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Supplemental Information

MYC Induces a Hybrid Energetics Program Early in Cell Reprogramming

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SUPPLEMENTAL INFORMATION

Supplemental information accompanying this paper includes:

- 6 Supplemental Figures:
  - S1, related to Figure 1.
  - S2, related to Figure 2.
  - S3 and S4, related to Figure 3.
  - S5, related to Figure 4
  - S6, related to Figures 5 and 6.
- Supplemental Experimental Procedures
- Supplemental References, related to Supplemental Experimental Procedures.
- 3 Supplemental Tables:
  - Table S1, primary antibodies used in this study.
  - Table S2, secondary antibodies used in this study.
  - Table S3, sequence of the primers used in this study.

SUPPLEMENTAL FIGURES

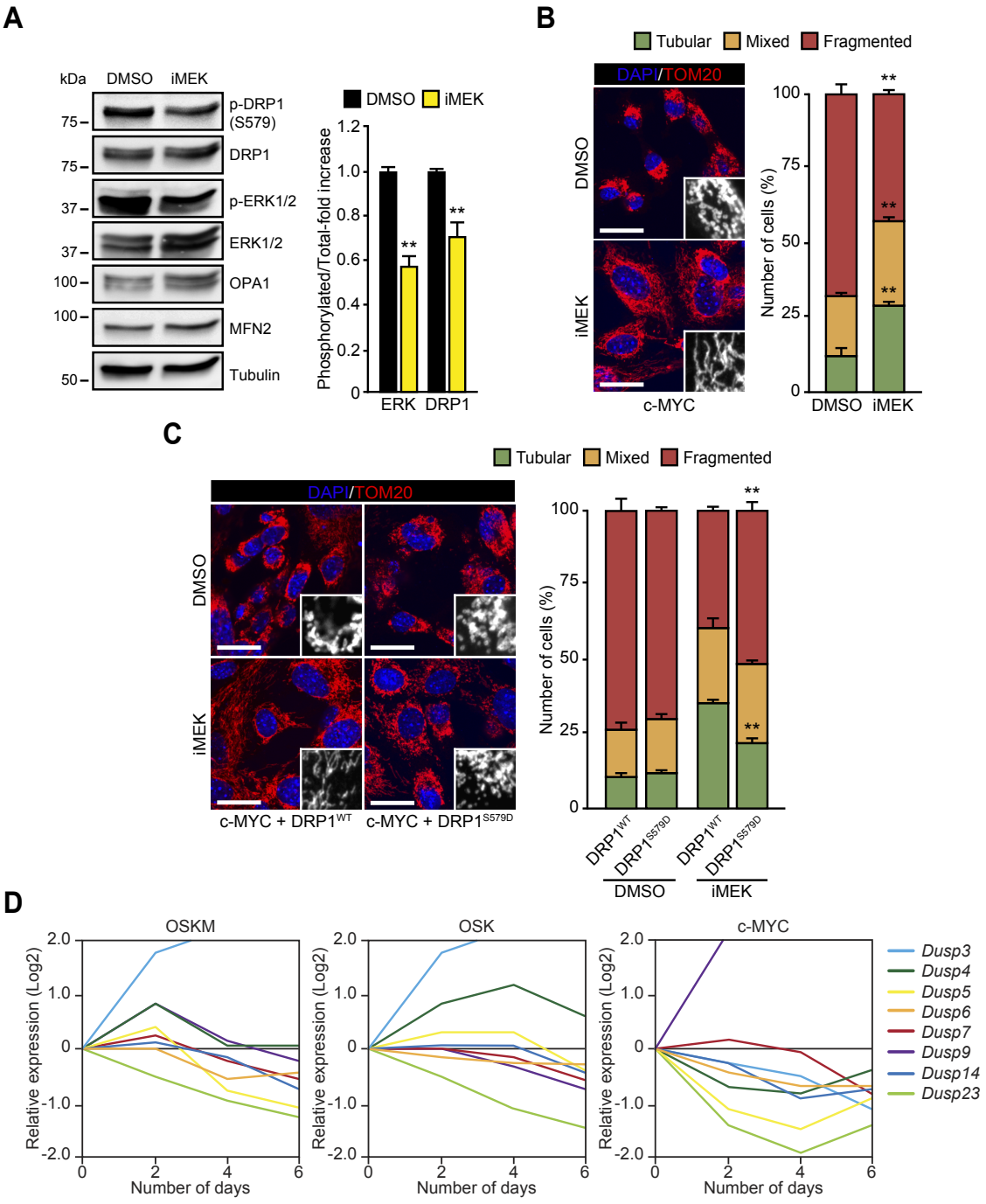

Figure S1. Role of c-MYC in ERK1/2-induced mitochondrial fission and DRP1 activation

(Related to Figure 1)

(A) Left panels, lysates from c-MYC-transduced MEFs for 3.5 days that were incubated during 10 hours with DMSO (as vehicle control) or the MEK1/2 inhibitor PD0325901 (1 μM) were analysed by immunoblotting using the indicated

antibodies. Right graph shows the quantification of ERK1/2 or DRP1 phosphorylation ratios in cells treated with either DMSO (black bars) or PD0325901 (yellow bars).

(B) C-MYC-transduced cells for 3.5 days were incubated with DMSO (as vehicle control) or the PD0325901 (1 μM) for 10 hours. Then, cells fixed and mitochondrial morphology assessed by immunofluorescence. Right panels, representative confocal images of MEFs stained with anti-TOM20 antibody (red). Inset shows a black and white magnification of the pictures. DAPI (blue) was used as a nuclear counterstaining. Graph on the left shows the quantification of the different mitochondrial morphologies observed.

