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Supplemental Information

Leptin Receptor Signaling Regulates Protein Synthesis Pathways and Neuronal Differentiation in Pluripotent Stem Cells

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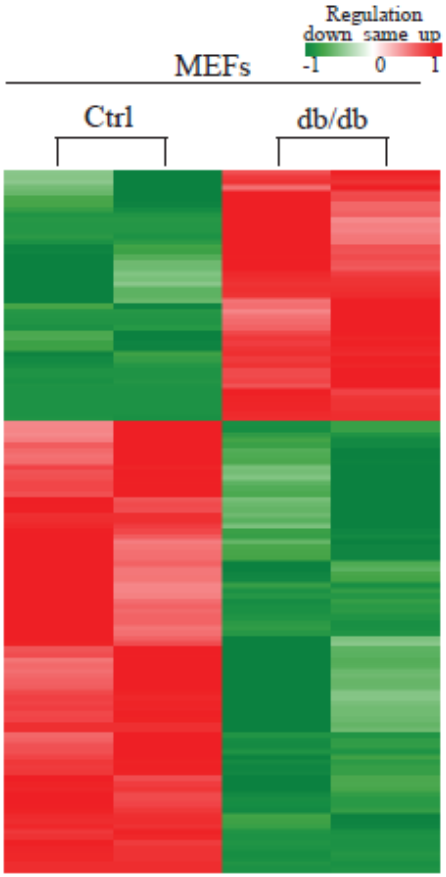
Figure S1 Supplemental Figures and Legends

A



Cellular movement
-ADAM15, CDKN2A, CRMP1,CYR61, DDR2, FST, GBP2, IGFBP7, LOXL2, NCAM1
Cell morphology
-ADAM15, CDKN2A, CRMP1, IGFBP7, LOXL2, TNC
Cell-to-cell signaling interaction
-CRMP1, NCAM1, SYNPO, TNC, VDAC1, VDAC3
Cellular development
-CYR61, FST, CDKN2A, NCAM1, TNC, WLS
Cellular assembly and organization
-CDKN2A, CYR61, VDAC1, NCAM1, SNCG, ADAM15, CYR61, NCAM1, Podxl

Cellular assembly and organization
-ATP5F1D, ATP5MF, ATP5PB, ATP5PO, FHL1, GSN, AUKRA, KIF11
Energy production
-ATP5F1D, ATP5MF, ATP5PB, ATP5PO, CAV1
Nucleic acid metabolism
-ATP5F1D, ATP5MF, ATP5PB, ATP5PO, CAV1, NUCB2, DCK
small molecule biochemistry
-ATP5F1D, ATP5MF, ATP5PB, ATP5PO, CAV1, GM2A, HEXA
cell cycle
-AUKRA, KIF11, ARPC1B, CAV1, DBI, DCK, HDAC1, PARP2

B

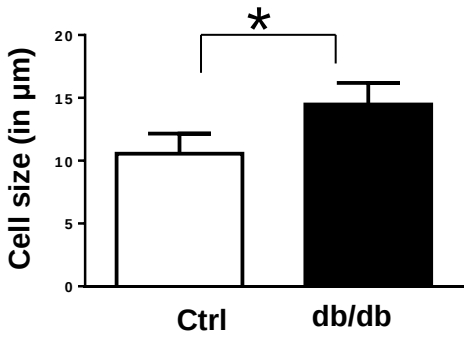
Mitochondrial dysfunction

Symbol	Identifier	Exp Val
	GenPept/UniProt/Swiss-Prot Ac	Log Ratio
ATP5D	Q9D3D9	↓-1.553
ATP5F1	Q9CQQ7	↓-1.289
ATP5I	Q06185	↓-1.737
ATP5J2	P56135	↓-2.752
ATP5O	Q9DB20	↓-1.744
NDUFA6	Q9CQZ5	↓-2.713
NDUFB10	Q9DCS9	↓-2.455
VDAC1	Q60932	↑1.657
VDAC3	Q60931	↑1.962

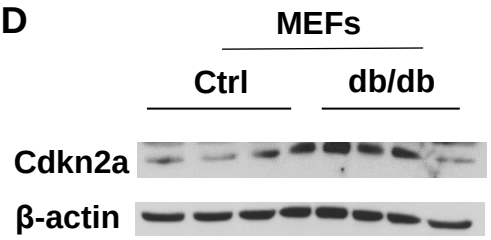
OXPHOS (Oxidative Phosphorylation)

Symbol	Identifier	Exp Val
	GenPept/UniProt/Swiss-Prot Ac	Log Ratio
ATP5D	Q9D3D9	↓-1.553
ATP5F1	Q9CQQ7	↓-1.289
ATP5I	Q06185	↓-1.737
ATP5J2	P56135	↓-2.752
ATP5O	Q9DB20	↓-1.744
NDUFA6	Q9CQZ5	↓-2.713
NDUFB10	Q9DCS9	↓-2.455

