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## Supplemental Data

# Superoxide Flashes in Single Mitochondria

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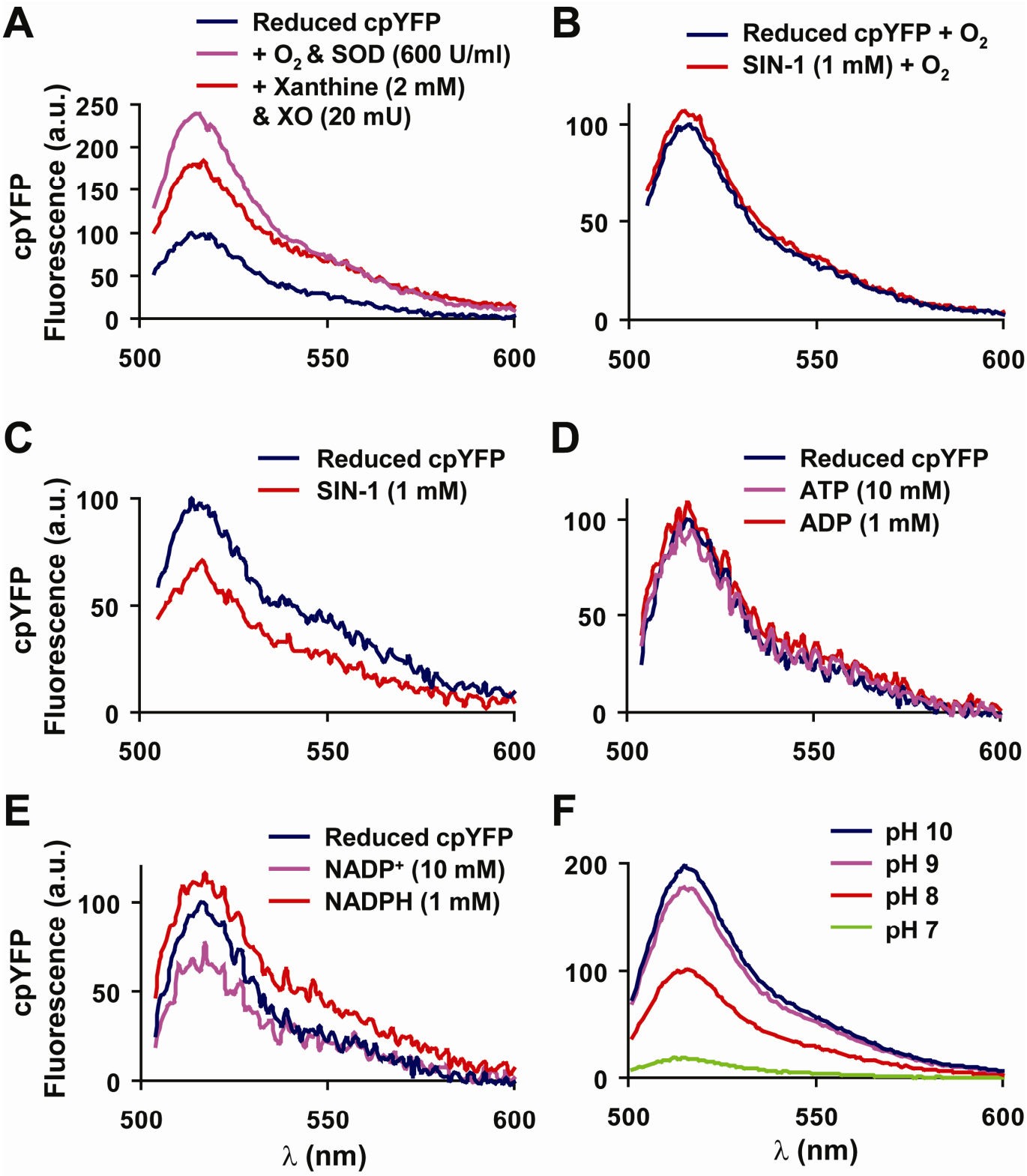

