SUPPLEMENTAL MATERIAL

Online Methods

Animal model and sample preparation. Animals were either paced from the right atrium for six weeks at ~200 bpm (DHF) or were subjected to three weeks of atrial pacing (dyssynchrony) followed by three weeks of bi-ventricular tachypacing at the same rate (CRT) as described previously[1]. Left bundle branch block (LBBB) was confirmed by intra-cardiac electrograms, with surface QRS widening from 50±7 to 104±7 ms ($p<0.001$). Bi-ventricular pacing was achieved by simultaneous lateral epicardial and right ventricular antero-apical free wall stimulation. All following operations were performed under low light conditions to preserve Cys modifications. At terminal study the hearts were extracted under cold cardioplegia anddissected into endocardial and mid/epicardial segments from the septum (i.e. LV and RV septum) and LV lateral wall. Tissue samples obtained from the upper third of the LV lateral wall were used in the present study. The desired tissue was cut into small pieces and rinsed briefly in cold Cys preservation buffer[2] (20 mmol/L NEM, 5 mmol/L EDTA, 1 mmol/L DTPA and 0.1 mmol/L neocuproine in PBS buffer) then snap-frozen in liquid nitrogen and stored at -80^{o}C until use.

Cell culture. HEK 293 cells used for exogenous expression of Cys mutants were cultured at 37^{o}C in a 5% CO_2 incubator in DMEM media (containing 4.5 g/L glucose, Mediatech) supplement with 10% FBS (HyClone).

Genetic manipulation. The human ATP5A1 and ATP5C1 full length cDNA clone in mammalian expression vector pCMV-Sport6 were from Human MGC verified FL cDNA collection of Open Biosystems (Thermo Scientific) with Clone ID 5582043 and 3445817, respectively. Human ATP5A1 and ATP5C1 ORF clones were from Ultimate™ ORF Clone collection of Open Biosystems (Thermo Scientific) with Clone ID IOH27858 and IOH2988, respectively. The ORF entry clones were integrated into C-terminally FLAG tagged expression vector FLAG-DEST under CAMV promoter by Gateway technology (Invitrogen). siRNAs are obtained from Ambion (Applied Biosystems) with si1767 (5'-ggaaaacctcaattgctatt-3') for ATP5A1 gene and si189 (5'-gcatgagtatctatgacgat-3') for ATP5C1 gene. Silent mutation for wild type siRNA resistance expression plasmid and Cys to Ser mutation in siRNA resistant expression plasmid was created by QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacture's protocol with the primers list in Table S1. The sequence of each mutant was further confirmed by DNA sequencing. Expression plasmids and siRNAs were introduced into HEK 293 cells by transient co-transfection with Lipofectamine™ RNAiMAX Reagent (Invitrogen) according to the manufacturer's protocol. After 3 days of transfection the cells were either treated with or without 1 mmol/L DTT or 100 μmol/L $CuCl_2$ in HBSS buffer (Invitorgen) for a further 15 min in the same incubator. At the end of treatment, the cells were rinsed with cold PBS and harvested for further analysis as described below.

Mitochondria preparation. Mitochondria were isolated by differential centrifugation protocol as described previously[3] with modifications to preserve endogenous Cys modification and avoid artificial ones, except the mitochondria used for in vitro treatment, which were isolated in the absence of NEM. All following operations were performed at 4^{o}C and under low light conditions. Briefly, about 250 mg frozen heart tissue were pulverized and then homogenized in 1mL of modified homogenization buffer (220 mmol/L mannitol, 70 mmol/L sucrose, 20 mmol/L NEM, 5 mmol/L EDTA, 1 mmol/L DTPA, 0.1 mmol/L neocuproine and 20 mmol/L HEPES, pH 7.4) plus EDTA-free protease inhibitor cocktail tablelet (Roche). The sample was centrifuged at 1100 x g for 5 min at 4°C and the pellet was resuspended in 500 μL of homogenization buffer and centrifuged as above. This step was repeated twice. Supernatants from each step were pooled and centrifuged at 7000 x g for 15 min at 4°C. The resulting pellet was resuspended in 1mL of the homogenization buffer and centrifuged at 20 000 x g for 15 min. This step was repeated, with 500 μL and then 250 μL of homogenization buffer. The pellet was then resuspended in

125 µL of homogenization buffer and centrifuged at 3000 x g for 5 min and the supernatant was collected and saved. The pellet was then resuspended in 75 µL of homogenization buffer and centrifuged at 3000 x g for 5 min. The supernatant was then combined with that from the previous step and centrifuged at 20,000 x g for 20 min. The final pellet was resuspended in 50 µL of homogenization buffer and protein concentration was determined by CB-X™ protein assay kit (G-Biosciences). Mitochondria were stored at -80^{o}C as 100 µg aliquots until use.

For the isolation of mitochondria from HEK cells, cells from 100 mm plate were washed twice with cold PBS and scraped into 2 mL cold PBS supplement with protease inhibitor cocktail. The samples were centrifuge at 470 x g for 5 min at 4°C and the pellets were homogenized in 100 µL homogenization buffer (220 mmol/L mannitol, 70 mmol/L Sucrose, 20 mmol/L HEPES, pH 7.4) with protease inhibitor cocktail. The sample was centrifuged at 600 x g for 5 min at 4°C and the pellet was resuspended in 100 µL of homogenization buffer and centrifuged as above. Supernatants from each step were pooled and centrifuged at 1000 x g for 5 min. The resulting supernatant was centrifuged at 20 000 x g for 20 min. The final pellet was resuspended in 50 µL of homogenization buffer and protein concentration was determined by CB-X™ protein assay kit (G-Biosciences). Mitochondria were stored at -80^{o}C as 20 µg aliquots until use.

Isolation of mitochondrial ATP synthase complex. Mitochondrial ATP synthase complex was isolated by ATP synthase immnocapture kit (Mitosciences) according to the manufacturer's protocol. Briefly, 200 µg of mitochondria were solubilized in 40 uL PBS buffer with 1% (w/v) n-dodecyl-β-D-maltoside supplement with EDTA-free protease inhibitor cocktail tablet (Roche) and incubated on ice for 30 min. The sample was centrifuged for 30 min at 22,000 x g at 4°C. The supernatant was mixed with 10 µL of antibody agarose bead and incubated on a nutator for 3h at room temperature. The supernatant was removed after a brief centrifugation and washed 3 times with 1 mL PBS containing 0.05% (w/v) n-dodecyl-β-D-maltoside. ATP synthase complexes were eluted with 30 µL elution buffer (0.mol/L glycine, pH 2.0) for 10 min with frequent agitation. The elutes were collected and the elution step was repeated twice with 20 µL elution buffer. The elutes were pooled and the pH was adjust to neutral pH by adding 15 µL 1 mol/L Tris Base

Induction of Ox-PTM in isolated mitochondria and ATP synthase complex with oxidants treatment. Isolated mitochondria (either under Cys modification preserving conditions or in the absence of NEM) were resuspended in homogenization buffer plus EDTA-free protease inhibitor cocktail tablet (Roche) at 1 µg/µl containing different reagents and were incubated at 37^{o}C for 15 min. After incubation the reagents were removed by centrifugation and the resulting mitochondrial pellets used for clear native PAGE (CNP) and in-gel ATPase activity assay, gel electrophoresis, or for the detection of S-nitrosation as described below. For induction of Ox-PTM in isolated ATP synthase complex, ATP synthase were mixed with different reagents and incubated as indicated in the Figure Legend.

Blue Native PAGE gels. Blue Native PAGE (BN-PAGE) was used to resolve the native, intact mitochondrial protein complexes.[4] Briefly, mitochondria samples were solubilized in BN-PAGE sample buffer (50mmol/L Tris-HCl, 50mmol/L NaCl, 10% glycerol w/v, 0.001% Ponceau S, pH 7.2 plus protease inhibitor cocktail) with 2% digitonin w/v at about 5 µg/µL and incubated on ice for 30 min. Each sample was centrifuged for 30 min at 22,000 x g at 4°C. The supernatant was collected and 50 µg of protein were mixed with 5% (w/v) Coomassie G250 to a final concentration of 0.4%, then loaded on a 3-12% NativePAGE Novex gel (1mm, Invitrogen) and run according to manufacturer's protocols. The gel was either transferred to PVDF membrane (Millipore, 45µm) for western blotting or separated by a second dimension SDS-PAGE (as described below).

