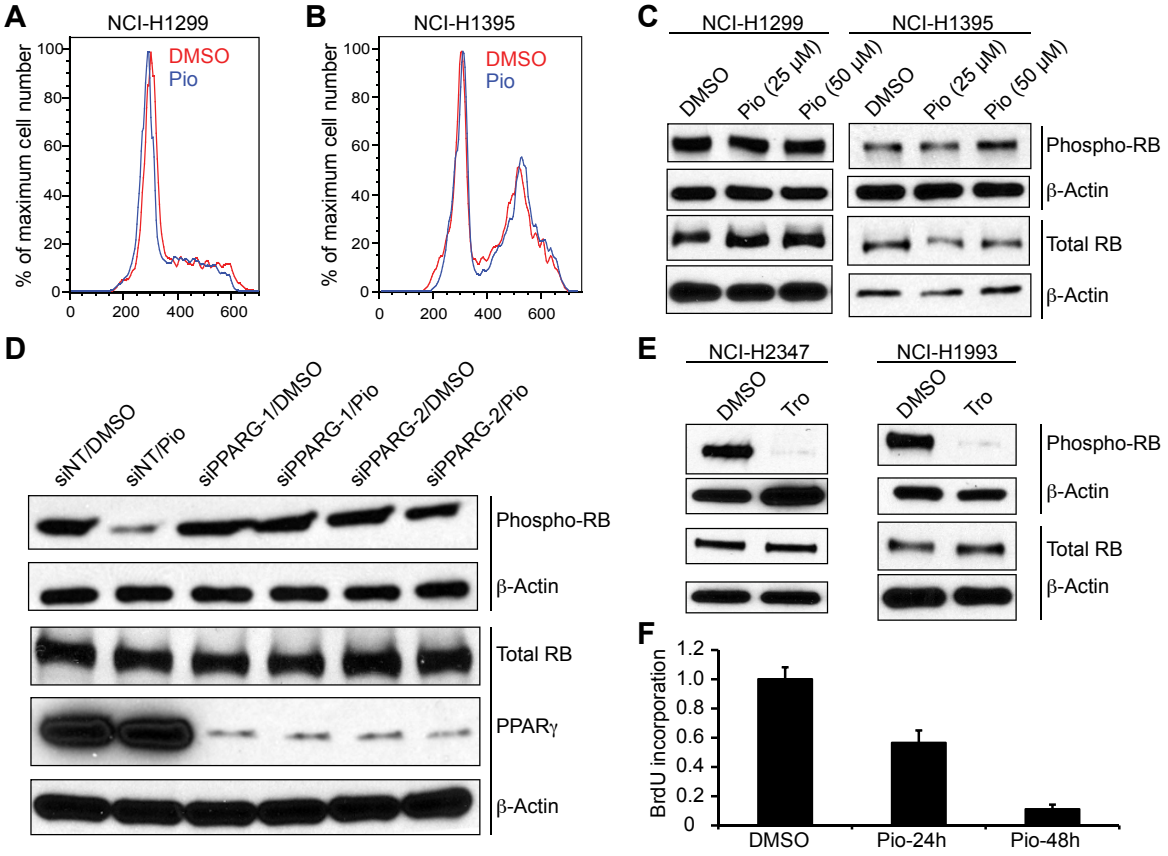
SUPPLEMENTAL FIGURES



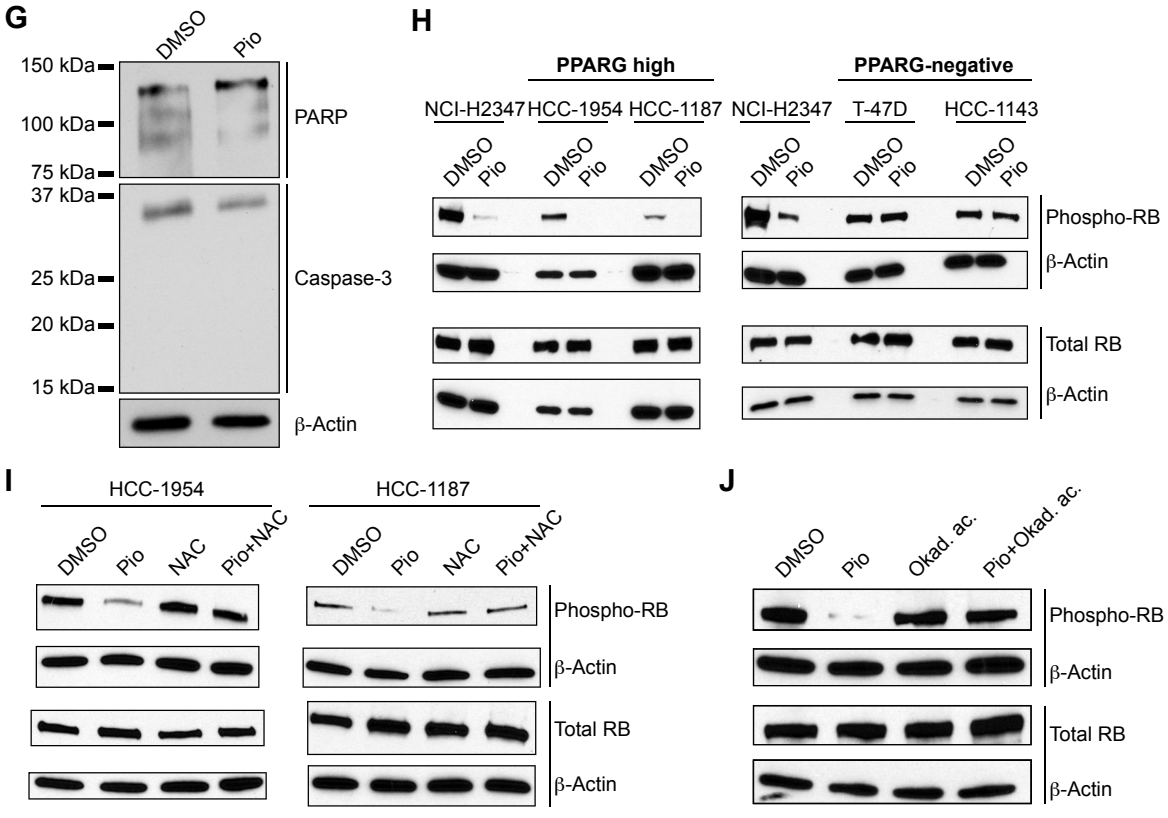


Figure S1 related to Figure 3: TZDs inhibits cell proliferation in PPARG-positive lung adenocarcinoma cells.

(**A and B**) DNA content analysis of PPARG-negative NCI-H1299 (**A**) and NCI-H1395 (**B**) cells after 24 hours of treatment with pioglitazone (Pio) or vehicle (DMSO) shows no effect on cell cycle progression.

(**C**) Pioglitazone treatment for 24 hours has no effect on the levels of phosphorylated RB in both NCI-H1299 and NCI-H1395 cells.

(**D**) PPARG knockdown by siRNAs prevents the decrease in phosphorylated RB upon pioglitazone treatment. NCI-H2347 cells were transfected with two siRNAs targeting PPARG or a non-targeting siRNA (siNT), after 72 hours the cells were treated with vehicle (DMSO) or pioglitazone for 24 hours and were analyzed by immunoblotting.

(**E**) Troglitazone treatment causes a decrease in the levels of phosphorylated RB in both NCI-H2347 and NCI-H1993 cells within 24 hours.

(**F**) Pioglitazone treatment reduces BrdU incorporation. NCI-H2347 cells were cultured in BrdU containing medium in the presence of pioglitazone for 24 or 48 hour, or vehicle (DMSO) prior to luminometric measurement of BrdU incorporation. Error bars represent s.d.

(**G**) Pioglitazone treatment for 24 hours has no effect on PARP and Caspase-3 cleavage in NCI-H2347 cells.

(**H**) Pioglitazone treatment for 24 hours reduces the levels of phosphorylated RB in both HCC-1954 and HCC-H1187 cells (breast cancer cells with high PPARγ expression) but has no effect on the PPARγ-negative cell lines T-47D and HCC-1143. For comparison RB phosphorylation for this treatment in NCI-H2347 cells is shown.

(**I**) NAC prevents the reduction in phosphorylated RB levels by pioglitazone in HCC-1954 and HCC-1187 cells 24 hours after treatment.

(**J**) Okadaic acid prevents the reduction in phosphorylated RB levels by pioglitazone in NCI-H2347 cells 24 hours after treatment.