A
Reduced cpYFP
+ O2 & SOD (600 U/ml)
+ Xanthine (2 mM)
& XO (20 mU)
cpYFP Fluorescence (a.u.)
250
200
150
100
50
0
500
550
600
B
Reduced cpYFP + O2
SIN-1 (1 mM) + O2
100
50
0
500
550
600
C
Reduced cpYFP
SIN-1 (1 mM)
cpYFP Fluorescence (a.u.)
100
50
0
500
550
600
D
Reduced cpYFP
ATP (10 mM)
ADP (1 mM)
100
50
0
500
550
600
E
Reduced cpYFP
NADP+ (10 mM)
NADPH (1 mM)
cpYFP Fluorescence (a.u.)
100
50
0
500
550
600
λ (nm)
F
pH 10
pH 9
pH 8
pH 7
200
100
0
500
550
600
λ (nm)

Figure S1

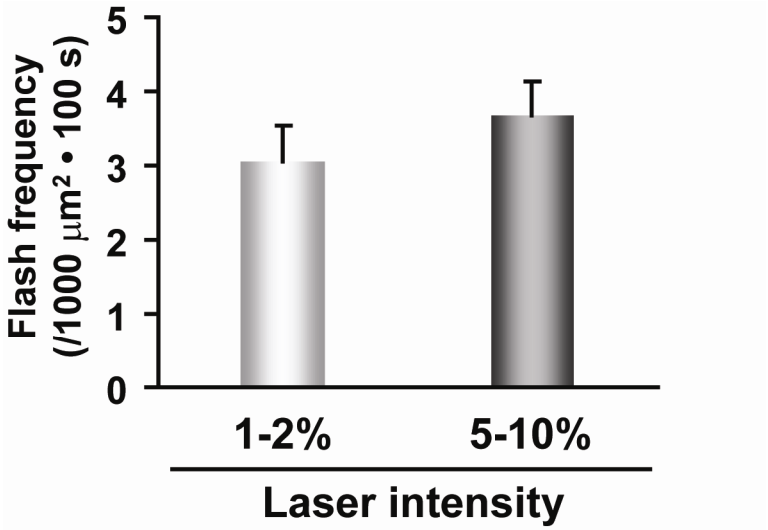
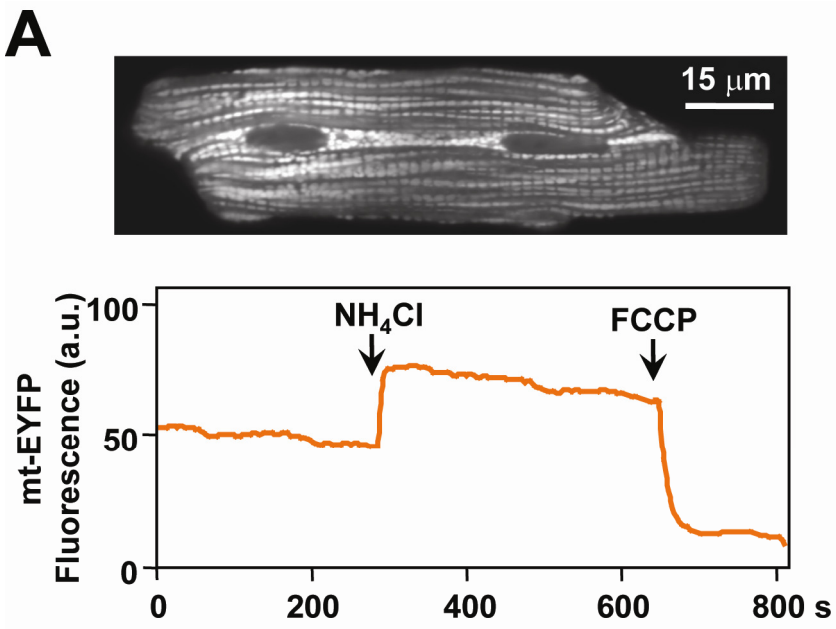