(C) Left panels show representative confocal images of MEFs expressing c-MYC, together with DRP1 wild type ($DRP1^{WT}$) or the phosphomimetic S579D mutation ($DRP1^{S579D}$), during 3.5 days. Cells were then treated, fixed and stained as in (C). DAPI (blue) was used as a nuclear counterstaining. Graph on the right shows the quantification of the indicated mitochondrial morphologies observed in the cells transduced and treated as indicated.

(D) Total RNA was extracted from OSKM, OSK or c-MYC-transduced cells at indicated days. Then, *Dusp3*, *Dusp4*, *Dusp5*, *Dusp6*, *Dusp7*, *Dusp9*, *Dusp14* or *Dusp23* gene expression was quantified by qPCR and represented as relative gene expression normalized to control MEFs (n = 3; each independent experiment was conducted in triplicate). Error bars are omitted from the graph for clarity.

Data represent mean ± SEM, one-tailed unpaired t-test (n = 3), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; **** $P < 0.0001$. Scale bars in (B) and (C) are 24 μm.

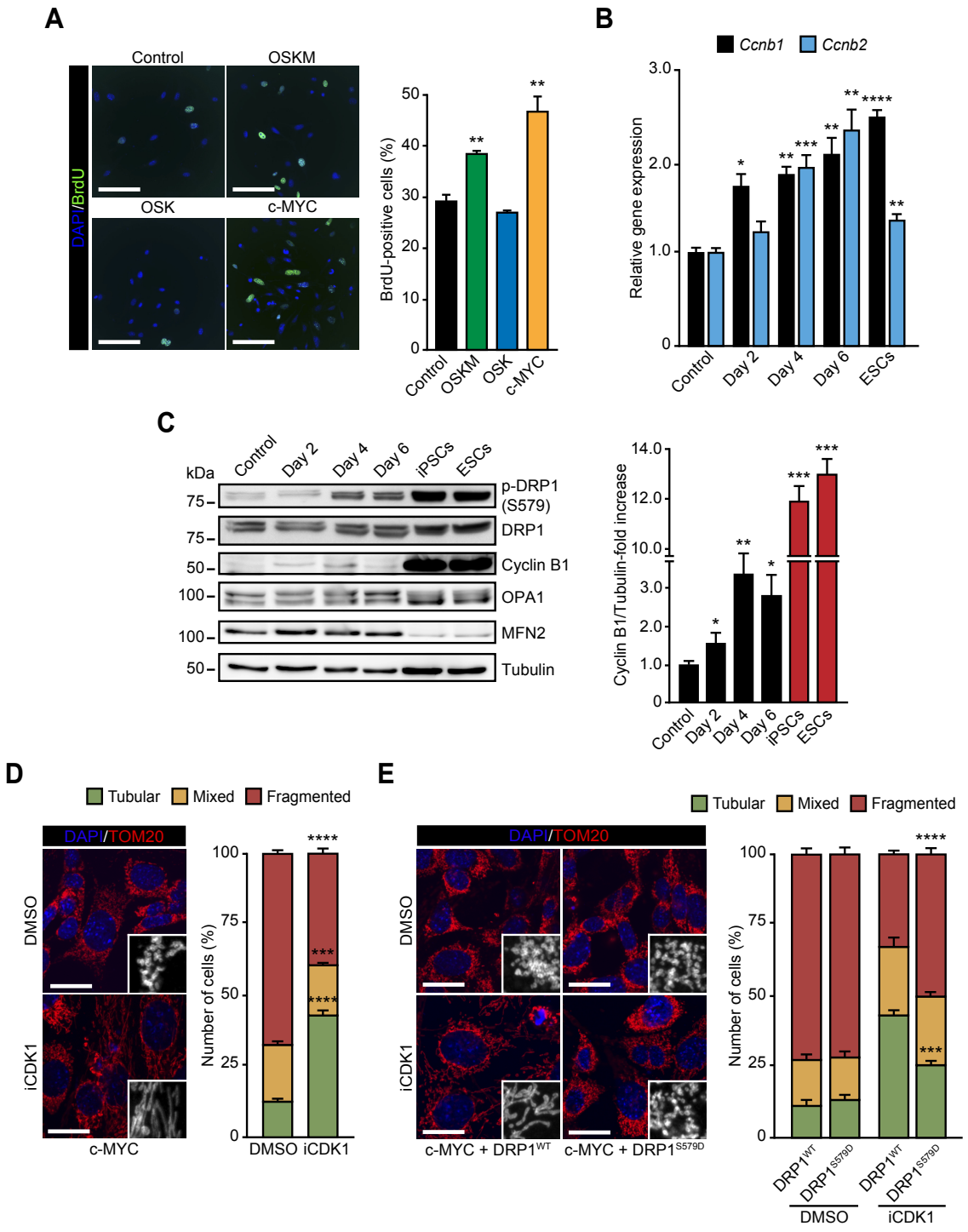

Figure S2. C-MYC induced proliferation early in cell reprogramming

(Related to Figure 2)

(A) MEFs were mock-infected (control) or transduced with the indicated factors. At day 4 post-transduction, cells were pulsed with 5-bromo-2'-deoxyuridine (BrdU) for 10 minutes, fixed and processed for immunofluorescence with anti-BrdU antibodies (green) and analysed by High-Content microscopy. Left panel,

representative immunofluorescence images (scale bars, 40 μm). Graph on the right shows the quantification of the data.

(B) Total RNA was extracted from MEFs left untreated (control) or OSKM-infected for the indicated days. *Ccnb1* (*Cyclin B1*, black bars) or *Ccnb2* (*Cyclin B2*, blue bars) gene expression was then assessed by qPCR and represented as relative gene expression normalized to control MEFs.

(C) Lysates of MEFs control or expressing OSKM for the specified days were analysed by immunoblotting using the displayed antibodies. Graphs on the right show the quantification of the data corresponding to Cyclin B1 protein expression.

