Recombinant Mitochondrial Transcription Factor A with N-terminal Mitochondrial Transduction Domain Increases Respiration and Mitochondrial Gene Expression in G11778A Leber's Hereditary Optic Neuropathy Cybrid Cells

Shilpa Iyer[1*], Ravindar R. Thomas[1*], Francisco R. Portell[2], Lisa D. Dunham[1], Caitlin K. Quigley[1] and James P. Bennett, Jr.[1a]

[1]Center for the Study of Neurodegenerative Diseases and Morris K. Udall Parkinson's Research Center of Excellence, University of Virginia;

[2]Gencia Corporation;

Charlottesville, Virginia

* These authors contributed equally to this work

[a] Corresponding Author :
James P. Bennett, Jr. MD PhD
PO Box 800394
Charlottesville, VA 22908
434-982-0602 (PH)
434-982-1726 (FAX)
bennett@virginia.edu

Diseases involving mitochondrial defects usually manifest themselves in high-energy, post-mitotic tissues such as brain, retina, skeletal and cardiac muscle and frequently cause deficiencies in mitochondrial bioenergetics[1, 2]. We have developed a scalable procedure to produce recombinant human mitochondrial transcription factor A (TFAM) [3-5] modified with an N-terminal protein transduction domain (PTD)[6] and mitochondrial localization signal (MLS) that allow it to cross membranes and enter mitochondria through its "mitochondrial transduction domain" (MTD,=PTD+MLS). *In vitro* studies in a classic mitochondrial disease cell model demonstrated that Alexa488-labeled MTD-TFAM rapidly entered the mitochondrial compartment. MTD-TFAM treatment of these cell lines reversibly increased oxygen consumption (respiration) rates 3-fold, levels of respiratory proteins and mitochondrial gene expression. *In vivo* results demonstrated that respiration increased to lesser degrees in mitochondria from tissues of mice injected with MTD-TFAM. MTD-TFAM can alter mitochondrial bioenergetics and holds promise for treatment of mitochondrial diseases involving deficiencies of energy production.

Mammalian mitochondrial DNA (mtDNA) is a ~16.6 kilobase circular genome that consists of a regulatory control region ("D-loop"), 13 genes for essential catalytic proteins of the ~87 proteins in the electron transport chain (ETC), 22 tRNA's and two ribosomal RNA's that facilitate translation of the mtDNA-encoded ETC proteins in the mitochondrial matrix[1]. The remainder of the ETC proteins and ~1200-1500 of the other mitochondrial catalytic and structural proteins are imported using multi-protein translocase complexes of the outer (TOM) and inner (TIM) mitochondrial membranes that direct protein precursors formed outside mitochondria to their appropriate location by means of specific N-terminal mitochondrial localization sequences [7]. After reaching their final destinations, the localization sequences are cleaved by mitochondrial proteases[7]. Because most catalytic ETC proteins coded by mtDNA are hydrophobic, special mitochondrial chaperones are believed to assist in proper folding and insertion into their respective ETC macrocomplexes[8, 9].

Although some of the basics of mtDNA replication and transcription are known, much is either controversial or remains to be discovered [5, 10-12]. Abnormalities of mtDNA replication and transcription (such as production of deleted species) or translation (due to mutations in tRNA or coding ETC genes) are responsible for illnesses present in childhood or early adulthood involving high energy, post-mitotic tissues such as brain, retina, heart and skeletal muscle [1, 2]. These "mitochondrial" diseases can display variable and overlapping phenotypes, and understanding their genotype-phenotype relationships remains a great challenge[1, 2].

Further insights into understanding mitochondrial genome replication and expression, in addition to development of novel therapies for mitochondrial diseases,

would benefit from technology that allows external manipulation of the mitochondrial genome. Mitochondrial transcription factor A (TFAM) is a member of the high-mobility group (HMG) of DNA-binding proteins that participate in mtDNA replication and transcription[3-5, 13-16]. Genetic deletion of TFAM is embryonic lethal[17], demonstrating its essential role in mitochondrial function. We report here the development of a technology to produce recombinant TFAM engineered with an N-terminal protein transduction domain (PTD), followed by a matrix mitochondrial localization sequence (MLS). We refer to the combination of PTD and MLS as "mitochondrial transduction domain" (MTD) (Figure 1A).

MTD-TFAM was produced initially as a N-terminal 6XHis-SUMO derivative to increase its intracellular solubility and with a rapid induction approach to minimize toxicity. The initial protein extract was treated with benzonase to remove contaminating DNA; 6XHis-SUMO-MTD-TFAM was isolated on a nickel column, eluted and treated with SUMO protease. Subsequent passage through a nickel column isolated the 6XHis-SUMO, and the eluted MTD-TFAM was purified further (Figure 1B)

We then exposed to MTD-TFAM SH-SY5Y neuroblastoma cybrid cells carrying a G11778A mtDNA mutation in the ND4 gene from a patient afflicted with Leber's Hereditary Optic Neuropathy (LHON), a cause of retinal ganglion cell degeneration and blindness in young adults[18, 19]. Incubation with Alexa488-labeled MTD-TFAM revealed rapid entry of MTD-TFAM into the mitochondrial compartment (Figure 1C).

