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Supplemental Information

mTORC1 Is a Major Regulatory Node in the FGF21 Signaling Network in Adipocytes

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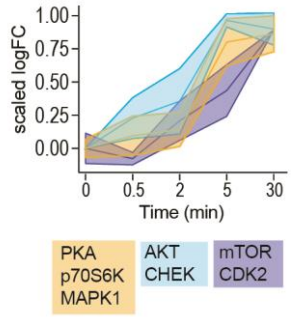
SUPPLEMENTAL FIGURES

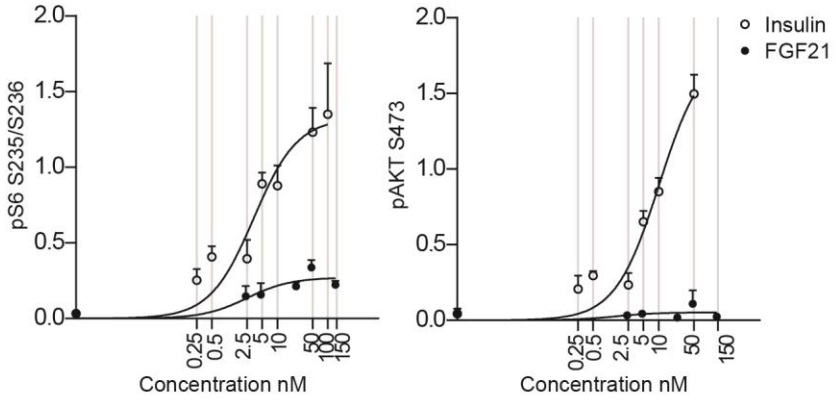
Supplemental Figure 1, related to Figure 1.

FGF21-regulated kinases were identified by KSR-LIVE and kinases with similar temporal profiles clustered together.



Supplemental Figure 2, related to Figure 2 and 3.

3T3-L1 adipocytes were serum-starved for 1.5 h then treated with insulin or FGF21 at indicated doses for 30 min. Cell lysates were immunoblotted for S6 S235/236 and Akt S473, which are indicative of mTORC1 and mTORC2 activity. Immunoblots were quantified and scaled (n=3, mean ± S.E.M).



SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse experiments

12 week old mice were injected intraperitoneally with either FGF21 (10 mg/kg) or saline as vehicle control.

Cell culture

3T3-L1 fibroblasts (Todaro and Green, 1963) were passaged at ~60% confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), GlutaMAX (Life Technologies), penicillin, streptomycin (Life Technologies) (FBS-DMEM P/S/G) at 37^{o}C with 10% CO_2. Differentiation was induced at 100% confluence by addition of 350 nM insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 250 nM dexamethasone and 400 nM biotin for 3 days. Cells were then incubated in the presence of 350 nM insulin for 3 d. Primary cells were isolated from white subcutaneous fat tissue from 8-10 weeks old C57BL6 mice as described before (Sugii et al., 2011). Adipocyte differentiation was induced by treating cells for 48 h in FBS-DMEM P/S/G 850 nM insulin, 0.5 mM IBMX, 1 µM dexamethasone, 1 nM T3, 125 nM indomethacin and 1 µM rosiglitazone (Caymen chemicals). After 48 h, cells were switched to FBS-DMEM P/S/G containing 850 nM insulin, 1 nM T3 and 1 µM rosiglitazone. Differentiated adipocytes were maintained in FBS-DMEM P/S/G. Adipocytes were used between days 10-12 after initiation of differentiation. For acute FGF21 or insulin treatments, adipocytes were serum starved in DMEM supplemented with 0.2% BSA (basal-DMEM) for 2 h before incubation with FGF21 or insulin. Inhibitors were added 30 min prior to hormone-stimulation, at the following concentrations: 500 nM rapamycin (LC-Laboratories), 10 µM MK2206 (Sellekchem), 10µM U0126 (Cell Signaling Technology).

Stable isotopically labelled amino acids in cell culture (SILAC) for measurement of FGF21 Phosphoproteome

SILAC DMEM (deficient in Lysine, Arginine and Leucine, Thermo Fisher) was supplemented with 10% dialysed FCS (Hyclone Laboratories), GlutaMAX (Life Technologies) and leucine. Arginine and Lysine was added in either light (Sigma), medium (Arg 6, Lys 4; Silantes) or heavy (Arg 10, Lys 8; Silantes) form. 3T3-L1 fibroblasts were passaged for six doublings in light or heavy SILAC DMEM and differentiated into adipocytes. Triple labelled SILAC 3T3-L1 adipocytes in 15cm dishes were serum-starved for 2 h in SILAC DMEM supplemented with 0.2% dialysed BSA, then treated with vehicle (PBS) or 23 nM FGF21 for either 0.5, 2, 5 of 30