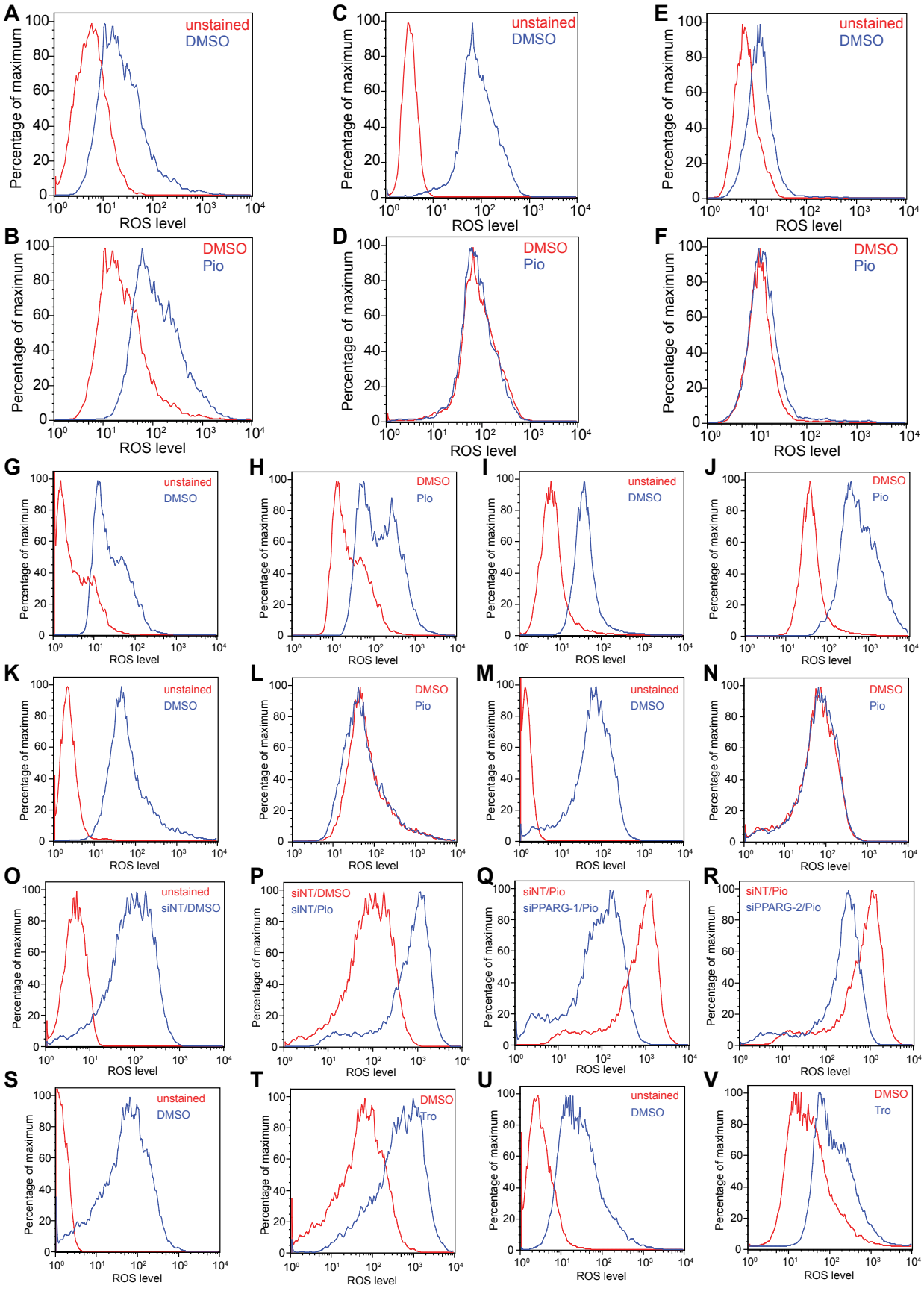

A
unstained
DMSO
B
DMSO
Pio
C
unstained
DMSO
D
DMSO
Pio
E
unstained
DMSO
F
DMSO
Pio
G
unstained
DMSO
H
DMSO
Pio
I
unstained
DMSO
J
DMSO
Pio
K
unstained
DMSO
L
DMSO
Pio
M
unstained
DMSO
N
DMSO
Pio
O
unstained
siNT/DMSO
P
siNT/DMSO
siNT/Pio
Q
siNT/Pio
siPPARG-1/Pio
R
siNT/Pio
siPPARG-2/Pio
S
unstained
DMSO
T
DMSO
Tro
U
unstained
DMSO
V
DMSO
Tro
Percentage of maximum
ROS level

Figure S2 related to Figure 3: TZDs increase ROS levels in PPARγ-positive lung adenocarcinoma cells.

(**A-F**) Pioglitazone treatment causes a marked increase in ROS levels in NCI-H1993 cells (**A and B**), but not in the PPARG-negative NCI-H1299 (**C and D**) and NCI-H1395 (**E and F**) cells. FACS with DCFDA was performed after 12 hour treatment with vehicle (DMSO) (**A,C,E**) or pioglitazone (**B,D,F**).

(**G-N**) Pioglitazone treatment causes a marked increase in ROS levels in the PPARγ-expressing HCC-1954 (**G and H**) and HCC-1187 cells (**I and J**), but not in the PPARγ-negative HCC-1143 (**K and L**) and T-47D cells (**M and N**). FACS with DCFDA was performed after 12 hour treatment with vehicle (DMSO) (**G,I,K,M**) or pioglitazone (**H,J,L,N**).

(**O-R**) PPARγ knockdown by siRNAs reduces the increase in ROS levels upon pioglitazone treatment. NCI-H2347 cells were transfected with a non-targeting siRNA (siNT) (**O and P**) or two siRNAs targeting PPARγ (**Q and R**), after 72 hours the cells were treated with vehicle (DMSO) or pioglitazone for 12 hours, stained with DCFDA and assayed by FACS.

(**S-V**) Troglitazone treatment causes a marked increase in ROS levels in NCI-H2347 (**S and T**) and NCI-H1993 cells (**U and V**). FACS with DCFDA was performed after 12 hours treatment with vehicle (DMSO) (**S and U**) or troglitazone (**T and V**).