(D) c-MYC-transduced cells for 3.5 days were incubated with DMSO (as vehicle control) or the CDK1 inhibitor RO-3306 (1 μM) for 10 hours (iCDK1). Cells were then treated, fixed and stained as in (Figure S1C). Graph on the right shows the quantification of the indicated mitochondrial morphologies observed in the cells transduced and treated as indicated.

(E) Left panels show representative confocal images of MEFs expressing c-MYC, together with DRP1 wild type ($DRP1^{WT}$) or the phosphomimetic S579D mutation ($DRP1^{S579D}$), during 3.5 days. Cells were then treated, fixed and stained as in (Figure S1C). Graph on the right shows the quantification of the indicated mitochondrial morphologies observed in the cells transduced and treated as indicated.

Data represent mean ± SEM, one-tailed unpaired t-test (n = 3), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; **** $P < 0.0001$. Scale bars in (A) 40 μm, in (D) and (E) 24 μm.

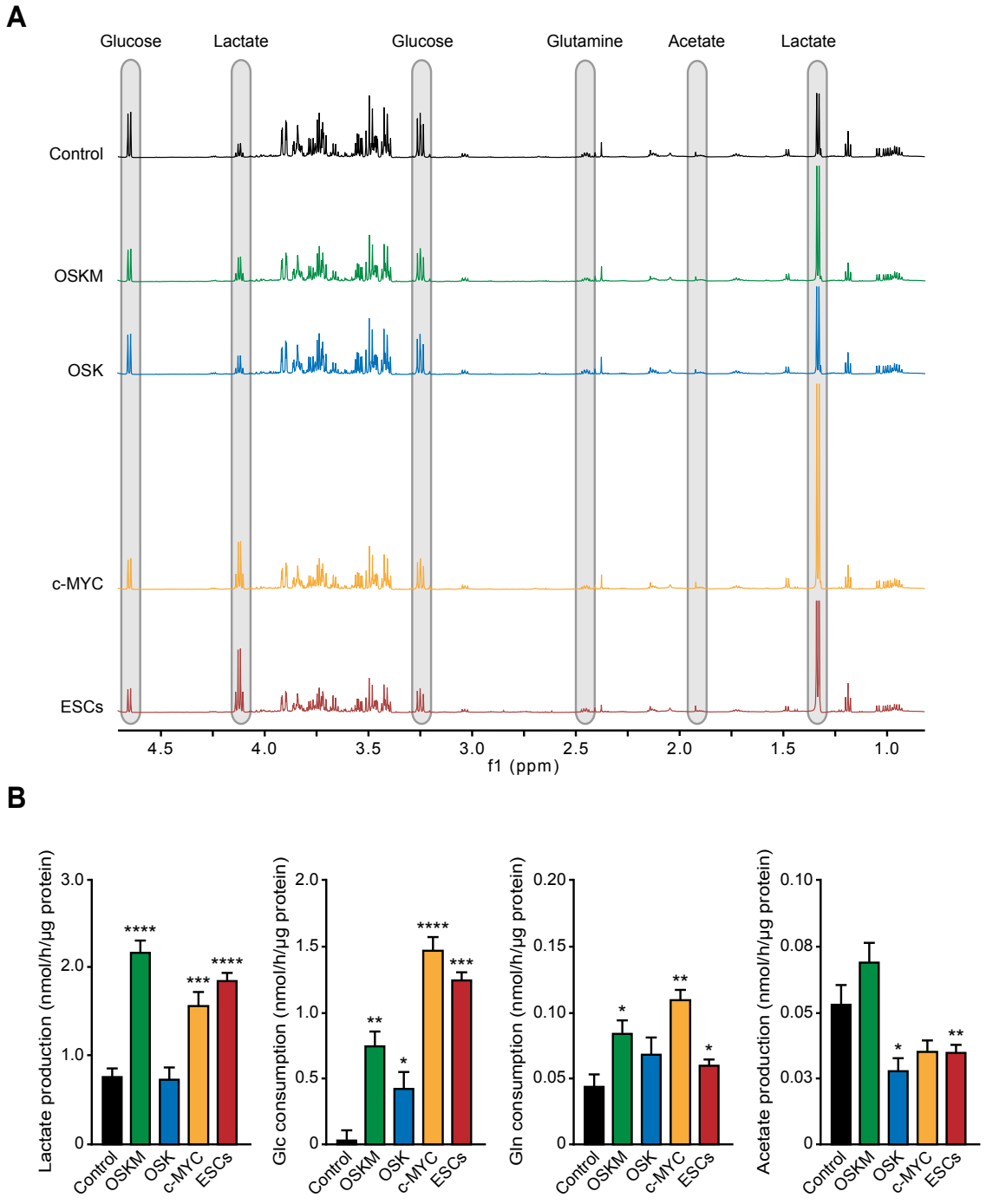

Figure S3. Induction of glycolysis by c-MYC early in cell reprogramming

(Related to Figure 3)

(A) Culture medium from MEFs mock-infected (control) or transduced with the indicated factors was changed at day 2.5 post-transduction. Thereafter, aliquots of conditioned media were taken at 10 (d3), 24 (d4), 48 (d5) or 72 (d6) hours and analyzed by NMR. Diagrams depict metabolomic footprints showing the ^{1}H NMR spectra of the indicated extracellular metabolites acquired at 500 MHz and 27 °C, at day 4 post-transduction with the factors specified on the left.

(B) Bars diagrams showing the kinetics of the metabolites shown in MEFs transduced with the indicated factors for four days, as assessed by NMR.

