Putative Model of Coupling of Mitochondrial Parameters Mediated via mitoCa²⁺ 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* mitoCa²⁺ changes. Generally, after increase in intracellular Ca²⁺ levels, mitoCa²⁺ 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 \rightarrow NADPH \rightarrow 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 mitoCa²⁺ uptake, depolarization will reduce the uptake thus limiting the damage induced by mitoCa²⁺ overload. Due to dependence of Ca²⁺ channels on redox environment, mitoROS changes could be transmitted into alterations in Ca²⁺ oscillations triggering changes in the NF- κ B and NFAT transcriptional activities in T cells.

Excessive mitoCa²⁺ accumulation induced by Ca²⁺ 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 mitoCa²⁺ (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 mitoCa²⁺ 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 mitoCa²⁺ 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, mito- Ca^{2+} uptake) may affect global Ca^{2+} signaling and activation of transcription factors via the modulation of Ca2+ 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 cytoCa²⁺, mitoCa²⁺, MMP, mitoROS, and mPTP

Cytoplasmic calcium levels were measured by loading the cells with 1 μ M 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 μ M 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 Jaggregate-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 μ M carbonyl cyanide mchlorophenylhydrazone (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 CoCl₂, 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. Nonfluorescent 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 $1 \,\mu M$ 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 Ca²⁺ (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⁺/Ca²⁺ exchanger (mNCX), was obtained from Sigma. Calcium Green-5N for the measurement of Ca²⁺ 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 μ g/ml Anti-Anti mixture (all from Gibco, Inc) at 37°C and 5% CO₂. 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)

SUPPLEMENTARY TABLE S1. (CONTINUED)