Flash frequency
(/1000 μm² • 100 s)
0
1
2
3
4
5
1-2%
5-10%
Laser intensity

**Figure S2**


A
15 μm
mt-EYFP Fluorescence (a.u.)
100
50
0
NH4Cl
FCCP
0
200
400
600
800 s

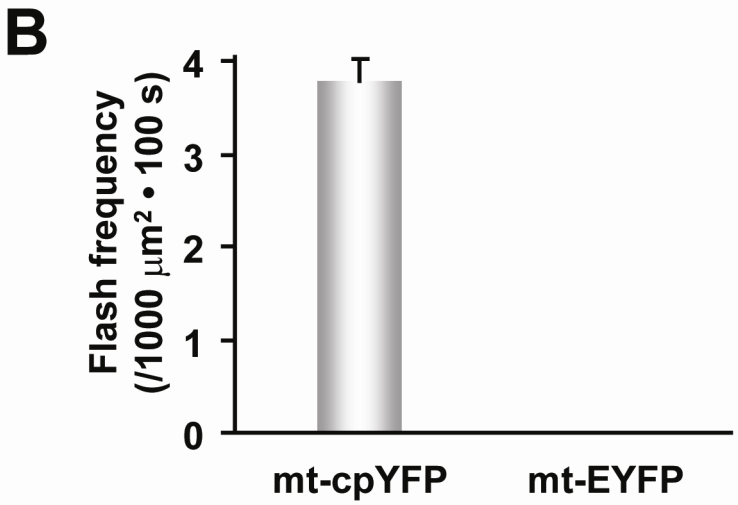

B
Flash frequency (/1000 μm² • 100 s)
4
3
