Supplementary Data

Putative Model of Coupling of Mitochondrial Parameters Mediated via *mitoCa^{2+} Changes in* Fus1 WT *and* Fus1 KO *Cells*

Based on the literature data and our results, we propose the following model of coupling of mitochondrial parameters mediated *via* mitoCa^{2+} changes. Generally, after increase in intracellular Ca^{2+} levels, mitoCa^{2+} uptake induces the Krebs cycle leading to the elevation of $\Delta\mu H^+$, reactive oxygen species (ROS) production, and stimulation of the redox chain NADH→NADPH→thiols. Balanced reduction of thiols maintains low ROS levels and protects cells from the oxidative stress.

Because $\Delta\mu H^+$ is a driving force behind mitoCa^{2+} uptake, depolarization will reduce the uptake thus limiting the damage induced by mitoCa^{2+} overload. Due to dependence of Ca^{2+} channels on redox environment, mitoROS changes could be transmitted into alterations in Ca^{2+} oscillations triggering changes in the NF-κB and NFAT transcriptional activities in T cells.

Excessive mitoCa^{2+} accumulation induced by Ca^{2+} overload leads to increased $\Delta\mu H^+$ followed by enhanced ROS production. Since ROS levels are determined by the balance of oxidation/reduction reactions, elevation of ROS will be accompanied by fast thiol oxidation and mitochondrial permeability transition pore (mPTP) opening. On the other hand, significantly decreased uptake and/or increased egress of mitoCa^{2+} (*e.g.,* in Fus1$^{-/-}$ cells) will not feed Krebs cycle enough to produce sufficient number of thiol reducing equivalents and produce the effects similar to excessive mitoCa^{2+} uptake, therefore contributing to ROS accumulation, $\Delta\mu H^+$ depolarization after redirection of reducing equivalents from H$^+$ accumulation on inner mitochondrial membrane to transhydrogenase/glutathionreductase (TH/GR) reactions, excessive thiol oxidation, and opening of mitochondrial channels (*e.g.,* IMAC, mPTP). In Fus1$^{-/-}$ splenocytes, Ca^{2+} uptake results in improved transduction of Ca^{2+} into $\Delta\mu H^+$ due to the loss of depolarizing effect of the reduction in Ca^{2+} accumulation. We propose that the decreased mitoCa^{2+} accumulation in Fus1$^{-/-}$ cells results in the slower transformation $\Delta\mu H^+ \rightarrow$ROS because of the reduced thiols due to the Krebs cycle stimulation. As a result, it prevents $\Delta\mu H^+$ from depolarization during coupled TH/GR reactions. All listed parameters ($\Delta\mu H^+$, ROS, mPTP, mitoCa^{2+} uptake) may affect global Ca^{2+} signaling and activation of transcription factors *via* the modulation of Ca^{2+} oscillations. In turn, alterations in transcription may affect cell differentiation (*e.g.,* Th cell polarization), proliferation, and other vital cellular processes.