Gene symbol

KO/WT fold change

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$\begin{array}{llllllllllllllllllllllllllllllllllll$	Clec4d	2.112152496	Aloxsap	1.034943833
$\begin{array}{llllllllllllllllllllllllllllllllllll$	Len2	2.091046291	CS12ID2 NHro5 (9)	1.034/81803
$\begin{array}{llllll} Lyn & 2.0580471 & Mi11400 & 1.63143391 \\ Zdhhc20 & 2.029025505 & Ddx60 & 1.650987274 \\ \mbox{Nup2101} & 2.003399307 & Tlr13 & 1.644923177 \\ \mbox{Adam4} & 1.988233227 & Ptpi (2 probes) & 1.644058916 \\ \mbox{Mir701} & 1.988233227 & Ptpi (2 probes) & 1.644058916 \\ \mbox{Mir701} & 1.988282136 & Plac8 & 1.6421353 \\ \mbox{Gd3} & 1.979451885 & MGC7817 & 1.63867369 \\ \mbox{Mir140} & 1.978560033 & Dnajb6 & 1.634680855 \\ \mbox{Igk} Igk 2 & 1.968426713 & Chi313 & 1.6346765052 \\ \mbox{Igk} Igk 3 & 1.925593264 & Gm5571 & 1.628473226 \\ \mbox{Offf} O & 1.912786695 & Ptpi & 1.627227538 \\ \mbox{Mir505} & 1.900689671 & Sz12 (5) & 1.624706363 \\ \mbox{Mir505} & 1.900689671 & Sz12 (5) & 1.624706363 \\ \mbox{Mir505} & 1.900689671 & Sz12 (5) & 1.6247076365 \\ \mbox{Gpr21} & 1.892420901 & Nup2101 & 1.623929231 \\ \mbox{Pranc} Igk 3 & 1.8892789532 & Myo1f & 1.623929231 \\ \mbox{Pranc} Igk 3 & 1.8892789532 & Myo1f & 1.62379716 \\ \mbox{Pranc} Igk 3 & 1.8892789532 & Myo1f & 1.623997037 \\ \mbox{Gpr21} & 1.892420901 & Nup2101 & 1.623929251 \\ \mbox{Pranc} Igk 3 & 1.855959 & Gm4787 & 1.616973461 \\ \mbox{Spef2} (8) & 1.873859779 & C5300300908Rik (2) & 1.614077666 \\ \mbox{Pranc} Igk 3 & 1.855982683 & Gm9982 & 1.607796706 \\ \mbox{Gm6683} & 1.855598683 & Gm9982 & 1.6007396706 \\ \mbox{Gm6683} & 1.855598683 & Gm9982 & 1.6007396706 \\ \mbox{Gm6683} & 1.85598683 & Gm9982 & 1.6007396706 \\ \mbox{Gm6683} & 1.85598246 & Fam196b & 1.600033195 \\ \mbox{Tr342} & 1.806319377 & I2rb & 1.59643078 \\ \mbox{Eid3} & 1.790513008 & Slc22a15 & 1.59643078 \\ \mbox{Eid3} & 1.790513008 & Slc22a15 & 1.59643078 \\ \mbox{Eid3} & 1.79456177 & Ebf1 & 1.592329725 \\ \mbox{Gm10008} & 1.766243192 & Plyrp1 & 1.592379275 \\ \mbox{Gm10008} & 1.76424192 & Plyrp1 & 1.5923804399 \\ \mbox{Gm10008} & 1.766243192 & Plyrp1 & 1.5923804554 \\ \mbox{Dock6} & 1.753191999 & Mir15a & 1.588628721 \\ \mbox{Bcb13} & 1.74587614 & Myo9a (4) & 1.588628721 \\ \mbox{Bcb13} & 1.74587614 & Myo9a (4) & 1.588628721 \\ \mbox{Bcb13} & 1.74587479 & Igk66 & 1.588768721 \\ \mbox{Bcb143} & 1.745868416 & Gm10551 & $	Субб	2.067724534	NIICJ (8) Min149b	1.031040307
Zdhc20 2.02902505 D0x00 1.630987274 Nup2101 2.003399307 Tlr13 1.6445923177 Adam4 1.988282136 Ptprj (2 probes) 1.644058916 Mir701 1.988588136 Ptprj (2 probes) 1.644058916 Mir701 1.988588136 Tyrobp 1.63800073 Cd63 1.979451885 MGC7817 1.63687369 Mir140 1.978360033 Dnajb6 1.634206165 Hspala (3) 1.945893834 Cd33 1.629765052 Gm5887 1.92593264 Gm55711 1.62473253 Mir505 1.900689671 Szt2 (5) 1.62470363 Fam129a 1.892420901 Nup2101 1.632992521 Gpr21 1.892420901 Nup2101 1.622397037 Gpr21 1.892420901 Nup2101 1.634206165 Pknc1 1.88516525 Gm4787 1.616973461 Spet2 (8) 1.873859779 C530030P08Rik (2) 1.614077666 Phan 1.85598683 Gm9822 1.6073461 Lyz2 1.835585099 Mir16-2 1.600033195 L	Lyn	2.0580471	MII[1460	1.031433391