C

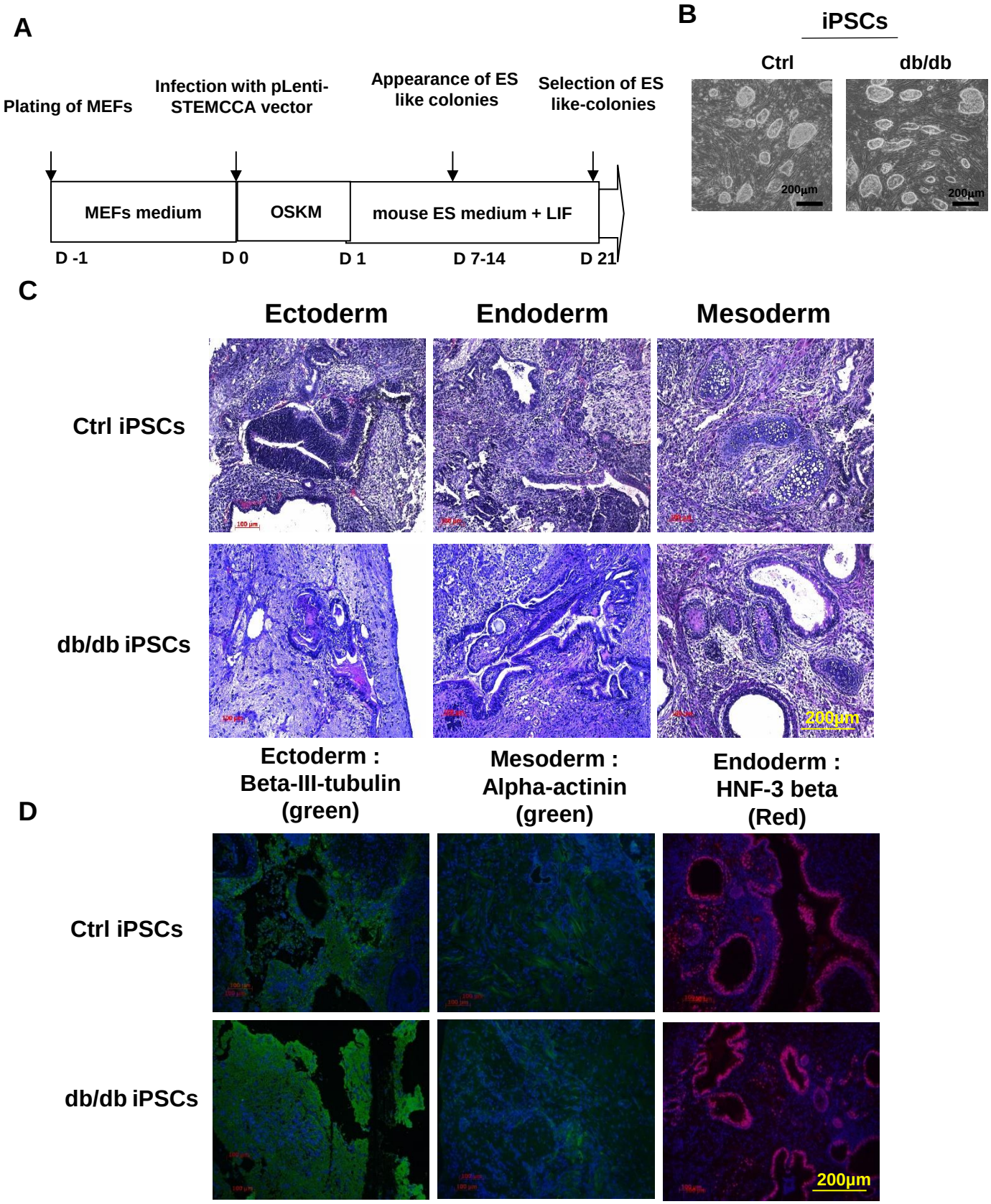


D



Supplemental Figure S1 : A. Heat map of differential regulated proteins between Ctrl and db/db MEFs :green represents downregulated proteins while red represents upregulated proteins in db/db MEFs; B. Differentially regulated proteins related to mitochondrial dysfunction (upper panel) and OXPHOS (lower panel); C. Quantification of Ctrl and db/db MEFs using cell counter; D. Cdkn2a protein level was upregulated in db/db MEFs. . n=3 independent experiments, data are shown as mean ± SD and student's t-test. (* $p<0.05$, ** $p<0.01$) (Related to Figure 1)

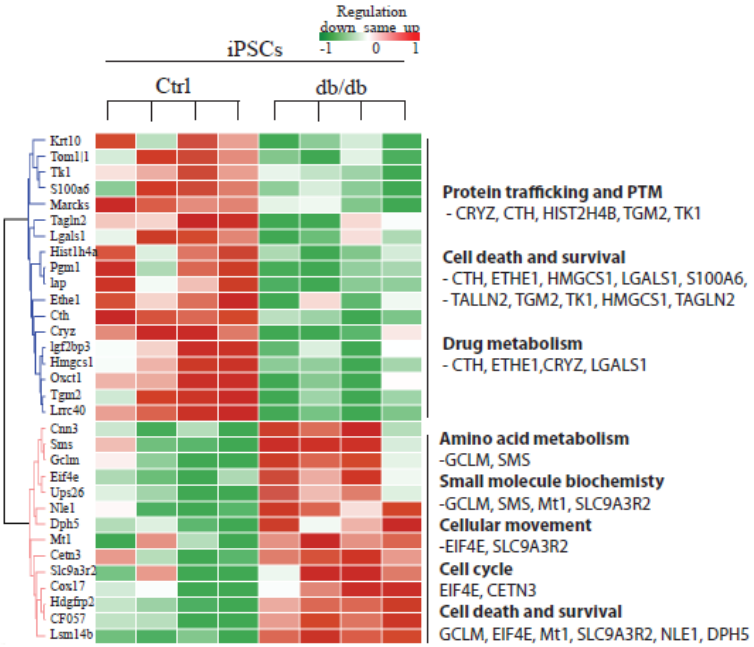
Figure S2



Supplemental Figure S2 : Generation and characterization of Ctrl and db/db iPSCs : A. Schematic reprogramming of mouse embryonic fibroblasts (MEFs) into induced pluripotent stem cells (iPSCs) B. Morphological images of Ctrl and db/db iPSCs , Scale bar-200µm; C. Ctrl and db/db iPSCs were injected into SCID or B6 mice and teratomas harvested after 4 weeks for analyses. H&E stainings of teratoma sections obtained from Ctrl and db/db iPSCs; D. Lineage marker staining's of teratoma sections showing the presence of the three lineage markers (Related to Figure 1) Scale bar-200µm and N=4. . n=3 independent experiments, data are shown as mean ± SD and student's t-test. (* $p<0.05$, ** $p<0.01$)