Data represent mean ± SEM, one-tailed unpaired t-test (n = 3), * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

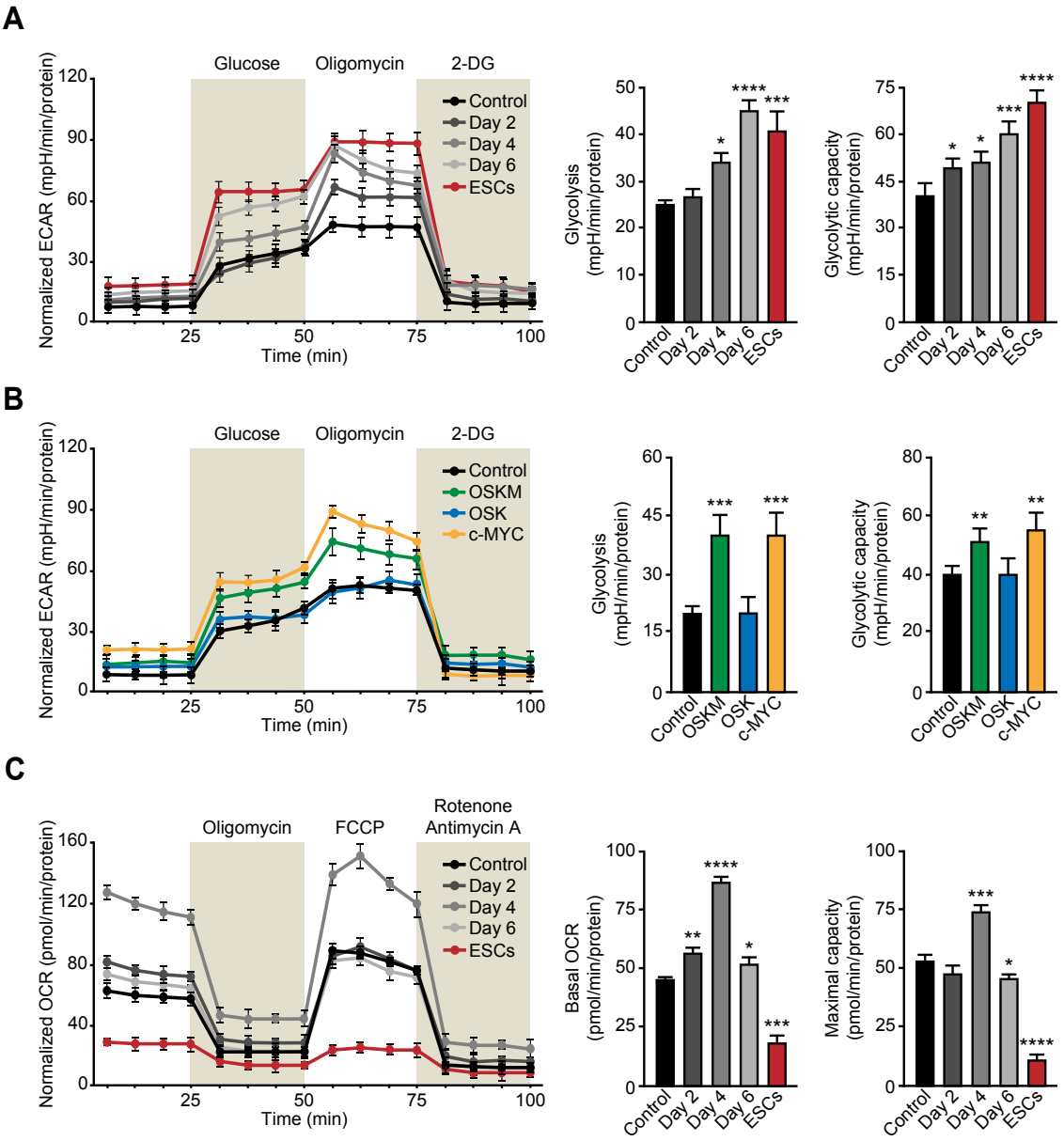

Figure S4. Metabolic profiles during early cell reprogramming

(Related to Figure 3)

(A) Graphs displaying representative extracellular acidification rate (ECAR) in ES cells (ESCs), or in control or OSKM-transduced MEFs for the indicated days.

(B) Graphs displaying representative extracellular acidification rate (ECAR) in MEFs control or transduced with the indicated factors at day 4 post-transduction.

(C) Graphs displaying representative Oxygen Consumption rates (OCR) in ES cells (ESCs), or in control or OSKM-transduced MEFs for the indicated days.

Data represent mean ± SEM, one-tailed unpaired t-test (n = 3, conducted in triplicate), * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. FCCP, Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone. 2-DG, 2-Deoxy-D-glucose.