Nup2102.00339930711115 1.643923127 Adam41.988233227Ptprj (2 probes) 1.644058916 Mir7011.981983566Tyrobp 1.63800073 Gdap101.981983566Tyrobp 1.6380073 Cd631.979451885MGC7817 1.63687369 Mir1401.978360033Dnajb6 1.634206165 Hspala (3)1.945893834Cd33 1.6229765052 Gm58871.925593264Gm5571 1.6224706363 Olir601.912786695Ptprj 1.622727538 Mir5051.900689671Szt2 (5) 1.6224706363 Fam129a1.892789532Myolf 1.623997037 Gpr211.89240901Nup2101 1.62392521 Phrc11.892789532Gm4787 1.616973461 Spe2 (8)1.8771862Ptgdh 1.608905956 Pinc11.885165259Gm4787 1.616973461 Spe2 (8)1.8757862Ptgdh 1.60033195 Ilr111.83509309Ccf4 1.60129502 Ilr121.83558809Mir16-2 1.60033195 Thbs11.83519309Ccf9 1.60033195 Thbs11.81648415Ermp1 1.600037401 Mir3401.83519309Ccf9 1.60033195 Thbs11.81648415Ermp1 1.60003819 Mir3421.806319377II2rb 1.593400439 Gm10008 1.766243192 Pigff 1.59340039 Gm10008 1.766243192 Pigff 1.59340039 Gm10008 1.76624	Zdhhc20	2.029025505	D0X00 T1-12	1.050987274
Adam4 1.988233227 PtpT (2 probes) 1.04403871 Mir701 1.98582136 Plac8 1.6421353 Gdap10 1.979451885 MGC7817 1.63687369 Cd63 1.979451885 MGC7817 1.634687369 Mir140 1.978360033 Dnajb6 1.634268165 Igk <v28< td=""> 1.968426713 Chi313 1.634206165 Hspala (3) 1.945893834 Cd33 1.629765052 Gm5887 1.925593264 Gm55711 1.628473226 Olfr60 1.912786695 Ptprj 1.627227538 Mir505 1.900689671 Szt2 (5) 1.624706363 Fam129a 1.892789532 Myolf 1.623997037 Gpr21 1.892308096 Cd24a 1.62139716 Phrnc1 1.885165259 Gm4787 1.616973461 Phrnc1 1.885165259 Gm4787 1.614077666 Phen 1.85771862 Phgdh 1.608905956 Gm6683 1.85598683 Gm9982 1.600796706 Mir340 1.81598246 Fam196b 1.600008812 Lyzz 1.83510</v28<>	Nup210l	2.003399307	$\frac{11113}{2}$	1.043923177
$\begin{array}{lllll} \begin{tabular}{l ll l$	Adam4	1.988233227	Plprj (2 probes)	1.044038910
Gdap10 1.981983566 1yrobp 1.63800073 Cd63 1.979451885 MGC7817 1.63867369 Mir140 1.978360033 Dnajb6 1.6342687369 Mir140 1.978360033 Dnajb6 1.634206165 Igk-V28 1.968426713 Chi313 1.629765052 Gm5887 1.925593264 Gm5571 1.628473226 Olfr60 1.912786695 Ptpj 1.622727538 Mir505 1.900689671 Szt2 (5) 1.624706363 Fam129a 1.892789532 Myo1f 1.623997037 Gpr21 1.892308096 Cd24a 1.621379716 Plxnc1 1.885165259 Gm4787 1.614077666 Spef2 (8) 1.873859779 C530030P08Rik (2) 1.6140776606 Ilrl1 1.847727492 Ms4a1/CD20 1.601129502 Lyz2 1.835588099 Mir16-2 1.60080161 Mir340 1.835109309 Ccr9 1.60073407 Mir341 1.81558246 Fam196b 1.600006759 Eid3	Mir701	1.985882136		1.0421353
Cd63 1.979451885 MGC 7817 1.63667369 Mir140 1.978360033 Dnajb6 1.6344860855 Hirl40 1.978360033 Dnajb6 1.6344206165 Hspala (3) 1.945893834 Cd33 1.629765052 Gm5887 1.92593264 Gm5571 1.622727338 Mir505 1.900689671 Szt2 (5) 1.624706363 Fam129a 1.892420901 Nup2101 1.623997037 Gpr21 1.89240901 Nup2101 1.623292521 Fam38a (2) 1.89240901 Nup2101 1.623292521 Spef2 (8) 1.873850779 C530030P08Rik (2) 1.616973461 Spef2 (8) 1.873859779 C530030P08Rik (2) 1.614077666 Pten 1.8571862 Phgdh 1.60129502 Lyz2 1.83585099 Mir16-2 1.600030161 Mir340 1.835109309 Ccr9 1.600133195 Thbs1 1.81648415 Ermp1 1.60006759 Mir342 1.806319377 112rb 1.59643078 Ei	Gdap10	1.981983566	I yrobp	1.63800073
$\begin{array}{llllllllllllllllllllllllllllllllllll$	Cd63	1.979451885	MGC/81/	1.0308/369
$\begin{array}{llllllllllllllllllllllllllllllllllll$	Mir140	1.978360033	Dhajbb	1.634680855
Hspa1a (3) 1.945893834 $Ca33$ 1.629765052 Gm5887 1.925593264 Gm5571 1.628473226 Olfr60 1.912786695 Ptprj 1.627227538 Mir505 1.900689671 Szt2 (5) 1.623997037 Gpr21 1.892789532 Myo1f 1.623397037 Gpr21 1.892308096 Cd24a 1.621379716 Plxnc1 1.885165259 Gm4787 1.616973461 Spef2 (8) 1.873859779 CS30030P08Rik (2) 1.614077666 Pren 1.85771862 Phagh 1.608905956 Gm6683 1.856598683 Gm9982 1.607796706 Ilrl1 1.835585099 Mir16-2 1.600098812 Lyz2 1.835585099 Mir16-2 1.600098812 Lyz2 1.835589466 Fam196b 1.600098812 Sestd1 1.81598246 Fam196b 1.59423028 Mir340 1.835109309 Ccr9 1.5043078 Mir342 1.806319377 $112rb$ 1.59423088 Eid3 1.790513008 Slc22a15 1.596132036 Plek 1.766243192 Plyrp1 1.59237275 Gpr52 1.761727758 $Tia27$ 1.593800439 Gm10008 1.766243192 Plyrp1 1.593800439 Gm10008 1.745376164 Myo9a (4) 1.5885628721 BC0184733 1.745083416 Gm10358 1.5885628721 Ly23 1.745083416 Gm103588 1.582688131	Igk-V28	1.968426713	Chi313	1.634206165