We next investigated if MTD-TFAM could alter the mitochondrial physiology in these LHON cybrid cells. Three consecutive independent experiments were carried out over several months in which LHON cybrid cells at the same initial passage numbers

were treated with MTD-TFAM or buffer control (CTL). The two groups of cells in each of the three independent experiments were passed in parallel to generate adequate cell densities to carry out multiple, simultaneous "high-resolution" oximetry-respiration experiments using intact cells metabolizing glucose[20]. In this approach, basal, ETC complex-dependent and incrementally uncoupled respiration rates were assayed in real time where all metabolic control systems were otherwise intact. Nine million living cells from individual experiments and their CTL were added to each 2 ml. respiration chamber. The basal respiration values were depicted as a function of the same number of live cells expressed as a percentage of the corresponding buffer control cell values at each of the time point. We observed that exposure to MTD-TFAM caused a time-dependent, reversible increase in basal respiration rates that reached a maximal ~3-fold increase over control samples at around 2 weeks (Figure 2A). Our second and third respiration experiments were designed to reproduce the apparent peak effect at ~2 weeks and then explore additional respiration responses before and after the time of apparent peak effect. We observed that peak respiration responses occurred between ~2-2.5 weeks after MTD-TFAM exposure and returned to CTL values over the next week.

Because TFAM is a recognized essential factor for mitochondrial genome replication and transcription, one possible explanation for this result is that MTD-TFAM exposure was increasing mitochondrial gene replication, transcription and translation into respiratory proteins. We used multiplex qPCR for several mitochondrial genes to monitor alterations in mitochondrial gene copy numbers in genomic DNA samples or mitochondrial gene expression in cDNA samples. We show averaged values for three mitochondrial genes (ND2, ND4, CO3) whose copy numbers were normalized to that of

nuclear DNA-encoded 18S rRNA in the genomic DNA or total RNA (cDNA) samples (Figures 2B and C). We found variable and occasionally substantial increases in mitochondrial gene copy numbers in both DNA and RNA (cDNA) samples following exposure of the LHON cybrid cells to MTD-TFAM. Overall the increases in mtDNA genes and gene expression tended to occur at earlier time points. It is currently unknown if the mtDNA gene copy number changes represent complete genomes, replication intermediates, or some combination.

In the third experimental series of LHON cybrid cell samples exposed to MTD-TFAM, we examined the levels of multiple individual ETC proteins with Western blots. We also studied assembly of ETC macrocomplexes with immunohistochemistry using antibodies directed against mtDNA-encoded catalytic subunits of complexes I and IV, compared to that of an antibody against a nuclear genome-encoded component of complex V (ATP synthase) as a marker for general mitochondrial distribution. Our Western blot analysis (Figures 2D and E) revealed that the relative mitochondrial mass in cells, expressed as a ratio of the outer mitochondrial membrane protein mitofilin to that of cytosolic beta actin, ~doubled (1.9-fold) in MTD-TFAM treated cells at the earliest time point examined (9 days) and was slightly below control cells by the last time point (20 days). The levels of a mtDNA-encoded (CIV, subunit 2) and multiple nuclear genome-encoded ETC proteins from several complexes also increased substantially and reversibly in the MTD-TFAM treated cells with the greatest overall increases observed in complex I at day 11 (Figure 3B). Confocal microscopy did not reveal any effects of exposure of the LHON cybrid cells to MTD-TFAM on the proportions of cells (97-

100%) with intact ETC complex I or complex IV macroassemblies (Supplemental Figure 1).

We then treated normal adult male mice with I.P. injections of MTD-TFAM or buffer control and assayed respiration in mitochondrial preparations from brain, heart, and liver. Mice injected twice, 48 hrs apart, with MTD-TFAM sufficient to bind ~100 ug of DNA in each injection showed increased respiration one week later. The greatest relative increase (compared to buffer control) was observed for respiration with complex I substrates (glutamate/malate) in heart mitochondria (Figure 3A). Respiration through several ETC complexes showed small increases for brain, heart and liver. In our mitochondrial preparations we observed substantial differences across tissues in the relative State 3 (+ADP) respiration rates for individual complexes (Figure 3B). Heart mitochondria showed the smallest relative proportion of complex I-mediated respiration, but also had the greatest relative increase in complex I-mediated respiration after MTD-TFAM treatment. It is not yet known whether these respiration changes derive from alterations in expression of mitochondrial ETC genes, mitochondrial mass, or some combination.