Figure S3

A



B

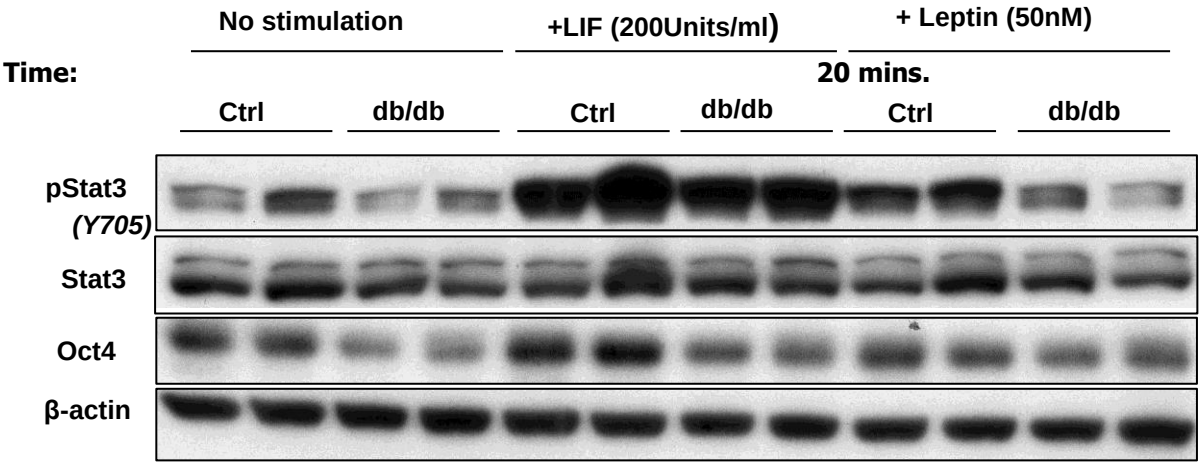
EIF2 signaling

Symbol	Identifier	Exp Val
	GenPept/UniProt/Swiss-Prot Ac	Log Ratio
EIF4E	P63073	↑1.287
RPL24	Q8BP67	↓-0.349
RPL28	P41105	↓-0.448
RPL10A	P53026	↓-0.266
RPL13A	P19253	↓-0.274
RPS7	P62082	↓-0.490

Oct4 signaling

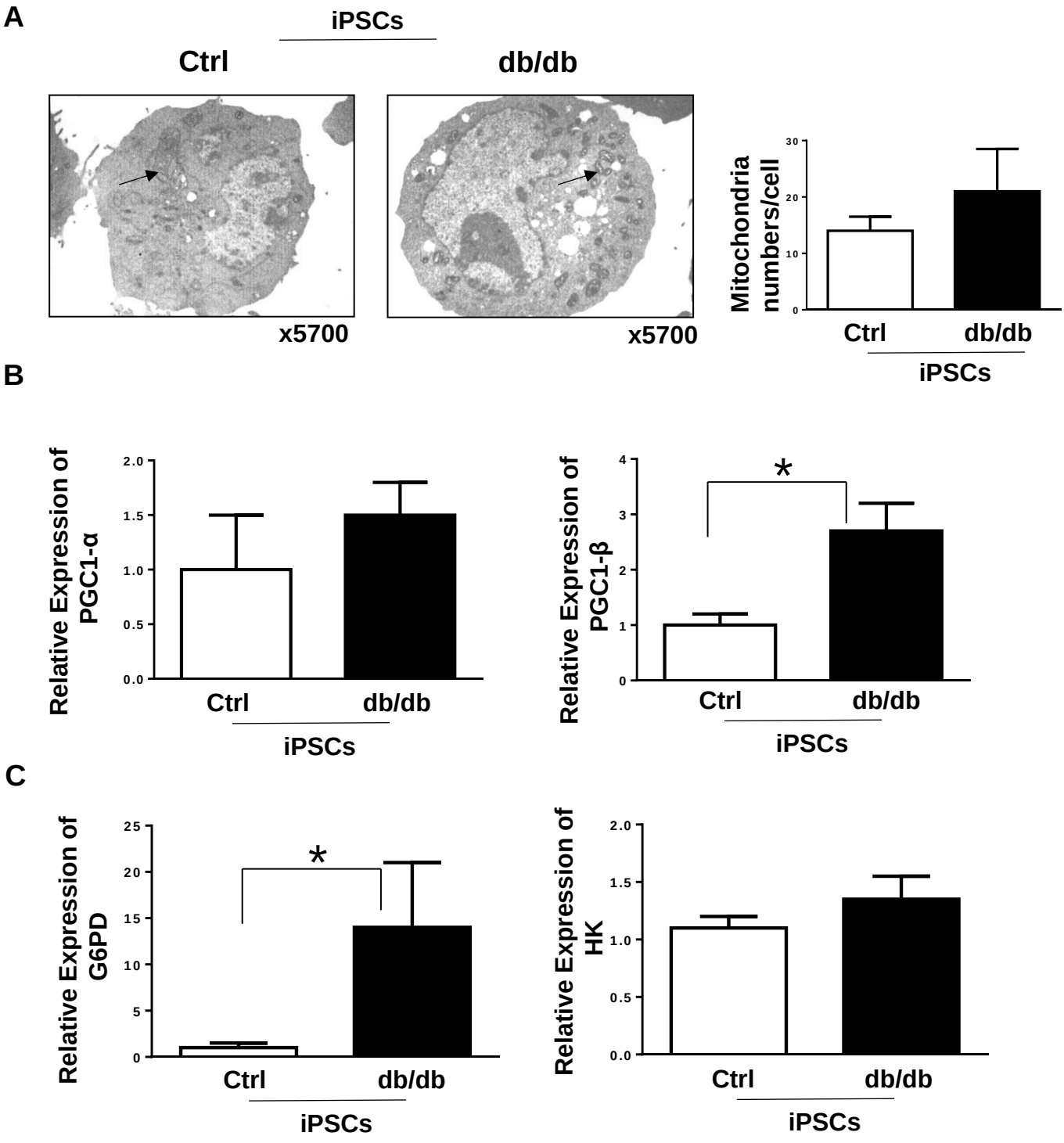
Symbol	Identifier	Exp Val
	GenPept/UniProt/Swiss-Prot Ac	Log Ratio
IGF2BP1	O88477	↓-0.598
POU5F1	P20263	↓-0.581
Tdh	Q8K3F7	↓-0.581