Gm 5887 1.925593264 Gm 5571 1.62847227538 Olfr60 1.912786695 Ptprj 1.62727538 Mir505 1.900689671 Szt2 (5) 1.624706363 Fam129a 1.892789532 Myolf 1.623997037 Gpr21 1.892420901 Nup2101 1.623292521 Fam38a (2) 1.892308096 Cd24a 1.621379716 Plxnc1 1.885165259 Gm 4787 1.616973461 Spef2 (8) 1.873859779 C530030P08Rik (2) 1.614077666 Pten 1.85771862 Phgdh 1.608905956 Gm 6683 1.856598683 Gm 9982 1.607796706 Ilt11 1.845771862 Migdh 1.60030161 Lyz2 1.835109309 Cr9 1.600133195 Thbs1 1.81598246 Fam196b 1.600068812 Sestd1 1.81598246 Fam196b 1.600008759 Mir342 1.806319377 Il2rb 1.59643078 Eid3 1.790513008 Slc22a15 1.59643078 Plek 1.774862177 Ebf1 1.594239081 Atp6v0a1 1.766243192 Pglyrp1 1.592379275 Gpr52 1.761727758 Tia72 1.590345254 Dock6 1.7537191999 1.582688131 1.582688131 BC018473 1.745083416 Gm 10551 1.58436737 U29423 1.74408691 Gm 10388 1.582688131	Hspala (3)	1.945893834	Ca33	1.629/65052
$\begin{array}{llllllllllllllllllllllllllllllllllll$	Gm5887	1.925593264	Gm55/1	1.6284/3226
Mir505 1.900689671 $S22 (25)$ 1.624706363 Fam129a 1.892789532 Myolf 1.623997037 Gpr21 1.892420901 Nup2101 1.623292521 Fam38a (2) 1.892308096 Cd24a 1.621379716 Plxnc1 1.885165259 Gm4787 1.616973461 Spef2 (8) 1.873859779 C530030P08Rik (2) 1.614077666 Pten 1.85771862 Phgdh 1.608905956 Gm6683 1.856598683 Gm9982 1.607796706 Illr11 1.847727492 Ms4a1/CD20 1.601129502 Lyz2 1.835585099 Mir16-2 1.600030161 Mir340 1.835109309 Ccr9 1.60008812 Sestd1 1.811598246 Fam196b 1.600006759 Mir342 1.806319377 $112rb$ 1.59643078 Eid3 1.790513008 $SIc22a15$ 1.59643078 Plek 1.774862177 Ebf1 1.593800439 Gm10008 1.766243192 Pglyrp1 1.592379275 Gpr52 1.761727758 Tiaf2 1.590345254 Dock6 1.753191999 Mir15a 1.58658721 Scs3 1.745376164 My09a (4) 1.585628721 BC018473 1.744086941 Gm103388 1.582688131	Olfr60	1.912786695	Ptprj	1.62/22/538
Fam129a 1.892789532 Myo17 1.623997037 Gpr21 1.892420901 Nup2101 1.623292521 Fam38a (2) 1.892308096 $Cd24a$ 1.621379716 Plxnc1 1.885165259 Gm4787 1.616973461 Spef2 (8) 1.873859779 $C530030P08Rik$ (2) 1.614077666 Pten 1.85771862 Phgdh 1.608905956 Gm6683 1.856598683 Gm9982 1.607796706 Illrl1 1.847727492 Ms4a1/CD20 1.600133195 Lyz2 1.835585099 Mir16-2 1.600078512 Sestd1 1.815598246 Fam196b 1.600008812 Mir340 1.835109309 Ccr9 1.59643078 Gid3 1.790513008 Slc22a15 1.59643078 Bid3 1.774862177 Ebf1 1.594239081 Atp6v0a1 1.76989405 Mir181a-1 1.592379275 Gpr52 1.761727758 Tiaf2 1.590345254 Ock6 1.753191999 Mir15a 1.58658721 Bool6683 1.745376164 Myo9a (4) 1.584336737 U29423 1.74408691 Gm10388 1.582688131	Mir505	1.900689671	Szt2 (5)	1.624/06363
Gpr211.892420901Nup21011.623292521Fam38a (2)1.892308096Cd24a1.621379716Plxnc11.885165259Gm47871.616973461Spef2 (8)1.8771862Phgdh1.608905956Gm66831.856598683Gm99821.601129502Lyz21.835585099Mir16-21.600030161Mir3401.835109309Ccr91.600006759Mir3421.811648415Ermp11.600006759Mir3421.806319377II2rb1.59643078Eid31.790513008Slc22a151.59643078Eid31.766243192Plyrp11.592379275Gpr521.761727758Tiaf21.590345254Dock61.753191999Mir15a1.5886019377Slc5a31.745376164Myo9a (4)1.585628721BC0184731.745083416Gm103511.584236737U294231.744086991Gm103881.582688131	Fam129a	1.892789532	Myolf	1.623997037