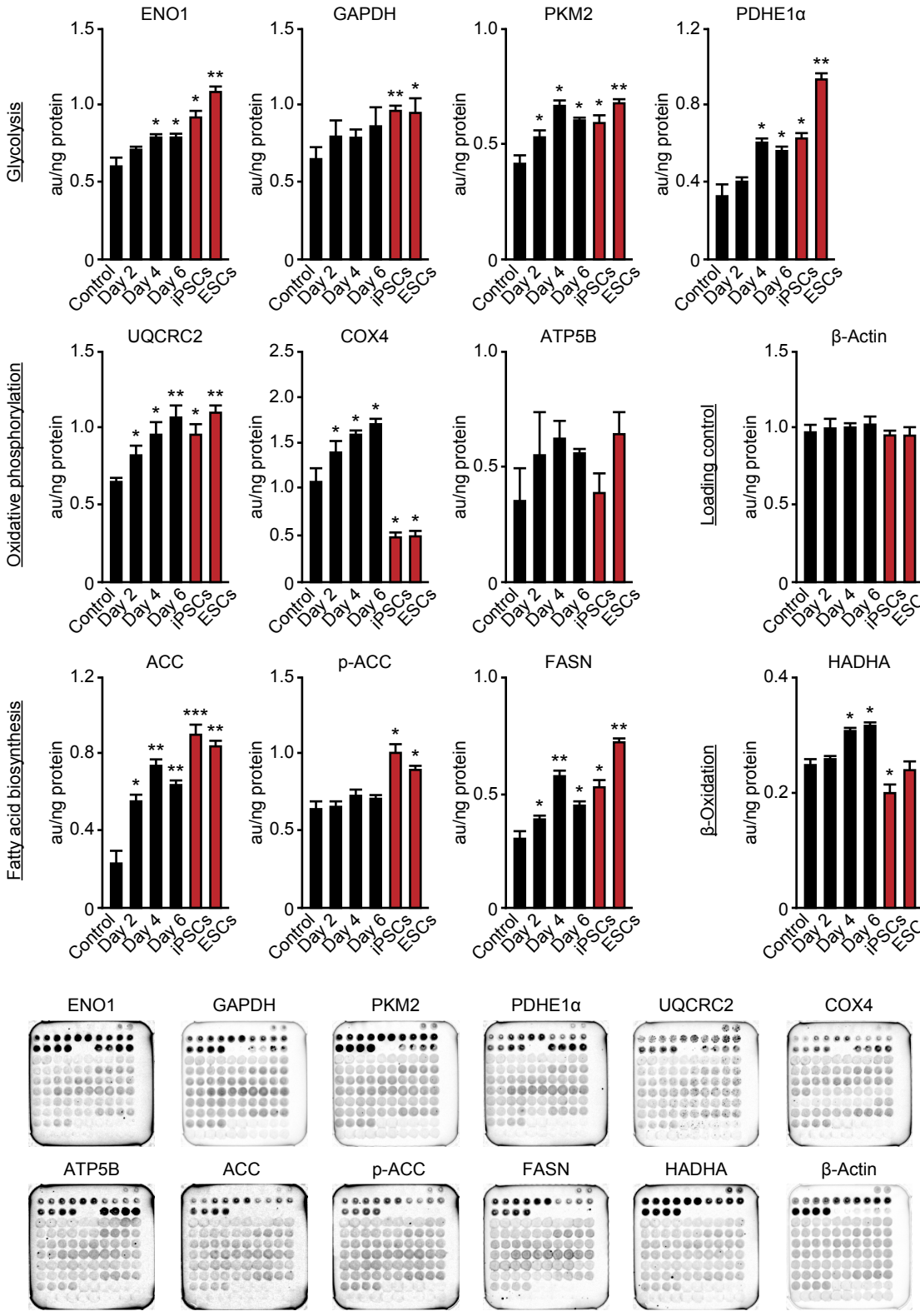

Figure S5. Reverse phase protein antibody microarray analysis of metabolic enzymes during early cell reprogramming

(Related to Figure 4)

Lysates of iPS (iPSCs) or ES (ESCs) cells, MEFs control or transduced with OSKM for the indicated days were analyzed using reverse phase protein arrays. Graphs show the quantification of the data that are represented as mean ± SEM. Bottom panels, representative images from the corresponding protein array pads.

Data represent mean ± SEM, one-tailed unpaired t-test (n = 3), * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