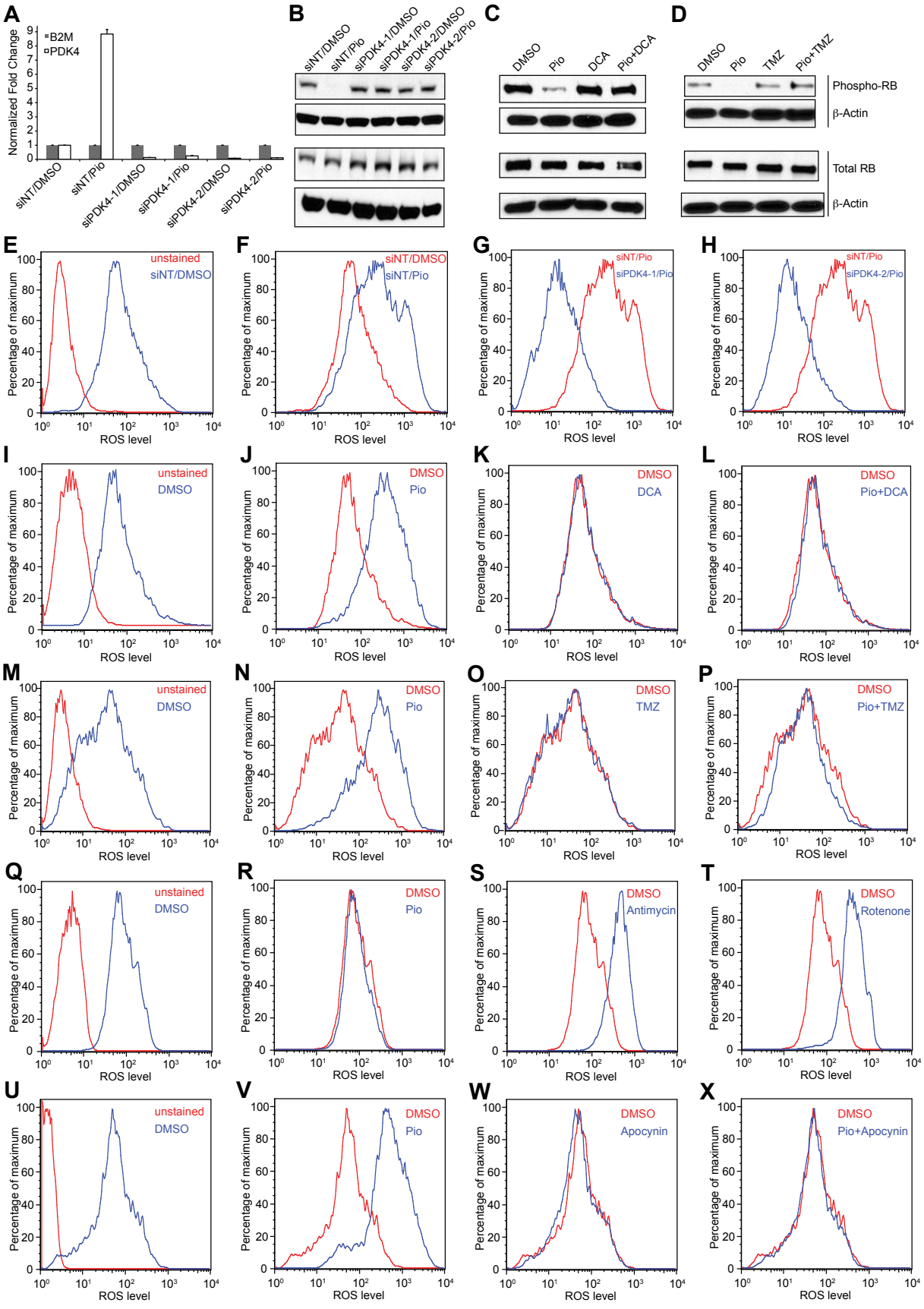

A
Normalized Fold Change
B2M
PDK4
siNT/DMSO
siNT/Pio
siPDK4-1/DMSO
siPDK4-1/Pio
siPDK4-2/DMSO
siPDK4-2/Pio
B
C
DMSO
Pio
DCA
Pio+DCA
D
TMZ
Pio+TMZ
Phospho-RB
β-Actin
Total RB
E
unstained
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
Antimycin
T
Rotenone
U
V
W
Apocynin
X
Pio+Apocynin
Percentage of maximum
ROS level

Figure S3 related to Figure 5: PDK4 and β-oxidation are required for the effects of pioglitazone on ROS levels and RB phosphorylation.

(**A**) siRNAs targeting PDK4 prevent upregulation of PDK4 by pioglitazone in NCI-H2347 cells. NCI-H2347 cells were transfected with two siRNAs targeting PDK4 or a non-targeting siRNA (siNT), after 48 hours the cells were treated with vehicle or pioglitazone for 24 hours. PDK4 transcript levels in NCI-H2347 cells were determined by quantitative RT-PCR using B2M as a calibrator for a pioglitazone treatment time course (n=3, error bars represent s.d.).

(**B**) PDK4 knockdown by siRNA prevents the decrease in phosphorylated RB by pioglitazone in NCI-H1993 cells. Cells were transfected with two siRNAs targeting PDK4 or a non-targeting siRNA (siNT), after 48 hours the cells were treated with vehicle or pioglitazone for 24 hours, and analyzed by immunoblotting.

(**C**) Co-treatment with the PDK inhibitor DCA prevents the decrease in phosphorylated RB levels in NCI-H1993 cells. Cells were treated with vehicle, pioglitazone, DCA, or pioglitazone and DCA for 24 hours, and analyzed by immunoblotting.

(**D**) Co-treatment with the β-oxidation inhibitor trimetazidine (TMZ) prevents the decrease in phosphorylated RB levels in NCI-H1993 cells. Cells were treated with vehicle, pioglitazone, trimetazidine, or pioglitazone and trimetazidine for 24 hours, and analyzed by immunoblotting.