Fam38a (2) 1.892308096 $Cd24a$ $1.6213/9716$ Plxncl 1.885165259 $Gm4787$ 1.616973461 Spef2 (8) 1.873859779 $C530030P08Rik$ (2) 1.614077666 Pten 1.85771862 Phgdh 1.608905956 Gm6683 1.856598683 Gm9982 1.607796706 Il1rl1 1.847727492 $Ms4a1/CD20$ 1.601129502 Lyz2 1.835585099 $Mir16-2$ 1.600030161 Mir340 1.835109309 $Ccr9$ 1.600098812 Sestd1 1.815598246 Fam196b 1.600006759 Mir342 1.81648415 Ermp1 1.600006759 Mir342 1.806319377 $II2rb$ 1.59643078 Eid3 1.790513008 $Slc22a15$ 1.59643078 Plek 1.774862177 Ebf1 1.593800439 Om 10008 1.766243192 Pglyrp1 1.592379275 Gpr52 1.761727758 Tiaf2 1.590345254 Dock6 1.774579 Igsf6 1.58961520797751 Slc5a3 1.745479 Igsf6 1.589637371 U29423 1.744086991 Gm10388 1.5826881371	Gpr21	1.892420901	Nup2101	1.623292521
Plxnc1 1.885165259 $Gm4/8/$ $1.6169/3461$ Spef2 (8) 1.873859779 $C530030P08Rik (2)$ 1.614077666 Pten 1.85771862 Phgdh 1.608905956 Gm6683 1.856598683 Gm9982 1.607796706 Illr11 1.847727492 $Ms4a1/CD20$ 1.600132195 Lyz2 1.835585099 $Mir16-2$ 1.600630161 Mir340 1.815598246 Fam196b 1.600098812 Sestd1 1.815598246 Fam196b 1.60000759 Mir342 1.81648415 Ermp1 1.600006759 Mir342 1.806319377 $II2rb$ 1.59643078 Eid3 1.790513008 Slc22a15 1.59643078 Atp6v0a1 1.766243192 Pglyrp1 1.593800439 Gm10008 1.766243192 Pglyrp1 1.592379275 Gpr52 1.761727758 Tiaf2 1.589690597 Dock6 1.745376164 $Myo9a (4)$ 1.585628721 BC018473 1.745083416 Gm10388 1.582688131	Fam38a (2)	1.892308096	Cd24a	1.6213/9/16
Spef2 (8) 1.873859779 $C530030P08Rik (2)$ $1.61407/666$ Pten 1.85771862 Phgdh 1.608905956 Gm6683 1.856598683 Gm9982 1.607796706 Ill11 1.847727492 Ms4a1/CD20 1.601129502 Lyz2 1.835585099 Mir16-2 1.600630161 Mir340 1.835109309 Ccr9 1.600133195 Thbs1 1.815598246 Fam196b 1.600098812 Sestd1 1.81648415 Ermp1 1.600006759 Mir342 1.806319377 $112rb$ 1.59643078 Eid3 1.790513008 Slc22a15 1.596132036 Plek 1.774862177 Ebf1 1.592379275 Gpr52 1.76127758 Tiaf2 1.590345254 Dock6 1.753191999 Mir15a 1.58960597 Slc5a3 1.745479 Igsf6 1.589515407 Cspp1 1.745083416 Gm10388 1.582688131	Plxnc1	1.885165259	Gm4787	1.6169/3461
Pten 1.85771862 Phgdh 1.608905956 Gm6683 1.856598683 Gm992 1.607796706 II1r11 1.847727492 $Ms4a1/CD20$ 1.601129502 Lyz2 1.835585099 Mir16-2 1.600630161 Mir340 1.835109309 Ccr9 1.600133195 Thbs1 1.815598246 Fam196b 1.600098812 Sestd1 1.811648415 Ermp1 1.600006759 Mir342 1.8106319377 $112rb$ 1.59643078 Eid3 1.790513008 Slc22a15 1.596132036 Plek 1.774862177 Ebf1 1.594239081 Atp6v0a1 1.766243192 Pglyrp1 1.592379275 Gpr52 1.761727758 Tiaf2 1.590345254 Dock6 1.773191999 Mir15a 1.589690597 Slc5a3 1.745479 Igsf6 1.589515407 Cspp1 1.745083416 Gm100551 1.584336737 U29423 1.744086991 Gm10388 1.582688131	Spef2 (8)	1.873859779	C530030P08Rik (2)	1.614077666
$\begin{array}{llllllllllllllllllllllllllllllllllll$	Pten	1.85771862	Phgdh	1.608905956
$\begin{array}{llllllllllllllllllllllllllllllllllll$	Gm6683	1.856598683	Gm9982	1.607/96706
Lyz2 1.835585099 Mir16-2 1.600630161 Mir340 1.835109309 Ccr9 1.600133195 Thbs1 1.815598246 Fam196b 1.600098812 Sestd1 1.811648415 Ermp1 1.600006759 Mir342 1.806319377 II2rb 1.59643078 Eid3 1.790513008 Slc22a15 1.596132036 Plek 1.774862177 Ebf1 1.594239081 Atp6v0a1 1.76989405 Mir181a-1 1.593800439 Gm10008 1.766243192 Pglyrp1 1.592379275 Gpr52 1.761727758 Tiaf2 1.589690597 Slc5a3 1.745479 Igsf6 1.589690597 Cspp1 1.745083416 Gm10551 1.584336737 U29423 1.744086991 Gm10388 1.582688131	[11r]1	1.847727492	Ms4a1/CD20	1.601129502