min. SILAC labels were switched between biological replicates. Acute stimulations were stopped by washing 4 x with ice-cold PBS. Cells were scraped in 1 mL buffer containing 6 M Urea, 2 M thiourea, protease inhibitors (Complete EDTA-free, Roche) and phosphatase inhibitors. Cells were homogenised by sonication (1 s x 30). Cellular debris was pelleted by centrifugation (21,000 g 10min) and lysate transferred to new tube. Protein content was assessed by the Bradford assay. Cell homogenates containing equal amounts of proteins were pooled so that 0, 0.5 and 5 min treated then 0, 2 and 30 min treated samples were always together. Following mixing, proteins were reduced, alkylated, acetone precipitated, resuspended in urea, and digested with trypsin (Promega). Peptides were desalted using SepPak tC18 cartridges, fractionated by Strong Cation Exchange (SCX) and enriched for phosphopeptides by TiO_2 enrichment as described previously (Humphrey et al., 2013).

Mass Spectrometry (MS) Bioinformatics analysis

LC-MS/MS analysis was carried out on an LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific) as described previously (Humphrey et al., 2013). Raw mass spectrometry data were processed using MaxQuant software (Cox and Mann, 2008) version 1.5.3.25 using default settings except for: Oxidised Methionine (M), Acetylation (Protein N-term) and Phospho (STY) which were selected as variable modifications, and Carbamidomethyl (C) as well as triple SILAC labels were set as fixed modifications. A maximum of two missed cleavages was permitted, 10 peaks per 100 Da, MS/MS tolerance of 20 ppm, and a minimum peptide length of 7. The “matching between runs” algorithm was enabled (time window = 2min) to transfer identifications between adjacent fractions. Database searching was performed using the Andromeda search engine integrated into MaxQuant environment (Cox et al., 2011) against the mouse Uniprot database, (January 2016). Protein, peptide and site FDR thresholds in MaxQuant were each set to a maximum of 1%.

Protein ratios were expressed relative to untreated cells. Only phosphorylation sites quantified in at least one replicate at each time point were retained for statistical analysis. Subsequently, the top 5% most variable phosphosites at each time point (determined by SD) were excluded from further analysis. Missing values were imputed using Amelia R package (Honaker et al., 2011). After missing value imputation, data were normalized by (1) fitting a locally weighted regression curve through the four time points and adjusting the median of the three subsequent time points based of the fitted values; (2) correcting for batch effect using SVA R package (Leek et al.); and (3) adjusting the median of the first time point using negative control sites including INSR 1337, INSR 1340, EPHA2 571 and PDGFRA 767.

Significantly regulated phosphopeptides were identified using a moderated t-test implemented in LIMMA package in R (Ritchie et al., 2015) and an adjusted p-value (false discovery rate (FDR) adjustment) cut-off of smaller than 0.01. Consensus amino acid motifs around FGF21-regulated phosphorylation sites at each time point were determined using Seq2Logo (Thomsen and Nielsen, 2012).

The phosphoproteomics data was analysed for enriched kinases using KSR-LIVE algorithm (Domanova et al., 2016). Phosphosites changed by greater than 1.5 fold were included in the analysis. A kinase substrate relationship (KSR) database was formed by integrating PhosphositePLUS (retrieved 06/2014), PhosphoELM (release 9.0), PhosphoPOINT (04/2014) and Human Protein Reference Database (release 9). A kinase was predicted to be active if the temporal profile of substrates which are exclusively assigned to that kinase formed a tight cluster. The exclusive substrates in this tight cluster formed the “core substrates” for a kinase. Tight clustering was done as described by Tseng et al. using default parameters. Random data was added to prevent the clustering algorithm splitting apart substrates which follow one time profile. Random data was generated from sampling a uniform distribution in the range of the original data. If a kinase had two or fewer substrates, clustering was not performed. To generate the characteristic temporal profile, exclusive and nonexclusive substrates of a kinase were clustered and the clusters which contained “core substrates” were taken as the characteristic temporal activity of the kinase.

Phosphoproteomics data generated from insulin stimulated adipocytes were processed as described previously (Humphrey et al., 2013). Kinase perturbation analysis (KinasePA) was used to investigate the coordinated regulation of kinase substrates with respect to 20 mins insulin treatment or 30 mins FGF21 treatment (Yang et al., 2016).

Statistical analysis

T-tests or ANOVAs were performed in GraphPad Prism version 6.01 for Windows (GraphPad Software).