(**E-H**) PDK4 knockdown by siRNA prevents the increase in ROS levels by pioglitazone in NCI-H1993 cells. Cells were transfected with a non-targeting siRNA (siNT) (**E and F**) or two siRNAs targeting PDK4 (**G and H**), after 48 hours the cells were treated with vehicle or pioglitazone for 12 hours, and stained with DCFDA and assayed by FACS.

(**I-L**) Co-treatment with DCA prevents the increase in ROS levels upon pioglitazone treatment in NCI-H1993 cells. Cells were treated with vehicle (**I**), pioglitazone (**J**), DCA (**K**), or pioglitazone and DCA (**L**) for 12 hours, and stained with DCFDA and assayed by FACS.

(**M-P**) Co-treatment with the β-oxidation inhibitor trimetazidine prevents the increase in ROS levels upon pioglitazone treatment in NCI-H1993 cells. Cells were treated with vehicle (**M**), pioglitazone (**N**), trimetazidine (**O**), or pioglitazone and trimetazidine (**P**) for 12 hours, and stained with DCFDA and assayed by FACS.

(**Q-T**) Pioglitazone treatment does not affect mitochondrial superoxide levels. NCI-H2347 cells were treated with vehicle (**Q**), pioglitazone (**R**), antimycin A (**S**), and rotenone (**T**) for 12 hours, and stained with MitoSOX and assayed by FACS.

(**U-X**) Co-treatment with apocynin prevents the increase in ROS levels upon pioglitazone treatment in NCI-H2347 cells. Cells were treated with vehicle (**U**), pioglitazone (**V**), apocynin (**W**), or pioglitazone and apocynin (**X**) for 12 hours, and stained with DCFDA and assayed by FACS.