C



Supplemental Figure S3 : Heat map of differentially regulated proteins between Ctrl and db/db iPSCs : A. Green represents downregulated proteins while red represents upregulated proteins in db/db iPSCs; B. Differentially regulated proteins related to eukaryotic initiation factor 2 (EIF2) (protein synthesis) and octamer- binding transcription factor 4 (Oct4) (pluripotency) signaling pathways; C. Western blot analysis showing reduced phospho-Stat3 and Oct4 in db/db iPSCs. β-actin was used as a loading control. n=3 independent experiments, data are shown as mean ± SD and student's t-test. (* $p<0.05$, ** $p<0.01$) (Related to Figure 1)

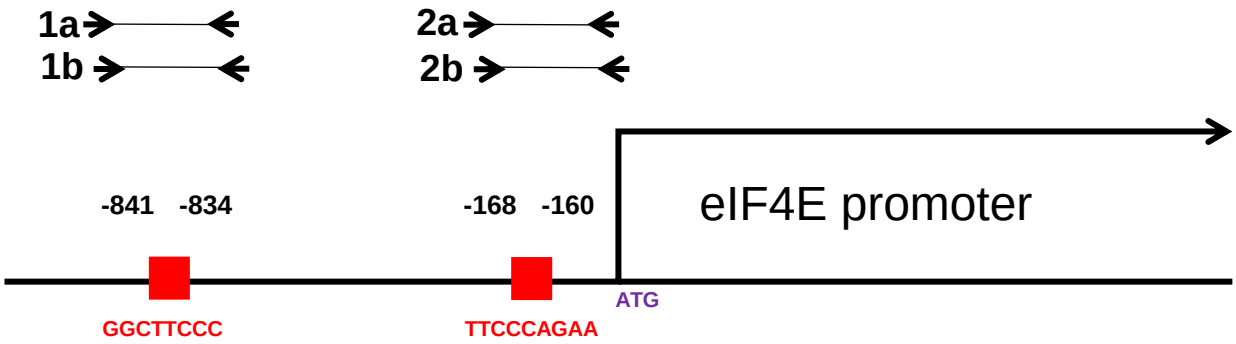
Figure S4



Supplemental Figure S4 : Mito OXPHOS characterization of Ctrl and db/db iPSCs: A. Electron microscopy images of iPSCs from both the groups; Black arrow indicates mitochondria inside the cell (left panel). Mitochondria quantification per cell using N=10 image each for Ctrl and db/db iPSCs (right panel) B. Real time PCR analysis of OXPHOS genes (peroxisome proliferator-activated receptor gamma Co-activator 1-alpha (PGC1-α) and peroxisome proliferator-activated receptor gamma Co-activator 1-beta PGC1-β). C Real time PCR analysis of glycolytic genes (glucose-6- phosphate dehydrogenase (G6PD) and hexokinase (HK) ,N=3 in Ctrl and db/db iPSCs. n=3 independent experiments, data are shown as mean ± SD and student's t-test. (* $p<0.05$, ** $p<0.01$) (Related to Figure 2).

