Supplemental Figures and Experimental Procedures

Pyruvate dehydrogenase complex (PDC) subunits moonlight as interaction partners of phosphorylated STAT5 in adipocytes and adipose tissue

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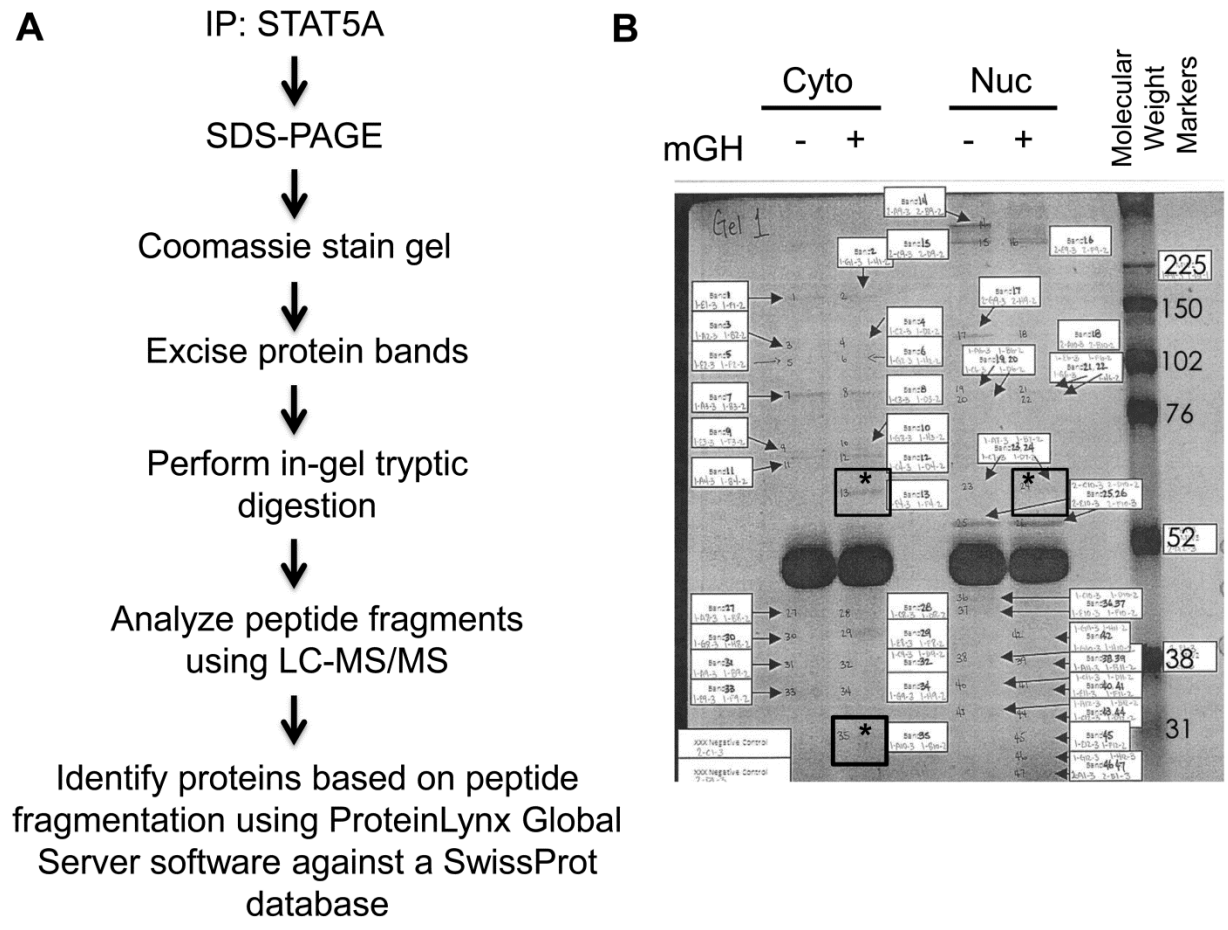

Figure S1. PDC subunits were identified as STAT5A-interacting proteins using a semi-non-biased MS-based approach. STAT5A was immunoprecipitated from cytosolic (Cyto) and nuclear (Nuc) fractions prepared from mature 3T3-L1 adipocytes under control and GH-stimulated conditions. A) Flow chart of the process we used to identify novel STAT5A-interacting protein partners in adipocytes. B) From this Coomassie-stained gel, protein bands were excised, digested and analyzed using LC-MS/MS. This analysis resulted in the identification of approximately a dozen STAT5A-binding proteins. Some of our most surprising hits were the E2 (70 kDa) and E1β (39 kDa) subunits of the pyruvate dehydrogenase complex (marked by *). These proteins were each identified from 8 – 13 peptide fragments analyzed using the ProteinLynx Global software against a SwissProt database (data not shown). The interactions between STAT5A and PDC subunits were also among the few clearly regulated by GH.