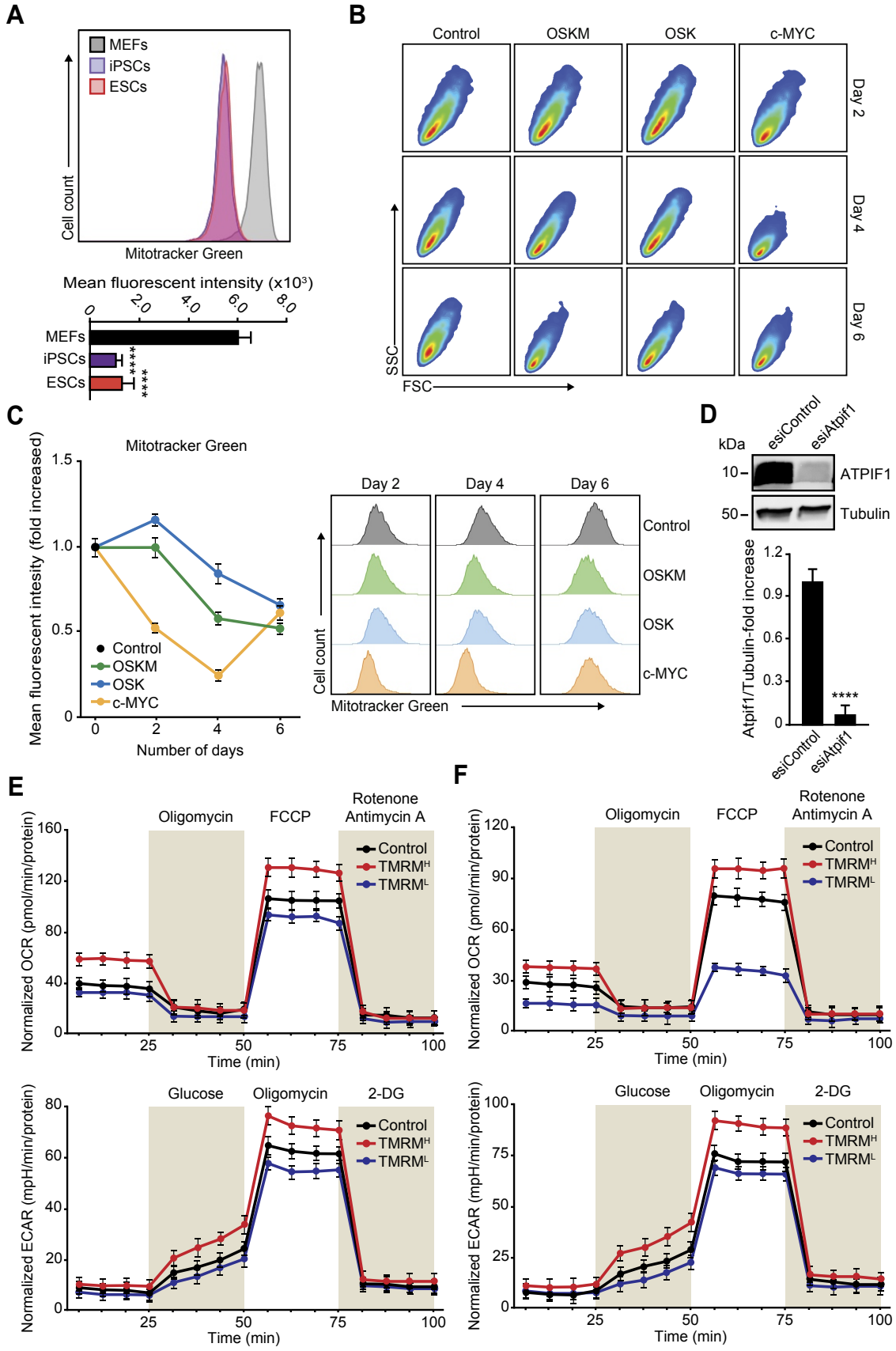

Figure S6. C-MYC increases mitochondrial membrane potential during cell reprogramming

(Related to Figures 5 and 6)

(A) Representative flow cytometry histograms of MEFs, iPS (iPSCs) and ES (ESCs) cells stained with Mitotracker Green FM, a mitochondrial membrane potential-independent probe, to assess mitochondrial mass. Graph underneath shows the quantification of the Mean Fluorescence Intensity of the histograms shown above.

(B-C) Representative flow cytometry histograms of non-transduced MEFs (Control) or transduced with OSKM, OSK or c-MYC-encoding retroviruses and stained with Mitotracker Green FM at the indicated days post-transduction for assessing Forward and Side scatter distributions (B) and mitochondrial mass (C). Graph on the left shows the quantification of the Mean Fluorescence Intensity of the histograms shown in (C).

(D) MEFs transduced with OSKM factors were transfected with esiRNAs targeting either e*GFP* (Control) or *Atpif1* at day 1 post-transduction. At day 6 post-transduction with OSKM retroviruses total lysates were analysed by immunoblotting using the indicated antibodies (upper panels). Graph underneath shows the quantification of the data.

(E-F) Graphs displaying representative oxygen consumption rate (OCR, upper graphs) or extracellular acidification rate (ECAR, bottom graphs) of OSKM-expressing cells sorted based on their TMRM staining intensity at day 6 (E) or 12 (F) post-transduction.

Data represent mean ± SEM, one-tailed unpaired t-test (n = 3), **** $P < 0.0001$. FCCP, Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone. 2-DG, 2-Deoxy-D-glucose.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture, reprogramming assays, reagents and plasmids

PlatE (Morita et al., 2000) and SNL (McMahon and Bradley, 1990) cells were grown in high glucose DMEM containing 10% heat-inactivated FBS. When indicated, SNL cells were mitotically inactivated by treatment with 10 μg/ml Mitomycin-C (Sigma-Aldrich) for 2.5 h at 37 °C.

Ecotropic retroviruses were produced in PlatE cells transfected using Polyethylenimine (PEI) "Max" (Mw 40,000) (Polysciences) exactly as described (Takahashi and Yamanaka, 2006; Prieto et al., 2016a; Prieto et al., 2016b). For cell reprogramming, 8 x 10^5 MEFs were plated per p100 mm the day before the assay. Next day (day 0), MEFs were incubated overnight with a mixture of mouse OCT4, SOX2, KLF4 and c-MYC retroviral supernatants supplemented with 4 μg/ml Polybrene (1:1:1:1 for OSKM, 1:1:1 for OSK and 1:4 for c-MYC). Next day, the supernatants were replaced with fresh media and cells were incubated for 3 more days (day 4). At day 2 media was changed to ES cell culture media containing 15 % FBS and LIF. Media was changed every other day. When indicated, cell reprogramming was conducted in the presence of DMSO (as control), 10 μM of c-MYC inhibitor 10058-F4 (Selleckchem), 10 μM of the CDK1 inhibitor RO-3306 (Merck), 1 μM of the MEK1/2 inhibitor PD0325901 (Millipore) or 0.5 μM Rotenone (Selleckchem). For assessing cell reprogramming efficiency (AP-staining) in the presence or absence of the MYC inhibitor (Figure 1A), OSKM-transduced cells for four days were plated (5 x 10^4 cells) on a confluent layer of mitotically-inactivated SNL feeders seeded the day before on gelatin-coated p60 mm at 2,5 x 10^6 cells per

dish. OSK-transduced cells were not plated on a confluent layer of feeders and were maintained in their original plate without reseeding. At day five post-transduction, MYC inhibitor or vehicle control were added to the plates and renewed every other day until the end of the experiment. AP-staining was performed at day 25.

Westernblot

Signals in western blots were detected using ECL prime (Amersham) and images automatically captured in an Alliance Mini HD9 digital imaging system equipped with a 16-bit (65536 grey levels) scientific grade camera with variable electronic shutter speed and 4.8 OD dynamic range (UVITEC). Acquired images were processed using Adobe Photoshop CS6 and analysed with ImageJ software.

High-content analysis

For the analysis, cells and nuclei were defined using the Hoechst 33258 staining. The nuclei were segmented using top-hat segmentation defining a minimum nucleus area of 50 μm^2. To define the cell segmentation a collar segmentation routine was used, specifying a ratio of 4 μm. To analyse the expression of BrdU by cell, the average intensity of pixels in the reference channel (AlexaFluor-549) within the defined nuclear region was measured. Once each cell had associated a nuclear intensity for the specific expression, a threshold filter defining positive and negative expressing cells was set. Threshold filter uses a histogram for data visualization. To specify the filter settings, the nuclear intensity measurement was selected. The threshold filter defines the cells with nuclear intensities above or below a given value as positive or negative respectively, for the expression of the protein. As a result, the program assigns to each cell the definition of positive or negative for the

expression of BrdU and generates a percentage of both cell populations (positive and negative) per well.

