membrane potential was assessed using JC-1 mitochondrial membrane potential assay kit (Abcam, Cambridge, MA), as per manufacturer's instructions. The values obtained by the assay were normalized to total protein.

2.7. Immunofluorescence

Cells were stained with β -actin antibody (Cell Signaling Technology, Danvers, MA) or Tom20 antibody (Santa Cruz Biotechnology, Dallas, TX) followed by Alexa Fluor[®]488 F(ab')₂ Fragment of Goat Anti-Rabbit IgG (H+L) antibody as per manufacturer's instructions. Mitochondria in cells were stained using MitoTracker[®] Red (Life technologies, Grand Island, NY), according to the manufacturer's instruction.

2.8. Western blotting

Cells were lysed with cell lysis buffer [50 mM HEPES, pH 7.6, 150 mM NaCl, 20 mM sodium pyrophosphate, 20 mM β -glycerophosphate, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Calbiochem), kept on ice for 30 min, and centrifuged at 14,000 rpm for 20 min. The protein concentration in the supernatant was determined by using Bio-Rad protein assay dye reagent (Bio-Rad,

Hercules, CA, USA). Western blotting was performed as previously described [22]. Antibodies to ClpP, ClpX, protein kinase R (PKR) and β -tubulin were from Sigma (St. Louis, MO). Antibodies to Hsp60, mitofusin 1 (Mfn1), Mfn2, OPA1, Fis1, Drp1 and ETC complex subunits were from Abcam (Cambridge, MA), P-eIF2 α , eIF2 α , general control nonderepressible 2 (GCN2) and Bip antibodies were from Cell Signaling Technology (Danvers, MA). Lon protease antibody was a gift from Dr. Luke Szveda (Oklahoma Medical Research Foundation, Oklahoma).

2.9. Transmission Electron Microscopy

The electron microscopy experiment was carried out in the Oklahoma Medical Research Foundation Imaging Facility using established procedures. Cells were fixed with 4% Paraformaldehyde (EM grade), 2% Gluteraldehyde (EM grade), in 0.1 M Sodium Cacodylate buffer overnight at room temperature on a rocker. Samples were then post fixed for 90 minutes in 1% Osmium tetroxide (OsO₄) in 0.1 M Sodium Cacodylate, and rinsed three times for five minutes each in 0.1 M Sodium Cacodylate buffer. This was followed by dehydrating the samples in a graded acetone series (50%, 60%, 75%, 85%, 95%, 100%) for 15 min each and two 15 min treatments in 100% Propylene Oxide. Next, the samples were infiltrated in a graded Epon/Araldite (EMS) resin/Propylene Oxide series (1:3, 1:1, 3:1) for 60 min, 120 min, and overnight, respectfully. The following day samples were further infiltrated with pure resin for 45 min, 90 min, and then overnight. The cells were then embedded in resin plus BDMA (accelerator) and polymerized at 60 °C for 48 h. Ultrathin sections were stained with Lead Citrate and Uranyl Acetate before viewing on a Hitachi H7600 Transmission Electron Microscope at 80 kV. From the obtained electron micrographs, mitochondrial area was assessed using NIH Image J software.

2.10. Statistics

All the data analysis was performed using Student's *t*-test. Difference were regarded as significant at the $p < 0.05$ level.

3. Results

3.1. Characterization of ClpP deficient cells and reduced response to UPR^{mt} induction

To study the role of ClpP in cells in culture, we reduced ClpP expression in C2C12 cells using shRNA or siRNA. We chose to study C2C12 muscle cells because skeletal muscle has a relatively high expression of ClpP compared to other tissues [23]. C2C12 muscle cells with a stable knockdown (KD) of ClpP were used to characterize the effect of ClpP deficiency on mitochondrial and cellular function. siRNA experiments were performed to validate the results obtained using ClpP KD cells to rule out the possibility of off-target effects in shRNA technology. Two clones each from control and ClpP KD cells were selected and characterized and data from one of the clones is presented. Expression of ClpP protein was decreased ~70% in ClpP KD cells compared to control cells, as determined by densitometric analysis of immunoblots (Fig. 1A and B). SiRNA approach also resulted in a similar (~68%) reduction in ClpP expression (data not shown). ClpX, the ATPase component of the ClpXP complex, was also downregulated to a similar extent, ~63% (Fig. 1A and B). Interestingly, the expression of mitochondrial chaperon Hsp60 that is induced in response to stress, and