2D BNP/SDS-PAGE. Upon completion of 1D BN-PAGE whole lanes were excised from the gel into 15 mL tubes for incubation in 1x NuPAGE LDS sample buffer (Invitrogen; 62 mmol/L Tris, 0.5% w/v LDS, 2.5% glycerol, 0.13 mmol/L EDTA, 0.55 mmol/L Coomassie G250 and 0.04 mmol/L phenol red, pH 8.5)

with or without 50 mmol/L DTT, for reducing and non-reducing PAGE, respectively, at 37°C for 30 min on a nutator. Gel slices were placed on the top of an 18 cm 10% SDS-PAGE gel (1.5mm, homemade) and overlaid with 0.5% agarose. The gels were run using MES running buffer (50mmol/L MES, 50mmol/L Tris–base, 0.1% w/v SDS, 1.0mmol/L EDTA, pH 7.3) at 70V (2 h) and 90V (16 h) . Gels were either fixed in 50% (v/v) methanol, 10% (v/v) acetic acid and silver stained for LC-MS or transferred to PVDF membrane for western blotting as described below.

In gel ATPase activity assay. Mitochondrial samples were gently solubilized and cleared as described above for BNP. Twenty micrograms of total protein from each sample were loaded directly onto a 3-12% NativePAGE Novex gel (1mm, Invitrogen) without coomassie blue and clear native PAGE (CNP) run according to published protocol[5] with 25 mmol/L imidazole/HCl, pH 7.0 as anode buffer and 50 mmol/L Tricine, 7.5 mmol/L imidazole, pH 7.0 supplement of 0.02% (w/v) sodium deoxychlorate and 0.05% (w/v) n-dodecyl-β-D-maltoside as cathode buffer. ATP hydrolysis activity was measured following CNP as described.[4] After completion of CNP the gel was briefly incubated in assay buffer (35 mmol/L Tris, 270 mmol/L glycine, 14 mmol/L $MgSO_4$, 0.2% (w/v) $Pb(NO_3)_2$, 8 mmol/L ATP, pH 8.3) at varying time courses as indicated in the results section. Gels were fixed in 50 % methanol and wash twice in ddH_2O for 10 min and then scanned and the lead precipitates quantified based on densitometry (Progenesis, Nonlinear Dynamics).

Measurement of Mitochondrial Respiration Tissue was obtained from the left ventricle endocardium of a normal dog. Mitochondria were isolated in normal Homogenization buffer as described above, in the presence of 0.1 mmol/L Neocuproine, 1 mmol/L DTPA and 5 mmol/L EDTA, in the dark. Following isolation, crude mitochondria were washed once with isolation buffer to remove these compounds prior to assay. Final preparations were resuspended in 20 mmol/L HEPES, 137 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 2 mmol/L KH_2PO_4, 0.5 mmol/L EGTA, 0.2% (w/v) BSA, pH 7.3 and were aliquoted into 96 wells (5 µg protein/well) of a polyethyleneimine-coated XF96 cell culture microplate (Seahorse Bioscience).[6] The plate was centrifuged at 3000 x *g* for 14 min at 4°C in an A-4-62 rotor. Plates, containing 200 µL of assay buffer per well, were used immediately. The cell culture microplate was incubated at 37°C and loaded into the Seahorse XF96 extracellular flux analyzer following the manufacturer's instructions. All experiments were performed at 37°C. Oxygen consumption data were acquired over 2 minutes (15 data points). Experiments were conducted in assay buffer alone or supplemented with either 20 mmol/L NEM or 1mmol/L DTT. The oxygen consumption rates were determined by using a compartment model-based 'deconvolution' algorithm which compensated for oxygen diffusion phenomena occurring around the entrapped volume, and for the response time of the probe.

Biotin switch assay. S-nitrosation was detected using biotin switch assay as described previously[7] and modified by our group.[8] For GSNO treatment, 100 µg of dog heart mitochondria isolated in the absence of NEM (mixture of three dogs from the same group) or 20 µg of rat heart mitochondrial ATP synthase were diluted to 0.8 µg/µL in HEN (250 mmol/L HEPES pH 7.7, 1 mmol/L EDTA and 0.1 mmol/L neocuproine) including 0.4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) and treated with different concentration of GSNO or mock treatment without GNSO for 15 min at 37 °C. All steps were performed in the dark or protected from light. Treatment compounds were removed using an HEN equilibrated 0.5 mL Zeba desalt spin column (Thermo Fisher Scientific) according to the manufacturer's protocol. The remaining free thiols were blocked with 100 mmol/L NEM in the presence of 2.5% (w/v) SDS and incubated for 30 min at 50 °C. Excess NEM was removed by methanol precipitation (five volumes) and washed an additional two times with methanol. SNO-modified thiols were reduced using 1 mmol/L ascorbate in five volumes of HENS (HEN plus 1% (w/v) SDS) and labeled with 0.8 mmol/L Biotin-HPDP (Thermo Fisher Scientific) for 1 hour at room temperature. Excess biotin-HPDP was removed by methanol precipitation (five volumes) and the resultant pellets were carefully washed with an additional volume of methanol. Pellets were resuspended to 1 µg/µL with HENS and diluted to 0.1 µg/µL in neutralization buffer (20 mmol/L HEPES, 150 mmol/L NaCl 1 mmol/L

EDTA, 0.5% (v/v) Triton X-100). Biotinylated proteins were captured by incubation with 10 of washed, packed ultralink immobilized streptavidin beads (Thermo Fisher Scientific) for 1 hour at room temperature. Beads were washed four times in 50-bead volumes of wash buffer (20 mmol/L HEPES, 600 mmol/L NaCl 1 mmol/L EDTA, 0.5% (v/v) Triton X-100) and twice with elution buffer (20 mmol/L HEPES pH 7.7, 100 mmol/L NaCl, 1 mmol/L EDTA). Captured proteins were eluted with 20 µL of elution buffer containing 100 mmol/L DTT, mixed with 8 µL of 4x LDS sample buffer, boiled, separated by SDS-PAGE and immunobloted with anti-Complex V alpha subunit antibody (Molecular probe). For detection of endogenous SNO in dog mitochondrial samples, 100 µg of mitochondria isolated under Cys modification preserving conditions (mixture of three dogs from the same group) were diluted to 0.8 µg/µL in HEN with 100 mmol/L NEM in the presence of 2.5% (w/v) SDS and incubated for 30 min at 50 °C and then followed essentially as in vitro assay as above.

Denaturing SDS-PAGE and Immunoblotting. Mitochondria or ATP synthase complex samples were solubilized and boiled in 1x LDS sample buffer either with or without 50 mmol/L DTT for reducing and non-reducing SDS-PAGE, respectively. The protein mixture was separated on a 4-12% NuPAGE gel (1 mm, Invitrogen). Samples were run at room temperature for 35 min at 200 V. For 2D non-reducing/reducing SDS/PAGE, whole lanes from the 1D non-reducing SDS-PAGE were excised from the gel and incubated in 1x LDS sample buffer with 50 mM DTT at 37^{o}C for 30 min on a nutator. Gel slices were placed on the top of a NuPAGE gel (1.5mm, 2D well, Invitorgen) and overlaid with 0.5% agarose. SDS-PAGE was run using MES running buffer at 80V (10 min), 150V (10 min) and 200V (30 min). Proteins were transferred to PVDF membrane in NuPAGE transfer buffer at 100 V for 1h. Membrane was stained with Direct Blue 71 (Sigma) and images recorded for subsequent luminescent signal normalization. Membrane was blocked overnight using 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBS-T: 100 mmol/L Tris-HCl, 0.9% (w/v) NaCl, 0.1% (v/v) Tween 20), incubated with primary antibody in TBS-T under gentle agitation for 2 h and then incubated with 0.03 µg/mL alkaline phosphatase-conjugated AffiniPure Goat Anti-Mouse or Anti-Rabbit (Jackson ImmunoResearch) in TBS-T under gentle agitation for 1 h. Chemiluminescence was performed using Immun-Star AP substrate pack (Bio-Rad) under manufacturer protocols and luminescence was detected with scientific imaging film (Kodak). Between blocking, antibody incubations and chemiluminescence detection, blots were washed 3 times for 10 min in TBS-T. The following primary antibody were used: 0.2 µg/mL anti-OxPhos complex V subunit α mouse IgG monoclonal 7H10 and 0.2 µg/mL anti-OxPhos complex V subunit β mouse IgG monoclonal 3D5 (Molecular Probes), Rabbit anti-ATPG polyclonal antibody (1: 1,000, Abcam), ATP synthase subunit OSCP monoclonal 4C11C10D12 (1:10,000, Mitosciences), Anti-FLAG monoclonal antibody (1:1,000, Sigma), Anti-GSH monoclonal antibody (1:1,000, Virogen).