Flow cytometric analysis of cytoCa^{2+}, mitoCa^{2+}, MMP, mitoROS, and mPTP

Cytoplasmic calcium levels were measured by loading the cells with 1 μM fluoro-3 acetoxymethyl ester (Fluo-3/AM) (excitation, 506 nm; emission, 526 nm; recorded in FL-1; Molecular Probes). Mitochondrial calcium level was estimated by loading the cells with 4 μM Rhod2/AM, which is compartmentalized into the mitochondria (44). Production of mitochondrial ROS (superoxide anion) was assessed fluorometrically using oxidation-sensitive fluorescent probes MitoSOX (Molecular Probes) as described elsewhere (41) $\Delta\psi$m was quantitated using a potential-dependent J-aggregate-forming lipophilic cation JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (Molecular Probes). JC-1 selectively incorporates into mitochondria, where it forms monomers (fluorescence in green, 527 nm) or aggregates, at high transmembrane potentials (fluorescence in red, 590 nm) (44). Cells were incubated with 0.5 μM JC-1 for 15–45 min at 37°C before flow cytometry. Co-treatment with a protonophore, 5 μM carbonyl cyanide m-chlorophenylhydrazone (Molecular Probes), for 15 min at 37°C resulted in decreased JC-1 fluorescence and served as a positive control for the disruption of $\Delta\psi$m (44). mPTP permeability was monitored through a release of fluorescent calcein from mitochondria *via* mPTP. The fluorescence from cytosolic calcein was quenched by the addition of $CoCl_2$, whereas the fluorescence from the mitochondrial calcein was maintained. Production of nitric oxide (NO) was estimated with dye 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM; Molecular Probes). Inside the cell, DAF-FM is deacetylated and retained in the cell. Non-fluorescent DAF-FM after reaction with NO forms fluorescent benzotriazole. For NO level estimation, cells were incubated with 1-5 μM DAF-FM for 1 h at 37°C. Excitation and emission maximum of DAF-FM are 495 and 515 nm, respectively (43). Oxidation of intracellular nonprotein thiols (primarily GSH) was measured using the Cell Tracker Green CMFDA (5-chloromethylfluorescein diacetate) probe, which has a chloromethyl group that, when reacting with thiols, gets converted into a fluorescent adduct. For GSH measurement, cells were incubated with 1 μM CMDFA for 30–60 min at 37°C. Excitation and emission maximum of CMDFA are 492 and 517 nm, respectively (21).

Antibodies and reagents

Conjugated mouse monoclonal antibodies, CD4-FITC, CD4-PE, IFNgamma-PE, IL-4-FITC, PD-1-FITC, and PD-L1-PE (Biolegend), were used for FACS. Mouse CD4$^+$ T-cell isolation kit II was purchased from Miltenyi Biotec. Probes for the measurement of cytosolic (Fluo-3) and mitochondrial Ca^{2+} (Rhod-2) were purchased from AnaSpec Inc. Probes for other mitochondrial parameters such as membrane potential (JC-1), ROS (MitoSOX), permeability transition pore assay, and mitochondrial morphology (MitoTracker Red) were all from Molecular Probes (Invitrogen). CGP37157, inhibitor of mitochondrial Na$^+$/Ca^{2+} exchanger (mNCX), was obtained from Sigma. Calcium Green-5N for the measurement of Ca^{2+} uptake by mitochondria in digitonin-permeabilized cells was purchased from Molecular Probes (Invitrogen).

Cell culture

Mouse immortalized kidney epithelial cells were maintained in DMEM/10% FBS medium with 100 μg/ml Anti-Anti mixture (all from Gibco, Inc) at 37°C and 5% CO_2.

Supplementary Table S1. List of Genes Upregulated at Basal Level in CD4+ T Cells Isolated from *Fus1 KO* Mice (mRNA Isolated from CD4+ T Cells of Five Mice per Group Was Pooled for Analysis)