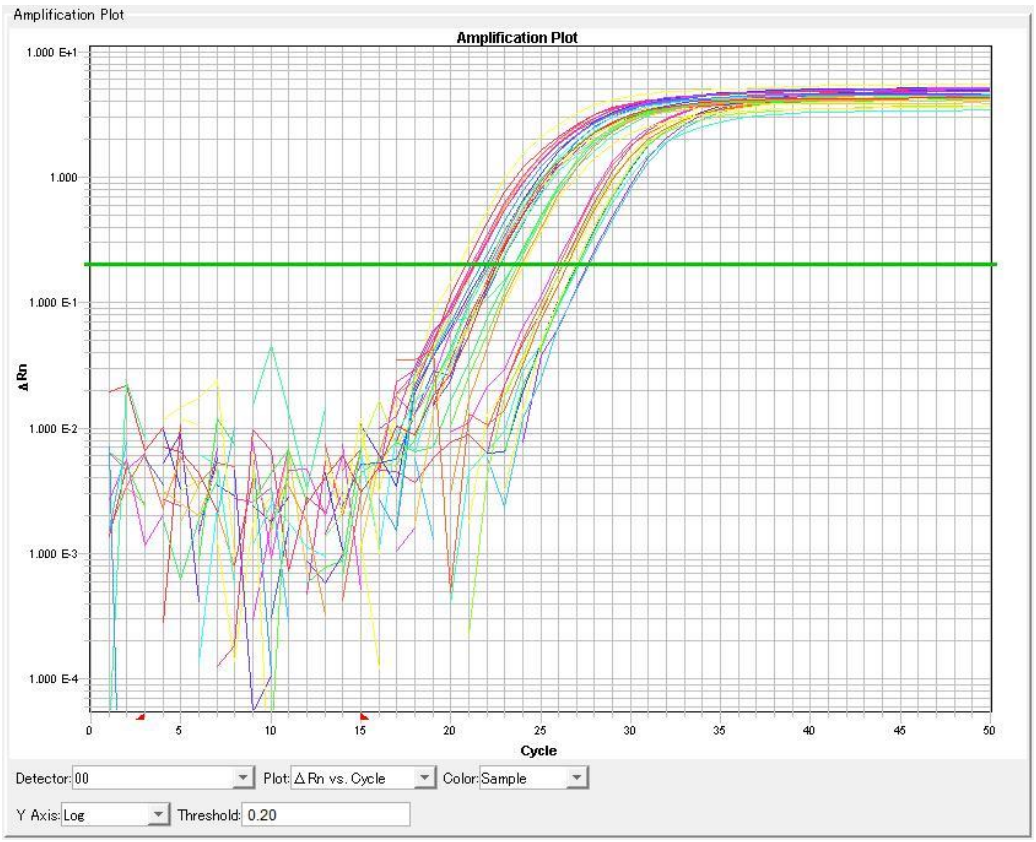
Figure S5

A



B

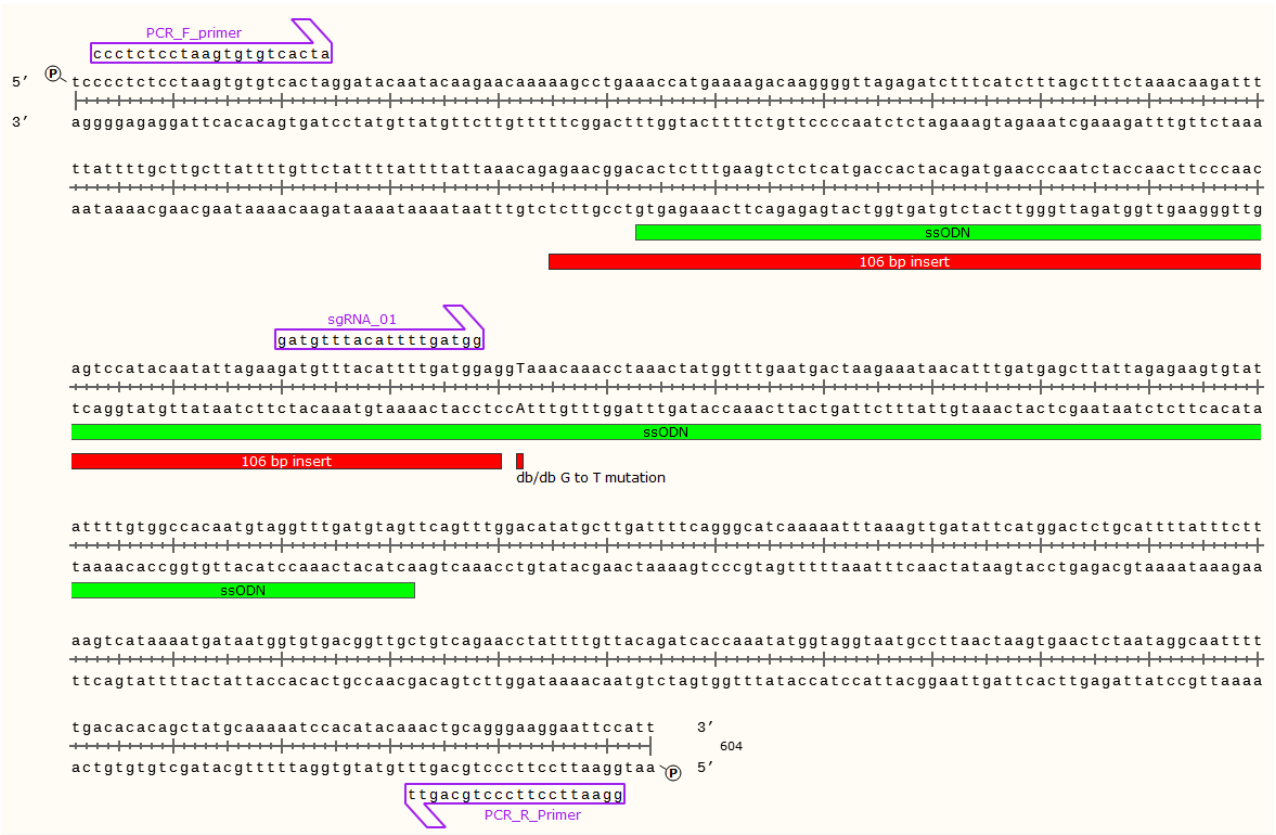
Dissociation curve for Stat3 primers 1a, 1b, 2a, 2b



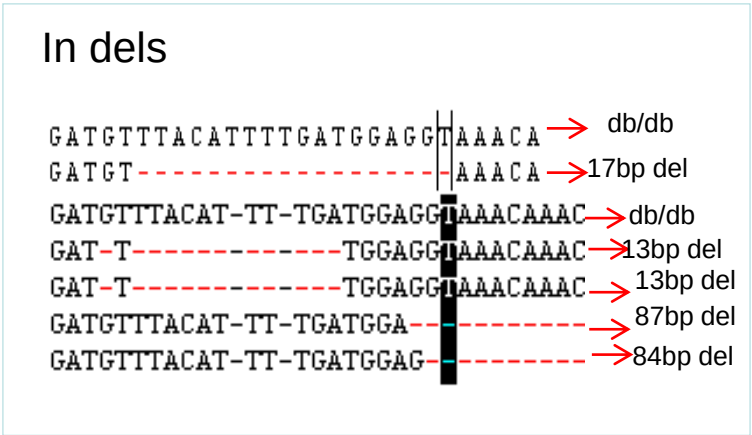
Supplemental Figure S5 : Signal transducer and activator of transcription 3 (Stat3) binding regions on the promoter of eukaryotic translation initiation actor 4E (eIF4E) : A. Two red boxes denote the Stat3 binding regions on the promoter of eIF4E ; B. Primers 1a ,1b were designed to amplify Stat3 binding region 1st while 2a, 2b were designed to amplify Stat3 binding region 2nd (Related to Figure 3 and 5)