Image Analysis and Quantification. Gel and immunoblot images were scanned at 200-300 dpi using an Epson Expression 10000XL device (Epson Electronics America, CA, USA). Protein spots from gels were then quantified using Progenesis Workstation 2005 software (Nonlinear Dynamics, New Castle-upon-Tyne, UK). Composite images of each treatment group were normalized by means of a total spot intensity comparison between sample groups. Quantified spot changes were within the linear range of silver stain. The same platform was used to acquire and analyze densitometry data generated through western blotting analysis. In this case, the luminescent signal generated by each sample band and recorded on the imaging film was normalized against the density of the correspondent lane stained with the general protein staining DB71.

Protein identification by Mass Spectrometry. Mass spectrometry analysis was performed as previously described.[9] Protein bands or spots were excised from 1D or 2D gel and destained according to a modified protocol.[9] Proteins were digested in 25 mmol/L ammonium bicarbonate, pH 8.0 with 10 µg/mL sequencing grade modified porcine trypsin (Promega) for 16-24 h at 37ºC or 10 µg/mL sequencing grade bovine chymotrypsin (Roche) overnight. Peptides were extracted twice with 50 µL of 50% acetonitrile

(ACN)/ 5% TFA for 60 min and then dried under vacuum. All samples were desalted with C18 Omix tips (Varian) according to manufacturer's protocol. Digested samples were analyzed using the an LTQ-Orbitrap (ThermoFinnigan) with a C18 column (PicoFrit™ 75μm column prepacked with BioBasic® C18, New Objectives) in gradient mode (inject at 8.5-30% 0.1% formic acid/90% acetonitrile (30min), 60% 0.1% formic acid/90% acetonitrile (18 minutes) and to 100% 0.1% formic acid/90% acetonitrile (22min)) with a flow rate of 300 nL/min. The electrospray voltage was 2.2 kV, precursor scans were taken from 350–1800 m/z and the top eight precursor ions were selected for MS/MS. MS/MS data were analyzed using Sorcerer 2™-SEQUEST® (Sage-N Research), with post-search analysis performed using Scaffold (Proteome Software). All raw data peak extraction was performed using Sorcerer 2™-SEQUEST® default settings. Data was searched using the IPI database (IPI_RAT_v3.62), using either a full trypsin or full chymotrypsin digestion, with the following criteria: variable modifications: acetylation (K), deamidation (N and Q) carbamidomethyl (C), NEM (C), d5NEM (C), oxidation (M); peptide mass tolerance set to 50 ppm. All MS/MS spectra were manually examined using Scaffold (Proteome Software). Protein name redundancy was removed and isoforms identified positively only if a peptide was observed to a unique peptide sequence.

Molecular Modeling. The three-dimensional structure of Bovine mitochondrial F_1 ATP synthase from protein data bank (PDB entry # 1E79) was modeled using PyMOL (Delano Scientific LLC).

Statistics. All data are expressed as means ± SD. Comparisons between different groups were performed via 2-tailed unpaired Student *t* test with $p<0.05$ being considered significant.

Online Figure Legends

Online Figure I. CRT increase mitochondrial ATPase activity. Mitochondria were isolated in normal Homogenization buffer without NEM and solubilized with 2% digtonin and clear native PAGE was run as described in Methods. A, Top: Representative images of in-gel ATPase activity assay on DHF, CRT and normal dogs. Bottom: ATP synthase complex was visualized with Coomassie blue staining after removing lead phosphate precipitate in destaining solution (20% Methanol, 10% Acetic acid). B, Quantification of in-gel ATPase activity showing CRT significantly recovers ATPase hydrolysis activity in DHF dogs and renders it comparable to normal dog levels. ATPase hydrolysis activity was normalized for ATP synthase complex protein content (n=3 DHF, 3 CRT, 3 Sham).

Online Figure II. Effect of NEM and DTT on mitochondrial respiration. Mitochondria were isolated in normal Homogenization buffer without NEM from normal dog. Oxygen consumption rates were determined in assay buffer alone (GM/SUCC) or supplemented with either 20 mmol NEM (GM NEM/SUCC NEM) or 1mmol DTT (GM DTT/SUCC DTT), n=4.

Online Figure III. Representative silver stained image of immunoprecipitated ATP synthase complex separated by 2D-nonreducing/reducing SDS-PAGE showing that the crosslinked products were disassociated into ATPα and ATPγ subunit after reducing with DTT. The identification of the components of the crosslinked products were confirmed by MS.

Online Figure IV. MS identification of the sites for Cys disulfide bond formation in ATPα and ATPγ. A, MS/MS spectrum of doubly protonated molecular ion, m/z = 700.88^{2+} of ATPα aa 242-252. d5NEM labeling of Cys244 was determined from tryptic fragement $_{242}$LYC*$_{244}$IYVAIGQK$_{252}$. The original MW for the tryptic fragment LYCIYVAIGQK is 1272.74 Da while that detected is 1401.76Da. The mass difference is 303 Da, corresponding to d5NEM. The MS/MS spectrum of the doubly protonated tryptic fragment ion at m/z 700.88 was obtained and the MW difference between fragment ions y8 and y9 of 130 Da compared to the native fragment ions, allow unequivocal assignment of the d5NEM labeling to Cys244. B, MS/MS spectrum of protonated molecular ion, m/z = 1126.41^{1+} of ATPα aa 292-300. d5NEM labeling of Cys294 was determined from chymotryptic fragement $_{252}$SGC*$_{294}$SM (+16, Oxidation)GEYF$_{300}$. The original MW for the Chymotryptic fragment SGCSM (+16, Oxidation)GEYF is 996.42 Da while that detected is 1126.41 Da. The mass difference is 130 Da, corresponding to d5NEM. The MS/MS spectrum of the doubly protonated tryptic fragment ion at m/z 1126.41^{1+} was obtained and the MW difference between fragment ions y6 and y7 of 130 Da compared to the native fragment ions, allowing unequivocal assignment of the d5NEM labeling to Cys294.

Online Figure V. MS identification of the sites of Cys S-Glu in ATPα. A, MS/MS spectrum of triply protonated molecular ion, m/z = 525.92^{3+} of aa 242-252. S-Glu Cys244 was determined from tryptic fragement $_{242}$LYC*$_{244}$IYVAIGQK$_{252}$. The original MW for the tryptic fragment LYCIYVAIGQK is 1272.74 Da while that detected is 1577.75Da. The mass difference is 305 Da, corresponding to GS. The MS/MS spectrum of the triply protonated tryptic fragment ion at m/z 525.92^{3+} was obtained and the MW difference between fragment ions b2 and b3 of 305 Da compared to the native fragment ions, allow unequivocal assignment of the S-Glu site to Cys244. B, MS/MS spectrum of triply protonated molecular ion, m/z = $1218.88^{3+}$of aa 271-302. S-Glu Cys294 was determined from tryptic fragment $_{271}$YITVVSATASDAAPLQ(+1, Deanimation)YLAPYSGC*$_{294}$SM(+16, Oxidation)GEYFR$_{302}$. The original MW for the tryptic fragment YITVVSATASDAAPLQ(+1, Deanimation)YLAPYSGCSM(+16, Oxidation)GEYFR is 3351.55 Da. while that detected is 3656.65 Da. The mass difference is 305 Da, corresponding to GS. The MS/MS spectrum of the triply protonated tryptic fragment ion at m/z 1218.88^{3+} was obtained and the MW difference between fragmented ions y7 and y8 of 305 Da, compared to the native fragment ions, allows unequivocal assignment of the S-Glu site to Cys294.

Online Figure VI. Molecular modeling of ATPα and ATPγ in F1 ATPase showing the sites of Cys Ox-PTMs. A, Side view showing C244 and C294 of ATPα are located on the surface of F_1 ATPase. B, Side view of F_1 ATPase in the orientation showing C103 of ATPγ is exposed to the solvent. C, Top view showing C294 of ATPα is located on the surface of F_1 ATPase. D, Close-up image show the detail of surrounding environment of C294 of ATPα. C294 is surrounded by several positive charge residues: Arg170, Arg301 and Arg351. Figures are produced by PyMol based on x-ray crystallographic determined protein structure of Bovine Mitochondrial F_1 ATP synthase (Protein Data Bank no. 1E79[18]).