In this study, we have shown that the naturally occurring TFAM protein which is essential for mtDNA expression and replication can be engineered with a protein transduction domain and mitochondrial localization signal (MTD-TFAM) so as to be able to enter rapidly into the mitochondrial compartment of cells. After developing a scalable production procedure, we found that incubation for only a few hours with MTD-TFAM, followed by return of cells to regular culture medium, substantially and reversibly increased cell respiration after an interval of ~2 weeks. We observed increases in

endogenous mitochondrial gene (DNA) and gene expression (cDNA) levels that varied in magnitude and timing in each of the three MTD-TFAM treatment experiments. Multiple nuclear genome-encoded and one mitochondrial genome-encoded ETC proteins also increased reversibly after MTD-TFAM exposure of cells. MTD-TFAM injection into mice also increased respiration to a lesser degree in mitochondrial preparations from several tissues.

Our *in vivo* findings with MTD-TFAM injections into mice contrast with a study of human TFAM expression in mice where mtDNA copy number increased without change in respiratory capacity[3]. In contrast, human TFAM expression in mice prevented loss of mitochondrial respiratory capacity in experimental myocardial infarction[21]. While our preliminary findings are encouraging for the therapeutic potential of MTD-TFAM to increase mitochondrial function *in vivo*, much remains to be explored in terms of whether human TFAM can reliably stimulate mouse mtDNA transcription, and the dose-response, timing and reversibility of the increased mitochondrial respiration we observed.

Most interestingly, we also found evidence in the cell culture experiments for a stimulation of mitochondrial biogenesis based on the increase in mitofilin/beta-actin ratios and increases in multiple ETC proteins coded for by the nuclear genome. These findings suggest that MTD-TFAM exposure is capable of triggering in cells a more substantial transcriptional response beyond that associated only with mtDNA replication and gene expression.

These encouraging initial results show that the human mitochondrial genome can now be manipulated from outside the cell to change expression so as to increase mitochondrial respiration, arguably the most essential physiological role of mitochondria.

However, many important questions remain to be answered about this technology and its mechanisms and potential therapeutic applications.

First, it is not known if exogenous MTD-TFAM that has migrated to the mitochondrial compartment has the same intra-mitochondrial localization as endogenous TFAM, which is believed to complex with mtDNA and multiple other proteins in mtDNA-protein complexes known as nucleoids [4, 22-25]. Our observations of increased mitochondrial gene expression after exposure to MTD-TFAM suggest that exogenous MTD-TFAM, which would be cleaved to native TFAM after mitochondrial importation, is capable of entering the matrix and nucleoids and positively regulating mitochondrial transcription and replication. Whether this occurs remains to be determined.

Second, since MTD-TFAM enters the mitochondrial compartment within minutes, based on colocalization observations through confocal microscopy, it is not obvious why there is a 1-2 week interval between MTD-TFAM exposure and increases in mitochondrial gene expression and respiration. Studies to determine more precisely the time courses of MTD-TFAM stimulation of mtDNA replication and gene expression are underway to address this question.

Third, our findings of an apparent global increase in mitochondrial ETC protein expression were unexpected. We have also observed the same phenomenon in cybrid cells made from mtDNA of patients with Parkinson's disease that were treated with MTD-TFAM and examined 10-11 weeks later (Keeney, et al, unpublished data). Further investigation is required on how exposure to a single mtDNA transcription factor can potentially activate a complex mitochondrial biogenesis program.

These initial observations demonstrate that MTD-TFAM can be produced in high purity in a scalable manner in quantities sufficient for *in vivo* studies, and that MTD-TFAM reversibly alters mitochondrial respiratory physiology. Much remains to be characterized about the mechanisms underlying our observations, and the therapeutic potential of MTD-TFAM for treating diseases associated with bioenergetic deficiency is worthy of further investigation. More importantly, our findings show that the mitochondrial genome is no longer an isolated site and can be manipulated from outside the cell with protein transduction technology.

Methods

Expression of MTD-TFAM

The nucleotide sequence corresponding to PTD-MLS-Tfam[i] was subcloned into PE-Sumo3 (Life Sensors). The construct was transformed into Tuner (DE3)pLysS cells (Novagen). Recombinant protein was expressed by the transformed bacteria cultured in Overnight Express TB medium (Novagen), an auto-induction media, supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol in a Bioflo 310 Fermentor/Bioreactor (New Brunswick). Growth and expression of the bacteria culture were performed at 37º C, dissolved oxygen 30%, with variable agitation and airflow. When culture achieved an optical density of 25 the bacteria were harvested and pelleted by centrifugation at 3500 g and stored at -80 degrees.