Figure S6

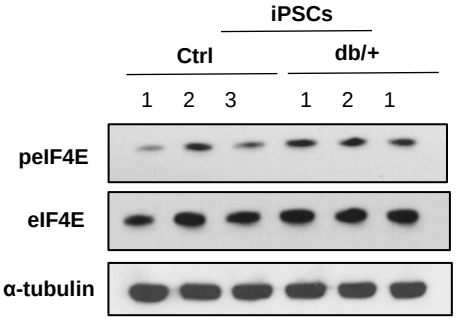
A



B



C



Supplemental Figure S6 : Designing the strategy for correction of db/db mutation: A. Guide RNAs specificto db/db mutation are designed along with Ctrl donor template. B. Many homozygous deletions (In dels) were found in CRISPR corrected db/db clones. C. Western blot analysis showing restoration of protein expression of peIF4E and total eIF4E in CRISPR-corrected db/db iPSCs. α-tubulin was used as a loading control. n=3 independent experiments, data are shown as mean ± SD and student's t-test. (* $p<0.05$, ** $p<0.01$). (Related to Figure 3)

Gupta et al, Supplemental materials

Experimental Procedures:

Lentiviral-mediated reprogramming and iPS cell generation and characterization

Briefly, MEFs (5 x 10^4) were plated in six well plates, and virally transduced with the lentiviral particles in the presence of 5 μg/ml Polybrene® (EMD Millipore) after 8-24 hours. The fibroblasts were washed three times with PBS and fed fresh 15% mouse embryonic stem (ES) cell media supplemented with leukemia inhibitory factor (LIF) (EMD millipore). On day 7-14, embryonic stem cell (ESC) like colonies were individually picked, cultured, expanded, frozen and subsequently characterized for pluripotency markers.

Gene expression analyses using quantitative RT-PCR and western immunoblotting

Expression of transcripts of interest was quantitated by SYBR Green-based reverse transcriptase-PCR (RT-PCR) using 2.0 μl of diluted cDNA and 8μl of SYBR Green Master Mix (Life Technologies) in a 10μl total reaction volume including primers at a concentration of 500nM each. Each PCR had three technical replicates per sample that were quantified using an ABI7600HT Sequence Detection System (Life Technologies). β-actin was used for normalization of expression of individual genes. The various antibodies were used in western blotting including OCT4 (Santa Cruz Bio.sc-5279), NANOG (Cell Signaling, 8785s), SATA3 (Santa Cruz Bio.sc-482), β-ACTIN (Santa Cruz Bio. sc-1616), pSTAT3 (Cell Signaling, 9145s), pEIF4E (Cell Signaling, 9741s), eIF4E (Cell Signaling, 9742s), pEIF4G (Cell Signaling, 2441s), EIF4G (Cell Signaling, 2469P), α-TUBULIN (Abcam, ab7291). The blots were developed using chemiluminescent substrate (low sensitivity, Pico reagent or high sensitivity, West Femto maximum sensitivity reagent) from ThermoFisher Scientific (Waltham, MA).

Immunohistochemistry

Cells were blocked by 5% donkey serum for 1 hour followed by the overnight incubation with primary antibodies chicken anti-NESTIN (1:1000, NB100-1604; Novus Biologicals), rabbit anti-NOGGIN (1:200, ab-16054, Abcam), rabbit anti-GFAP (1:1000, NB300-141, Novus Biologicals). Next day, cells were washed three times and incubated with secondary antibodies anti-chicken Alexa 488 for NESTIN, anti-rabbit Alexa 488 for GFAP, and anti-rabbit Alexa 594 for NOGGIN ; 1:1000 ,Invitrogen for 1-2 hour. DAPI was used to stain nuclei.

Teratoma sections were fixed in paraformaldehyde, processed and embedded in paraffin. All sectioning and histopathology procedures were performed at the DF/HCC Research Pathology Core. Slides were analyzed by hematoxylin and eosin staining or immunostaining with specific primary antibodies

Metabolic profiling:

Briefly, cells were cultured in non-buffered DMEM with 4.5g/l glucose. A bioenergetics profile, comprised of basal mitochondrial respiration, ATP turnover, H^+ leak, mitochondrial respiratory capacity and non-mitochondrial respiration, was determined by measuring oxygen consumption rate (OCR) in the basal state and following sequential spaced injections of oligomycin (10 µM), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (1 µM) and rotenone (5 µM). For evaluation of glycolysis, cells were processed in non-buffered KHB buffer, followed by determination of basal respiration and the glycolytic capacity was assessed by measuring OCR in the basal state, followed by injection of 25 mM glucose and injection of 25 mM 2-deoxyglucose.

Mass spectrometry analyses

The details of the procedures used for proteomics analyses are available in Supplemental information. Briefly, cells were lysed and prepared for analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Peptides from each cell line were labeled using isobaric tags (Tandem Mass Tags, TMT, Thermo Fischer Scientific, Bremen, Germany) and multiplexed in equal amounts, experimental design in Supplemental information. Each multiplexed sample was subjected to fractionation prior to LC-MS/MS analysis on an LTQ Orbitrap Elite coupled to a Dionex Ultimate NCS-3000 LC system (both Thermo Fischer Scientific, Bremen, Germany).

Details regarding the mass spectrometry data interpretation are available in Supplemental information. Briefly, raw mass spectrometry data were converted using MSConvert as part of the ProteoWizard (Chambers et al. 2012) package, and searched with OMSSA (Geer et al. 2004) and X! Tandem (Craig and Beavis 2004) *via* SearchGUI (Vaudel et al. 2011). Search engine results were processed using PeptideShaker (Vaudel et al. 2015). Notably, so-called protein ambiguity groups were built based on the unicity of peptide sequences in proteins as described in (Nesvizhskii and Aebersold 2005). Proteins identified by mass spectrometry will implicitly refer to protein ambiguity groups. All identification results were validated at a 1% False Discovery Rate (FDR) threshold estimated using the target/decoy strategy (Elias and Gygi 2010). Proteins abundances were inferred from the reporter ion intensities using Reporter (http://compomics.github.io/projects/reporter.html).