Online Figure VII. Effects of Cysteine mutants (C244S, C294S of ATPα and C103S of ATPγ) on ATPase activity in HEK cell line. C-terminally FLAG-tagged CDS of WT and C244S, C294S and C103S mutant plasmids were co-tranfected into HEK cells with corresponding siRNA (si1767 for ATPα and si189 for ATPγ) to replace the endogenous ones. A, In-gel ATPase activity of different Cys mutants. Mitochondrial were isolated 3 days after transfection and ATPase hydrolysis activity was measured as described in Methods. ATP synthase was visualized with Coomassie blue staining after removing lead phosphate precipitate in destaining solution (20% Methanol, 10% Acetic acid). ATPα, ATPβ and ATPγ were determined by western blot in total mitochondria from the same transfection. B. Quantification of in-gel ATPase activity based on densitometry. ATP synthase activity is normalized to its corresponding CV protein content. N=3 independent transfection experiments.

Online Figure VIII. Effects of oxidizing and reducing reagents on ATPase activity of Cys mutants in HEK cells. Full length cDNA of WT and C244S, C294S and C103S mutant plasmids were co-transfected into HEK cells along with the corresponding siRNA for the native subunit (si1767 for ATPA and si189 for ATPG) to knockdown the endogenous ones. Oxidizing and reducing reagent treatment following transfection were carried out as described in Methods. A, Representative images show the effect of control (NT), oxidizing (100 μMol $CuCl_2$) and reducing reagents (1 mMol DTT) on in-gel ATPase activity (top image) of Cys mutants . ATP synthase complex (bottom image) was visualized with Coomassie blue staining after removing lead phosphate precipitate in destaining solution (20% Methanol, 10% Acetic acid). B. Quantification of in-gel ATPase activity based on densitometry. ATP synthase activity is normalized to its corresponding CV protein content. n=3 independent transfections for each treatment.

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Online Table I.

Primers used for site directed mutagenesis

Site for mutation	Primers
si1767-resistance	5'-GATTATTGGTGACCGACAGACTGGGAAGACTTCGATCGCTATTGACACAATCATTAACC-3' 5'-GGTTAATGATTGTGTCAATAGCGATCGAAGTCTTCCCAGTCTGTCGGTCACCAATAATC-3'
si189-resistance	5'-TTGCAAGTGCTGACAGCATGAGCATATACGATGATATTGATGCTGACGTGCTGC-3' 5'-GCAGCACGTCAGCATCAATATCATCGTATATGCTCATGCTGTCAGCACTTGCAA-3'
ATPα-C244S	5'-CTGATGAAAAGAAGAAGCTGTACAGTATTTATGTTGCTATTGGTCAA-3' 5'-TTGACCAATAGCAACATAAATACTGTACAGCTTCTTCTTTTCATCAT-3'
ATPα-C294S	5'-GCTCCTTACTCTGGCAGTTCCATGGGAGAGT-3' 5'-ACTCTCCCATGGAACTGCCAGAGTAAGGAGC-3'
ATPγ-C103S	5'-CCTCAGATCGAGGACTGAGTGGTGCTATTCATTCC-3' 5'-GGAATGAATAGCACCACTCAGTCCTCGATCTGAGG-3'

Online Table II.

MSMS data for the identification of proteins involved in Cys Ox-PTM

MS/MS sample name	Corresponding Figures	Protein name	Protein accession numbers	Protein molecular weight (Da)	Protein identification probability	Number of unique peptides	Peptide sequence	Best Peptide identification probability	Best SEQUEST XCorr score
120 kD spot (ATPα-ATPα)	Figure 2A	ATP synthase alpha chain	P15999	59,755.20	100%	24	VLSIGDGIAR	95%	2.7426
							RLTDADAMK	95%	2.9356
							AVDSLVPIGR	95%	2.8047
							GYLDKLEPSK	95%	2.8746
							HALIIYDDLSK	95%	3.6292
							TSIAIDTIINQK	95%	3.4518
							GIRPAINVGLSVSR	95%	4.1619
							TGTAEmSSILEER	95%	3.6957
							QGQYSPMAIEEQVAVIYAGVR	95%	4.892
							EAYPGDVFYLHSR	95%	3.0599
							ILGADTSVDLEETGR	95%	4.9025
							LKEIVTNFLAGFEP	95%	4.1956
							TGAIVDVPVGDELLGR	95%	4.877
							NVQAEEMVEFSSGLK	95%	5.0012
							VVDALGNAIDGKGPVGSK	95%	4.288
							DNGKHALIIYDDLSK	95%	2.2362
							RTGAIVDVPVGDELLGR	95%	4.5033
							GmSLNLEPDNVGVVVFGNDK	95%	6.2909
							PAINVGLSVSR	95%	2.4617
							YTIVVSATASDAAPLQYLAPYSGcSmGEYFR	95%	4.9977
							QGQYSPMAIEEQVAVIYAGVR	95%	5.7477
							EVAAFAQFGSDLDAATQQLLSR	95%	7.3506

							FESAFLSHVVSQHQSLLGNIR	95%	6.6733
							LYclYVAIGQK	95%	1.9474
100 kD spot (ATPα-ATPγ)	Figure 2A	ATP synthase alpha chain	P15999	59,755.20	100%	15	VLSIGDGIAR	95%	2.4204
							VVDALGNAIDGK	95%	3.7335
							HALIIYDDLSK	95%	3.6873
							GIRPAINVGLSVSR	95%	3.8853
							TGTAEmSSILEER	95%	3.3142
							EAYPGDVFYLHSR	95%	3.208
							EVAAFAQFGSDLDAATQQLLSR	95%	5.3277
							ILGADTSVDLEETGR	95%	4.7915
							LKEIVTNFLAGFEP	95%	2.6153
							FESAFLSHVVSQHQSLLGNIR	95%	5.8761
							TGAIVDVPVGDELLGR	95%	2.5071
							NVQAEEmVEFSSGLK	95%	3.894
							GmSLNLEPDNVGVVVFGNDK	95%	6.3655
							QGQYSPmAIEEQVAVIYAGVR	95%	4.9404
							EVAAFAQFGSDLDAATQQLLSR	95%	7.1378
							EVMIVGIGEK	95%	3.0622
		ATP synthase gamma chain	P35435	30,191.00	100%	9	GLcGAIHSSVAK	95%	3.744
							HLIIGVSSDR	95%	3.0421
							KPPTFGDASVIALELLNSGYEFDEGSIIFNQFK	95%	6.9847
							LTLTFNR	95%	1.689
							NASDmIDKLTLTFNR	95%	4.3139
							NDmAALTAAGK	95%	3.4991
							THSDQFLVSFK	95%	3.4421
							VYGTGSLALYEK	95%	3.236
55 kD spot (ATPα-ATPα)	Figure 2C	ATP synthase alpha chain	P15999	59,755.20	100%	28	APGIIPR	95%	1.5659
							AVDSLVPIGR	95%	3.0136
							EAYPGDVFYLHSR	95%	2.942
							ELIIGDR	95%	1.8256
							EPMQTGIK	95%	1.6297

							EVAAFAQFGSDLDAATQQLLSR	95%	7.2245
							FESAFLSHVVSQHQSLLGNIR	95%	6.3238
							GIRPAINVGLSVSR	95%	3.9805
							GmSLNLEPDNVGVVVFGNDK	95%	6.3637
							GYLDKLEPSK	95%	2.961
							HALIIYDDLSK	95%	3.5123
							ILGADTSVDLEETGR	95%	4.4387
							LELAQYR	95%	2.32
							LKEIVTNFLAGFEP	95%	4.1763
							LTDADAMK	95%	1.9194
							LTELLK	95%	1.7771
							NVQAEEmVEFSSGLK	95%	4.7048
							PAINVGLSVSR	95%	3.4606
							QGQYSPMAIEEQVAVIYAGVR	95%	5.5986
							RLTDADAMK	95%	2.7343
							RSTVAQLVK	95%	2.5234
							RTGAIVDVPVGDELLGR	95%	4.2767
							TGAIVDVPVGDELLGR	95%	5.0384
							TGTAEMSSILEER	95%	3.4682
							TSIAIDTIINQK	95%	3.5082
							VLSIGDGIAR	95%	2.7201
							VVDALGNAIDGKGPVGSK	95%	3.2867
							YTIVVSATASDAAPLQYLAPYSGcSmGEYFR	95%	4.26
55 kD spot (ATPα-ATPγ)	Figure 2C	ATP synthase alpha chain	P35435	31,191.00	100%	24	VLSIGDGIAR	95%	2.7426
							RLTDADAMK	95%	2.9356
							AVDSLVPIGR	95%	2.8047
							GYLDKLEPSK	95%	2.8746
							HALIIYDDLSK	95%	3.6292
							TSIAIDTIINQK	95%	3.4518
							GIRPAINVGLSVSR	95%	4.1619
							TGTAEmSSILEER	95%	3.6957