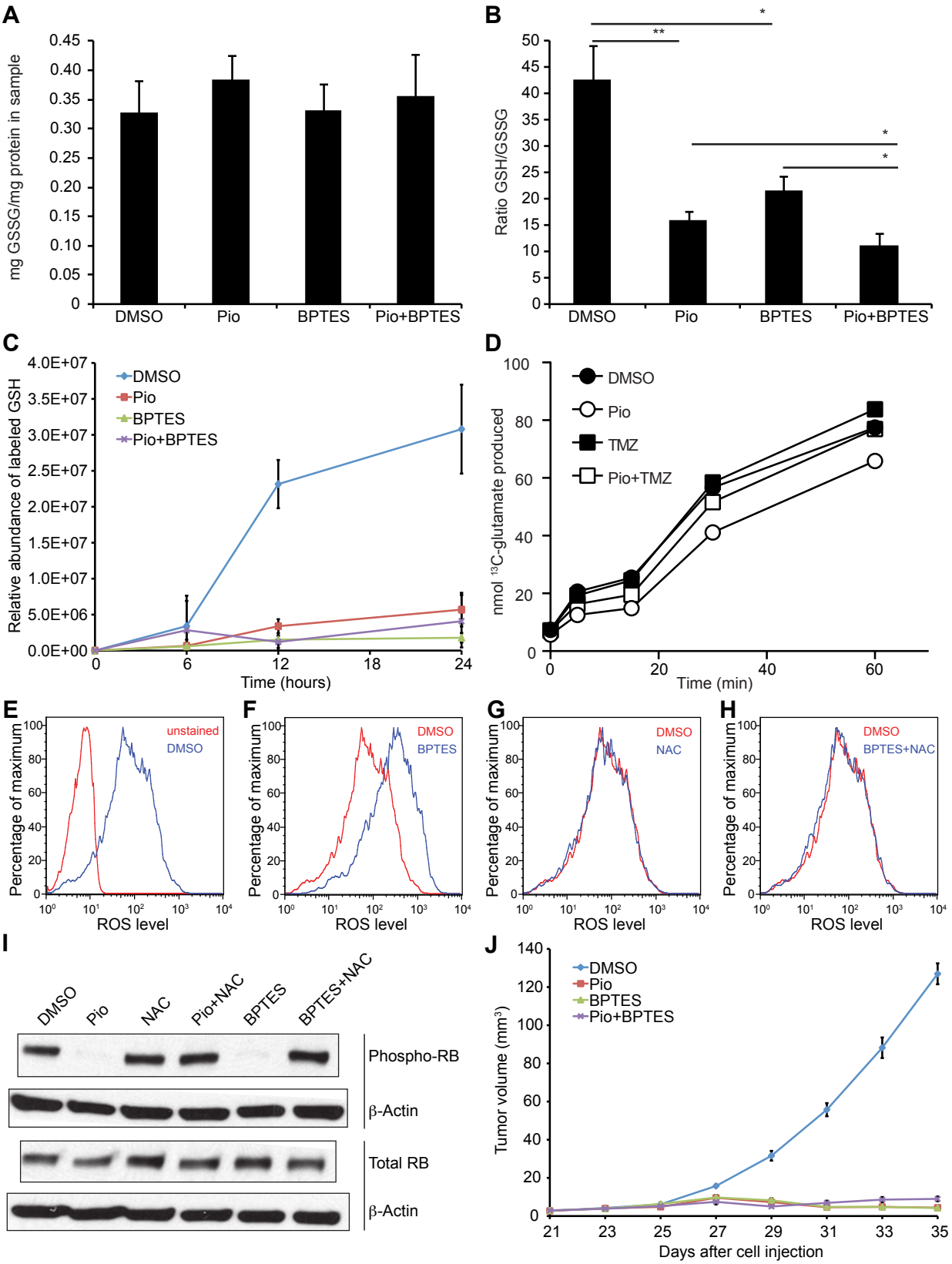

A
mg GSSG/mg protein in sample
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
DMSO
Pio
BPTES
Pio+BPTES
B
Ratio GSH/GSSG
50
45
40
35
30
25
20
15
10
5
0
*
**
*
*
DMSO
Pio
BPTES
Pio+BPTES
C
Relative abundance of labeled GSH
4.0E+07
3.5E+07
3.0E+07
2.5E+07
2.0E+07
1.5E+07
1.0E+07
5.0E+06
0.0E+00
DMSO
Pio
BPTES
Pio+BPTES
0
6
12
18
24
Time (hours)
D
nmol 13C-glutamate produced
100
80
60
40
20
0
DMSO
Pio
TMZ
Pio+TMZ
0
20
40
60
Time (min)
E
Percentage of maximum
100
80
60
40
20
0
unstained
DMSO
10^0
10^1
10^2
10^3
10^4
ROS level
F
Percentage of maximum
DMSO
BPTES
ROS level
G
Percentage of maximum
DMSO
NAC
ROS level
H
Percentage of maximum
DMSO
BPTES+NAC
ROS level
I
DMSO
Pio
NAC
Pio+NAC
BPTES
BPTES+NAC
Phospho-RB
β-Actin
Total RB
β-Actin
J
Tumor volume (mm3)
140
120
100
80
60
40
20
0
DMSO
Pio
BPTES
Pio+BPTES
21
23
25
27
29
31
33
35
Days after cell injection

Figure S4 related to Figure 7: Effect of pioglitazone treatment on glutathione levels in NCI-H2347 cells.

(**A and B**) Oxidized Glutathione (GSSG) (**A**) abundance and GSH/GSSG ratio (**B**) in control cells (vehicle, DMSO) and in cells treated with pioglitazone, BPTES, or pioglitazone and BPTES. Data is the mean of three independent cultures. Error bars represent s.d.; *, $p<0.05$; **, $p<0.005$.

(**C**) Pioglitazone and BPTES treatment reduce the flux from extracellular [U-^{13}C] glutamine to intracellular GSH. Relative abundance of labeled (m+5) GSH in vehicle, pioglitazone, BPTES and pioglitazone/BPTES treated NCI-H2347 cells cultured with [U-^{13}C] glutamine. Data is the mean and of three independent cultures. Error bars represent s.d. The differences for vehicle vs. pioglitazone, BPTES or BPTES/pioglitazone treatment are highly significant after 12 hours ($p<0.001$).

(**D**) Time-dependent production of [U-^{13}C]glutamate in vehicle, pioglitazone, trimetazidine, and pioglitazone/trimetazidine treated cells cultured with [U-^{13}C]glutamine. Each time point is derived from an independent culture.

(**E-H**) BPTES treatment causes an increase in ROS levels in NCI-H2347 cells that is prevented by cotreatment with NAC. FACS with DCFDA was performed after 12 hour treatment with vehicle (DMSO) (**E**) or BPTES (**F**) or NAC (**G**) or BPTES and NAC (**H**).

(**I**) NAC prevents the reduction in phosphorylated RB levels by BPTES (and pioglitazone) in NCI-H2347 cells after 24 hours of treatment.

(**J**) BPTES treatment phenocopies the growth inhibiting effect of pioglitazone *in vivo*. Mice xenografted with NCI-H2347 cells were treated upon appearance of tumors with DMSO (vehicle), pioglitazone, BPTES, or a combination of pioglitazone and BPTES (i.p.) every other day beginning on day 25 after cell injection. Error bars represent s.e.m. N = 10 for all treatment groups.

NOTE: The data shown in Figures 6A and 6B and S4J were obtained from a single experimental series and the individual graphs contain therefore the same curves for DMSO and pioglitazone treatment.