Supplemental methods for the Proteomic analyses

Chemicals

Bicinchoninic acid assay (BCA) protein assay kit was purchased from Novagen® (EMD Chemicals, San Diego, CA, USA). TMT sixplex™ Isobaric Mass Tagging Kit, including Pierce™ Trypsin Protease (MS grade), were purchased from Thermo Scientific (Rockford, IL, USA). Water, acetonitrile (ACN), and formic acid (FA), all MS-grade, were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell lysis and protein digestion

Cells were lyzed using M-Per mammalian protein extraction buffer and protein concentration was determined using a BCA protein assay kit. Dry aliquots containing an estimated amount of 50 µg of proteins were reduced, alkylated and digested overnight according to the Tandem Mass Tagging (TMT) kit vendor's instructions. Briefly, samples were re-suspended in 8 M urea, followed by addition of 100 mM Triethyl Ammonium Bicarbonate (TEAB). The volume was adjusted with milliQ water to reach a final protein concentration of 5 µg/µL. Protein disulfide bonds were reduced by the addition of 200 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and incubated for one hour at room temperature (RT). Free sulfhydryl groups were subsequently alkylated by the addition of 375 mM Iodoacetamide (IAA) dissolved in 100 mM TEAB, for 30 min in the dark at RT. The protein mixture was diluted 6 times with pre-chilled acetone (-20 °C) to allow for precipitation for 16 h at -20 °C. After centrifugation at 8,000 x g for 10 min at 4 °C, the acetone was gently removed and the sample dried. Proteins were re-suspended in 50 µL 100 mM TEAB and digested overnight (37 °C) using trypsin, at a protease to protein ratio of 1:40.

Tandem Mass Tag (TMT) 6-plex Labeling

Tryptic digests were labeled with Tandem Mass Tag TMT 6-plex reagents according to the manufacturer's protocol, and combined. Three TMT 6-plex labeling sets were used for the twelve samples included in this study, samples were randomly assigned to four labels TMT^6-128-131. Two common reference samples containing equal amounts from each individual sample were labeled TMT^6-126 and TMT^6-127. Sample repartition is detailed in Table 1.

Sample Fractionation

Labeled peptide samples were pre-fractionated by mixed mode reversed phase-anion exchange (MM RP-AX) as described in (Phillips et al. 2010), using a Promix MP 250 mm x 2.1 mm id, pore size 300Å column (SIELC Technologies, Prospect Hights, IL, USA) connected to an Agilent Technology 1260 off-line LC-system. Each of the three combined samples (Set 1, 2, 3) was re-suspended in 120 µL solvent A (20mM ammonium formate/ 3% acetonitrile (ACN), pH 6.5) and loaded onto the column. Column flow was set to 50 µL/min and gradient length was 70 min. From 0-45 min solvent B (2mM ammonium formate/ 80% ACN) increased linearly from 15% to 60%, from 45-55 min 60% B, from 55-65 min 100% B and from 65-70 min 15% B. The samples were separated during equal time intervals into 60 fractions. (The eight first fractions containing TMT-reagent debris were discarded. Fractions at each end of the gradient were combined to give the total number of representative fractions of 39. Fractions 9-11 and 12-14 were combined, as well as fractions 49-51, 52-54, and 55-60).

LC-MS/MS analysis

In total, 117 fractions were obtained from the twelve initial protein lysates (39 fractions x 3). From each of these, 0.5 µg was dissolved in 1% aqueous formic acid (FA) prior to LC-MS/MS analysis on an LTQ-Orbitrap Elite mass spectrometer, equipped with a nanospray Flex ion source, and coupled to a Dionex Ultimate NCS-3000 LC system (all from Thermo Fischer Scientific, Bremen, Germany).

The samples were loaded and desalted on a pre-column (Acclaim PepMap 100, 2 cm x 75 µm i.d. nanoViper column, packed with 2 µm C18 beads) at a flow rate of 5 µL/min for 6 min using an isocratic flow of 0.1% FA and 2% ACN. Peptides were separated during a biphasic ACN gradient from two nanoflow UPLC pumps with a flow rate of 280 nL/min on the analytical column (Acclaim PepMap 100, 15 cm x 75µm i.d. nanoViper column, packed with 2µm C18 beads). Solvent A was 0.1% FA and 2% ACN. Solvent B was 0.1% FA and 90 % ACN. The gradient was 0-61.5 min ramp from 8–38% B, 61.5-64.5 min ramp from 38–90% B, 64.5-69.5 min 90% B followed by column conditioning for 12 minutes with 5% B.

The ten most intense ions in every survey scan were subjected to higher energy collision dissociation (HCD) with a normalized collision energy of 40%, a default charge state of 2 and an activation time of 0.100 ms, accounting for a dynamic exclusion of 25 s. Survey full scan MS spectra (from *m/z* 300-2000) were acquired at a resolution of 60,000 at *m/z* 400. MS data were acquired over 90 minutes.

Data interpretation

The acquired raw data files were converted to peak lists using MSConvert as part of the ProteoWizard (Kessner et al. 2008) package, and searched using OMSSA (Geer et al. 2004) version 2.1.9 and X!Tandem (Craig and Beavis 2004) version Sledgehammer (2013.09.01.1) *via*

SearchGUI (Vaudel et al. 2011) version 1.16.0 against a concatenated target/decoy version of the *Mouse musculus* reviewed complement of the UniProtKB (Apweiler et al. 2004) database (downloaded November 2013, 16, 656 target sequences) where decoy sequences are the reversed version of the target as generated by SearchGUI. The search settings were: carbamidomethylation of Cys (+57.021464 Da), TMT 6-plex on peptide N-term peptide and Lys (+229.162932 Da) as fixed modifications; oxidation of Met (+15.994915 Da) as variable modification; precursor mass tolerance 10.0 ppm; fragment mass tolerance 0.5 Da; trypsin as enzyme allowing maximum of two missed cleavages. All other settings were set to the defaults of SearchGUI.