							QGQYSPMAIEEQVAVIYAGVR	95%	4.892
							EAYPGDVFYLHSR	95%	3.0599
							ILGADTSVDLEETGR	95%	4.9025
							LKEIVTNFLAGFEP	95%	4.1956
							TGAIVDVPVGDELLGR	95%	4.877
							NVQAEEMVEFSSGLK	95%	5.0012
							VVDALGNAIDGKGPVGSK	95%	4.288
							DNGKHALIIYDDLSK	95%	2.2362
							RTGAIVDVPVGDELLGR	95%	4.5033
							GmSLNLEPDNVGVVVFGNDK	95%	6.2909
							PAINVGLSVSR	95%	2.4617
							YTIVVSATASDAAPLQYLAPYSGcSmGEYFR	95%	4.9977
							QGQYSPMAIEEQVAVIYAGVR	95%	5.7477
							EVAAFAQFGSDLDAATQQLLSR	95%	7.3506
							FESAFLSHVVSQHQSLLGNIR	95%	6.6733
							LYcIYVAIGQK	95%	1.9474
55 kD spot (ATPα-OSCP)	Figure 2C	ATP synthase alpha chain	P15999	59,755.20	100%	16	EAYPGDVFYLHSR	95%	3.208
							EIVTNFLAGFEP	95%	1.9918
							EVAAFAQFGSDLDAATQQLLSR	95%	7.1378
							FESAFLSHVVSQHQSLLGNIR	95%	5.6777
							GIRPAINVGLSVSR	95%	3.8853
							GmSLNLEPDNVGVVVFGNDK	95%	6.3376
							HALIIYDDLSK	95%	3.6873
							ILGADTSVDLEETGR	95%	4.6996
							LELAQYR	95%	1.809
							LKEIVTNFLAGFEP	95%	2.6153
							NVQAEEmVEFSSGLK	95%	3.894
							QGQYSPmAIEEQVAVIYAGVR	95%	4.9404
							TGAIVDVPVGDELLGR	95%	2.5071
							TGTAEmSSILEER	95%	3.3142
							VLSIGDGIAR	95%	2.4204

30 kD spot (ATPα-ATPγ)	Figure 2C	ATP synthase gamma chain	P35435	31,191.00	100%	9	VVDALGNAIDGK	95%	3.6368
							EVmIVGIGEK(I)	95%	2.7065
							GLcGAIHSSVAK	95%	3.9601
							KHLIIGVSSDR	95%	3.3678
							LTLTFNR	95%	1.7548
							NASDmIDKLTLTFNR	95%	3.4726
							NDmAALTAAGK	95%	3.9176
							SVISYK	95%	1.0947
							THSDQFLVSFK	95%	3.7985
							VYGTGSLALYEK	95%	3.5167
31 kD spot (ATPγ-ATPγ)	Figure 2C	ATP synthase gamma chain	P35435	31,191.00	100%	2	HLIIGVSSDRGLcGAIHSSVAK	95%	3.7785
							THSDQFLVSFK	95%	3.5758
31 kD spot (ATPγ-OSCP)	Figure 2C	ATP synthase gamma chain	P35435	31,191.00	100%	2	THSDQFLVSFK	95%	3.5542
							HLIIGVSSDR	95%	3.0313
21 kD spot (ATPα-OSCP)	Figure 2C	ATP synthase Oligomycin sensitivity conferral protein	Q06647	23,398.00	100%	3	FSPLTANLmNLLAENGR	95%	4.0342
							GEVPcTVTTAFPLDEAVLSELK	95%	5.5232
							GQILNLEVK	95%	2.6291
21 kD spot (ATPγ-OSCP)	Figure 2C	ATP synthase Oligomycin sensitivity conferral protein	Q06647	23,398.00	100%	2	LGNTQGVISAFSTImSVHR	95%	3.8617
							LVRPPVQVYGIEGR	95%	4.377
55 kD spot (ATPα-ATPα)	Figure S1	ATP synthase alpha chain	P15999	59,755.20	100%	29	AVDSLVPIGR	95%	3.0799
							DNGKHALIIYDDLSK	95%	3.8717
							EAYPGDVFYLHSR	95%	3.1262
							EIVTNFLAGFEP	95%	2.9053
							EVAAFAQFGSDLDAATQQLLSR	95%	7.5483
							FESAFLSHVVSQHQSLLGNIR	95%	6.2253
							FNDGTDEK	95%	2.8759
							GIRPAINVGLSVSR	95%	3.4554
							GmSLNLEPDNVGVVVFGNDK	95%	6.0585
							GYLDKLEPSK	95%	2.852
							HALIIYDDLSK	95%	3.9081

							ILGADTSVDLEETGR	95%	4.782
							ISVREPmQTGIK	95%	2.601
							ITKFESAFLSHVVSQHQSLLGNIR	95%	5.3854
							LKEIVTNFLAGFEP	95%	4.1789
							LTDADAmK	95%	2.1065
							LYcIYVAIGQK	95%	3.51
							NVQAEEMVEFSSGLK	95%	4.5882
							PAINVGLSVSR	95%	3.7312
							QGQYSPmAIEEQVAVIYAGVR	95%	4.9203
							RSTVAQLVK	95%	2.7258
							RTGAIVDVPVGDELLGR	95%	3.6478
							STVAQLVK	95%	2.3519
							TGAIVDVPVGDELLGR	95%	5.1034
							TGTAEMSSILEER	95%	3.9229
							TSIAIDTIINQK	95%	3.3719
							VGSAAQTR	95%	2.1424
							VLSIGDGIAR	95%	2.5852
							VVDALGNAIDGK	95%	3.7023
55 kD spot (ATPα-ATPγ)	Figure S1	ATP synthase alpha chain	P15999	59,755.20	100%	18	APGIIPR	95%	1.5159
							AVDSLVPIGR	95%	1.6537
							EAYPGDVFYLHSR	95%	3.0889
							EIVTNFLAGFEP	95%	2.8563
							EVAAFAQFGSDLDAATQQLLSR	95%	7.2335
							FESAFLSHVVSQHQSLLGNIR	95%	6.4421
							GIRPAINVGLSVSR	95%	4.0077
							GmSLNLEPDNVGVVVFGNDK	95%	6.4425
							GYLDKLEPSK	95%	2.8014
							HALIIYDDLSK	95%	3.6965
							ILGADTSVDLEETGR	95%	4.5912
							LELAQYR	95%	1.8779
							LTDADAMK	95%	1.3226

							QGQYSPmAIEEQVAVIYAGVR	95%	4.8712
							TGAIVDVPVGDELLGR	95%	4.5132
							TGTAEmSSILEER	95%	3.2765
							VLSIGDGIAR	95%	1.4765
							VVDALGNAIDGK	95%	3.3351
30 kD spot (ATPα-ATPγ)	Figure S1	ATP synthase gamma chain	P35435	31,191.00	100%	5	EVmIVGIGEK(I)	95%	2.7065
							KHLIIGVSSDR	95%	3.3678
							NASDmIDKLTLTFNR	95%	3.4726
							THSDQFLVSFK	95%	3.7985
							VYGTGSLALYEK	95%	3.5167
d5NEM labeling (ATPα-C244)	Figure S2	ATP synthase alpha chain	P15999	59,755.20	100%	38	APGIIPR	95%	1.3489
							AVDSLVPIGR	95%	2.8781
							DNGKHALIIYDDLSK	95%	4.1946
							EAYPGDVFYLHSR	95%	3.3131
							EIVTNFLAGFEA	95%	3.6765
							ELIIGDR	95%	1.748
							EPMQTGIKAVDSLVPIGR	95%	2.017
							EVAAFAQFGSDLDAATQQLLSR	95%	7.9702
							FEHAFLAHVISQHQALLGNIR	95%	6.7836
							FNDGTDEK	95%	2.6578
							GIRPAINVGLSVSR	95%	4.1464
							GmSLNLEPDnVGVVVFGnDK	95%	6.6646
							GYLDKLEPSKITK	95%	2.485
							HALIIYDDLSK	95%	3.9478
							ILGADTSVDLEETGR	95%	4.8392
							ISEQSDAKLK	95%	3.3934
							LELAqYR	95%	3.2336
							LIKEGDIVKR	95%	2.8852
							LKEIVTnFLAGFEA	95%	4.9688
							LTDADAMK	95%	2.5073
							LTELLKQGQYSPmAIEEQVAVIYAGVR	95%	5.1579