Immunoblotting

Cells were washed 3 times with ice-cold PBS and solubilised in 2% SDS in PBS containing protease inhibitors (Roche), and phosphatase inhibitors (1mM sodium pyrophosphate, 2mM sodium orthovanadate, 10mM sodium fluoride). Cells were lysed by sonication for 12 s. Insoluble material was removed by centrifugation at 21,000×g. Protein concentration was determined by bicinchoninic acid method (Thermo Scientific). 10 µg of protein was resolved by SDS-PAGE and transferred to PVDF membranes. Where indicated, membranes were

stained with Sypro Ruby (Thermo Scientific). Then membranes were blocked in 5% skim milk powder in Tris-buffered saline (TBS) buffer for 1 h, followed by an overnight incubation at 4^{o}C with specific primary antibody solutions. Antibodies specific for S235/S236 pS6, T389 pS6K, S473 pAkt, pT642 pAS160, T246 pPRAS40, Adiponectin were from Cell Signalling Technology; αtubulin from Sigma; 14-3-3β from Santa Cruz. Membranes were incubated with an appropriate secondary for 1 h before signals were detected using ECL (Thermo Scientific or Millipore) on the Chemidoc MP (Bio-Rad). In some cases, IRDye700- or 800-conjugated secondary antibodies were used and then scanned at the 700nm and 800nm channel using the Odyssey IR imager. Densitometry analysis of immunoblots was performed using ImageJ software version 1.47 (Rasband, 1997-2015).

2-Deoxyglucose (2DOG) Uptake Assay

3T3-L1 adipocytes in 24-well plates were serum starved in basal-DMEM for 2 h. Cells were then washed 3 times in warm PBS and incubated in Krebs Ringer buffer (KRP) (0.6 mM Na2HPO4, 0.4 mM NaH2PO4, 120 mM NaCl, 6 mM KCl, 1 mM CaCl2, 1.2 mM MgSO4 and 12.5 mM Hepes (pH 7.4)) with 0.2 % BSA. [^{3}H]-2DOG (PerkinElmer Life Sciences) 0.125 µCi/well and 50 µM unlabelled 2DOG were added for 5 min. Glucose uptake was terminated with three rapid washes in ice-cold PBS, after which cells were solubilized in 1% (w/v) Triton X-100 in PBS on a shaker for 1 h and assessed for radioactivity by scintillation counting using a β-scintillation counter. To determine non-specific glucose uptake, cells were treated with 25 µM cytochalasin B 30 min prior to addition of 2DOG. To measure insulin-sensitive glucose uptake, cells were stimulated with 10 nM insulin for 20 min and 2-DOG added in the final 5 min of insulin stimulation. All data was normalised to protein content. Each condition was performed in triplicate.

Lipolysis

3T3-L1 adipocytes in 24-well plates were serum starved in basal-DMEM for 2 h. Cells were washed 3 times in warm PBS and incubated in KRP supplemented with 3.5% free fatty acid BSA (Sigma Aldrich) and 5 mM glucose for 2 h. Cells were treated with or without isoproterenol, insulin or FGF21 for 1 h as indicated. Aliquots of media were taken to assay for glycerol content using Sigma glycerol reagent according to manufacturer's instructions. The cells were next lysed in 1% (w/v) Triton X-100 in PBS and assessed for protein concentration. Glycerol release was normalised to cellular protein content. Each sample was assayed in duplicate, and the average of the duplicates was considered as one biological replicate.

[^{3}H]Leucine incorporation assay for Protein Synthesis

3T3-L1 adipocytes in 24-well plates were washed twice and incubated with leucine-free DMEM (Sigma-Aldrich) supplemented with 0.2 % BSA and 20 mM HEPES, pH 7.4 for 2 h. [^{3}H] Leucine (5 µCi/ml) (PerkinElmer Life Sciences) was added at the same time as indicated doses of FGF21 or insulin for 1 h. To determine background leucine incorporation, 5 µM cycloheximide was added for 10 min before addition of [^{3}H] leucine. Leucine incorporation was terminated with 3 rapid washes in ice-cold PBS followed by incubating cells with ice-cold 10% TCA for 10 min to precipitate protein. Pellets were washed 3 times in ice cold 10% TCA to remove free [^{3}H] leucine. Pellets were resuspended in 50 nM NaOH with 1% Triton X-100 at 65^{o}C for 20 min. Samples were assessed for radioactivity, and results were normalised for protein content. Assays were performed in triplicate and the average of the triplicate was considered as one biological replicate.

qPCR

Total RNA was isolated from cells using Nucleospin RNA columns (Macherey-Nagel). cDNA was prepared from total RNA using the first strand cDNA synthesis kit from Thermo Scientific. qPCR reactions were performed on the LightCycler 480 PCR machine (Roche) using SYBR-Green fluorescent dye (Invitrogen). Relative mRNA expression was determined by ΔΔ-Ct method using Tata-Binding Protein (TBP) levels as endogenous control. The primer sets used were: UCP1-F: CTG CCA GGA CAG TAC CCA AGC G; UCP1-R: TCT GTC TGG ACT TCA TCA GC; FGF21-F: AGC TCT CTA TGG ATC GCC TCA CTT; FGF21-R: ACA CAT TGT AAC CGT CCT CCA GCA; TBP-F: ACC CTT CAC CAA TGA CTC CTA TG; TBP-R: TGA CTG CAG CAA ATC GCT TGG.

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