Gene symbol	*KO/WT fold change*
Gnl2	3.309226408
Acadl	3.233942438
Retnlg	2.913616505
Mmp8	2.911357488
S100a9	2.815958358
S100a8	2.738101638
F630028O10Rik	2.608049045
Ltf	2.605519406
Igkv12-47	2.496804738
Il1f9	2.487706007
Trdn (5)	2.469390505
Slc7a11	2.359272399
Mmp9	2.323977337
Wfikkn2	2.304386961
Gp49a	2.213599158
Slc15a2	2.203234415
Lilrb4	2.188555545
Ly6g	2.160803677
Hmox1	2.156630474
Clec4d	2.112152496
Lcn2	2.091046291
Cybb	2.067724534
Lyn	2.0580471
Zdhhc20	2.029025505
Nup210l	2.003399307
Adam4	1.988233227
Mir701	1.985882136
Gdap10	1.981983566
Cd63	1.979451885
Mir140	1.978360033
Igk-V28	1.968426713
Hspa1a (3)	1.945893834
Gm5887	1.925593264
Olfr60	1.912786695
Mir505	1.900689671
Fam129a	1.892789532
Gpr21	1.892420901
Fam38a (2)	1.892308096
Plxnc1	1.885165259
Spef2 (8)	1.873859779
Pten	1.85771862
Gm6683	1.856598683
Il1rl1	1.847727492
Lyz2	1.835585099
Mir340	1.835109309
Thbs1	1.815598246
Sestd1	1.811648415
Mir342	1.806319377
Eid3	1.790513008
Plek	1.774862177
Atp6v0a1	1.76989405
Gm10008	1.766243192
Gpr52	1.761727758
Dock6	1.753191999
Slc5a3	1.745479
Cspp1	1.745376164
BC018473	1.745083416
U29423	1.744086991
Adam8	1.741591172
Ube2v1	1.736494691
9430025M13Rik	1.733589115
Irf4	1.732515188
Csf2rb	1.730731587
Tanc2	1.722102482
Csf3r	1.720947397
Itgae	1.719522508
Mir29b-1	1.719310366
Phxr4	1.718917138
Clec4n	1.715120571
Tbrg3	1.709054022
Map3k12	1.696129119
Ctsw	1.694390024
Rnf213 (8)	1.691076588
Arhgap20	1.681693746
Notch2	1.677314241
Gm15800 (2)	1.675823256
Hmgb3 (2)	1.675316878
Nrp1	1.675093934
Snord118 (2)	1.670202466
1700031F05Rik	1.669317063
9530009G21Rik	1.663618923
Alox5ap	1.654945835
Csf2rb2	1.654781805
Nlrc5 (8)	1.651646567
Mir148b	1.651455391
Ddx60	1.650987274
Tlr13	1.645923177
Ptprj (2 probes)	1.644058916
Plac8	1.6421353
Tyrobp	1.63800073
MGC7817	1.63687369
Dnajb6	1.634680855
Chi3l3	1.634206165
Cd33	1.629765052
Gm5571	1.628473226
Ptprj	1.627227538
Szt2 (5)	1.624706363
Myo1f	1.623997037
Nup210l	1.623292521
Cd24a	1.621379716
Gm4787	1.616973461
C530030P08Rik (2)	1.614077666
Phgdh	1.608905956
Gm9982	1.607796706
Ms4a1/CD20	1.601129502
Mir16-2	1.600630161
Ccr9	1.600133195
Fam196b	1.600098812
Ermp1	1.600006759
Il2rb	1.59643078
Slc22a15	1.596132036
Ebf1	1.594239081
Mir181a-1	1.593800439
Pglyrp1	1.592379275
Tiaf2	1.590345254
Mir15a	1.589690597
Igsf6	1.589515407
Myo9a (4)	1.585628721
Gm10551	1.584336737
Gm10388	1.582688131

(*continued*)

Supplementary Table S1. (Continued)

Gene symbol	KO/WT fold change
Cd74	1.581548721
Mir421	1.579363222
Snord53	1.578831272
Plcg2	1.577008023
I830127L07Rik	1.576884508
Il23r	1.572251487
Plxdc2	1.57146049
Gtf3c2	1.569655545
Igf1r	1.568950678
Cd19	1.565847811
Atp6v0d2	1.563643906
Lrrc6	1.561713704
Anxa2	1.560476899
D130062J21Rik	1.560400104
Clic4	1.559301599
Lrrk2	1.558559249
1700081L11Rik	1.557594829
Adam19	1.556395808
Wnk1	1.548084263
Ikzf2	1.547457728
Cdkl3	1.547272177
Foxp3	1.547039465
Ern1	1.54637155
Htt	1.546251506
4932438A13Rik	1.543946755
Rangrf (2)	1.543626803
Tet3 (2)	1.543034159
Gnrh1	1.542832027
Kcnip2	1.541432805
Snord85	1.539856594
Ptpn3	1.539430782
Dennd4a (2)	1.535551608
Raph1	1.534081362
Diap2	1.532536038
Gm340	1.53231935
1300014I06Rik	1.529835997
Mll2 (2)	1.526301526
Cpd	1.524853878
Hipk2	1.520921824
Mll1	1.51834538
LOC630837	1.516613006
Fscn1	1.51533209
Pilra	1.515311083
Mcpt8	1.514646368
Arrdc4	1.510313453
Mir19b-1	1.509836156
Arhgap26	1.509254393
Plbd1	1.507180286
Tnfrsf1b	1.506103588
Cdk6	1.503428231
Nup210l	1.499262499
5430427O19Rik	1.499071296
Spib	1.496298489
Ncf1	1.495406802
Bcl2a1c	1.495382961
Pou2f2	1.495218164
Atrnl1	1.494269117
Mir30e	1.49148038
Endod1	1.491130992
Tmem176b	1.488837192
Vav2	1.487904571
Cacnb3	1.48498669