Printing and processing of reverse phase protein arrays

For printing, serially diluted protein extracts (0–1 μg/μl) derived from HCT116 colocarcinoma and BT-549 breast cancer cell lines were also prepared to assess printing quality and the linear response of protein recognition by the antibodies used. A standard curve of BSA (0-1 μg/μl) and mouse IgGs (0-0.6 ng/ml) were also prepared for printing as internal negative and positive controls, respectively. 1 nl volume of each sample was spotted in duplicate onto nitrocellulose-coated glass slides containing 16 pads (FAST Slides, Scheleicher & Schuell BioScience, Inc.) using a BioOdyssey Calligrapher MiniArrayer printer (Bio-Rad) equipped with a solid pin (MCP310S) at constant humidity (RH 45 %) and 10 °C and 16 °C for the plate and chamber, respectively.

After printing, arrays were allowed to dry and further blocked in PBS-T containing 5 % skimmed milk. After, each pad in the array was incubated overnight at 4 °C with the indicated concentrations of highly specific primary monoclonal or polyclonal antibodies. Spotted samples in one of the pads were fixed with XFCF buffer (10% acetic acid, 30% ethanol) for 1h, stained with 0.0001% Fast Green FCF (Sigma-Aldrich) in XFCF for 5 minutes and washed 5 times with XFCF in order to quantify the total protein amount of each spot. The other pads, after incubation with the primary antibodies, were washed with PBS-T and further incubated with a goat anti-mouse highly cross-adsorbed antibody conjugated with CF-647 (Sigma-Aldrich, 1:500) or, in the case of rabbit antibodies, a donkey anti-rabbit secondary antibody

conjugated with alexa-647 (1:500) (Invitrogen). Microarrays were scanned using a Typhoon 9410 scanner (GE Healthcare). The mean fluorescent intensity of the spots was quantified using GenePix Pro 7 software system and converted into arbitrary units of expressed protein per ng of protein in the sample, using the expression obtained in the standard curve of the HCT116 cell line and normalized to the protein amount in the sample obtained from the FCF stained pad. Representative technical variances of the β-Actin arrays, calculated by the squared coefficient of variation (SCV = σ*100/|x|), were 4.8 ± 0.7. Details of the used antibodies are provided in Supplemental Tables 1 and 2.

Metabolomic footprinting

For NMR analysis, 60 µL of D_2O (Eurisotop) containing 5 mM sodium 3-(trimethylsilyl) propionate-2,2,3,3-d4 (TSP) (Eurisotop) for chemical shift reference and 92.50 mM formate (Sigma) for peak quantification, reference was added to 540 µL of medium. The concentration of the formate stock solution was previously calibrated with a quantitative 1D NMR experiment, applying the Eretic Signal (Bruker Biospin). Samples were then centrifuged for 5 min at 10,000 X*g* and the supernatant transferred to 5 mm NMR tubes (DeuteroGmB). ^{1}H NMR analyses were carried out on a Bruker Ultrashield Plus 500 MHz spectrometer with a TBI probe. A NOESY mixing time of 0.01 s was applied for improving the baseline and the water signal was suppressed with pre-saturation. The spectra were acquired over a width of 30 ppm with 96 k data points and an acquisition time of 2.7 s. 64 scans were accumulated for each spectrum and the relaxation delay was set to 4 s. Spectra were processed with an exponential line broadening of 0.5 Hz and a zero filling to 128 k. The baseline was corrected manually and the spectra referenced to

the TSP peak (0.00 ppm) using MestReNova 8.1 software (MestRelab Research). Metabolite assignment was performed with the help of the Human Metabolome database and published literature. Metabolite concentrations in basal media were subtracted from concentrations of metabolites in 10-to-72 h conditioned media for the calculation of net fluxes. The resulting rates were normalized to total cellular protein content determined by a BCA protein assay (Pierce, Thermo-Scientific)

Extracellular metabolic flux analysis

Basal and uncoupled oxygen consumption rates (OCR), or extracellular acidification rates (ECAR), were measured using a Seahorse bioanalizer (XF96) and the Mito or Glycolysis stress test kits (both from Seahorse Bioscience, Millipore), respectively. Cells (20,000-30,000 cells per well) were plated the day before the measurements on XF96 culture microplates. Next day, media was changed to unbuffered XF Base medium supplemented with 2 mM Glutamine (glycolysis stress kit), or 25 mM glucose, 1 mM Sodium Pyruvate and 2 mM Glutamine (mitochondria stress kit) and equilibrated for 1 hour at 37 ˚C without a CO_2 supply. For OCR assessment, Oligomycin (1 μM), FCCP (1 μM) and Antimycin A/Rotenone (0.5 μM) were used. For ECAR assessment, glucose (10 mM), oligomycin (1 μM) and 2-Deoxy-D-glucose (50 mM) were used. Measurements were taken every 5 minutes after addition of the drugs (a total of 4 readings per reagent added) and results were normalized to the total cellular protein content determined by a BCA protein assay. Each experiment was conducted in triplicate and repeated at least 3 times.

SUPPLEMENTAL REFERENCES

McMahon AP and Bradley A (1990) The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 62:1073-1085.

Morita S, Kojima T and Kitamura T (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther 7:1063-1066.

SUPPLEMENTAL TABLES

Table S1. Primary antibodies used in this study.