[1] **SUMO3-11Arg PTD – SODMLS - MatureTFAM**
MGHHHHHHGGMSEEKPKEGVKTENDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLS
MRQIRFRFDGQPINETDTPAQLEMEDEDTIDVFQQQTGGRRRRRRRRRRRGEGDIMG
EWGNEIFGAI AGFLGGE MLSRAVCGTSR QLPPVLGYLGSRQ SSVLASCPKKPVSSYLR
FSKEQLPIFK AQNPDAKTTELIRRIAQRWR ELPDSKKKIYQDAYRAEWQVYKEEISRFKE
QLTPSQIM SLEKEIMD KHLKRKAM TKKKELTLLGKPKRPRSAYN VYVAERFQEA
KGDSPQEKLK TVKENWKNLS DSEKELYIQH AKEDETRYHN EMKSWEEQ MIEVGRKD
LLRRTIKKQR KYGAEEC) KGDSPQEKLK TVKENWKNLS DSEKELYIQH AKEDETRYHN
EMKSWEEQ MIEVGRKD LLRRTIKKQR KYGAEEC

Protein Purification

Cell pellets were resuspended in modified Bugbuster (Novagen) lysis buffer (1 M TBS, 1M Urea, 250mm Sucrose, 15% glycerol, 80mM imidazole). Benzonase (Novagen) was then added to the lysate (250 U/ml) and allowed to incubate under agitation at room temperature for six hours. The lysate was then clarified by centrifugation at 35,000 g for 35 minutes and then 0.2 μ filtered to remove micro-aggregates. Isolation of the vector protein was performed on a GE AKTA purifier utilizing Histrap HP 5mL columns. Imidazole was removed from the eluted protein utilizing Slide-a-lyzer (Pierce) dialysis cassette (20 K MWCO) in dialysis buffer (1M TBS, 1M Urea, 250 mM sucrose, 250 mM NaCl, 20% glycerol.) Sumo protease was then added to the elution to allow for cleavage of the sumo-fusion fragment from the target protein vector. The solution was then applied to a 1mL Histrap (GE) column and the sumo fusion , and other nickel binding contaminants were removed from the solution. The flow-through was collected, dialyzed to remove NaCl (.5M TBS, 1M Urea, 250 mM sucrose) and applied to a Hitrap heparin column to ensure proper conformation of the target vector protein and remove remaining contaminants from the preparation. The elution was then screened via SDS page analysis for proper size, complete removal of sumo fusion, and purity. Western blot analysis was also performed utilizing TFAM antibodies (Santa Cruz) to verify the success of target protein purification. Each lot of protein vector was tested for DNA binding using EMSA (Electrophoretic Mobility Shift Assay) as a measure of biological activity. Protein was stored in 50% glycerol at -20 degrees.

DS9 LHON cell culture

DS9 cybrid cells containing the G11778A LHON mutation in high abundance were a kind gift of Dr. Russell Swerdlow and were created in a SH-SY5Y rho0 cell line by fusion with platelets from a 42 year old male with LHON. Cells were grown in DMEM containing 10% FCS and passed with trypsin.

Exposure to MTD-TFAM

DS-9 cells were grown to ~50% confluency in T25 flasks. MTD-TFAM sufficient to bind ~10 ug of mtDNA, based on EMSA (typically ~100 uL of protein in 50% glycerol solution) was mixed with 50 uL of Roche Expand Long Template PCR buffer #3, water added to 500 uL and incubated for 30 min at 37 degrees. The resulting solution was mixed with 4.5 ml of DMEM and added to DS9 cells in T25 flasks that had regular media removed and had been rinsed with DMEM. The cells were incubated with MTD-TFAM (or buffer control) for 5 hours at 37 degrees, then the MTD-TFAM solution was removed and replaced with regular media.

Whole cell respiration

MTD-TFAM and buffer control cells were passed in parallel, expanded in culture and harvested with trypsin. 4.5 million Trypan-blue excluding cells/ml of DMEM with glucose were added to each chamber of an Oroboros Oxygraph 2 respirometer and studied intact with “high resolution respirometry”. After assay of basal respiration rates, ATP synthase was inhibited with oligomycin and mitochondria were incrementally uncoupled with FCCP injection to estimate maximal uncoupled respiration that was sequentially inhibited with rotenone to inhibit complex I followed by antimycin A/myxothiazole to inhibit complex III.

RT-qPCR analysis of mitochondrial gene expression

Total genomic DNA and total RNA were isolated from cell pellets using All-Prep kits from Qiagen and amounts assayed with Quant-IT DNA and RNA assays (InVitrogen). 1 ug of total RNA was reverse transcribed into cDNA using iScript (BioRad). Levels of 18S rRNA were assayed using SybrGreen detection (BioRad) in genomic DNA or cDNA samples and Roche human genomic DNA as standard. Copy numbers of 12S rRNA, ND2, CO3 and ND4 mitochondrial genes were assayed in a multiplex qPCR assay (BioRad Powermix) using a full-length PCR product of human mtDNA as standard. All RT-qPCR was carried out in an iQ5 thermocycler (BioRad) using primers and probes designed with Beacon Designer software. Levels of mitochondrial genes were normalized to 18S rRNA in either DNA or RNA (cDNA) samples.

*Sfa*N1 restriction analysis

A PCR product in the ND4 gene spanning the *Sfa*N1 site removed by the G11778A mutation was amplified from genomic DNA or cDNA and digested with *Sfa*N1. Digestion products were analyzed using automated electrophoresis (Experion, BioRad).