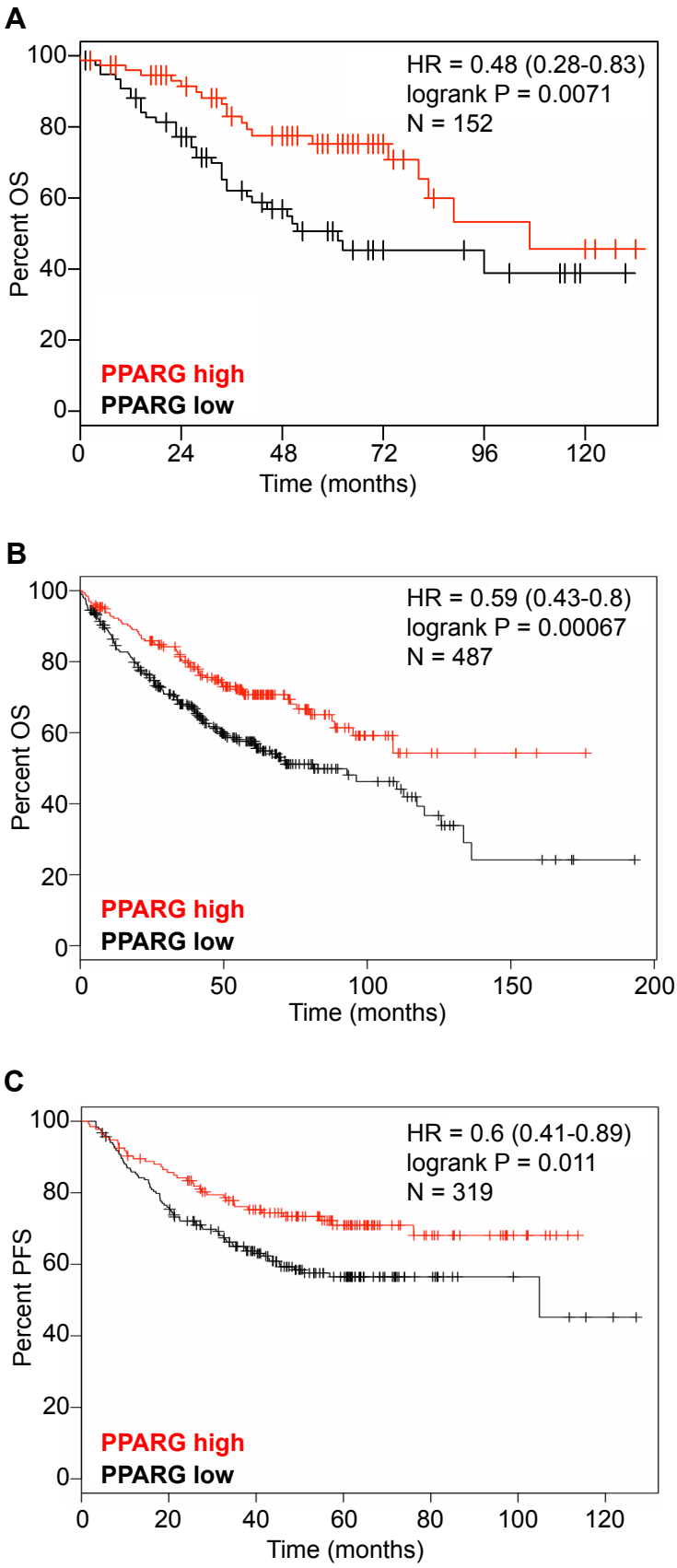

A
HR = 0.48 (0.28-0.83)
logrank P = 0.0071
N = 152
Percent OS
PPARG high
PPARG low
Time (months)
B
HR = 0.59 (0.43-0.8)
logrank P = 0.00067
N = 487
Percent OS
PPARG high
PPARG low
Time (months)
C
HR = 0.6 (0.41-0.89)
logrank P = 0.011
N = 319
Percent PFS
PPARG high
PPARG low
Time (months)

Figure S5 related to Figure 7: PPARγ expression in early stage lung adenocarcinomas predicts clinical outcome.

(A-C) Kaplan-Meier analysis was performed for overall survival (OS) and/or progression-free survival (PFS) in the UT Southwestern/MD Anderson PROSPECT cohort (NCBI GEO accession no. GSE41271) (**A**) and a meta-analysis cohort (Gyorffy et al., 2013) (**B and C**). PPARγ mRNA expression in tumor biopsy samples were used to group patients by median into PPARG high and PPARG low groups. *P* values were obtained with the logrank test.

SUPPLEMENTAL TABLES

Table S1 related to Figure 1: Genomic coordinates of PPARγ binding regions in NCI-H2347 and NCI-H1993 cells.

This table is provided as a separate file.

Table S2 related to Figure 1: *de novo* motifs elicited in PPARγ binding regions in NCI-H2347 and NCI-H1993 cells.

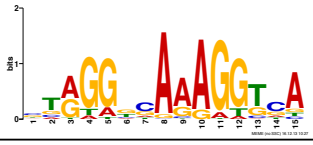
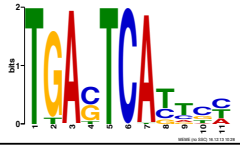
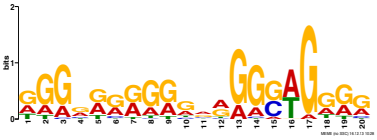
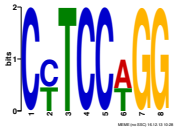
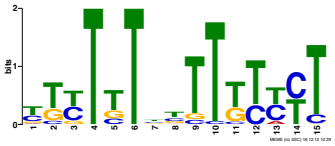
Motif	Motif Logo	E-value	Tomtom match	Match P-value
Motif 1		2.1e-474	PPARG::RXRA	2.22e-16
Motif 2		1.1e-101	AP1/Fos	1.54e-05
Motif 3		7.9e-067	SP1	6.32e-06
Motif 4		2.6e-002	STAT3	3.23e-05
Motif 5		5.9e-001	Zfp105	1.45e-03

Table S3 related to Figure 1: Enriched known transcription factor motifs in PPARγ binding regions in NCI-H2347 and NCI-H1993 cells.

This table is provided as a separate file.

Table S4 related to Figure 2: Significant gene expression changes in NCI-H2347 cells after 12, 24, and 48 hours of pioglitazone treatment.