Mir340 1.835109309 Ccr9 1.600133195 Thbs1 1.815598246 Fam196b 1.600098812 Sestd1 1.811648415 Ermp1 1.600006759 Mir342 1.806319377 II2rb 1.59643078 Eid3 1.790513008 Slc22a15 1.596132036 Plek 1.774862177 Ebf1 1.594239081 Atp6v0a1 1.76989405 Mir181a-1 1.593800439 Gm10008 1.766243192 Pglyrp1 1.592379275 Gpr52 1.761727758 Tiaf2 1.589690597 Dock6 1.753191999 Mir15a 1.589690597 Slc5a3 1.745479 Igsf6 1.589515407 Cspp1 1.745083416 Gm10551 1.584336737 U29423 1.744086991 Gm10388 1.582688131	Lyz2	1.835585099	Mirl6-2	1.600630161
Thbs11.815598246Fam196b1.600098812Sestd11.811648415Ermp11.600006759Mir3421.806319377II2rb1.59643078Eid31.790513008Slc22a151.596132036Plek1.774862177Ebf11.594239081Atp6v0a11.76989405Mir181a-11.593800439Gm100081.766243192Pglyrp11.592379275Gpr521.761727758Tiaf21.589690597Slc5a31.745479Igsf61.589515407Cspp11.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Mir340	1.835109309	Ccr9	1.600133195
Sestd11.811648415Ermp11.600006759Mir3421.806319377II2rb1.59643078Eid31.790513008Slc22a151.596132036Plek1.774862177Ebf11.594239081Atp6v0a11.76989405Mir181a-11.593800439Gm100081.766243192Pglyrp11.592379275Gpr521.761727758Tiaf21.590345254Dock61.753191999Mir15a1.589690597Slc5a31.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Thbs1	1.815598246	Fam196b	1.600098812
Mir3421.806319377Il2rb1.59643078Eid31.790513008Slc22a151.596132036Plek1.774862177Ebf11.594239081Atp6v0a11.76989405Mir181a-11.593800439Gm100081.766243192Pglyrp11.592379275Gpr521.761727758Tiaf21.590345254Dock61.753191999Mir15a1.589690597Slc5a31.745479Igsf61.589515407Cspp11.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Sestd1	1.811648415	Ermpl	1.600006759
Eid31.790513008Slc22a151.596132036Plek1.774862177Ebf11.594239081Atp6v0a11.76989405Mir181a-11.593800439Gm100081.766243192Pglyrp11.592379275Gpr521.761727758Tiaf21.590345254Dock61.753191999Mir15a1.589690597Slc5a31.745479Igsf61.589515407Cspp11.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Mir342	1.806319377	ll2rb	1.59643078
Plek1.774862177Ebf11.594239081Atp6v0a11.76989405Mir181a-11.593800439Gm100081.766243192Pglyrp11.592379275Gpr521.761727758Tiaf21.590345254Dock61.753191999Mir15a1.589690597Slc5a31.745479Igsf61.589515407Cspp11.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Eid3	1.790513008	SIc22a15	1.596132036
Atp6v0a11.76989405Mir181a-11.593800439Gm100081.766243192Pglyrp11.592379275Gpr521.761727758Tiaf21.590345254Dock61.753191999Mir15a1.589690597Slc5a31.745479Igsf61.589515407Cspp11.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Plek	1.774862177	Ebf1	1.594239081
Gm100081.766243192Pglyrp11.592379275Gpr521.761727758Tiaf21.590345254Dock61.753191999Mir15a1.589690597Slc5a31.745479Igsf61.589515407Cspp11.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Atp6v0a1	1.76989405	Mir181a-1	1.593800439
Gpr521.761727758Tiaf21.590345254Dock61.753191999Mir15a1.589690597Slc5a31.745479Igsf61.589515407Cspp11.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Gm10008	1.766243192	Pglyrp1	1.592379275
Dock61.753191999Mir15a1.589690597Slc5a31.745479Igsf61.589515407Cspp11.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Gpr52	1.761727758	Tiaf2	1.590345254
Slc5a31.745479Igsf61.589515407Cspp11.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Dock6	1.753191999	Mir15a	1.589690597
Cspp11.745376164Myo9a (4)1.585628721BC0184731.745083416Gm105511.584336737U294231.744086991Gm103881.582688131	Slc5a3	1.745479	Igsf6	1.589515407
BC018473 1.745083416 Gm10551 1.584336737 U29423 1.744086991 Gm10388 1.582688131	Cspp1	1.745376164	Myo9a (4)	1.585628721
U29423 1 744086991 Gm10388 1.582688131	BC018473	1.745083416	Gm10551	1.584336737
1./ 11000//1	U29423	1.744086991	Gm10388	1.582688131