the mitochondrial matrix Lon protease that degrades oxidized proteins, remained unaffected by the changes in ClpP protein levels (Fig. 1A and B). Since induction of UPR^{mt} under stress conditions can up-regulate ClpP and Hsp60, we tested how ClpP KD cells respond to mitochondrial-specific stress. Control and ClpP KD cells were treated with doxycycline (DOX), an antibiotic that inhibits mitochondrial translation and up-regulates UPR^{mt} components ClpP and Hsp60 [9]. While DOX increased expression of Hsp60 by ~1.5-fold in control cells, such a response to DOX was blunted in ClpP KD cells, indicating that mitochondrial stress-mediated induction of Hsp60 is dependent on ClpP (Fig. 1C).

3.2. A decrease in ClpP alters mitochondrial morphology

Loss of mitochondrial proteases has previously been shown to result in altered mitochondrial morphology. For example, mitochondria from WI-38 VA-13 human lung fibroblasts exposed to Lon antisense that reduced Lon protein levels resulted in aberrant mitochondrial morphology characterized by loss of cristae, electron dense inclusions and giant vacuoles [24]. In mouse embryonic fibroblasts, YME1L1 shRNA was shown to result in increased mitochondrial fragmentation and reduced fission/fusion rates [25]. Consistent with these findings, we found that reduced ClpP protein in C2C12 muscle cells can significantly alter mitochondrial morphology. Mitochondria in control cells appeared mostly elongated or ovoid-shaped as observed by electron microscopy, while mitochondria in ClpP KD cells were more rounded and appeared smaller than mitochondria in control cells (Fig. 2A). Assessment of mitochondrial area showed that mitochondria from ClpP KD cells are significantly smaller than control cell mitochondria and the percentage of smaller mitochondria is higher in ClpP KD cells (Fig. 2B). However, we did not see an obvious change in mitochondrial number between control and ClpP KD cells. Mitochondria are dynamic organelles that continually undergo fusion and fission, which in turn maintains their shape, size, number and physiological function. Since ClpP KD cells have smaller mitochondria, we used immunofluorescence techniques to test whether they undergo fragmentation. In control cells mitochondria appear more localized near nucleus, while in ClpP KD cells mitochondria appeared more scattered throughout the cell (Fig. 2C). However, a definite conclusion about fragmentation is hard to draw from the immunofluorescence images alone. Since we observed a change in mitochondrial morphology for ClpP KD cells by electron microscopy and a possibility of fragmentation by immunofluorescence, we tested whether mitochondrial fission/fusion protein levels are altered in ClpP KD cells. In mammals, mitofusin 1 (Mfn1) and Mfn2 regulate outer membrane fusion, while OPA1 is involved in inner membrane fusion. Two proteins, Drp1 and Fis1, regulate mitochondrial fission [26]. Expressions of Mfn1, Mfn2 and OPA1 were similar in control and ClpP KD cells (Fig. 2D). Among the fission proteins, Fis1 protein level was similar in control and ClpP KD cells while Drp1 was elevated in ClpP KD cells, compared to control cells (Fig. 2D). Thus, altered mitochondrial morphology in ClpP KD cells could be attributed to changes in mitochondrial fission.

3.3. Mitochondrial respiration is decreased and glycolysis is increased in ClpP KD cells

Accumulation of unfolded proteins within mitochondrial matrix leads to transcriptional up-regulation of ClpP in mammalian cells [8]. However, it is not known whether ClpP is essential for maintaining mitochondrial function under normal conditions. Because