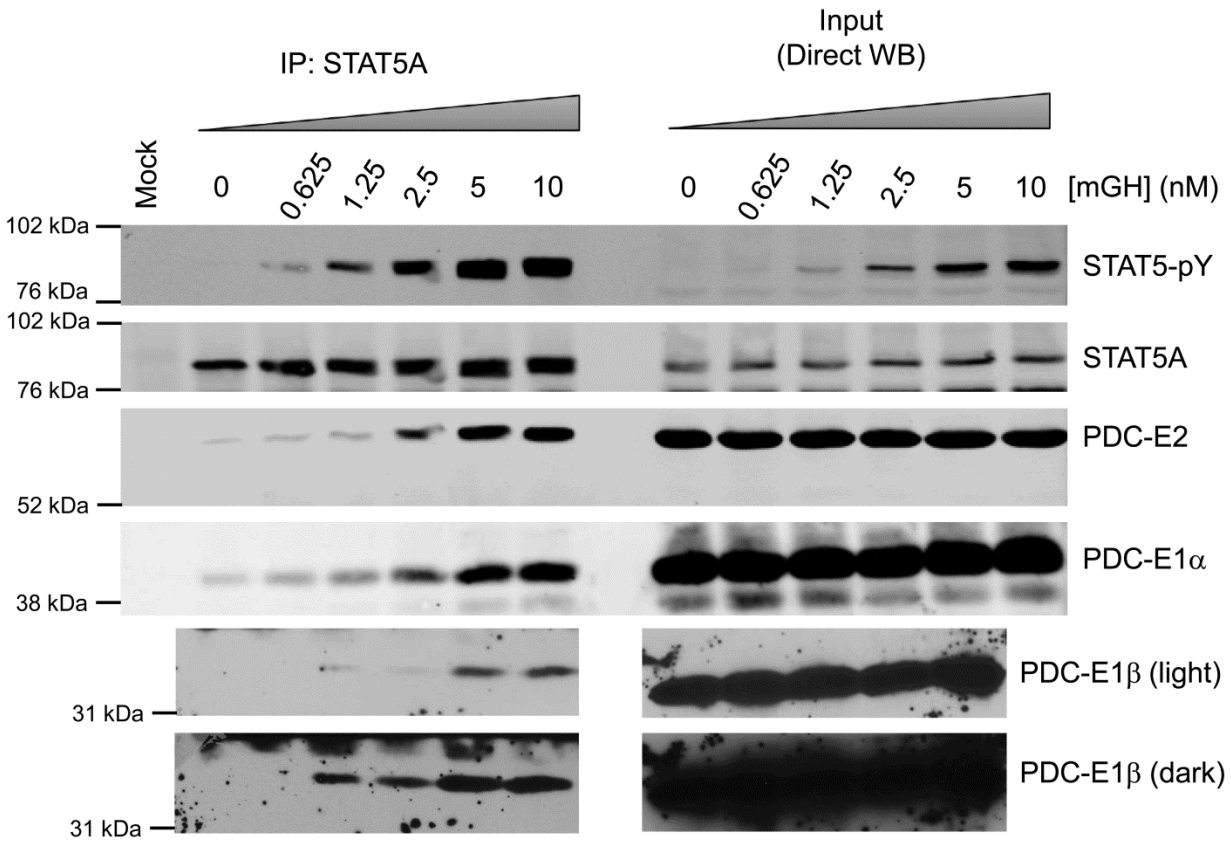

Figure S2. GH induces the interaction between STAT5A and multiple PDC-E1 subunits (α and β) in a dose-dependent manner. Fully differentiated 3T3-L1 adipocytes were untreated (0) or treated with 0.625 – 10 nM mGH for 15 min. Monolayers were scraped into immunoprecipitation (IP) lysis buffer and whole-cell lysates were prepared. IP experiments were performed using the anti-STAT5A antibody and 300 μg of lysate protein. The mock experiment contained IP antibody but no protein lysate. Western blotting (WB) was used to examine the protein content of the immunoprecipitates (IP: STAT5A; left) and inputs (Direct WB; right). Molecular weight markers are shown to the left of each strip, while western blot targets are identified on the right. E1α (43 kDa), E1β (39 kDa), and E2 (70 kDa) are subunits of PDC. The expected molecular weight of STAT5A/STAT5^{pY} is 95 kDa. The rabbit polyclonal anti-PDC-E1β antibody was purchased from Abcam (Cat #: ab126203).