Western blot analysis of ETC proteins

100 ug of total cell protein were loaded onto 4-12% Bis-Tris CriterionTM precast gels (BioRad) and separated. The proteins were then transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen). Complex I subunits were detected by immunoblotting using the following antibodies purchased from Mitosciences: MS111 against subunit NDUFA9 at 1.125 µg/mL, MS110 against subunit NDUFS3 at 0.5 µg/mL, MS109 against an 8kDa subunit at 1 µg/mL, MS107 against subunit NDUFB4 at 0.5 µg/mL, and MS105 against subunit NDUFB8 followed by an IRDye® 800 goat anti-mouse secondary at 1:15,000 (Li-cor). Subunits from complexes I-V were detected by

immunoblotting using the Mitoprofile® human total OXPHOS complexes detection kit at 1:575 (Mitosciences) followed by an IRDye® 800 goat anti-mouse secondary at 1:25,000 (Li-cor). Mitofilin was assayed as an estimate of mitochondrial mass in each sample and was detected by immunoblotting using the MSM02 antibody at 2 µg/mL purchased from Mitosciences followed by an IRDye® 800 goat anti-mouse secondary (Li-cor). Beta-actin was used as a loading control and was detected by immunoblotting using a polyclonal beta-actin antibody purchased from Abcam followed by an IRDye® 680 goat anti-rabbit secondary at 1:15,000 (Li-cor). The membranes were visualized and bands quantiated using the Odyssey infrared imaging system (Li-cor).

Immunohistochemical analysis of ETC macrocomplex assembly

Cells were grown to subconfluence (70-80%) in polylysine coated Mattek dishes, fixed with 4% parafomaldehyde in 0.1M PBS for 20min., rinsed in PBS and stained following the protocol recommended by Mitosciences (www.mitosciences.com) Briefly, fixed cells were treated with antigen retrieval buffer (5% urea in 100mM Tris, pH 9.5) at 95°C for 20 min, followed by 3 washes with PBS, 0.2% Triton-X-100 in PBS for 15 min and 3 more washes with PBS. Cells were blocked in 10% goat serum for one hour at room temp.; primary antibodies (Mitosciences) were added to each dish in 10% goat serum: anti-complex 1 (MS602-1 NDUFB4) 50µg/ml; anti-complex 4, subunit 1 (MS602-IV) 6.25µg/ml; anti-complex 5α control (MS602-CVα) 1.25µg/ml and incubated at 4°C overnight. Cells were washed in 1% goat serum; secondary antibodies were added to each as recommended by the protocol and incubated at room temperature for 2 hours. Following 3 washes in 1% goat serum, dishes were rinsed twice in PBS, then 45nM TOTO-3 (Invitrogen, USA) was added for 10 min. at room temp. to stain nuclei. Cells

were washed twice in PBS and mounted with Vectashield (Vector Labs, Burlingame, CA). Single plane confocal images were made using 60x objective on an Olympus IX70 microscope with Oympus Confocal Laser Scanning System and argon ion and helium/neon ion lasers.

MTD-TFAM injections and mitochondrial fraction respiration

MTD-TFAM capable of binding ~100ug of DNA (~0.45 mg purified protein in 1 ml of 50% glycerol solution) was dialyzed against 5% glycerol in PBS then concentrated to ~0.5 ml using Amicon centrifuge filters. Testing of the Amicon flow-through revealed no DNA binding capacity. Adult male mice were injected IP with the dialyzed, concentrated MTD-TFAM (or buffer control). Each mouse received 2 IP injections 48 hrs apart of either MTD-TFAM or buffer and was sacrificed 7 days after the first injection. In parallel brain, heart, and equal weights of liver were removed, weighed and homogenized in a Teflon-glass homogenizer. P2 pellets were kept on ice until resuspension in "MiRO5" mitochondrial respiration media (http://www.oroboros.at/index.php?id=524#857). One fourth of each tissue's P2 pellet from MTD-TFAM or buffer control mouse was added to the respirometer chamber, followed by sequential additions of substrates to assay State 3 respiration (+ ADP) through complex I (glutamate/malate), complex II/III (rotenone + succinate) and complex IV (antimycinA/myxothiazole+ascorbate/TMPD+ cytochrome C) followed by sodium azide.

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Figure Legends

Figure 1. (a) schematic of MTD-TFAM. PTD=11 arginine protein transduction domain. SOD2=mitochondrial localization sequence for matrix enzyme SOD2; HMG=high mobility group domain. (b) (left) Coumassie stained gel showing purification of recombinant MTD-TFAM. Lanes 1=SUMO-TFAM; 2= after removal of SUMO; 3, 4= dialysis; 5, 6=further column purification. (right) agarose gel EMSA of circular human mtDNA incubated with increasing amounts of purified MTD-TFAM. (c) MTD-TFAM was labeled with Alexa488 and incubated with LHON cybrid cells where mitochondria were stained with MitoTracker Red. Shown are images obtained 39 minutes after exposing the cells to Alexa488-MTD-TFAM.