2
1
0
T
mt-cpYFP
mt-EYFP

**Figure S3**

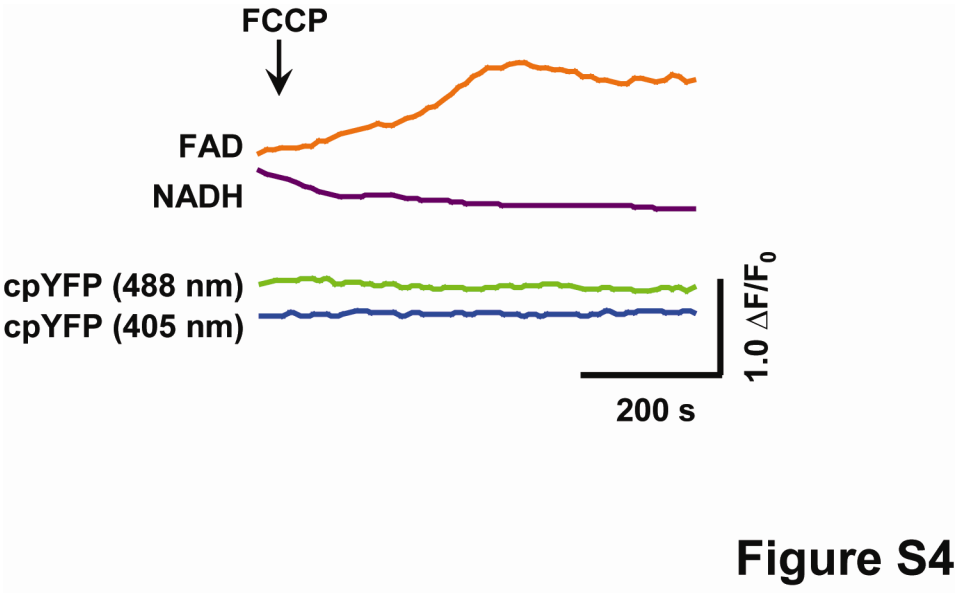



**Figure S4**

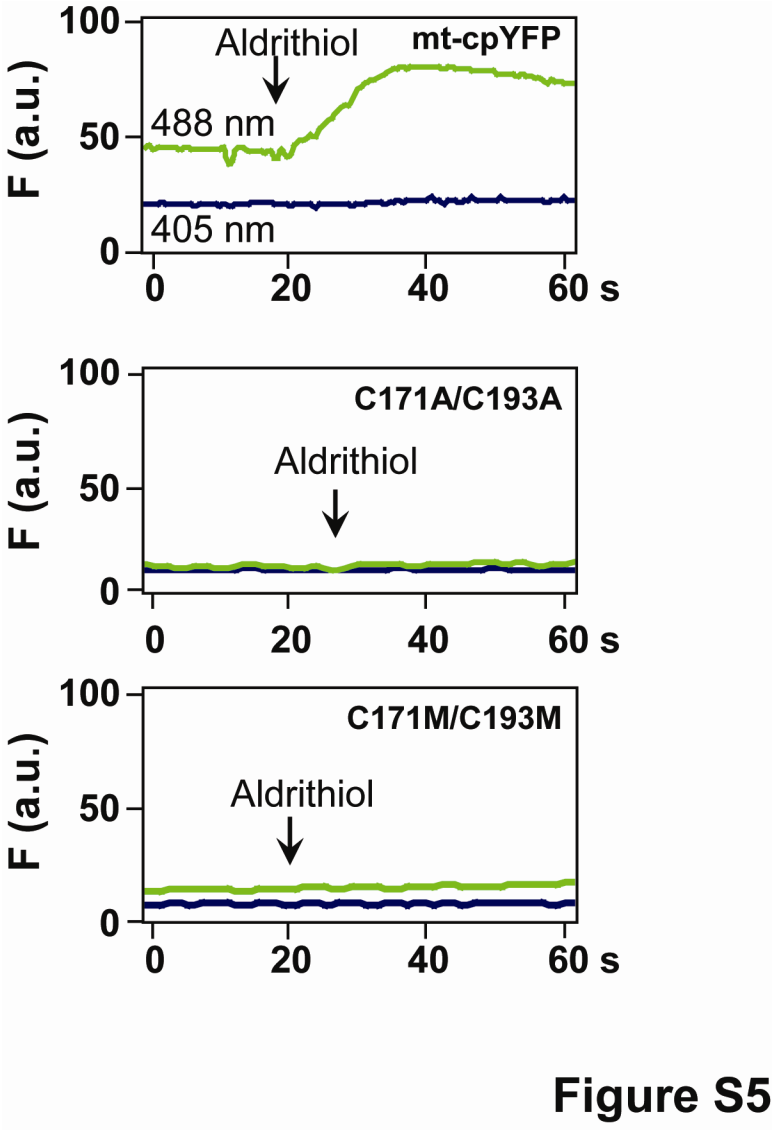

100
50
0
F (a.u.)
Aldrithiol
mt-cpYFP
488 nm
405 nm
0
20
40
60 s
C171A/C193A
Aldrithiol
C171M/C193M
Aldrithiol