							Lyc(d5NEM)IYVAIGQK	95%	3.2883
							Lyc(NEM)IYVAIGQK	95%	3.4234
							NVqAEEMVEFSSGLK	95%	6.1064
							PAInVGLSVSR	95%	4.3618
							qGQYSPMAIEEQVAVIYAGVR	95%	6.0312
							QTGKTSIAIDTIINQK	95%	3.4024
							RLTDADAMK	95%	2.909
							RSTVAQLVK	95%	2.5419
							RTGAIVDVPVGEELLGR	95%	3.8727
							RVGLKAPGIIPR	95%	2.9758
							STVAqLVK	95%	3.1759
							TDGkISEQSDAK	95%	3.9635
							TGAIVDVPVGEELLGR	95%	5.0877
							TGTAEVSSILEER	95%	4.0023
							TSIAIDTIInQK	95%	4.7536
							VGSAAQTR	95%	1.827
							VLSIGDGIAR	95%	3.2334
							VVDALGNAIDGKGPVGSK	95%	4.4578
							YTIVVSATASDAAPLqYLAPYSGc(NEM)SmGEYFR		
d5NEM labeling (ATPα-C294)	Figure S2	ATP synthase alpha chain	P15999	59,755.20	100%	8	DDLSKqAVAY	95%	2.3648
							GGGSLTALPVIETqAGDVSAY	95%	5.1965
							IPTNVISITDGQIF	95%	3.7358
							LAGFEA	95%	1.4509
							LDKLEPSKITKF	95%	4.5564
							NDGTDEKKKLY	95%	3.0621
							RDNGKHALIIY	95%	2.5562
							SGc(d5NEM)SMGEYF	95%	2.1353
							SGc(NEM)SMGEYF	95%	1.9656
d5NEM labeling (ATPγ-C103)	Figure S2	ATP synthase gamma chain	P35435	31,191.00	100%	11	ADIKVPEDKK	95%	3.3623
							EVMLVGIGDK	95%	3.079
							GLc(d5NEM)GAIHSSVAK	95%	3.4929

							GLc(NEM)GAIHSSVAK	95%	3.1747
							KHLLIGVSSDR	95%	3.986
							KPPTFGDASVIALELLNSGYEFDEGSIIFNR	95%	6.0447
							MTAMDnASK	95%	3.3255
							NASEmIDKLTLTFNR	95%	4.3307
							nEVATLQAAGK	95%	3.366
							QMKNEVATLQAAGK	95%	3.0464
							THSDQFLVSFKEVGR	95%	4.4431
							VYGVGSLALYEK	95%	4.0552
55 kD band Glutathiony-lation	Figure S3	ATP synthase alpha chain	P15999	59,755.20	100%	29	APGIIPR	95%	1.3243
							AVDSLVPIGR	95%	3.2115
							EAYPGDVFYLHSR	95%	3.3256
							EPMQTGIK	95%	1.6896
							EVAAFAQFGSDLDAATQQLLSR	95%	7.497
							FESAFLSHVVSQHQSLLGNIR	95%	5.8887
							GIRPAInVGLSVSR	95%	4.2934
							GmSLNLEPDNVGVVVFGnDK	95%	6.6572
							GYLDKLEPSK	95%	2.8295
							HALIIYDDLSK	95%	3.6833
							ILGADTSVDLEETGR	95%	4.8259
							ISEQSDAkLK	95%	2.6263
							LELAqYR	95%	3.1055
							LKEIVTnFLAGFEP	95%	4.6336
							LTDADAMK	95%	2.0018
							Lyc(GLU)IYVAIGQK	95%	3.7852
							NVqAEEMVEFSSGLK	95%	6.1377
							PAInVGLSVSR	95%	4.3925
							QGQYSPMAIEEQVAVIYAGVR	95%	5.5783
							RLTDADAMK	95%	2.5176
							RSTVAQLVK	95%	2.4779
							RTGAIVDVPVGDELLGR	95%	3.9762

							STVAqLVK	95%	2.5067
							TGAIVDVPVGDELLGR	95%	4.4569
							TGTAEMSSILEER	95%	3.8415
							TSIAIDTIInQK	95%	4.9325
							VLSIGDGIAR	95%	2.7933
							VVDALGnAIDGK	95%	4.9614
							YTIVVSATASDAAPLqYLAPYSGc(GLU)SmGEYFR	95%	5.0649

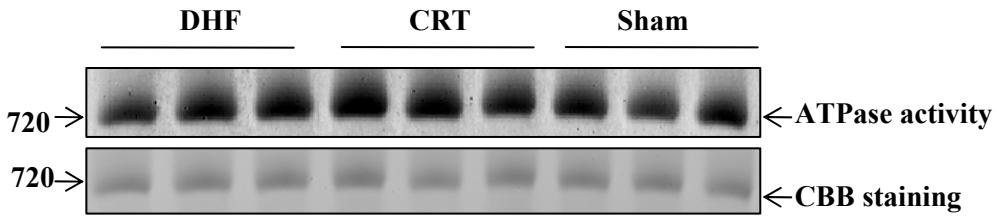
Online Table III.

Optimization for Rescue ATPase activity by in vitro expression ATPγ in endogenous gene knockdown HEK 293 cells

Parameter	Transfection reagent		Expression plasmid			siRNA	
	Co-transfection	Sequential transfection	Quantity (6 Well plate)	Construct	Combination	Target sequence	Concentration
Optimization	Lipofectamine 2000	RNAimax (siRNA, 1st day)	100 ng	ORF with C-term Flag tag	gamma	si189 (CDS)	5 nM
	Lipofectamine RNAimax	Lipofectamine 2000 (plasmid, 2nd day)	600 ng	ORF without tag	gamma+alpha	SI02654484 (3UTR)	10 nM
			1000 ng	Full length cDNA	gamma+beta	SI00306894 (3UTR)	20 nM
			1500 ng		gamma+beta+alpha		30 nM
			2500 ng				