Figure 2. Effects of MTD-TFAM on respiration and mitochondrial gene expression. (a) In three independent, consecutive experiments G11778A LHON cells were incubated with 32 ng of MTD-TFAM in 4 ml of DMEM media (or DMEM media with buffer salts) for 4 hours, then rinsed and passed in parallel in regular DMEM growth media. At varying times after MTD-TFAM incubation, aliquots of cells were harvested with trypsin and utilized for intact cell respiration at densities of

4.5 million live cells/ml. Shown are basal respiration rates in glucose for MTD-TFAM exposed cells expressed as percentages of simultaneously assayed CTL cells. (b,c) Aliquots of cells used for respiration experiments in (a) were extracted for genomic DNA and total RNA using All-Prep kits; 1 ug RNA was reverse transcribed to cDNA. DNA and cDNA samples were assayed using qPCR and multiplex TaqMan probes for ND2, ND4 and CO3 genes with a linear complete mtDNA PCR product serving as external standard. Levels of 18S rRNA assayed using SyberGreen qPCR and Roche genomic DNA as external standard served as the normalization gene. Shown are 18S rRNA normalized averaged copy numbers (ND2, ND4, CO3) for MTD-TFAM expressed as percentages of CTLs at each time point. (d) Aliquots of cells from the third MTD-TFAM experiment were analyzed for ETC proteins using a panel of mouse monoclonal antibodies from MitoSciences. Beta actin served as loading control. (e) Quantitation of Western blot data from (d) for the cells exposed to MTD-TFAM and expressed as percentage of buffer CTL.

Figure 3. Effects of MTD-TFAM injected in vivo on mitochondrial respiration in mouse organs. 0.45 mg of MTD-TFAM was dialyzed against 5% glycerol in PBS, concentrated to ~0.5 ml using an Amicon filter and injected IP on days 1 and 3. On day 7 mice were sacrificed and organs from MTD-TFAM or buffer CTL injected mice were processed in equal weights in parallel to make P2 pellets, which were studied for substrate-specific State 3 (+ADP) respiration. (a) Shown are averaged (n=3 pairs) respiration rates for MTD-TFAM injected mice for brain,

heart, and liver mitochondria expressed as percentages of simultaneously assayed CTLs. CI=complex I (rotenone-sensitive malate/glutamate); CII=complex II (antimycinA/myxathiazole-sensitive succinate); CIV=complex IV (azide-sensitive ascorbate/TMPD/cyt C). (b) ratios of CII and CIV respiration to CI presented as averages from 5-6 CTL mitochondrial preparations.

Supplemental Figure 1. ETC complex assembly remains unaltered in LHON cybrid cells treated with MTD-TFAM A: LHON cybrid cells treated with MTD-TFAM or buffer control (CTL) were analyzed for Complex I subunit assembly (shown in green) by immunocytochemistry. B: In panel B, the same set of LHON cybrid cells were analyzed for Complex IV subunit assembly shown in green. In both the panels (A and B), the nuclei in blue are stained with TOTO3 and Complex V-α in red, has been used as a control for mitochondria. Overlay in yellow indicates ETC complex subunits overlapping with the mitochondria. Scale bars, 10μm.