**Figure S5**

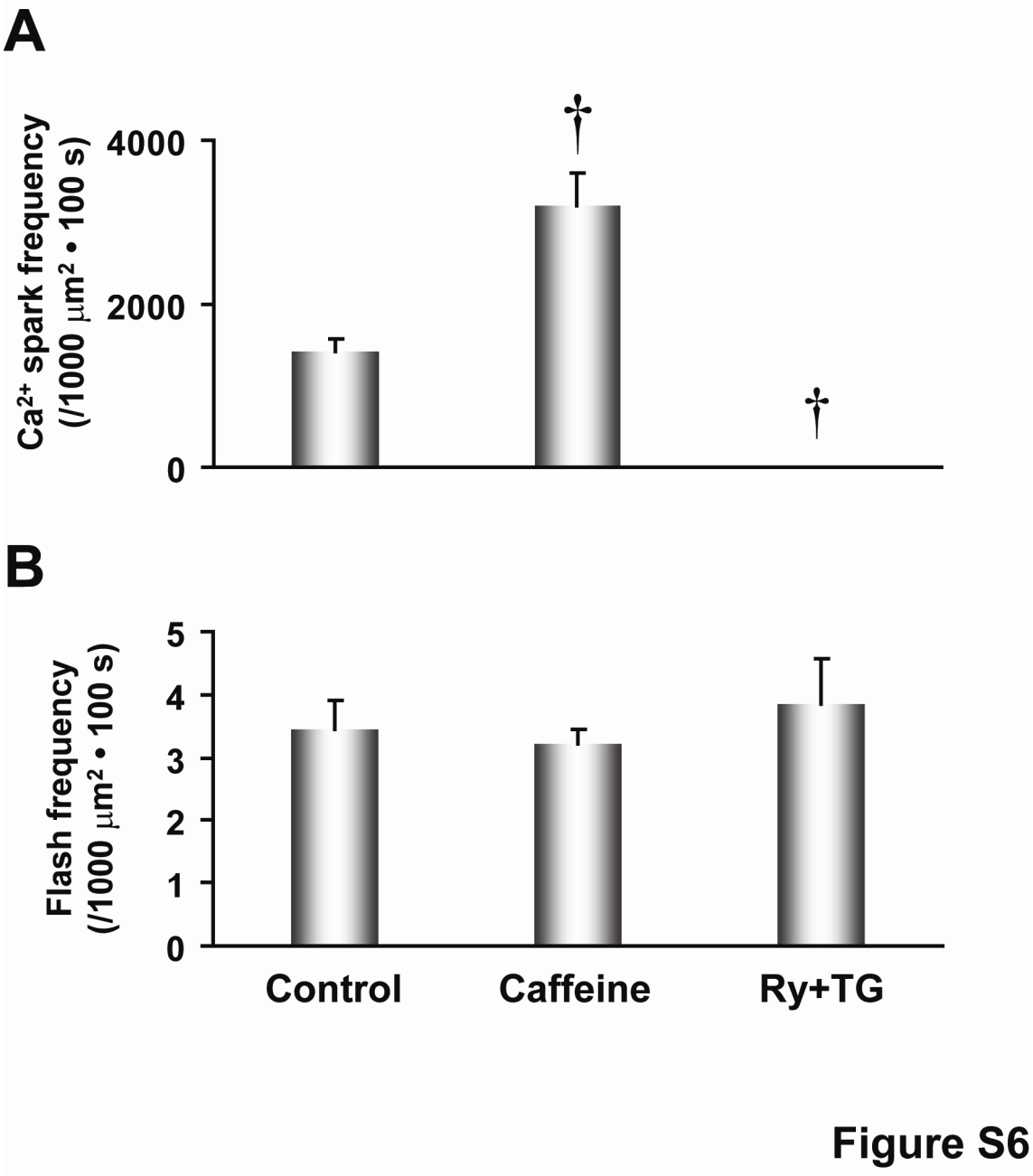

A
Ca2+ spark frequency
(/1000 μm2 • 100 s)
4000
2000
0
B
Flash frequency
(/1000 μm2 • 100 s)
5
4
3
2
1
0
Control
Caffeine
Ry+TG

Figure S6

## SUPPLEMENTAL FIGURE LEGENDS

**Figure S1. cpYFP Responses to (A) Superoxide in the Presence of SOD, (B) Peroxynitrite, (C) Nitric Oxide, (D) ADP/ATP , (E) $NADP^+$/NADPH and (F) pH**

Pre-incubation of cpYFP with Cu/Zn-SOD (600 U/ml) under aerobic conditions abolished the increase in cpYFP fluorescence (488 nm excitation) to xanthine (2 mM) plus xanthine oxidase (20 mU). Peroxynitrite was produced by dissolving SIN-1 (1 mM) in an aerobic solution and nitric oxide was produced by dissolving SIN-1 (1 mM) in an anaerobic solution. The other metabolites used were ADP (1 mM), ATP (10 mM), $NADP^+$ (10 mM), and NADPH (1 mM).

**Figure S2. Superoxide Flash Activity Is not Induced by Photostimulation**

Average superoxide flash frequency was not different between normal (1-2% transmission) and 5-fold higher laser intensity (5-10% transmission). Data are mean ± SEM. n = 12-18 cells.

**Figure S3. The mt-EYFP pH Biosensor Does not Detect Flash-Like Events**

(A) Mitochondrial expression pattern of mt-EYFP in cardiac myocytes (upper panel) and mt-EYFP emission following alkalization (extracellular application of 30 mM $NH_4Cl$) and subsequent acidosis (addition of 40 μM FCCP). (B) Lack of transient mt-EYFP events indicating that flash activity does not reflect brief periods of matrix alkalization. n = 12-53 cells.

**Figure S4. Change in Mitochondrial Redox Potential Does not Alter mt-cpYFP Fluorescence**

Mild mitochondrial uncoupling with FCCP (50 nM) resulted in mitochondrial oxidation as validated by elevated FAD fluorescence and decreased NADH fluorescence (upper panel). However, uncoupling had no effect on mt-cpYFP fluorescence at either 488 or 405 nm excitation (lower panel).

**Figure S5. Cysteine-null Variants of mt-cpYFP (C171A/C193A and C171M/C193M) Are Insensitive to Aldrithiol (1 mM)**

Aldrithiol (1 mM) was applied to cardiac cells expressing either wild type mt-cpYFP (top panel), C171A/C193A (middle panel), or C171M/C193M (lower panel). Note the low basal probe fluorescence in cardiac myocytes expressing the cysteine-null variants. Similar results were observed in 15 cells across 3 independent sets of experiments.