Online Figure I

A



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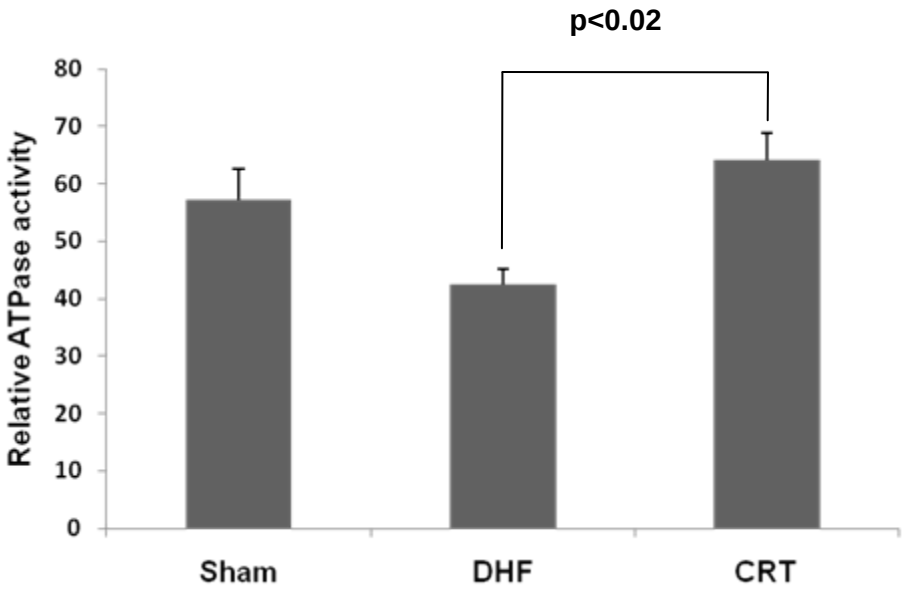
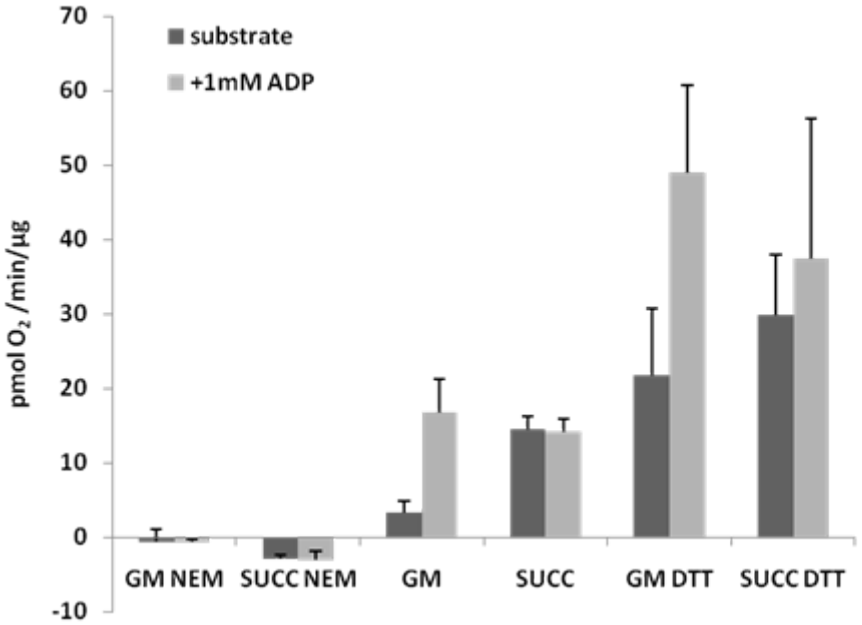
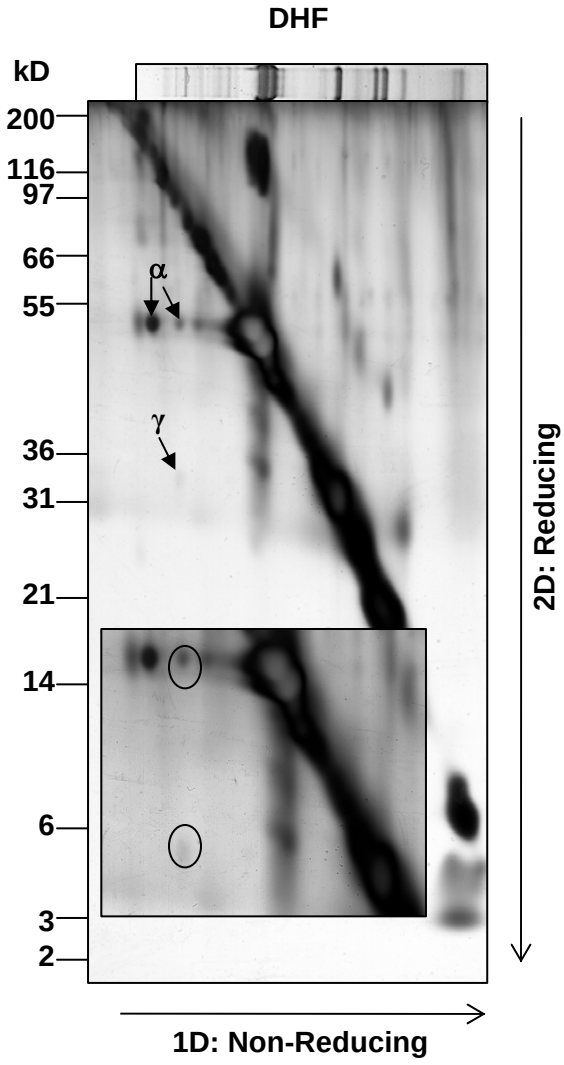


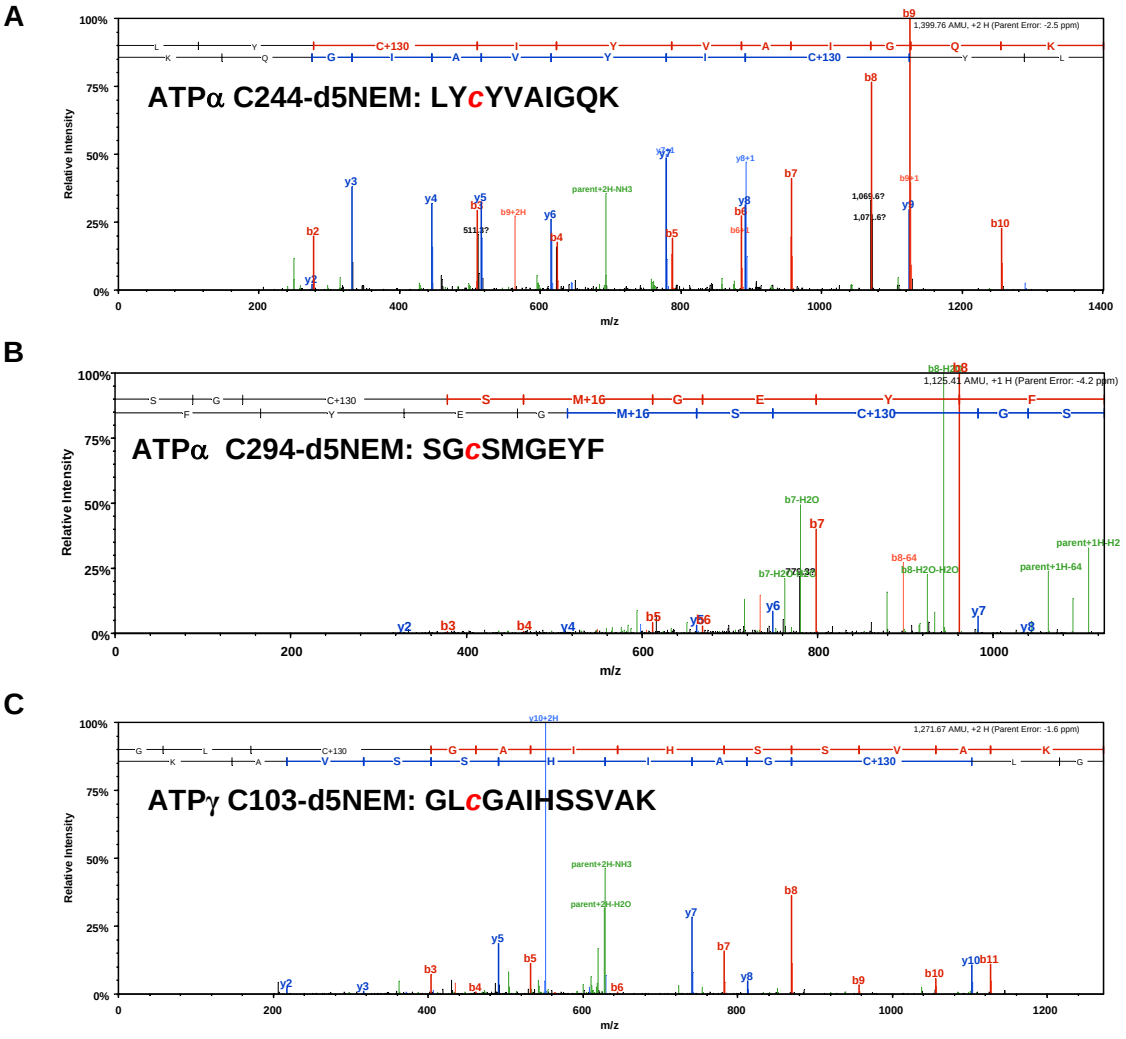
Online Figure II



Online Figure III



Online Figure IV



Online Figure V

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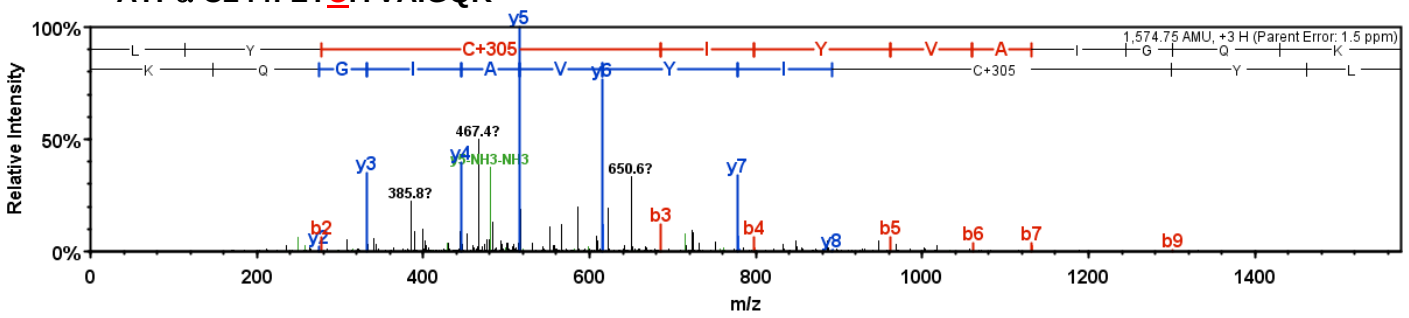