mitochondria are the major site of ATP production, we tested whether a reduction in ClpP affects mitochondrial respiration. Respiration rate in control and ClpP KD cells was measured using the Seahorse Bioscience XF24 Extracellular Flux Analyzer. Oxygen consumption rates obtained from individual wells were normalized to protein concentration per well in control and ClpP KD cells. Due to lower proliferation rates, ClpP KD cells have lower protein concentration per well (~14 mg) compared to control cells (~23 mg). Basal respiration in the resting state without the addition of ETC inhibitors or uncouplers was reduced by ~23% in ClpP KD cells compared to control cells (Fig. 3A). In addition, we observed a decrease in ATP-linked respiration (~32%), maximal respiration (~54%), reserve capacity (~80%) and non-mitochondrial respiration (~30%) and an increase in proton leak (~51%) in ClpP KD cells compared to control cells. Similar results were obtained using ClpP siRNA that resulted in ~68% decrease in the expression of ClpP (data not shown). Together, these data suggest that decrease in ClpP lowers mitochondrial respiration capacity. Since maximal respiration represents how a system reacts to increased ATP demand, a decrease in maximal respiration might be associated with reduced energy availability in ClpP KD cells.

We also tested whether the glycolytic pathway is induced to compensate for reduced ATP production in these cells, using a glycolysis stress assay in a Seahorse Bioscience XF24 Extracellular Flux Analyzer. ECAR was recorded when cells were metabolically perturbed by the sequential injections of glucose, oligomycin and 2-deoxyglucose. Basal ECAR representing non-glycolytic acidification due to other metabolic pathways was increased by ~50% in ClpP KD cells (Fig. 3B). Similarly, glucose-induced glycolysis was higher by ~20% in ClpP KD cells. However, treatment with oligomycin to inhibit mitochondrial ATP production and thereby shift the energy production to glycolysis was surprisingly lower in ClpP KD cells. Glycolytic capacity (the difference between oligomycin-induced ECAR and basal ECAR) and glycolytic reserve (the difference between oligomycin-induced and glucose-induced ECAR) were attenuated by ~27% and ~59%, respectively, in ClpP KD cells compared to control cells. These results show that even though ClpP KD cells are able to produce ATP through glycolysis when provided with glucose, they have lower glycolytic capacity and glycolytic reserve.

3.4. Mitochondrial alterations in ClpP KD cells

An inhibition or decline in ETC activity or ETC subunit levels could contribute to decreased respiration in mitochondria. Since ClpP KD cells exhibited a decrease in basal and maximal respiration, we tested whether activity of ETC chain complexes were affected by the decrease in ClpP protein expression. Respiration in intact cells was measured using a Seahorse XF 24 analyzer with mitochondrial respiratory substrates I and II, an indirect way for measuring complex activity. With complex I substrates glutamate and malate, OCR was reduced by ~40% and using complex II substrate succinate a decrease of ~22% was observed for ClpP KD cells (Fig. 4A). Similar results were obtained when respiration was measured using a Clarke's electrode (data not shown). These findings suggest that decreased levels of ClpP protein could attenuate activities of ETC complexes that in turn could affect mitochondrial respiration.

We also measured the expression of a select number of nuclear DNA-encoded ETC subunits. Protein levels of complex I subunit (30 kDa, NDUFS3), complex II subunit (70 kDa, SDHA), complex III subunit (UQCRC2) and complex V subunit (56.5 kDa, ATP5A) were similar in control and ClpP KD cells (Fig. 4B). In contrast, protein content of complex I (20 kDa subunit, NDUFS7) and complex IV-4 was decreased by ~55% and ~60% in ClpP KD cells compared to control cells (Fig. 4B). Similarly, protein levels of complex IV-1, a mtDNA-encoded subunit of the ETC, was decreased by ~30% in ClpP KD cells. These findings suggest that decreased levels of ClpP protein could alter ETC subunit levels and attenuate the activity of ETC complexes that in turn could affect mitochondrial respiration. How deficiency of ClpP decreases the level and activity of ETC complexes needs to be investigated.