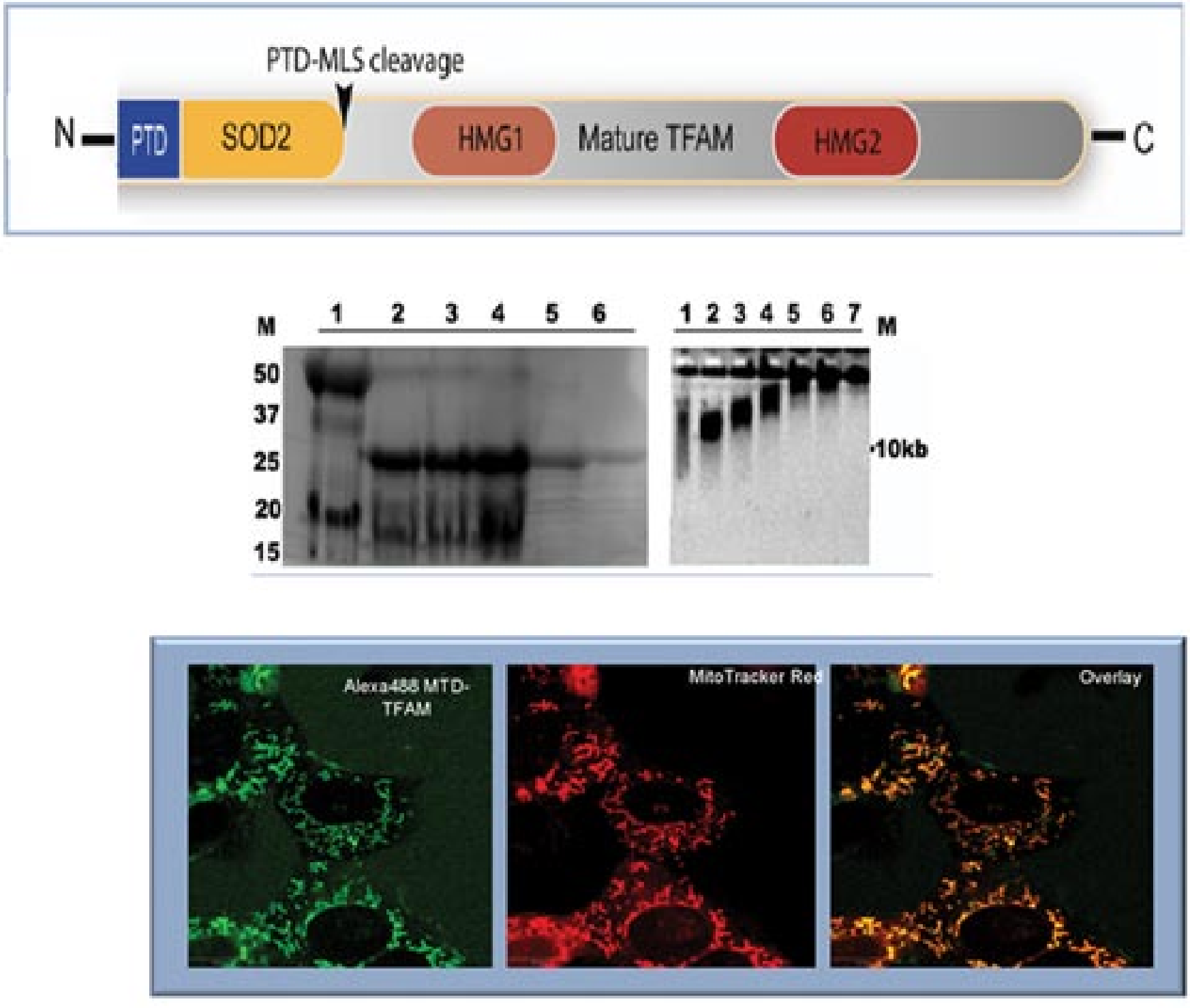

Figure 1- Iyer, et al

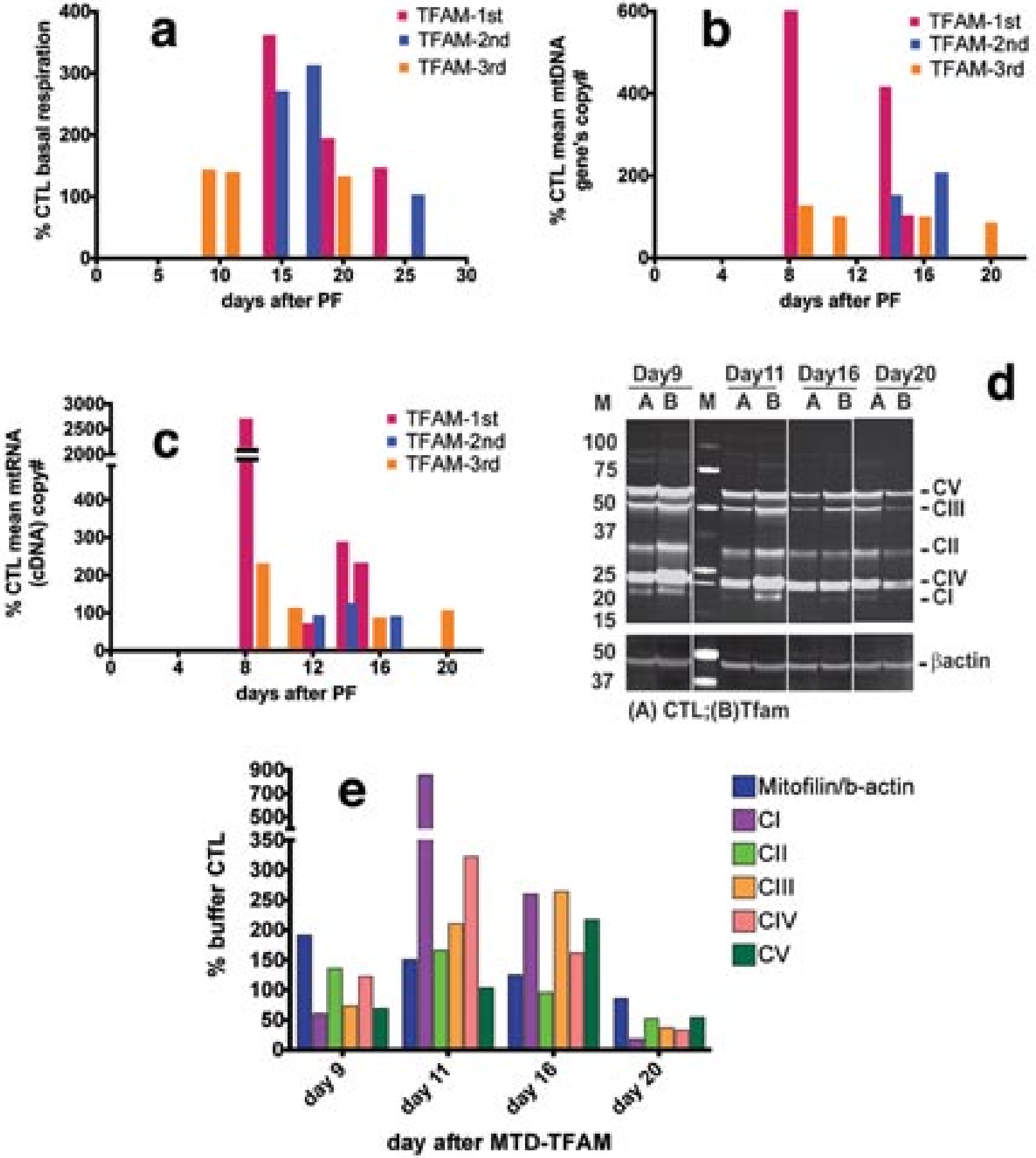