**Figure S6. Lack of a Correlation between $Ca^{2+}$ Spark Frequency and Superoxide Flash Frequency in Cardiac Ventricular Myocytes**

(A) $Ca^{2+}$ spark frequencies in control, in the presence of 1 mM caffeine, and after treatment with ryanodine plus thapsigargin (Ry+TG, 10 μM, 15 min) in cardiac myocytes loaded with the $Ca^{2+}$ indicator, fluo-4. †, $P<0.001$ versus control. $Ca^{2+}$ spark frequency observed in confocal linescan images was expressed as events per 1000 $\mu m^2$ per 100 s by assuming a lateral spark-registering band of 2 μm during line scan imaging. (B) Mitochondrial superoxide flash frequency recorded under the same conditions as (A). Data are mean ± SEM. n = 10-11 cells.

## Supplemental Experimental Procedures

### cDNA Constructs

mt-cpYFP was constructed from mitochondrial targeted ratiometric pericam (rpericamMT) cloned into pcDNA3 (Nagai et al., 2001) by removing nucleotide sequences encoding calmodulin (nt 886-1323) and M13 (nt 49-126) using the gene splicing by overlap extension (SOE) technique (Horton et al., 1989). The final PCR product was digested with HindIII/XbaI and cloned into pcDNA3. cpYFP was constructed from mt-cpYFP by removing nucleotide sequences encoding the 11 amino acid (LSLRQSIRFFK) mitochondrial targeting sequence of cytochrome oxidase subunit IV. Double cysteine-to-alanine and cysteine-to-methionine substitutions in mt-cpYFP (C171A/C193A, and C171M/C193M) were constructed using a standard two-step site-directed mutagenesis strategy. mt-EYFP was from Clontech.

### Generation of Cardiac Specific mt-cpYFP Transgenic Mice

The cardiac specific mt-cpYFP expression vector was constructed by inserting mt-cpYFP between HindIII and EcoRV sites and after the mouse α-MHC promoter in pBKSII-α-MHC-SV40 vector (a kind gift from Dr. Jeffery Robbins, University of Cincinnati). The linearized expression vector was injected into the pronucleus of fertilized mouse oocytes. Genotyping was performed by PCR using specific upstream (5'-AGTTAACCAGGTGAGAATGTT-3') and downstream (5'-CCGATGCCGCTGCGCTT-3') primers.

**RNA Interference**

Two sets of distinct siRNA sequences for cyclophilin D (encoded by *Ppif*) knockdown were designed by using "BLOCK-iT™ RNAi Designer" and transfected into neonatal cardiac myocytes by using lipofectamine RNAiMAX (Invitrogen). Time dependent inhibition of cyclophilin D expression was achieved 3-5 days after gene transfer of either siRNA construct. The sequences are: 5'-GGAAGACAUCUAAGAAGAUTT-3' (sense) and 5'-AUCUUCUUAGAUGUCUUCCTT-3' (anti-sense) for siRNA1, and 5'-GCUUUCCUGACGAGAACUUTT-3' (sense) and 5'-AAGUUCUCGUCAGGAAAGCTT-3' (anti-sense) for siRNA2. The scrambled siRNA sequences: 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (anti-sense) were used as control.

**Spectral Analysis of cpYFP**

cpYFP cDNA (807 bp) was cloned into a prokaryotic expression vector (pRSET) and transferred into *E. coli.* cells (BL21(DE3)LysS) for large-scale protein expression. *In vitro* redox calibration of cpYFP fluorescence was carried out using methods described previously (Hanson et al., 2004). Briefly, under an inert environment, purified cpYFP protein (1 μM) was incubated with either 10 mM reduced DTT or 1 mM aldrithiol for at least 3 hours. Reduced DTT was removed from the solution to enable measurement of cpYFP responses to various ROS and metabolites. The calibration solution contained (in mM) HEPES 75, KCl 125, and EDTA 1 (pH=8.0). Emission and excitation spectra of reduced and oxidized cpYFP in the presence of designated reagents were obtained with a spectrofluorimeter (Model: CM1T10I, HORIBA Jobin Yvon, Inc.) continuously purged

with nitrogen gas.

## Supplemental References

Hanson, G.T., Aggeler, R., Oglesbee, D., Cannon, M., Capaldi, R.A., Tsien, R.Y., and Remington, S.J. (2004). Investigating mitochondrial redox potential with redox-sensitive green fluorescent protein indicators. J Biol Chem *279*, 13044-13053.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene *77*, 61-68.

Nagai, T., Sawano, A., Park, E.S., and Miyawaki, A. (2001). Circularly permuted green fluorescent proteins engineered to sense $Ca^{2+}$. Proc Natl Acad Sci U S A *98*, 3197-3202.