ETC complexes are important sources of reactive oxygen species (ROS) generation in the mitochondria and elevated levels of ROS contributes to different pathological conditions including aging [27]. Since inhibition of ETC complex activity has been associated with increased ROS production, the rate of mitochondrial H₂O₂ production in control and ClpP KD cells were measured. The rate of H₂O₂ production was measured using Amplex Red, which in the presence of horseradish peroxidase reacts with hydrogen peroxide to produce the red fluorescent oxidation product, resorufin. The rate of H₂O₂ production for ClpP KD cells with complex I-linked substrate (glutamate/malate) and complex II-linked substrate (succinate/rotenone) were elevated by ~4.6- and ~2.4-fold, respectively, compared to control cells (Fig. 4C). Similar results were obtained using ClpP siRNA (data not shown). Catalase inhibited complex I-linked substrate (glutamate/malate) mediated H₂O₂ production by 93% in both control and ClpP KD cells, while complex II-linked substrate (succinate/rotenone) mediated H₂O₂ production was reduced by only ~40%. We also found that activity of aconitase, a mitochondrial matrix enzyme that is susceptible to oxidative stress, was reduced by ~25% in ClpP KD cells (Fig. 4D) [3]. This suggests that a decline in ClpP levels could lead to elevated levels of ROS production and a decrease in ETC complex activities potentially contributes to this effect.

In order to further characterize the mitochondrial defects that lead to a decline in oxygen consumption in ClpP KD cells, we tested whether mitochondrial membrane potential is affected due to a decrease in ClpP. MitoTracker Red is a fluorescent dye that is efficiently taken up by mitochondria in live cells. However, its uptake into mitochondria is membrane-potential dependent. Staining of control and ClpP KD cells with Mitotracker Red showed that ClpP KD cells have decreased fluorescent intensity compared to control cells (Fig. 5A), indicating a decrease in membrane potential for ClpP KD cells. The decline in membrane potential in ClpP KD cells was quantified using JC-1 membrane potential assay kit. JC-1 is a lipophilic, cationic dye that can selectively enter into mitochondria and reversibly change color depending on membrane potential. Consistent with the observation using Mito Tracker red, a ~15% decrease in membrane potential was observed in ClpP KD cells, compared to control cells at basal level (Fig. 5B).

3.5. A decrease in ClpP alters cell morphology, reduces cell proliferation and impairs differentiation

The ClpP KD cells were morphologically different from control cells. ClpP KD cells exhibited a flattened cell appearance, compared to control cells, as observed by actin immunostaining (Fig. 6A). Next, we evaluated the proliferation rate of control and ClpP KD cells by seeding equal number of control and ClpP KD cells and monitoring cell number over the next 3 days. Compared to control cells, the cell number in ClpP KD cells was significantly lower on days 1, 2 and 3 after seeding (Fig. 6B). On day 1, number of control cells was ~3.7-fold higher than in ClpP KD cells and on day 3 it was ~5.1-fold higher. A similar result was obtained using siRNA for ClpP (data not shown). The decrease in cell number in ClpP KD cells was likely not due to increased cell death since we did not observe an increased number of floating cells and there was no change in caspase 3 activity to indicate apoptosis. Thus, the reduction in cell number of ClpP KD cells was more likely due to decreased proliferation. To test whether elevated ROS levels contribute to decreased proliferation, control and ClpP KD cells were pre-treated with 200 U or 500 U of PEG-catalase (Sigma, St. Louis, MO), and proliferation rate was assessed. However, PEG-catalase did not affect the proliferation rate of ClpP KD cells suggesting that increased ROS is not a casual factor for reduced cell proliferation (data not shown).

To test whether knockdown of ClpP affects differentiation, myoblasts were grown in low serum medium to differentiate them to myotubes. While control myoblasts differentiated into myo-tubes, ClpP KD cells exhibited impaired differentiation. Even prolonged incubation in the differentiation medium did not result in differentiation into ClpP KD cells. Western blot analysis showed that myosin heavy chain (MHC) and myogenin, markers of myocyte differentiation were up-regulated during differentiation in control cells, but not in ClpP KD cells (Fig. 6C) [28]. We also found that ClpP and PGC1- α (the transcription factor that regulates mitochondrial biogenesis) were also elevated with differentiation in control cells, but not in ClpP KD cells (Fig. 6C). Taken together, these findings suggest that ClpP plays an important role in cell proliferation and muscle cell differentiation.

