Science Signaling
AAAS

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Supplementary Materials for

Stress-induced dynamic regulation of mitochondrial STAT3 and its association with cyclophilin D reduce mitochondrial ROS production

Jeremy A. Meier, Moonjung Hyun, Marc Cantwell, Ali Raza, Claudia Mertens, Vidisha Raje, Jennifer Sisler, Erin Tracy, Sylvia Torres-Odio, Suzana Gispert, Peter E. Shaw, Heinz Baumann, Dipankar Bandyopadhyay, Kazuaki Takabe, Andrew C. Larner*

*Corresponding author. Email: andrew.larner@vcuhealth.org

Published 28 March 2017, *Sci. Signal.* **10**, eaag2588 (2017)
DOI: 10.1126/scisignal.aag2588

This PDF file includes:

Supplemental Figure 1

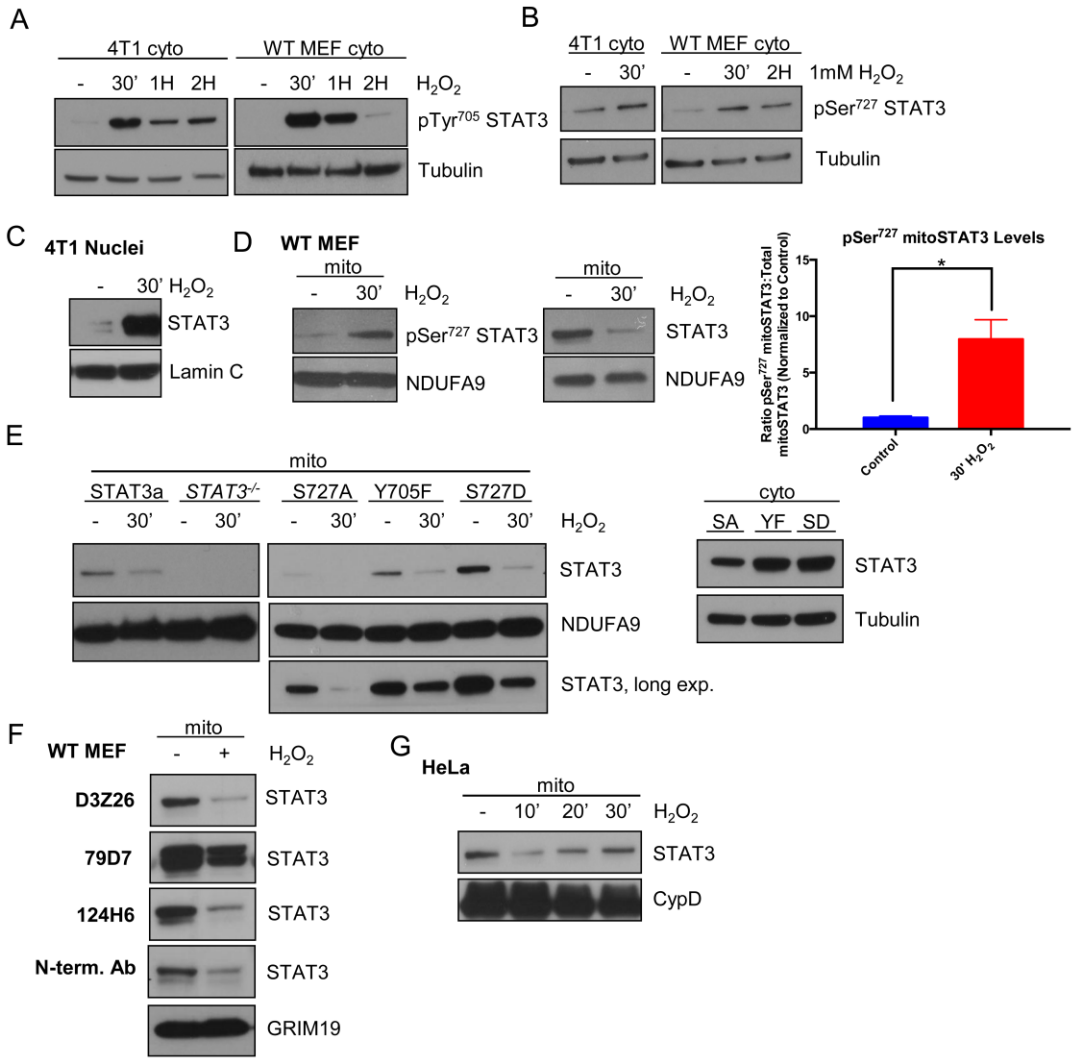

Fig. S1. Ser^{727} and Tyr^{705} of STAT3 are not required for H_2O_2-induced mitoSTAT3 loss. 4T1 or wild-type (WT) MEF cytosolic extracts were probed for pTyr^{705} (**A**) or (**B**) pSer^{727} STAT3. Blots are representative of 2 independent experiments. (**C**) 4T1 cells untreated or treated for 30' with H_2O_2 were collected and nuclear extracts were immunoblotted for STAT3. Blots are representative of 2 independent experiments. (**D**) Mitochondrial lysates were probed for