Figure 2- Iyer, et al

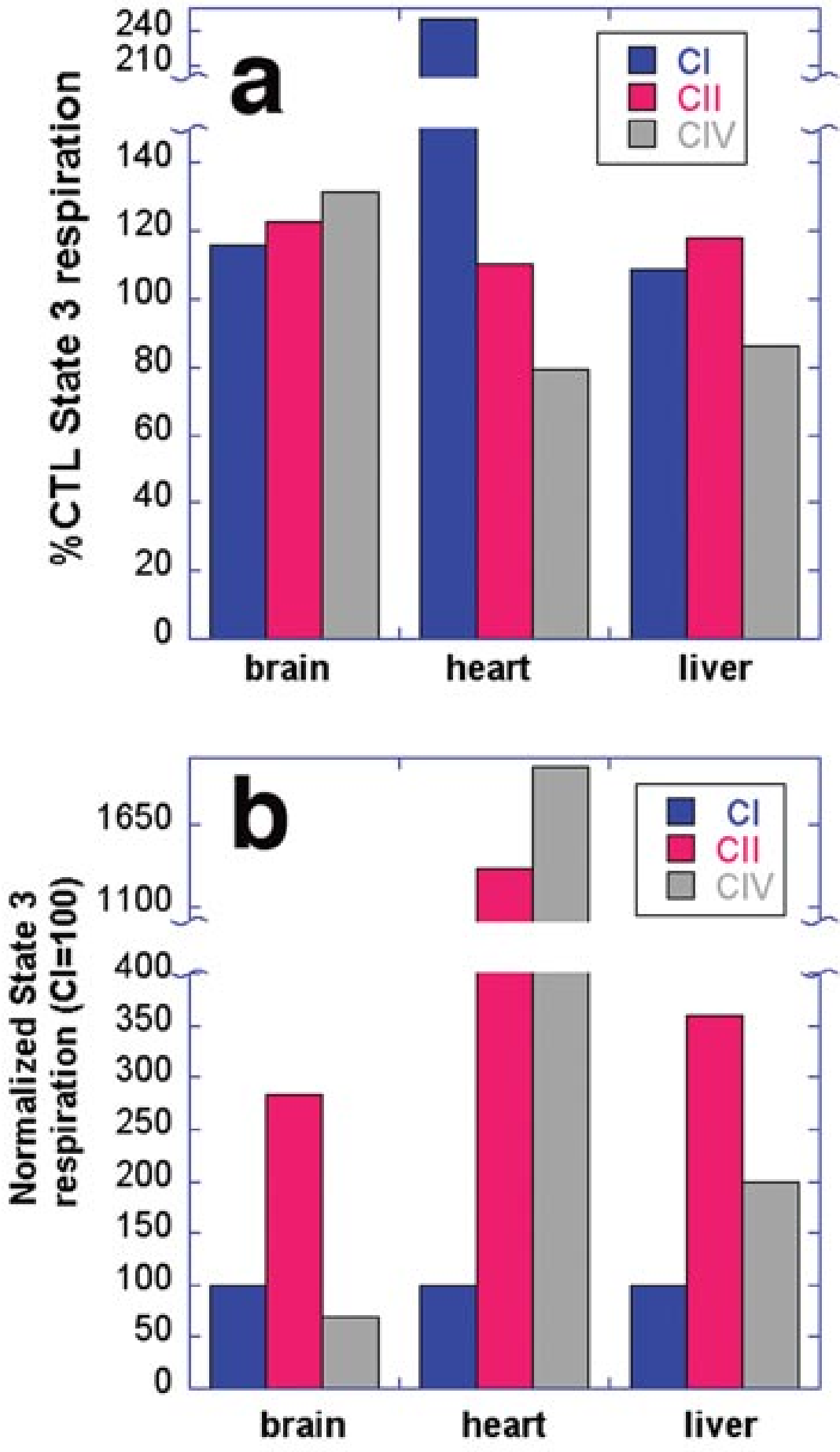

Figure 3-Iyer, et al