3.6. A decline in ClpP inhibits protein translation

Previous studies have shown that mitochondrial respiration inhibitors can decrease protein translation through phosphorylation of eIF2 α [29]. Since ClpP KD cells show significant mitochondrial dysfunction, we tested whether ClpP KD cells also exhibit translation attenuation. Phosphorylation of eIF2 α at Ser 51 was dramatically elevated (~20-fold) in ClpP KD cells compared to control cells, while basal eIF2 α levels remained unchanged, indicating an inhibition of protein translation (Fig. 7A). Phosphorylation of eIF2 α is executed by one of the four kinases of eIF2 α protein kinase R (PKR) in presence of dsRNA, PRKR-like endoplasmic reticulum kinase (PERK) under ER stress, general control nonderepressible 2 (GCN2) on amino acid deprivation or heme-regulated inhibitor kinase (HRI) during heme deprivation. To test whether ER stress is up-regulated in ClpP KD cells the expression level of molecular chaperone Bip/Grp78, a marker for ER stress was tested (Fig. 7B). Expression of Bip protein was similar in control and ClpP KD cells indicating that PERK may not be the kinase for eIF2 α in ClpP KD cells. Surprisingly, GCN2 levels were decreased (~60%) in ClpP KD cells showing that GCN2 is also not likely to be responsible

for increased phosphorylation of eIF2 α (Fig. 7B). In contrast, expression of PKR was elevated by 40% in ClpP KD cells compared to control cells suggesting that PKR is responsible for the increased phosphorylation of eIF2 α in ClpP KD cells.

4. Discussion

ClpP and Lon protease are the two primary quality control proteases in the mitochondrial matrix. The role of Lon protease in eliminating denatured and oxidized proteins has been well characterized in mammalian cells [3,30]. However, relatively little is known about the function of ClpP in mammalian cells, other than its role in degrading unfolded proteins in the mitochondrial matrix and as a key component of the mitochondrial unfolded protein response pathway that helps to maintain mitochondrial protein homeostasis [6]. While it is known that ClpX ATPase that assembles with ClpP to form functional ClpXP protease complex is redox regulated, it is not known whether ClpP the ATP-dependent serine protease is redox regulated. [31]. The goal of the current study was to obtain a better understanding of the importance of ClpP by measuring a number of aspects of mitochondrial function in cells made deficient in ClpP. Our study demonstrates that in addition to its specific role in UPR^{mt}, loss of ClpP leads to altered mitochondrial function and may also be involved in other cellular processes. Specifically, we show that reduced levels of ClpP alters mitochondrial and cellular morphology, reduced cell proliferation, and results in a decline in mitochondrial respiration, lower OCR in response to ETC complex I and II substrates and increased ROS production.

Several previous studies support a role for mitochondrial proteases in the maintenance of mitochondrial function. For example, reduction of Lon protease in human WI-38 VA-13 lung fibroblasts and in B16F10 melanoma cells causes mitochondrial dysfunction characterized by impaired respiration, altered mitochondrial morphology, diminished membrane potential and shift to anaerobic energy metabolism [24,32]. A decline or alteration in mitochondrial inner and intermembrane proteases such as PARL, AFG3L2, YME1L1 and HtrA2 has also been associated with mitochondrial respiration impairments of varying intensities [33–35]. Thus, the maintenance of mitochondrial protein homeostasis in both the matrix and the intermembrane space is critical for maintaining mitochondrial integrity and function. To assess mitochondrial function in response to loss of ClpP *in vitro*, we measured mitochondrial respiration, glycolytic activity, electron transport complex activity and generation of H₂O₂ in C2C12 muscle cells with a 70% reduction in ClpP. We found significant reductions in basal, ATP-linked and maximal mitochondrial respiration in cells with reduced level of ClpP. This is consistent with reduced mitochondrial respiration previously reported in mitochondria isolated from liver, heart and skeletal muscle tissue from ClpP null mice, although these alterations were reported to be relatively mild [14]. We also found that ClpP reduction in the C2C12 muscle cells resulted in altered mitochondrial morphology, diminished mitochondrial membrane potential and a shift to anaerobic energy metabolism. Interestingly, we also observed a selective reduction in ETC complex I and IV subunits. While the mechanism by which reduced ClpP alters mitochondrial function is not known, it is possible that deficiency of ClpP can increase the overall protein load in the mitochondria that in turn can lead to improper assembly or maintenance of ETC supercomplexes. Consistent with this, a decrease in ETC supercomplexes was observed in

liver and heart of mice deficient in ClpP [14]. It is also possible that reduced clearance of misfolded proteins could reduce protein turnover and increase the number of proteins with submaximal function, including ETC proteins.