pSer^{727} STAT3 and total STAT3 abundance and quantified by densitometry analysis. *p=0.0229. Blots are representative of 4 independent experiments. (**E**) *STAT3*$^{-/-}$ MEFs were reconstituted with WT STAT3 (STAT3a) or Ser^{727} (S727A and S727D) and Tyr^{705} (Y705F) mutants. Mitochondrial extracts from untreated cells or cells treated with H_2O_2 for 30' were immunoblotted for STAT3 or NDUFA9

(loading control). Cytosolic amounts of STAT3 Ser^{727} and Tyr^{705} mutants are shown as a control (right panel). Blots are representative of 2 independent experiments. (**F**) Mitochondrial extracts from WT MEFs untreated or treated for 30' with H_2O_2 were probed with multiple STAT3 antibodies and GRIM19 (loading control). D3Z2G (Cell Signaling, rabbit monoclonal, C-terminus), 79D7 (Cell signaling, rabbit monoclonal, C-terminus), 124H6 (Cell Signaling, mouse monoclonal, C-terminus), N-term Ab (BD Biosciences, mouse monoclonal, N-terminus). Blots are representative of 2 independent experiments. (**G**) Mitochondria were isolated from HeLa cells treated for the indicated times with H_2O_2 and lysates were resolved by SDS-PAGE and blotted for STAT3. Blots are representative of 2 independent experiments.