(*continued*)

Supplementary Table S1. (Continued)

Gene symbol	KO/WT fold change
Cxcr2	1.484834359
Nfat5	1.481434664
Gm9766	1.48114204
ND6	1.481112267
Rora	1.480894638
H2-Aa	1.477826598
4932438A13Rik	1.47673402
Gabarapl2	1.476380922
Olfr798	1.475314977
Nt5e	1.475164661
Bglap	1.474376519
Cep350	1.472246251
Tiam1	1.466200091
Cd93	1.464405401
Abcc1	1.464125275
H2-Eb1	1.462254065
Kcnrg	1.461125398
Eif2c2	1.459505857
BC049807	1.459447182
Hells	1.459411776
B230325K18Rik	1.459405706
Iigp1	1.459380417
Gcnt1	1.459379406
St6galnac3	1.459180141
Ctsh	1.459108331
LOC100046275	1.458807983
Tsc22d1	1.458530949
Neil2	1.457548611
Rnf157	1.457538508
Nbeal1	1.457105159
Cd180	1.456393291
Anxa4	1.454948411
Gm5415	1.454752777
Swap70	1.45262569
Gm16970	1.451414914
Ikzf4	1.449870453
Olfr776	1.449856383
Slamf7	1.449504689
Cyfip1	1.448632854

Numbers within parentheses indicate when genes are represented by multiple probes. Proteins implicated in Ca^{2+}-binding or Ca^{2+}-dependent activities are shown in bold.

KO, knockout; WT, wild type.

Splenocyte isolation, CD4$^+$ T-cell purification and activation

CD4$^+$ cells were purified from the whole splenocyte fraction using CD4$^+$ isolation kit II (Miltenyi Biotec). CD4$^+$ cells were activated, unless otherwise indicated, with plate-bound α-CD3 (1.0 μg/ml) and soluble α-CD28 (1.0 μg/ml; both from Biolegend) and cultured at a density of $\sim 1 \times 10^6$ cells/ml in 24-well plates in RPMI-1640 medium supplemented with Anti-Anti mixture and 10% FBS).

Mitochondrial morphology imaging

Epithelial cells at $\sim$ 70%–80% confluency were treated with Ca^{2+} and Ca^{2+}/ionomysin for 15 mins and with Mitotracker Red. Cells were then washed and fixed in 3.7% formaldehyde for 15 mins in the dark, permeabilized, and mounted with DAPI-containing mounting media. Zeiss LSM

Supplementary Table S2. List of Genes (Left Column) Activated by CD3/CD28 Stimulation of CD4$^+$ T Cells for 12 h