Mitochondria are important sources ROS and excessive amounts of ROS can cause deleterious effects. We found that decline in ClpP resulted in an elevated production of ROS in C2C12 cells. A decline in mitochondrial protease Lon, PARL and HtrA2 have also been shown to increase ROS production [36–39]. Surprisingly, in our study we found that only 40% of Amplex Red oxidation was inhibited by catalase when using succinate/rotenone substrates. While we cannot explain this discrepancy, one potential source of this peroxidase activity in ClpP KD cells is a contribution from the microperoxidase activity of cytochrome c. For example, it has been shown that in Parkinson's disease that cytochrome c released from the mitochondrial membrane is activated to acquire peroxidase activity by cellular proteases and H₂O₂ produced by dysfunctional mitochondria [40,41]. Hence, it is possible that high levels of ROS in ClpP KD cells could induce peroxidase activity of cytochrome c that could account for the remaining 60% of Amplex Red oxidation.

In addition to mitochondrial quality control mediated by proteases at the molecular level, mitochondrial dynamics also play a critical role in the quality control of mitochondria at the organelle level. Mitochondrial fission and fusion are essential for maintaining mitochondrial morphology, stability of mtDNA, respiratory capacity, the response to cellular stress and apoptosis [26]. Several previous studies have shown that the loss of mitochondrial proteases can alter mitochondrial dynamics. For example, a decrease in PARL decreases fusion regulators Mfn2 and OPA1 and increases the fission protein Fis1 [38]. Loss of YME1L1 and AFG3L2 have been shown to result in mitochondrial fragmentation [25,42]. Furthermore, YME1L1 and OMA1 directly regulate mitochondrial dynamics through cleavage of OPA1 to balance fission and fusion [43]. In our study, we found that reduction of ClpP in C2C12 muscle cells resulted in smaller mitochondria and elevated expression of mitochondrial fission protein Drp1, potentially to protect mitochondrial quality. However, it is not clear whether smaller mitochondria in ClpP KD cells are a consequence of Drp1 expression. Previous studies have shown that ClpP is involved in phosphatase and tensin homolog-induced kinase 1 (PINK1) degradation in mammalian cells. PINK1 is a protein involved in mitophagy, the selective degradation of mitochondria by autophagy following damage or stress, suggesting a role for ClpP in mitophagy [13]. Taken together this suggests that ClpP can protect mitochondrial quality through changes in mitochondrial dynamics and degradation. However, the mechanism by which reduced levels of ClpP lead to increased Drp1 levels in ClpP KD cells needs further investigation.

Stress-mediated chaperone expression in mitochondria has been demonstrated to be an important consequence of the UPR^{mt} in *C. elegans* [44]. In *C. elegans* as well as in mammalian cells, an increase in ClpP and Hsp60 expression occurs in response to accumulation of unfolded proteins in the mitochondrial matrix through UPR^{mt} [6,8]. ClpP RNAi in *C. elegans* blunted Hsp60 induction by mitochondrial stress, indicating that ClpP is essential for UPR^{mt} induction [6]. Our findings in C2C12 cells suggest that ClpP is also critical for UPR^{mt} induction in mammalian cells, since ClpP deficient cells failed to elevate the expression of Hsp60 in response to UPR^{mt} inducer. It is surprising that the basal level of

Hsp60 is unaltered in ClpP KD cells; however, the lack of induction of Hsp60 and Lon protease in our ClpP deficient cells is consistent with previously reported findings in tissues from *ClpP*^{-/-} mice [14]. Thus, our study demonstrates that ClpP is essential for UPR^{mt} induction in response to stress.