Supplemental Figure 2

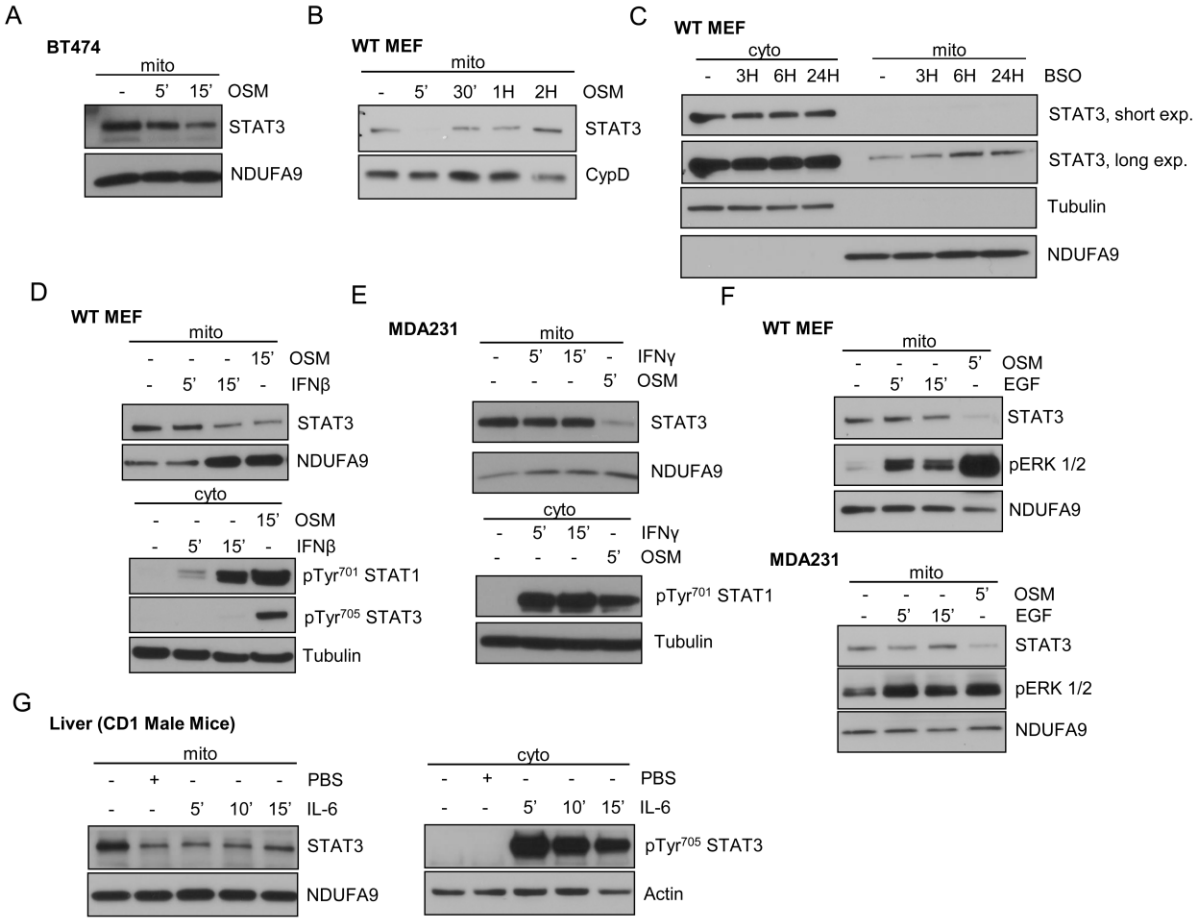

Fig. S2. Selectivity of the mitoSTAT3 signaling pathway and relevance in vivo. (**A**) BT474 [$ER^-/PR^-/HER2^+$] human breast cancer cells were treated with OSM for the indicated times and mitochondrial extracts were probed for STAT3. Blots are representative of 2 independent experiments. (**B**) Mitochondria from WT MEFs treated with OSM for the indicated times were isolated and extracts immunoblotted for STAT3. Blots are representative of 2 independent experiments. (**C**) WT MEFs were treated with Buthionine Sulfoximine (BSO) for the indicated times and mitochondrial extracts were immunoblotted for mitoSTAT3. Blots are representative of 2 independent experiments. (**D**) WT MEFs were treated for the indicated times with IFNβ and immunoblotted for STAT3 (mito fraction, top panel) or $pTyr^{701}$ STAT1 and $pTyr^{705}$ STAT3 (cyto fraction, bottom panel). Blots are representative of 2 independent experiments. (**E**) Mitochondrial extracts from MDA-231 cells were treated with IFNγ and mitochondrial fractions were

probed for STAT3 (top panel). Cytosolic extracts (bottom panel) were probed for pTyr^{701} STAT1, Blots are representative of 2 independent experiments. (**F**) Mitochondrial extracts from WT MEFs (top panel) or MDA-231 cells (bottom panel) were treated with epidermal growth factor (EGF) and immunoblotted for STAT3. Blots are representative of 2 independent experiments. (**G**) 8-12 week old CD1 male mice were tail vein injected with either PBS or IL-6 (500μg/kg) and liver mitochondrial (left panel) or cytosolic fractions (right panel) were immunoblotted for STAT3 and pTyr^{705} STAT3 respectively. A non-injected control liver extract is presented in Lane 1. Blots are representative of 2 independent experiments.