Antigen	Host[a]	Company, reference[b]	Dilution, (application)[c]
ACC	Rb	CST, #3676	1:1000 (WB)
p-ACC (Ser79)	Rb	CST, #11818	1:1000 (WB)
β-Actin	Ms	SA, A2547	1:500 (PMA)
ATP5B	Ms	In house (J.M. Cuezva)	1:150 (PMA)
ATPIF1	Rb	SCBT, sc-134962	1:500 (WB)
BrdU	Rt	ID, OBT0030	1:500 (IF/HCA)
Cyclin B1	Ms	SCBT, sc-245	1:100 (WB)
COX4	Ms	Abcam, ab14744	1:100 (PMA)
DRP1	Rb	CST, #8570	1:1000 (WB); 1:50 (IF)
p-DRP1 (Ser616)	Rb	CST, #3455	1:1000 (WB)
ENO1	Rb	Abgent, AM2192b-ev	1:200 (PMA)
ERK1/2	Rb	CST, #9102	1:1000 (WB)
p-ERK1/2 (Thr202/Tyr204)	Ms	CST, #9106	1:2000 (WB)
FASN	Rb	CST, #3180	1:1000 (WB)
GAPDH	Rb	CST, #5174	1:1000 (WB)
GAPDH	Ms	In house (J.M. Cuezva)	1:250 (PMA)
HADHA	Rb	Abcam, ab54477	1:1000 (PMA)
HK1	Rb	CST, #2024	1:1000 (WB)
HK2	Rb	CST, #2867	1:1000 (WB)
LDHA	Rb	CST, #3582	1:1000 (WB)
MFN2	Rb	SA, AV42420	1:1000 (WB)
NDUFS3	Ms	SCBT, sc-374282	1:200 (IF)
OPA1	Ms	BD, #612606	1:1000 (WB)
PDH	Rb	CST, #3205	1:1000 (WB)

PDHE1α	Ms	TFS, 459400	1:200 (PMA)
PKM1/2	Rb	CST, #3190	1:1000 (WB)
PKM2	Ms	In house (J.M. Cuezva)	1:150 (PMA)
SDHA	Rb	SCBT, sc-11998	1:300 (WB)
TOM20	Rb	SCBT, sc-11415	1:50 (IF); 1:500 (WB)
TOM20	Ms	SA, WH0009804M1	1:500 (IF)
α–Tubulin	Ms	SCBT, sc-32293	1:5000 (WB)
UQCRC2	Ms	Abcam, ab14745	1:500 (PMA)

(*a*) Rb, rabbit; Ms, Mouse, Rt, Rat.

(*b*) SCBT, Santa Cruz Biotechnology; CST, Cell Signaling Technologies; ID, Immunological Direct; SA, Sigma-Aldrich; BD, BD Biosciences.

(*c*) IF, immunofluorescence; HCA, High-Content analysis; PMA, reverse phase protein microarrays; WB, Western blot.

Table S2. Secondary antibodies used in this study.

Antigen	Host[a]	Conjugated[b]	Company, reference[c]	Dilution, (application)[d]
Mouse IgG	Dk	AF–488	TFS, A-21202	1:1000 (IF)
Mouse IgG	Dk	AF–555	TFS, A-31570	1:1000 (IF)
Mouse IgG	Dk	AF–647	TFS, A-31571	1:1000 (IF)
Mouse IgM	Dk	AF–555	TFS, A-21426	1:1000 (IF)
Rabbit IgG	Dk	AF–488	TFS, A-21206	1:1000 (IF)
Rabbit IgG	Dk	AF–555	TFS, A-31572	1:1000 (IF)
Rabbit IgG	Dk	AF–647	TFS, A-31573	1:1000 (IF)
Rat IgG	Dk	AF–488	TFS, A-11006	1:1000 (IF)
Mouse IgG	Gt	HRP	TFS, 31432	1:5000 (WB)
Rabbit IgG	Gt	HRP	TFS, 31460	1:5000 (WB)
Mouse IgG	Gt	CF–647	SA, SAB4600183	1:500 (PMA)
Rabbit IgG	Dk	AF–647	TFS, A-31573	1:500 (PMA)

(*a*) Dk, donkey; Gt, goat.

(*b*) AF, AlexaFluor; CF, cyanine-based fluorescent dye; HRP, Horseradish Peroxidase

(*c*) SA, Sigma-Aldrich; TFS, Thermo Fisher Scientific.

(*d*) FC, flow cytometry; IF, Immunofluorescence; PMA, reverse phase protein microarrays; WB, Western blot.

Table S3. Sequences of the primers used in this study for qPCR analysis employing SYBR Green.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Atpif1	*ACCATTCGAAGGAGATAGAG*	*ATTCAAAGCCATGTGTCTAC*
Ccnb1	*ACATGACTGTCAAGAACAAG*	*CAGATGTAGCAGTCTATTGG*
Ccnb2	*GCCAAGGAAAATGGAATTTG*	*TACGGTTGTCATTGACTTTC*
Dusp3	*CACCACAAACCAAAAACATC*	*CATGCTATCCTTGACCTTTC*
Dusp4	*TATTGAGTGGAGAGGGAAAG*	*ATTCCTGTGAGTTCAACAAC*
Dusp5	*AAATCCTTCCCTTCCTCTAC*	*CTTGAAAGTGGGAGCTAATG*
Dusp6	*ATGATGAGGTCTTCAGTCTC*	*CAAAATACCCCTTGAGACAC*
Dusp7	*GAGAGAGAGAGTGTTCTCAG*	*AGGTAGGAAATGGTAGAACC*
Dusp9	*CGTCTGTGACTGTTACTTTC*	*TGTGGCAAAGAATGATACAC*
Dusp14	*CATCATCCCAGACGTTTATG*	*CATCTCTTAAACCCGAACAC*
Dusp23	*CATTGAGACGTATGAACAGG*	*AGTACTTTAGTTCACTCCCC*