ClpP deficiency in C2C12 cells decreased cell proliferation and also altered cell morphology. Consistent with our observation, mouse embryonic fibroblasts from *ClpP*^{-/-} mice also exhibited decreased population doubling and reached replicative senescence earlier compared to wild-type cells [14]. Decline in Lon protease in WI-38 VA-13 human lung fibroblasts exhibited a more severe phenotype characterized by inhibition of cell proliferation and survival [24]. In addition to reduced proliferation, ClpP KD myo-blast have an impaired ability to differentiate. This is consistent with the fact that mitochondria play an important role in the regulation of myogenesis, and impairments in mitochondrial function inhibit myocyte differentiation [45]. However, the mechanism mediating this effect in ClpP KD cells is not clear. We also have evidence of reduced protein translation in ClpP KD cells as indicated by inhibition of eIF2 α . Phosphorylation of eIF2 α inhibits eIF2 α activity and initiation of translation. While eIF2 α reduces global translation, it induces preferential translation of specific mRNAs that aid in the regulation of genes involved in metabolism and apoptosis [46]. Unfolded proteins, nutrient and metabolic deficiency or mitochondrial dysfunction have all been reported to trigger eIF2 α phosphorylation and thereby inhibit translation, as part of the adaptive integrated stress response. Consistent with this, inhibition of mitochondrial respiration using chemicals have also been shown to inhibit protein translation [29,47]. Thus, it is possible that the reduction in translation could be a survival mechanism for the cells in response to mitochondrial dysfunction. It is also possible that a decline in cellular translation could contribute to reduced proliferation rate in ClpP KD cells.

In conclusion, our findings demonstrate that ClpP is an important peptidase in the mitochondrial matrix that is essential for normal mitochondrial functions, maintaining mitochondrial morphology and induction of mitochondrial stress response pathway, UPR^{mt}. Our findings are important because we show for the first time that ClpP deficiency can significantly impair mitochondrial function, even under non-stressed conditions. The effect of ClpP deficiency on other cellular functions such as cell proliferation and differentiation suggest a more global role for ClpP protease in cellular function. It is also not known how reduced level of ClpP can affect the expression of nuclear encoded genes in the regulation of cellular function. One intriguing possibility is that ClpP-mediated retrograde signaling is essential for mitochondrial-nuclear crosstalk to coordinate cellular functions. In support of this, in *C. elegans*, efflux of mitochondrial peptides generated by ClpP to the cytosol is key to the retrograde signaling [48]. Our future studies will focus on whether a similar mechanism exists in mammalian cells and on identifying the players of mitochondrial-nuclear cross-talk initiated by ClpP.

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Abbreviations

ClpP	caseinolytic peptidase P
UPR^{mt}	mitochondrial unfolded protein response
ETC	electron transport chain
ROS	reactive oxygen species
eIF2α	eukaryotic initiation factor 2 alpha
QC	quality control
Hsp60	heat shock protein 60
shRNA	short hairpin RNA
OCR	oxygen consumption rate
FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine
DMEM	Dulbecco's modified Eagle's medium
ECAR	extracellular acidification rate
H₂O₂	hydrogen peroxide
PKR	protein kinase R
Mfn	mitofusin
GCN2	general control nonderepressible 2
DOX	doxycycline
mtDNA	mitochondrial DNA
MHC	myosin heavy chain
TFAM	mitochondrial transcription factor A
PINK1	phosphatase and tensin homolog-induced kinase 1

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Fig. 1. Decrease in ClpP levels inhibits Hsp60 induction in response to DOX. (A) Immunoblots showing the expression level of ClpP, ClpX, Lon and HSp60 in control and ClpP KD cells. β-Tubulin was used as a loading control. (B) Quantification of ClpP, ClpX, Lon and HSp60 to β-tubulin is represented graphically. White and gray bars represent control and ClpP KD cells, respectively. (C) Control and ClpP KD cells were treated with DMSO (DOX-) or DOX (DOX+) (15 mg/ml) for 48 h and changes in the expression level of ClpP and Hsp60 were assessed by western blotting. Quantification of Hsp60 to β-tubulin is represented graphically on the right panel. Error bars represent $\pm$ SEM from three independent experiments, *** p <0.001, ** p <0.01.

Fig. 2.

A decrease in ClpP level alters mitochondrial morphology. (A) Electron micrographs of control and ClpP KD cells (magnification 25,000 $\times$). White arrows indicate mitochondria. (B) Quantification of mitochondrial area (left panel) and distribution of mitochondria based on size (right panel) in control (open bars) and ClpP KD cells (gray bars) using Image J software (left panel). (C) Tom20 immunofluorescence images of control and ClpP KD cells by confocal microscopy. Scale bar=10 μM . (D) Immunoblots showing expression level of Mfn1, Mfn2, OPA1, Fis1, Drp1 and ClpP in control and ClpP KD cells (left panel). β -Tubulin was used as a loading control. Quantification of protein levels normalized to β -Tubulin is shown in the right panel. All the experiments were repeated three times and error bar in graphs represent $\pm$ SEM from three independent experiments, *** p <0.001.