Supplemental Figure 3

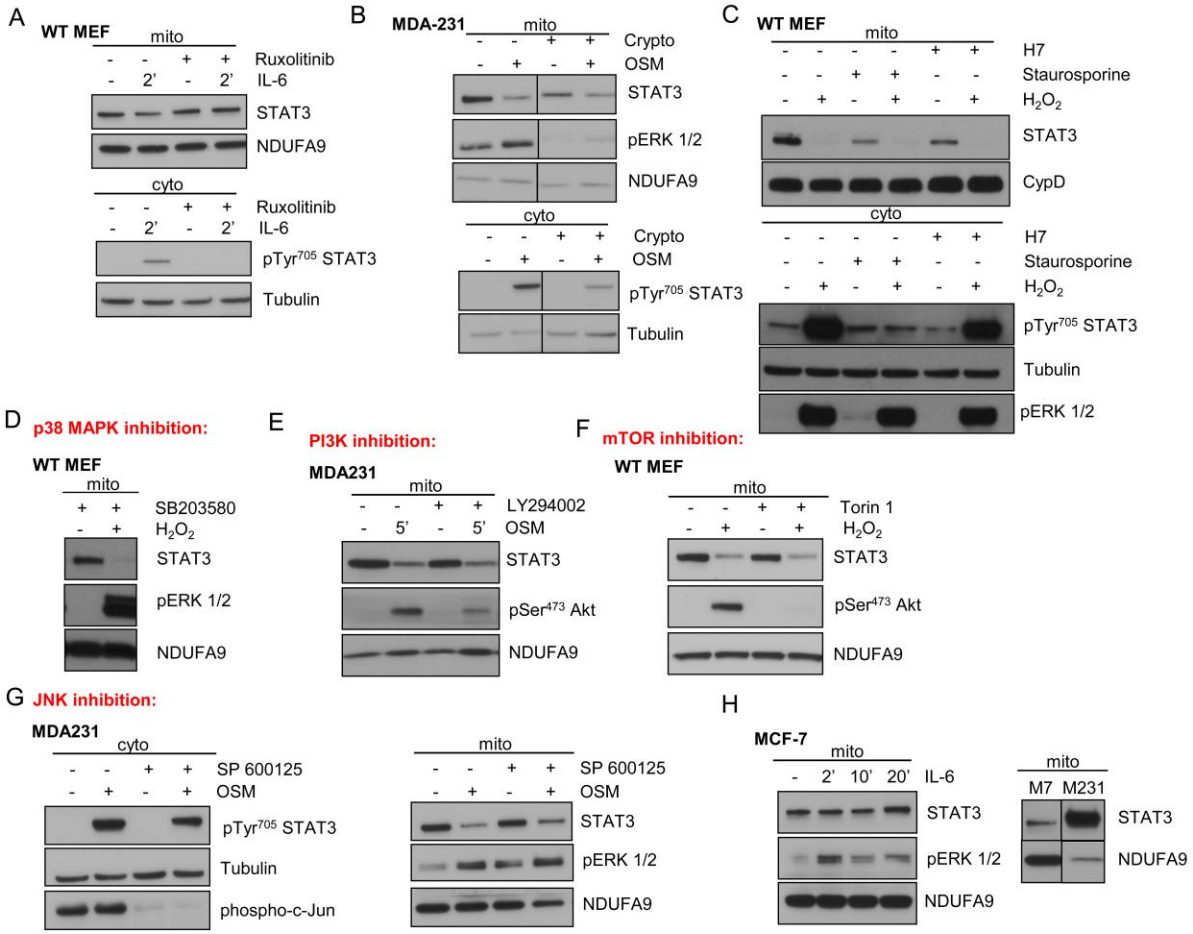

Fig. S3. Inhibition of relevant kinase pathways does not affect stimulation-induced decreases in mitoSTAT3. (**A**) WT MEFs were treated with IL-6 either in the presence or absence of Ruxolitinib and mitochondrial and cytosolic extracts were evaluated by western blotting. Blots are representative of 2 independent experiments. (**B**) MDA-231 cells were pre-treated with the STAT3 inhibitor cryptotanshinone for 1H prior to OSM stimulation (5' treatment). Mitochondrial and cytosolic extracts were immunoblotted for STAT3 and pTyr^{705} STAT3 respectively. Blots are representative of 2 independent experiments. (**C**) WT MEFs were incubated with Staurosporine or H7 Dihydrochloride 30' prior to H_2O_2 treatment and mitochondrial (top panel) and cytosolic (bottom panel) fractions were immunoblotted. Blots are representative of 2 independent experiments. (**D** to **G**) WT MEFs or MDA-231 cells were pre-incubated with inhibitors to p38 MAPK (**D, SB203580**), PI3K (**E, LY294002**), mTOR (**F, Torin 1**), or JNK (**G, SP600125**) and