#. Gene name	*Activation index*	
	WT	*KO*
1. Irf4	12.3231314	7.5318747
2. Itih5	6.82112641	4.4342527
3. Cd69	5.43508651	3.884853
4. Il2	4.97878253	3.4303814
5 Ccl22	4.36408439	3.5443228
6. Socs2	4.33369013	3.0510842
7. Gbp5	3.91431538	2.5991286
8. Tnf	3.49319456	2.6839113
9. Gpr83	3.3371904	2.3182899
10. Iigp1	3.21244777	1.824061
11. Nfkbid	2.86361988	2.1490395
12. Clic4	2.75261912	2.039259
13. Bcat1	2.66750135	1.8072599
14. Gm12250	2.59756527	1.5286806
15. Gm129	2.56008374	1.7132765
16. Gbp2	2.53934779	1.741293
17.E430024C06Rik	2.4293915	1.7021427
18. Cd83	2.42835274	1.9056366
19. Bcl2a1c	2.3530272	1.5129623
20. Fcer2a	2.34646835	1.1004488
21. Rrs1	2.30439495	1.5762889
22. Tgtp1 (2)	2.25670838	1.4218507
23. Myb	2.17504462	1.560054
24. Gbp1	2.1727437	1.6590588
25. Irgm1	2.15698329	1.408188
26. Bcl2a1a	2.14358853	1.6992275
27. Irgm2	2.11152306	1.5346599
28. Gbp3	2.0939892	1.6267979
29. Odc1	2.06805564	1.6446094
30. Cd200	2.05993241	1.5080163
31. Rgs1	2.03068434	1.5311366
32. Pim2	1.9926385	1.3690261
33. Rgs16	1.98996768	1.5624045
34. Mpa2l	1.96120453	1.4460025
35. Serpinb9	1.94931738	1.2539241
36. Stat1	1.94603816	1.3548283
37. Ccr8	1.92169982	2.4724168
38. Cth	1.92101529	1.534994
39. Gas5	1.9026326	0.8414182
40.Nop58	1.90125494	1.4965381
41.Rnf213	1.89197234	1.0120356
42. Apex1	1.88733562	1.1718371
43. Ttc27	1.86722269	1.4652786
44. Hspd1	1.86482468	1.5459568
45. Gm4759	1.8556672	1.0056728
46. Gnl3	1.85325191	1.4933465
47. Ebi3	1.84830648	1.3738971
48. Swap70	1.83020722	1.1431463
49. Dnahc7b	1.82472114	1.4806945
50. Lilrb4	1.82399655	1.2254096
51. Gvin1	1.82268721	1.3510396
52. Gpt2	1.80460114	1.1846509
53. Gp49a	1.80054418	1.2338004
54. Gm5772	1.79634817	1.250045
55. Gm13235	1.78177479	0.6577367
56. Tsen15	1.78106232	1.3933995
57. Nlrc5	1.77719994	1.0263452
58. Ecm1	1.77396679	1.4163129
59. Snord1c	1.77325006	1.1835052
60. Rrp15	1.77150557	1.3168628

(*continued*)

Supplementary Table S2. (Continued)

#. Gene name	*Activation index*	
	WT	*KO*
61. Apol7c	1.74540278	1.0197539
62. Rtp4	1.73695575	1.1549401
63. Fscn1	1.73445571	1.0413529
64. Gbp4	1.73056125	1.3432613
65. Snhg1	1.66413561	1.0402895
66. Shmt1	1.66260793	1.2436952
67. Nup210l	1.66105633	0.9412344
68. Zfp640	1.64673796	1.1902792
69. Mrpl42	1.62451267	1.1938197
70. Gm8995	1.62229929	1.2399999
71. Gbp6	1.62041124	1.2723715
72. Ms4a4c	1.61439096	1.2347115
73. Il2rb	1.60515209	1.1176202
74. Zfp600	1.60413438	1.068329
75. Mrps36	1.59112921	1.1779963
76.2810021G02Rik	1.59014685	0.8284864
77.1810029B16Rik	1.58788342	1.2941824
78. Snord38a	1.58652032	1.0149756
79. Atp13a3	1.58334868	0.8990199
80. Zfp455	1.58070922	0.955783
81. Akr1c18	1.57804572	1.0165795
82. Dkc1	1.57621791	1.1843726
83. Gm5921	1.57587161	1.2149189
84. Pus7	1.57198015	1.0921023
85. Cmpk1	1.57002116	0.9486403
86. Irf9	1.56850486	1.0862303
87. Chchd4	1.56580223	1.1182037
88. Dleu2	1.56456218	1.2228742
89. Samsn1	1.55176598	1.2096117
90. Trdn	1.54395104	1.0980015
91. St6galnac4	1.53248824	1.270867
92. Nudt11	1.52517522	0.8827299
93.2610044O15Rik	1.52402651	1.0611135
94. Tnip3	1.52258102	1.1098056
95. Anp32-ps	1.52207875	0.9594782
96. Chd7	1.51762989	1.1702656
97. Gm8639	1.51541297	1.1077712
98. Ltn1	1.51384448	1.0685067
99. Dip2c	1.51088096	0.9273855
100. St13	1.51031659	1.1261352
101. Ipo7	1.50496402	1.0958649
102. Dnajc13	1.49571157	0.9562309
103. Gcsh	1.48657989	1.1327371
104. Ewsr1	1.48511639	0.9691541
105. Rpl22l1	1.48209816	1.0958862
106. Sft2d3	1.4812714	1.0461836
107. H2-T24	1.47696946	1.0999165
108. Slc7a11	1.4699866	1.1291374
109. Olfr800	1.4679227	1.1890208
110. Utp18	1.46726047	1.1459777
111. Idi1	1.46641759	1.0678056
112. Olfr798	1.46236455	0.7831464
113. Eif2b3	1.45793965	1.1478531
114. Naa15	1.45494034	1.2112528
115. Snord58b	1.45452491	1.010018
116. Id3	1.45199753	1.1335154
117. Nampt	1.45183852	1.1519379
118. Nol11	1.45159702	1.1383151
119. E2f5	1.4501187	0.9939554