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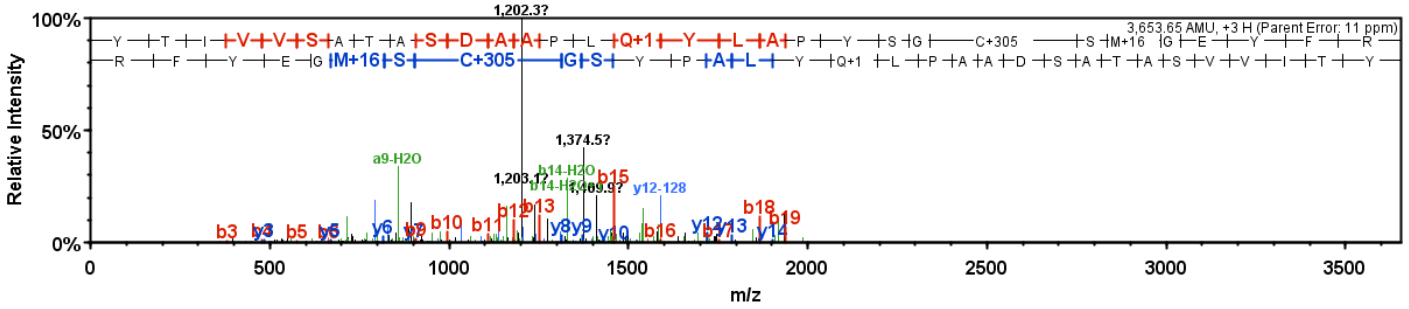
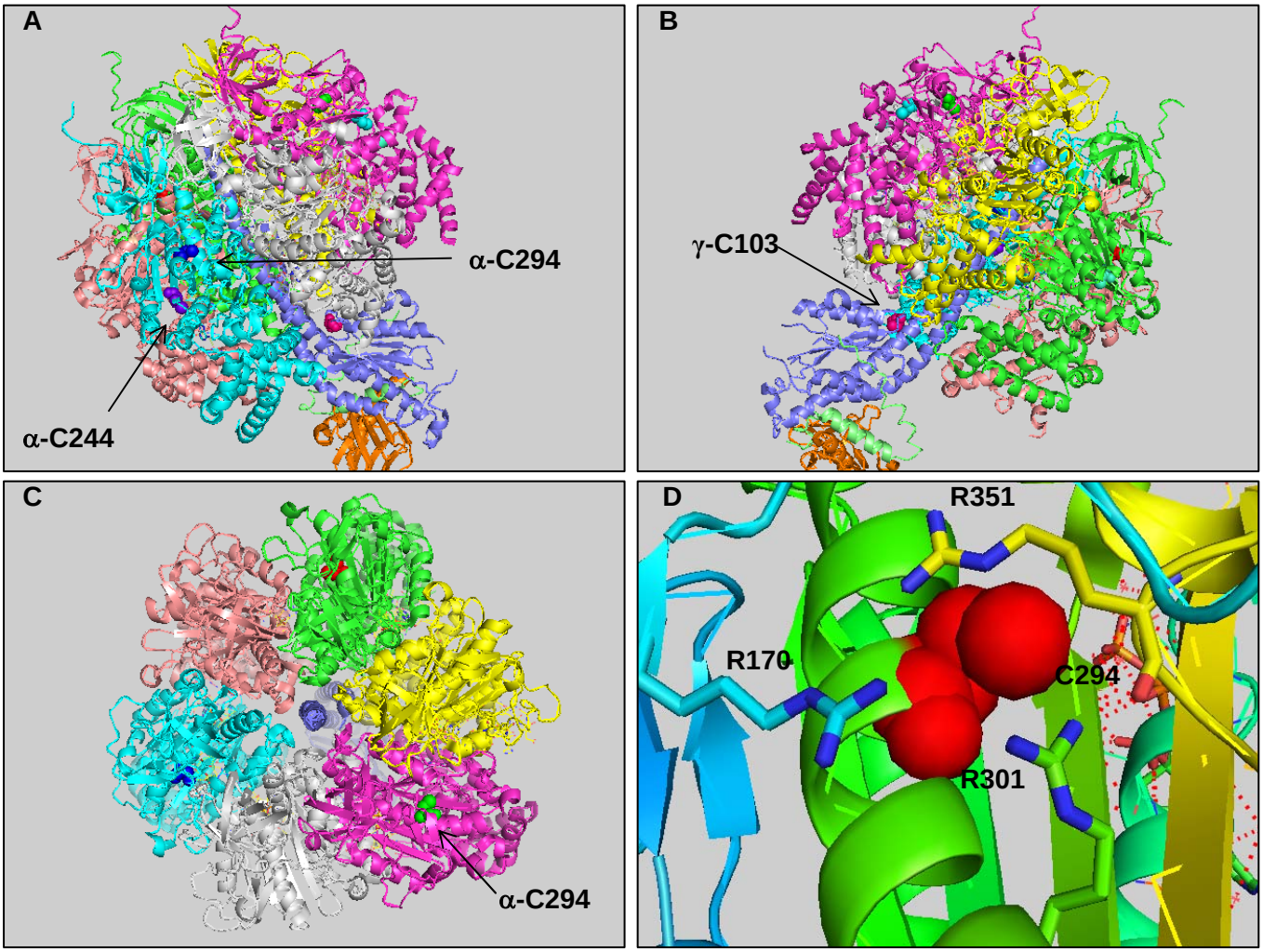
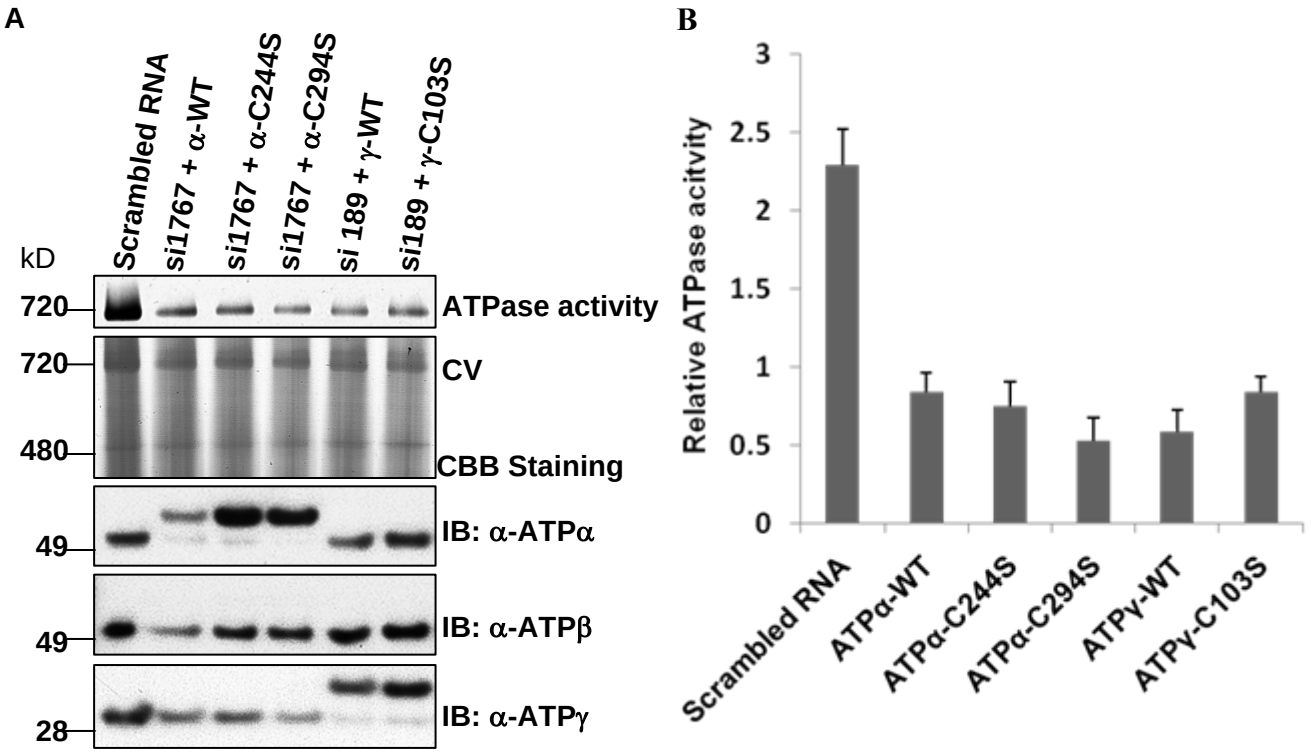


Online Figure VI



Online Figure VII



Online Figure VIII