treated with either OSM (5') or H_2O_2 (30'). Mitochondrial and/or cytosolic extracts were probed as indicated. Blots are representative of 2 independent experiments. (**H**) MCF-7 cells were treated with IL-6 for the indicated times and mitochondrial extracts were probed for STAT3, pERK1/2, or NDUFA9 (left panel). Comparison of mitochondrial STAT3 amounts in MCF-7 compared to MDA-231 cells (right panel). Blots are representative of 3 independent experiments.

Supplemental Figure 4

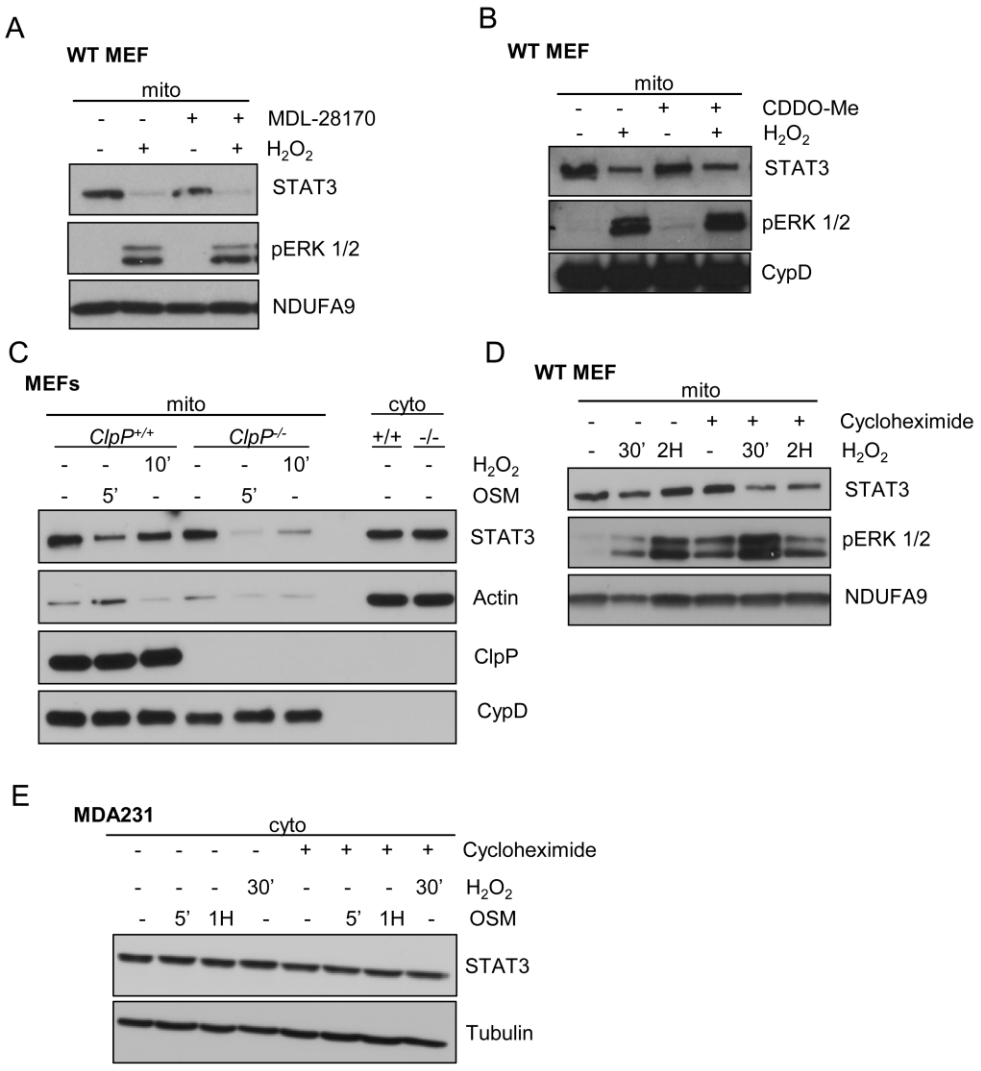

Fig. S4. Inhibition of mitoproteases does not affect proteolysis of mitoSTAT3. (**A**) WT MEFs were pretreated with the calpain inhibitor MDL-28170 and subjected to H_2O_2 treatment for 30'. Mitochondrial extracts were probed for STAT3. Blots are representative of 2 independent experiments. (**B**) Mitochondrial lysates from CDDO-Me pre-treated WT MEFs were subjected to western blotting following treatment with H_2O_2 for 30'. Blots are representative of 2 independent experiments. (**C**) $ClpP^{+/+}$ or $ClpP^{-/-}$ MEFs were treated with either OSM or H_2O_2 and mitochondrial and cytosolic fractions were probed for STAT3, Actin, ClpP, and CypD. Blots are representative of 2 independent experiments. (**D**) WT MEFs were treated with cycloheximide for 2H prior to H_2O_2 stimulation and isolated mitochondrial extracts were immunoblotted. Blots are

representative of 2 independent experiments. (**E**) Cytosolic extracts from MDA-231 cells treated with or without cycloheximide were stimulated with OSM or H_2O_2 and subjected to western blotting. Blots are representative of 3 independent experiments.