Right columns present activation indices (expression level after stimulation/basal expression level) for these genes in *Fus1 WT* and *Fus1 KO* CD4$^+$ T cells.

510 META Upright Confocal Microscope was used for analyzing mitochondrial morphology and making images.

Measurement of mitochondrial Ca^{2+} uptake capacity of digitonin-permeabilized cells

Extramitochondrial free Ca^{2+} was monitored in the presence of digitonin-permeabilized cells as described in (42, 48) Briefly, cells (2×10^6 cells/ml) were resuspended in KCl medium (125 m*M* KCl, 2 m*M* K_2HPO_4, 1 m*M* $MgCl_2$, 20 m*M* Hepes, pH 7.0) containing 5 m*M* glutamate, 5 m*M* malate, and 5 m*M* succinate as oxidable substrates and 0.5 μM Ca^{2+} green-5N. The plasma membranes were then selectively permeabilized with digitonin (0.01%wt/vol final). Fluorescence (Ex506/Em531 nm) was monitored at room temperature using a SynergyMX (BioTek) fluorescence spectrophotometer. The involvement of mNCX in the Ca^{2+} homeostasis was measured by the replacement of Na-containing buffer with Na-free buffer or by the addition of benzodiazepine CGP37157 (10–20 μM), a blocker of mNCX, to challenged cells.

$CD4^+$ T-cell proliferation assay

Proliferation of $CD4^+$ T cells was measured by flow cytometry after a CFSE dilution assay as described elsewhere (36). Splenocytes were stained with CFSE (1 μM), stimulated with CD3/CD28 (1 μg/ml of each) or left unstimulated, harvested after 1, 2, and 3 days, stained for a surface CD4 expression with anti-mouse CD4 antibodies labeled with PerCP-Cy5.5 (Biolegend), and acquired on FACS Calibur flow cytometer (Beckman Coulter).

Intracellular cytokine staining

Cytokine staining and analysis was performed as described elsewhere (54). The antibodies used were anti-mouse IFNγ-FITC (1/100 μl permeabilization buffer) and α-IL4-PE (1/100 μl permeabilization buffer) (Biolegend).

Apoptosis T-cell assays

Apoptosis was monitored by flow cytometry after concurrent staining with FITC-conjugated Annexin V (Annexin V-FITC; R&D Systems) (FL-1) and 7-AAD (FL-3) as described elsewhere (44, 44). Apoptosis rates were expressed as a shift in Annexin V binding in PI-negative cells.

$CD4^+$ T cells RNA isolation and microarray analysis

RNA from unstimulated and stimulated for 12 h with CD3/CD28 antibodies $CD4^+$ T cells collected from five animals per group was isolated with the RNAeasy mini RNA isolation kit (Qiagen), mixed together to obtain sufficient for hybridization RNA amount, and subjected to differential expression analysis using gene 1.0 microarray platform, which was performed in the Genomic Core Facility at the Vanderbilt University.

Statistical analysis

Results are presented as mean ± SE. Comparisons between the two groups were performed using the Student's *t*-test. When analyzing statistical differences between the KO and WT mice, $p < 0.05$ was considered significant.