Search engine results were processed using PeptideShaker version 0.25.0 (Vaudel et al. 2015) Briefly, peptide spectrum matches (PSMs) were assembled into peptides and proteins and the identification results were validated at a 1% False Discovery Rate (FDR) threshold estimated using the target and decoy distributions of matches (Elias and Gygi 2010), a confidence level is provided for every match as complement of the Posterior Error Probability (PEP) estimated using the target and decoy distributions of matches (Nesvizhskii 2010). Protein ambiguity groups were built based on the unicity of peptide sequences in proteins as described in (Nesvizhskii and Aebersold 2005), and a representative protein was chosen for every group based on the evidence level provided by UniProt. In the following, proteins identified by mass spectrometry will implicitly refer to protein ambiguity groups.

Proteins were quantified using Reporter version of November 2013 (http://compomics.github.io/projects/reporter.html). Briefly, for every validated protein, the TMT reporter ions were extracted from spectra of validated PSMs and deisotoped using the isotope abundance matrix (Vaudel et al. 2010) provided by the manufacturer. Intensities were

normalized using the median intensity in order to limit the ratio deviation (Vaudel et al. 2014) and peptide and protein ratios were estimated using maximum likelihood estimators (Burkhart et al. 2011). The median of the reference channels was used as reference and sample to reference ratios were estimated for all samples. Ratios were $\log_2$ converted and normalized to the median to avoid inter-sample bias. Only those proteins presenting two or more validated and quantitated peptides were retained for further analysis. Standard contaminants were excluded from downstream statistical analysis.

Unsupervised hierarchical clustering

Unsupervised hierarchical were performed in Perseus (v.1.6.2.3) on z-normalized abundance values. The parameters for clustering were average linkage and euclidean correlation as distance measurement, prepossessed with k-means.

Statistical analysis

The significance of the protein regulation between different cell types was evaluated using a student's t-test and a p-value <0.05 was considered significant. Volcano plots were created by plotting the p-value against the regulation level. Significantly regulated proteins were separated into three groups according to the measured regulation level: (1) background (ratio between 0.5:1 and 2:1); (2) moderately regulated (ratio between 0.1:1 and 0.5:1 or 2:1 and 10:1); and strongly regulated (ratio lower than 0.1:1 or higher than 10:1). Hierarchical clustering was conducted using Perseus as part of the MaxQuant software suite (Cox and Mann 2008).

Supplemental methods for the RNAseq analyses

Data

RNA-seq raw reads are downloaded from DNA Link, which are reversely-stranded paired-end reads. The counts table tx2gene_counts_mmu.rds is generated by aligning reads to the mouse transcriptome (Ensembl version 94) using kallisto (Bray et al. 2016) and converting transcript counts to gene counts using tximport (Soneson et al. 2015).

The phenotype information is in table pheno_mouse.csv.

Filtering, normalization, and transformation

To filter out low expressing genes, we keep genes that have counts per million (CPM) more than 1 in at least 4 samples. There are 14245 genes after filtering. We then normalize counts by weighted trimmed mean of M-values (TMM) (Robinson and Oshlack 2010). The normalization factors are between 0.93 and 1.08.

In order to use linear models in the following analysis, we perform Voom transformation (Law et al. 2014) to transform counts into logCPM, where CPM = 1e+6 * count of a gene / (total counts of the sample * normalization factor of the sample). Voom transformation also estimates the mean-variance relationship and use it to compute appropriate observation-level weights, so that more read depth gives more weights.

PCA

To get an overall view of the similarity and/or difference of the samples, we perform principal component analysis (PCA). The PCA plot pca.pdf shows that samples are clustered by groups and there is an outlier aB6_A5_2 (shown in the labeled PCA plot pca_labeled.pdf). Therefore we remove the outlier and redo the PCA pca_no_outlier.pdf.

Linear modeling

To discover the differential genes, we use limma, an R package that powers differential expression analyses (Ritchie et al. 2015). We account for the correlation of technical replicates in the analysis. We perform moderated t-test to detect genes that are differentially expressed between mutants and controls.

In the p-value distribution pval_dist.pdf, the Q-Q plot shows that we observe more significant p-values than expect, and the histogram shows that there is substantial enrichment of significant p-values and the proportion of the true null hypothesis is less than 50%.

Gene statistics table for all genes gene_stats.csv. The table contains the average logCPM of each group, p-values, FDR, log fold-change, fold-change, and gene annotation. Genes with FDR < 0.25 are considered significantly changed.

Plots

In the volcano plots volcanoes.pdf, the top genes of either smallest p-values or largest logFC are labeled.

In the dot plots top_genes_dotplots.pdf, top genes (according to p-values) are selected and the logCPM are plotted.

In the heatmap top_genes_heat.pdf, the same set of top genes are used as in dot plots. The z-scores of the logCPM are plotted.

Pathway analysis

We obtain the Gene sets from the MSigDB Collections. We select the gene sets that belong to the canonical pathways (CP), gene motif (transcription factor targets or microRNA targets), and gene ontology (GO).

We then use the Fry function of the Rotation Gene Set Test (Roast) in the limma R package to perform pathway analysis (Wu et al. 2010). This will tell us if all the genes in a pathway gene set are directionally or undirectionally (i.e. mixed) changed. The results are in tables cp_fry.xlsx; motif_fry.xlsx; go_fry.xlsx.

We also use the Fisher Exact test to determine if the gene lists in Gene list for heat maps in db project.csv are over presneted in pathway gene sets.

Supplemental References:

Apweiler R, Bairoch A, Wu CH, Barker WC, Boeckmann B, Ferro S, Gasteiger E, Huang H, Lopez R, Magrane M et al. 2004. UniProt: the Universal Protein knowledgebase. *Nucleic Acids Res* **32**: D115-119.

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