Supplemental Figure 5

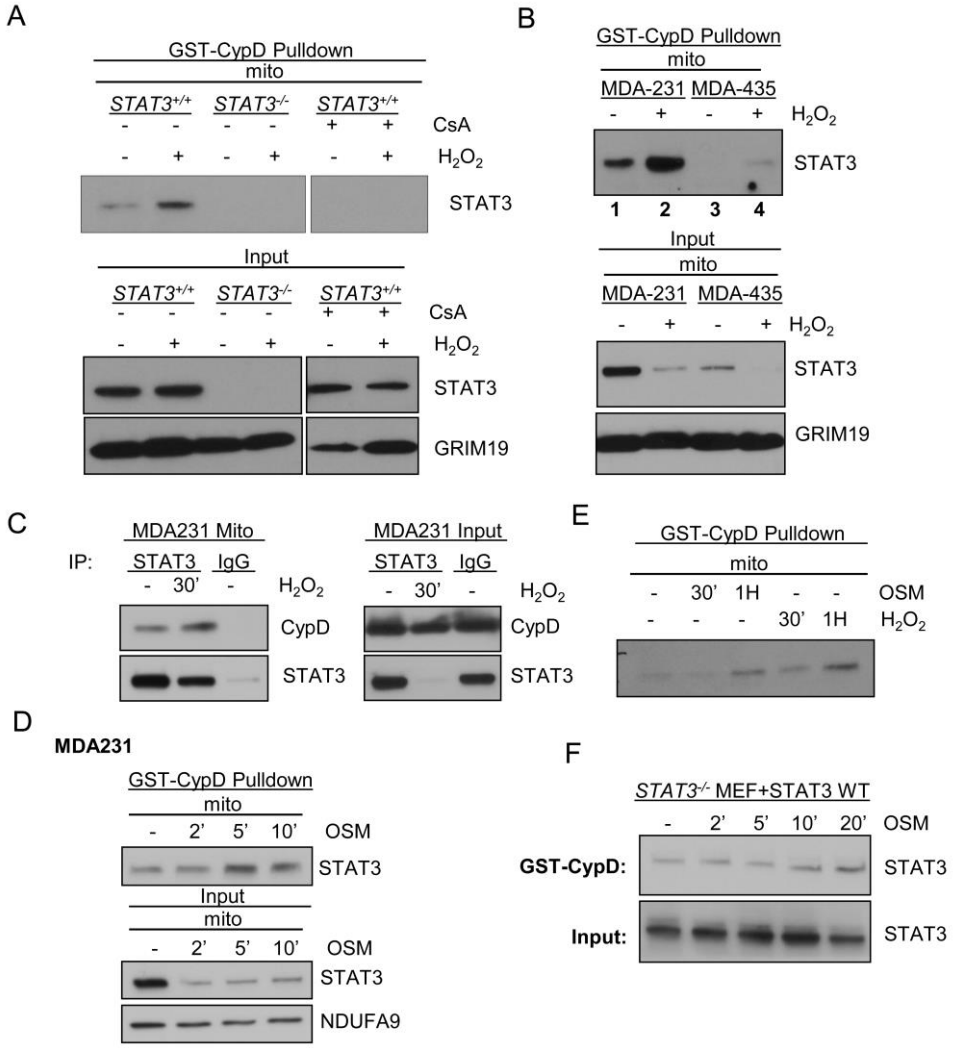

Fig. S5. mitoSTAT3 inducibly binds to CypD after H_2O_2 or cytokine stimulation. (**A**) Mitochondrial lysates from *STAT3*$^{+/+}$ or *STAT3*$^{-/-}$ MEFs untreated or treated with H_2O_2 were incubated with GST-CypD in the presence or absence of CsA and probed for STAT3. Input is shown in bottom panel. Blots are representative of 2 independent experiments. (**B**) MDA-231 [ER$^-$/PR$^-$/HER2$^-$] or MDA-435 [ER$^-$/PR$^-$/HER2$^+$] human breast cancer cells were treated for 4H with H_2O_2 and purified mitochondria were subjected to GST-CypD pulldown (top panel). Lower panels show the input (10% of that used for the pull downs). Blots are representative of 2 independent experiments. (**C**) Immunoprecipitation of STAT3 or control IgG from purified mitochondria from MDA-231 cells untreated or treated for 30' with H_2O_2 and probed for STAT3 and CypD. Lower

panels show the input (10% of that used for the immunoprecipitation) Blots are representative of 2 independent experiments. (**D** to **F**) GST-CypD pulldown of STAT3 from mitochondrial extracts following OSM treatment for the indicated times in MDA-231 cells (**D**), 4T1 cells (**E,** input presented in Fig. 2C), and in *STAT3*$^{-/-}$ MEFs re-constituted with WT STAT3 (**F**). Blots are representative of 2 independent experiments.