(continued)

(continued)

SUPPLEMENTARY TABLE S1. (CONTINUED)

SUPPLEMENTARY TABLE S1. (CONTINUED)

Gene symbol	KO/WT fold change	Gene symbol	KO/WT fold change
Cd74	1.581548721	Cxcr2	1.484834359
Mir421	1.579363222	Nfat5	1.481434664
Snord53	1.578831272	Gm9766	1.48114204
Plcg2	1.577008023	ND6	1.481112267
I830127L07Rik	1.576884508	Rora	1.480894638
Il23r	1.572251487	H2-Aa	1.477826598
Plxdc2	1.57146049	4932438A13Rik	1.47673402
Gtf3c2	1.569655545	Gabarapl2	1.476380922
Igf1r	1.568950678	Olfr798	1.475314977
Cd19	1.565847811	Nt5e	1.475164661
Atp6v0d2	1.563643906	Bglap	1.474376519
Lrrc6	1.561713704	Cep350	1.472246251
	1.5604/6899	Tiam I	1.466200091
D130062J21R1K	1.560400104		1.464405401
Clic4	1.559301599		1.404125275
LITK2	1.558559249	H2-EDI Kanna	1.402234003
1/00081L11KIK Adam10	1.557594829	Kening Eif2a2	1.401123398
Adam19 Wnk1	1.530595808	EI12C2 BC040807	1.439303837
WIIKI Uzafa	1.540004205	BC049807 Halls	1.439447182
Cdk13	1.547457726	B230325K18Rik	1 459405706
Foxn3	1.547039465	lign1	1 459380417
Frn1	1 54637155	Gent1	1 459379406
Htt	1 546251506	St6galnac3	1 459180141
4932438A13Rik	1 543946755	Ctsh	1 459108331
Rangrf (2)	1.543626803	LOC100046275	1.458807983
Tet3 (2)	1.543034159	Tsc22d1	1.458530949
Gnrh1	1.542832027	Neil2	1.457548611
Kcnip2	1.541432805	Rnf157	1.457538508
Snord85	1.539856594	Nbeal1	1.457105159
Ptpn3	1.539430782	Cd180	1.456393291
Dennd4a (2)	1.535551608	Anxa4	1.454948411
Raph1	1.534081362	Gm5415	1.454752777
Diap2	1.532536038	Swap70	1.45262569
Gm340	1.53231935	Gm16970	1.451414914
1300014I06Rik	1.529835997	Ikzf4	1.449870453
Mil2 (2)	1.526301526	Olfr776	1.449856383
Cpd	1.524853878	Slamf7	1.449504689
Hipk2	1.520921824	Cyfipl	1.448632854
MIII LOG(20927	1.51834538	Numbers within parentheses indicate w	when genes are represented
LUC030837	1.516013006	by multiple probes Proteins implicated	in Ca^{2+} -binding or Ca^{2+}
FSCIII Dilro	1.51535209	-dependent activities are shown in bold.	in our binding of ou
Filla Ment8	1.515511065	KO, knockout; WT, wild type.	
Arrde	1 510313453		
Mir19h-1	1 509836156		
Arhgan?6	1 509254393	Splenocyte isolation CD4 ⁺ T-cell pu	rification
Plbd1	1.507180286	and activation	inication
Tnfrsf1b	1.506103588		
Cdk6	1.503428231	CD4 ⁺ cells were purified from	the whole splenocyte
Nup2101	1.499262499	fraction using CD4 ⁺ isolation kit II (1	Miltenyi Biotec). CD4 ⁺
5430427O19Rik	1.499071296	cells were activated, unless otherwis	se indicated, with plate-
Spib	1.496298489	bound α -CD3 (1.0 μ g/ml) and solut	ble α -CD28 (1.0 μ g/ml;
Ncf1	1.495406802	both from Biolegend) and cultured a	at a density of $\sim 1 \times 10^6$
Bcl2a1c	1.495382961	cells/ml in 24-well plates in RPMI	-1640 medium supple-
Pou2f2	1.495218164	mented with Anti-Anti mixture and	10% FBS).
Atrn11	1.494269117	······································	/ -
Mir30e	1.49148038	Mitochondrial morphology imaging	
Endod I	1.491130992		
1 mem1 /6b	1.488837192	Epithelial cells at $\sim 70\%$ –80% c	confluency were treated
vav2 Caaph3	1.48/9045/1	with Ca ²⁺ and Ca ²⁺ /ionomysin for	15 mins and with Mito-
Caciilos	1.48498009	tracker Red. Cells were then wash	ned and fixed in 3.7%
	(continued)	formaldehyde for 15 mins in the da	ark, permeabilized, and
		mounted with DAPI-containing mound	nting media. Zeiss LSM

SUPPLEMENTARY TABLE S2.	LIST OF GENES
(Left Column) Activated	BY CD3/CD28
STIMULATION OF CD4 ⁺ T C	Cells for 12 h

SUPPLEMENTARY TABLE S2. (CONTINUED)