Fig. 3.

Mitochondrial respiration is decreased and glycolysis is increased in ClpP KD cells. (A) Cellular bioenergetics in control and ClpP KD cells were measured using the Seahorse Bioscience XF24 Extracellular Flux Analyzer mitostress assay. Control (open bars) and ClpP KD cells (gray bars) are represented graphically. (B) Glycolysis in control and ClpP KD cells were measured using the Seahorse Bioscience XF24 Extracellular Flux Analyzer glycolysis stress assay. Control (open bars) and ClpP KD cells (gray bars) are represented graphically. OCR obtained from each well is normalized with total protein concentration in the well. Error bar in graphs represent $\pm$ SEM from three independent experiments.

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Fig. 4. ETC complex activities are lower and ROS generation is higher in ClpP KD cells. (A) Activities of complexes I and II were measured using the Seahorse Bioscience XF24 Extracellular Flux Analyzer. Glutamate/malate (complex I substrate) or succinate (complex II substrate) were injected at a concentration 10 mM and changes in OCR was measured in control (open bars) and ClpP KD (gray bars). (B) Immunoblots of complex I (20 kDa subunit), complex I (30 kDa subunit), complex IV-4, complex II (70 kDa subunit), complex III subunit, complex IV-4, complex IV-1 and complex V (56.5 kDa subunit) and β-tubulin (loading control) in control and ClpP KD cells (left panel). Quantification of protein expression to β-tubulin is shown in the right panel. (C) H₂O₂ production in control (open bars) and ClpP KD (gray bars) cells with complex I-linked substrates (glutamate/malate) and

complex II-linked substrate (succinate+rotenone). (D) Aconitase activity represented in percentage in control and ClpP KD cells. Error bars represent $\pm$ SEM obtained from three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Fig. 5. Reduction in mitochondrial membrane potential in ClpP KD cells. (A) Staining of mitochondria in control (left) and ClpP KD (right) cells using mitotracker. Magnification (20x). (B) Mitochondrial membrane potential measured using JC-1 in control (open bar) and ClpP KD (gray bar) cells (20,000 cells). ** $p < 0.01$. The experiments were repeated at least three times and the data represents $\pm$ SEM obtained from three independent experiments. ** $p < 0.01$.

Fig. 6.

A decrease in ClpP alters cell morphology, reduces cell proliferation and attenuates protein translation. (A) β -actin immunostaining of control (left) and ClpP KD (right) cells. Magnification 20x. (B) Cell number of control and ClpP KD cells on days 1, 2 and 3 after seeding equal number of cells. (C) Immunoblots of MHC, myogenin, ClpP, PGC1- α and β -tubulin in control and ClpP KD cells at differentiation days 1–4 (left panel). Quantification of proteins to β -tubulin is shown in the right panel. (D) Immunoblots showing expression level of P-eIF2 α (Ser51) and eIF2 α in control and ClpP KD cells. Quantification of P-eIF2 α /eIF2 α is shown in the right panel. (D) Immunoblots showing expression level of GCN2, Bip and PKR in control and ClpP KD cells. β -tubulin was used a loading control. Quantification of GCN2, Bip and PKR to β -tubulin is shown in the right panel. The experiments were repeated three times and the data represents $\pm$ SEM obtained from the experiments. *** p <0.001, ** p <0.01, * p <0.05.

Fig. 7.

A decrease in ClpP attenuates protein translation. (A) Immunoblots showing expression level of P-eIF2α (Ser51) and eIF2α in control and ClpP KD cells. Quantification of P-eIF2α/eIF2α is shown in the right panel. (B) Immunoblots showing expression level of GCN2, Bip and PKR in control and ClpP KD cells. β-Tubulin was used as a loading control. Quantification of GCN2, Bip and PKR to β-tubulin is shown in the right panel. The data represents $\pm$ SEM from three independent experiments. * $p < 0.05$.