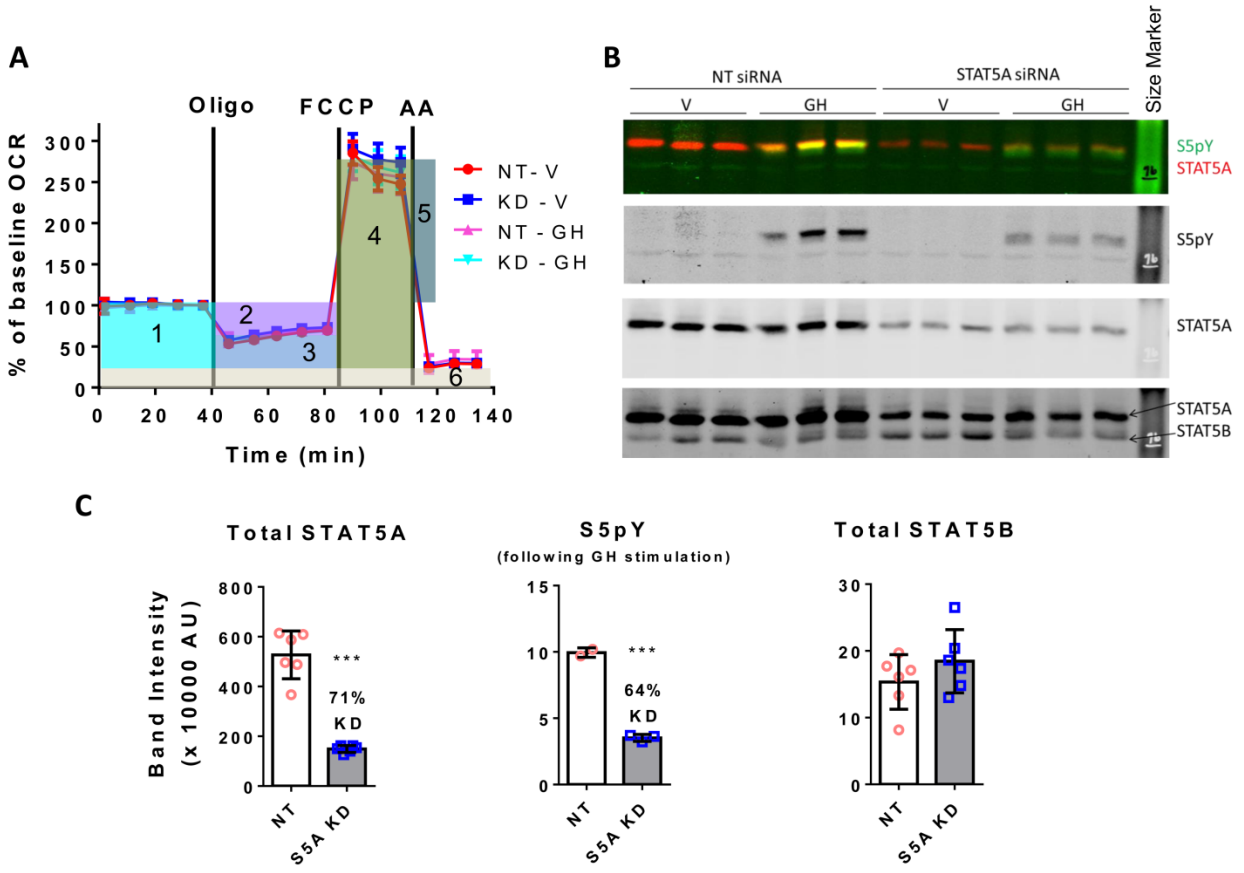

Figure S3. Growth hormone stimulation and STAT5A knockdown do not alter cellular respiration in adipocyte mitochondria. A) Oxygen consumption rate (OCR) was measured from mature 3T3-L1 adipocytes using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Basal respiration **(1)** was measured. Then mitochondrial function was perturbed using Oligomycin (Oligo; ATP synthase inhibitor), FCCP (mitochondrial uncoupler), and Antimycin A (AA; complex III inhibitor) to examine ATP production **(2)**, proton leak **(3)**, maximal respiration **(4)**, and spare respiratory capacity **(5)**. After shutting down the flow of electrons through the electron transport chain, the remainder of the OCR results from non-mitochondrial respiration **(6)**. None of these six aspects of basal respiration were altered by growth hormone (GH) stimulation or by knocking down STAT5A in mature adipocytes. Adipocytes were transfected with non-targeting (NT) or STAT5A-targeted (KD) siRNA 5 days prior to overnight treatment with vehicle (V; 10 uM $NaHCO_3$) or GH (10 nM) for 16 hours. OCR was measured in the morning following overnight treatment. OCR data are plotted as mean ± standard deviation (n = 5 wells per group). This experiment was performed one time. **B)** In a parallel experiment, mature 3T3-L1 adipocytes were transfected with siRNA as described for Figure S3A for ~6 days prior to treatment with V or GH for 20 minutes. Adipocytes were harvested in IP buffer and analyzed by Western blotting. The expected molecular weight of STAT5A is 95 kDa and STAT5B is 92 kDa. GH induced STAT5 tyrosine phosphorylation (S5pY), and phosphorylated STAT5 run at ~95 kDa. Similar levels of STAT5A knockdown

were observed from experiments on at least 3 independent batches of cells. **C)** Band intensities for total STAT5A, tyrosine phosphorylated STAT5 (S5pY), and total STAT5B in Figure S3B were quantified using Image Studio Lite Ver. 4 (LI-COR Biotechnology). Data are plotted as mean ± standard deviation (n = 2 – 6 per group as shown by individual data points in each graph). We used t-tests to assess statistical significance, and *** represents $p \leq 0.0002$ relative to NT control. Percent knockdown (KD) relative to cells transfected with NT siRNA is shown for STAT5A and S5pY. STAT5B was not knockdown by STAT5A-targeted siRNA.