Activation index

Act		n index	#. Gene name	WT	KO
#. Gene name	WT	КО	61. Apol7c	1.74540278	1.0197539
			62. Rtp4	1.73695575	1.1549401
1. Irf4	12.3231314	7.5318747	63. Fscn1	1.73445571	1.0413529
2. Itih5	6.82112641	4.4342527	64. Gbp4	1.73056125	1.3432613
3. Cd69	5.43508651	3.884853	65. Snhgl	1.66413561	1.0402895
4. Il2	4.97878253	3.4303814	66. Shmt1	1.66260793	1.2436952
5 Ccl22	4.36408439	3.5443228	67. Nup2101	1.66105633	0.9412344
6. Socs2	4.33369013	3.0510842	68. Zfp640	1.646/3/96	1.1902/92
7. Gbp5	3.91431538	2.5991286	69. MIRP42	1.02451207	1.1938197
8. Thf	3.49319456	2.6839113	70. GIII8993	1.02229929	1.23999999
9. Gpr83	3.3371904	2.3182899	71. $GOPO$	1.02041124	1.2/23/13
10. ligp1	3.21244///	1.824061	72. $1/154a4c$ 73. $1/2rb$	1.01439090	1.2347113
11. NIKDIO	2.86361988	2.1490395	73. 11210 74. 7fp600	1.60/13/38	1.1170202
12. Clic4	2.75261912	2.039259	75 $Mrps 36$	1 50112021	1 1770063
15. $DCall = 14. Cm 12250$	2.00730133	1.60/2399	76 2810021G02Rik	1 59014685	0.8284864
14. GIII12230 15. Gm120	2.39730327	1.3280800	77 1810029B16Rik	1.597011005	1 2941824
15. Ghn2	2.50006574	1.7132703	78 Snord38a	1.58652032	1 0149756
17 E430024C06Bik	2.33934779	1.741293	79. Atp13a3	1.58334868	0.8990199
18 Cd83	2.4273713	1.9056366	80. Zfp455	1.58070922	0.955783
19 Bel^{2}	2 3530272	1 5129623	81. Akr1c18	1.57804572	1.0165795
20 Fcer2a	2.34646835	1 1004488	82. Dkc1	1.57621791	1.1843726
21 Rrs1	2.30439495	1 5762889	83. Gm5921	1.57587161	1.2149189
22. $Tgtp1$ (2)	2.25670838	1.4218507	84. Pus7	1.57198015	1.0921023
23. Myb	2.17504462	1.560054	85. Cmpk1	1.57002116	0.9486403
24. Gbp1	2.1727437	1.6590588	86. Irf9	1.56850486	1.0862303
25. Irgm1	2.15698329	1.408188	87. Chchd4	1.56580223	1.1182037
26. Bcl2a1a	2.14358853	1.6992275	88. Dleu2	1.56456218	1.2228742
27. Irgm2	2.11152306	1.5346599	89. Samsn1	1.55176598	1.2096117
28. Gbp3	2.0939892	1.6267979	90. Trdn	1.54395104	1.0980015
29. Odc1	2.06805564	1.6446094	91. St6galnac4	1.53248824	1.270867
30. Cd200	2.05993241	1.5080163	92. Nudt11	1.52517522	0.8827299
31. Rgs1	2.03068434	1.5311366	93.2610044015Rik	1.52402651	1.0611135
32. Pim2	1.9926385	1.3690261	94. Inip3	1.52258102	1.1098056
33. Rgs16	1.98996768	1.5624045	95. Anp52-ps	1.32207873	0.9394782
34. Mpa21	1.96120453	1.4460025	90. Clid7 07 Gm8630	1.51702969	1.1702030
35. Serpinb9	1.94931738	1.2539241	97.0110039 08 L tn 1	1.513844297	1.1077712
30. Stat1	1.94603816	1.3548283	99 Din2c	1 51088096	0.9273855
37. CCr8	1.92109982	2.4/24108	100 St13	1.51031659	1 1261352
30. Cui	1.92101329	0.8414182	101 Ipo7	1.50496402	1.0958649
40 Non58	1.9020320	1 / 965381	102. Dnaic13	1.49571157	0.9562309
41 Rnf213	1 89197234	1.0120356	103. Gcsh	1.48657989	1.1327371
42 Apex1	1.88733562	1 1718371	104. Ewsr1	1.48511639	0.9691541
43. Ttc27	1.86722269	1.4652786	105. Rpl2211	1.48209816	1.0958862
44. Hspd1	1.86482468	1.5459568	106. Sft2d3	1.4812714	1.0461836
45. Gm4759	1.8556672	1.0056728	107. H2-T24	1.47696946	1.0999165
46. Gnl3	1.85325191	1.4933465	108. Slc7a11	1.4699866	1.1291374
47. Ebi3	1.84830648	1.3738971	109. Olfr800	1.4679227	1.1890208
48. Swap70	1.83020722	1.1431463	110. Utp18	1.46726047	1.1459777
49. Dnaĥc7b	1.82472114	1.4806945	111. Idi1	1.46641759	1.0678056
50. Lilrb4	1.82399655	1.2254096	112. Olfr798	1.46236455	0.7831464
51. Gvin1	1.82268721	1.3510396	113. Eif2b3	1.45793965	1.14/8531
52. Gpt2	1.80460114	1.1846509	114. Naal5	1.45494034	1.2112528
53. Gp49a	1.80054418	1.2338004	115. Snord58b	1.45452491	1.010018
54. Gm5772	1.79634817	1.250045	110. Ia5	1.45199/55	1.1333154
55. Gm13235	1.78177479	0.6577367	117. Nampt 118. No111	1.43183832	1.13193/9
56. Tsen15	1.78106232	1.3933995	110. F2f5	1.45159702	0.0020554
5/. NIRCS	1.///19994	1.0263452	117. E213	1.400110/	0.9939334
50. Ecml	1.//3966/9	1.4163129	Right columns present	activation indices (expre	ession level after
J7. SHOPUIC	1.77150557	1.1833032	stimulation/basal expression	on level) for these genes	in Fusl WT and
00. KIP15	1.//10000/	1.3108028	Fusl KO CD4 ⁺ T cells.		

(continued)

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

Measurement of mitochondrial Ca²⁺ uptake capacity of digitonin-permeabilized cells

Extramitochondrial free Ca²⁺ was monitored in the presence of digitonin-permeabilized cells as described in (42, 48) Briefly, cells (2×10⁶ cells/ml) were resuspended in KCl medium (125 m*M* KCl, 2 m*M* K₂HPO₄, 1 m*M* MgCl₂, 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 μ *M* Ca²⁺ 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 Ca²⁺ homeostasis was measured by the replacement of Na-containing buffer with Na-free buffer or by the addition of benzodiazepine CGP37157 (10– 20 μ *M*), 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 μ M), stimulated with CD3/CD28 (1 μ g/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 IFN γ -FITC (1/100 μ l permeabilization buffer) and α -IL4-PE (1/100 μ l 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 \pm 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.