Supplemental Figure 6

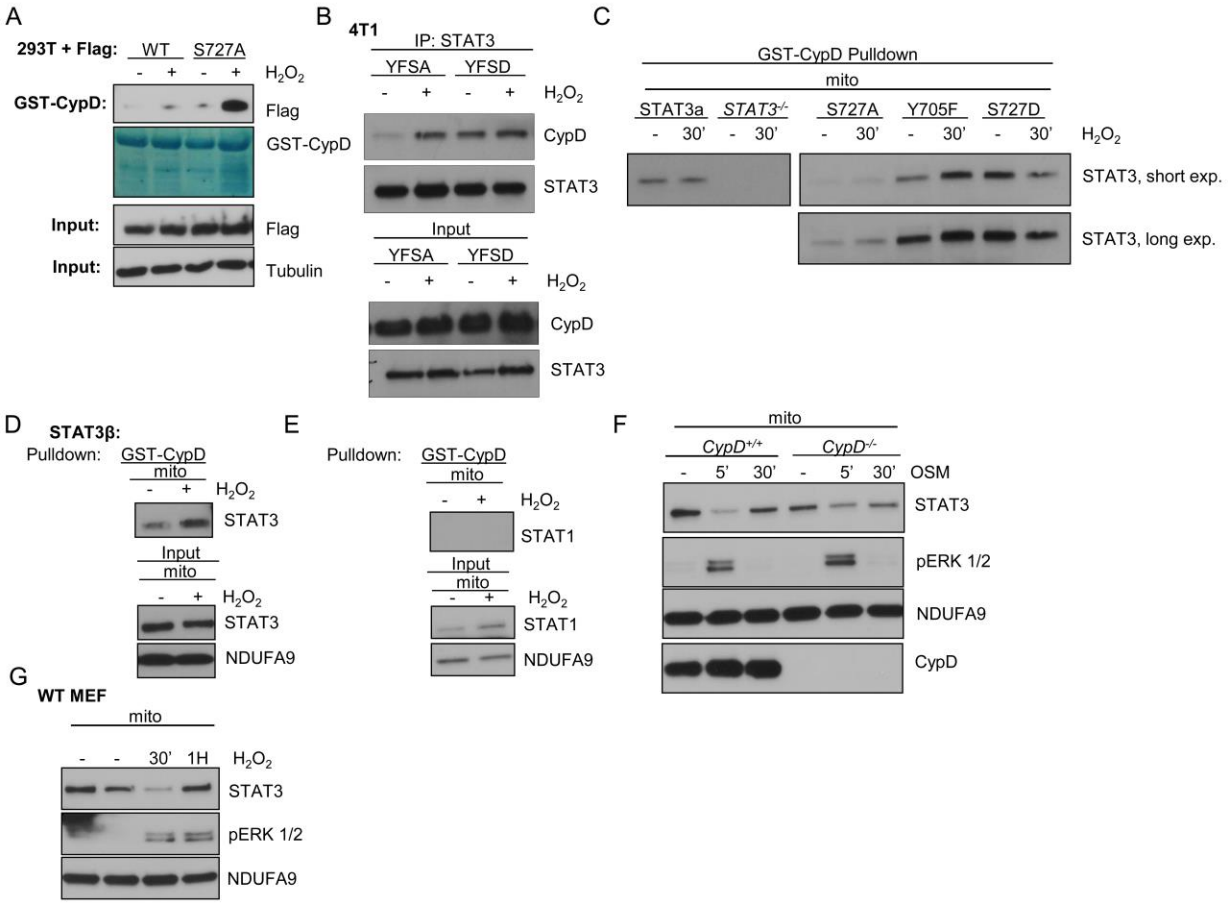

Fig. S6. Ser^{727} is dispensable for the mitoSTAT3-CypD interaction. (**A**) Expression of Flag-STAT3 WT or -S727A STAT3 in 293T cells was followed by H_2O_2 treatment and resulting whole cell extracts were incubated with GST-CypD and probed for Flag. Coomassie stain of GST-CypD input is shown. Blots are representative of 2 independent experiments. (**B**) 4T1 cells expressing the mutant STAT3 isoforms Y705F/S727A (YFSA) or Y705F/S727D (YFSD) were treated with H_2O_2 and STAT3 was immunoprecipitated from mitochondrial extracts and probed for CypD. Blots are representative of 2 independent experiments. (**C**) *STAT3*$^{-/-}$ MEFs were transduced with retroviral constructs to drive expression of WT STAT3 (STAT3α) or the following STAT3 mutant constructs: S727A, Y705F, or S727D. Immunoblots of mitoSTAT3 following GST-CypD Pulldown of mitochondrial extracts isolated from cells after 30' H_2O_2 treatment. Input as presented in fig. S1E. Blots are representative of 2 biological replicates. (**D**) *STAT3*$^{-/-}$ MEFs expressing STAT3β, a C-terminal truncation mutant that

lacks the Ser^{727} site, were treated with H_2O_2 (30') and subjected to GST-CypD pulldown with blotting for STAT3. Blots are representative of 2 independent experiments. (**E**) Mitochondria from WT MEFs treated for 30' with H_2O_2 were incubated with GST-CypD and probed for mitochondrial STAT1. Blots are representative of 2 independent experiments. (**F**) $CypD^{+/+}$ MEFs and $CypD^{-/-}$ MEFs were treated for the indicated times with OSM and isolated mitochondrial lysates were immunoblotted for STAT3, pERK1/2, NDUFA9 (loading control) and CypD. Blots are representative of 2 independent experiments. **(G**) WT MEFs were pre-treated with CsA (4H) then treated with H_2O_2 for the indicated times and mitochondrial lysates were probed for STAT3. Blots are representative of 2 independent experiments.