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Small interfering RNA (siRNA)-mediated knockdown of STAT5A – Mature 3T3-L1 adipocytes, 6 – 14 days post-MDI, were detached from the culture plate using trypsin and re-plated in typical 24-well plates (protein analysis) or 24-well Seahorse assay plates (OCR measurement) at a density of 1.16 x 10^5 cells/cm^2. Seahorse assay plates were coated with 0.2% gelatin (Sigma-Aldrich, Cat #: G1393) prior to plating trypsinized adipocytes. While cells were adhering they were transfected with 100nM siRNA (Thermo-Dharmacon; non-targeting siRNA Cat #: D-001810-10-50; STAT5A siRNA Cat #: LQ-063202-00-0002) using DharmaFECT Duo transfection reagent (Thermo-Dharmacon, Cat #: T-2010-03) in OptiMEM (Thermo, Cat #: 11058-021). Antibiotic-free medium (10% FBS/DMEM) was changed after 24 hours, and every 24 - 48 hours thereafter until day 5 or 6 when used for Seahorse XF24 assay or harvested in IP buffer for protein to assess knockdown efficiency.

Seahorse XF24 Assay to measure oxygen consumption rate (OCR) – The XF24 assay was performed using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience) according to the manufacturer's instructions. Briefly, mature 3T3-L1 adipocytes that had been previously transfected with NT or STAT5A-targeted siRNA were used to measure OCR. Prior to the assay, the medium was changed to 1% calf serum/DMEM containing vehicle ($NaHCO_3$) or 10 nM mGH, and the cells were incubated for 16 hours overnight. For the assay, the medium was changed to XF assay medium containing 5mM glucose, 1mM sodium pyruvate, and vehicle or 10 nM mGH. Mitochondrial function was perturbed by injecting 2 uM Oligomycin, 4 uM FCCP, and 4 uM Antimycin A at defined times during the assay. Percent baseline values were calculated in excel and graphed using Prism 6.05 (GraphPad Software, Inc.).