This table is provided as a separate file.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies and reagents

The following antibodies were used: Phospho-RB (Serine 807/811) (Cell Signaling Technology, #9308), Total RB (Cell Signaling Technology, #9969), β-Actin (Cell signaling technology, 3700), PPARγ (Cell Signaling Technology, #2443), PARP (Cell Signaling Technology, #9532), Caspase-3 (Cell Signaling Technology, #9665), Anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, #7074), Anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology, #7076).

The following reagents were used: Pioglitazone/Pio (E6910), Troglitazone/Tro (T2573), N-acetylcysteine/NAC (A9165), L-Ascorbic acid/Vitamin C (A4403), H_2O_2 (MKBF6355), Dichloroacetate/DCA (707066), Trimetazidine/TMZ (653322), Propidium iodide (P4864), Rotenone (R8875), Antimycin A (A8674), and Okadaic acid/Okad. ac. (O9381) from Sigma-Aldrich; Apocynin (Sc203321) from Santa Cruz Biotechnology, CM-H2DCFDA (C6827), MitoSOX (M36008), Antibiotic-anti-mycotic (15240-062), Puromycin (A1138-03) from Life Technologies, Bovine Serum Albumin (001-000-173) from Jackson ImmunoResearch; non-fat dry milk powder (170-6404) from BioRad; RPMI (SH 30027FS), RPMI with Phenol red (17-105-CV) and Trypsin (SH3004201) from Fisher, Fetal bovine serum (S11150) and Charcoal stripped serum (S11650) from Atlanta Biologicals. BPTES was a gift from Takashi Tsukamoto.

siRNAs/shRNAs

The following ON-TARGET *plus* siRNAs were obtained from Dharmacon: siPDK4-1 (CAACGCCUGUGAUGGAUAA), siPDK4-2 (GACCGCCUCUUUAGUUAUA), siPPARG-1 (GACAUGAAUUCCUUAAUGA), siPPARG-2 (GAUAUCAAGCCCUUCACUA), ON-TARGET *plus* non-targeting pool, siNT (D-001810-10-50). The following shRNA vectors were obtained from OpenBiosystems: shPPARG-1 (TRCN0000001673, CAGCATTTCTACTCCACATTA), shPPARG-2 (TRCN0000001674, GACAACAGACAAATCACCATT), shNT (pLKO.1 Vector Control, #RHS4080).

Chromatin immunoprecipitation (ChIP)

We generated PPARγ-LAP BAC transgenic NCI-H2347 and NCI-H1993 cell lines using the BAC-transgenesis approach previously described (Hua et al., 2009; Kittler et al., 2013; Poser et al., 2008). Cells at 80% confluency (~1-1.5x10^7) were cross-linked with 1% formaldehyde for 10 minutes at 37°C, and quenched with 125 mM glycine at room temperature for 5 minutes. The fixed cells were washed twice with cold PBS, scraped, and transferred into 1 ml PBS containing protease inhibitors (Roche). After centrifugation at 700 g for 4 minutes at 4°C, the cell pellets were resuspended in 100 µl ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl [pH 8.1] with protease inhibitors) and sonicated at 4°C with a Bioruptor (Diagenode) (30 seconds ON and 30 seconds OFF at highest power for 12 minutes). The sheared chromatin with a fragment length of ~200 – 600 bp) was centrifuged at 10,000 g for 10 minutes at 4°C). 100 µl of the supernatant was used for ChIP or as input. A 1:10 dilution of the solubilized chromatin in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl 16.7 mM Tris-HCl [pH 8.1]) was incubated at 4°C overnight with 6 µg/ml of a goat anti-GFP (raised against His-tagged full-length eGFP and affinity-purified with GST-tagged full-length eGFP). Immunoprecipitations were carried out by incubating with 40 µl pre-cleared Protein G Sepharose beads (Amersham Bioscience) for 1 hour at 4°C, followed by five washes for 10 minutes with

1ml of the following buffers: Buffer I: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl; Buffer II: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl; Buffer III: 0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]; twice with TE buffer [pH 8.0]. Elution from the beads was performed twice with 100 µl ChIP elution buffer (1% SDS, 0.1 M NaHCO3) at room temperature (RT) for 15 minutes. Protein-DNA complexes were de-crosslinked by heating at 65°C in 192 mM NaCl for 16 hours. DNA fragments were purified using QiaQuick PCR Purification kit (Qiagen) and eluted into 30 µl H_2O according to the manufacturer's protocol after treatment with RNase A and Proteinase K.

ChIP-seq

Barcoded libraries of ChIP and input DNA were generated with the SOLiD Fragment Library Barcoding Kit (Applied Biosystems), and 35-nt single-end reads were generated with the SOLiD4 HQ system (Applied Biosystems). The primary sequencing data were directly translated from color space to mapped DNA sequence reads in the human reference genome (NCBI37/hg19 assembly) by using LifeScope (v.2.10). During the alignment, three filter steps were applied to remove low quality, ambiguous, and redundant reads. PPARγ-bound regions were identified as genomic regions with a significant read enrichment and binding peak profile in the PPARγ reads over the input reads by using the Model-based Analysis of ChIP-seq (MACS) (Poser et al., 2008) software tool (v.1.4.2) with 1% FDR. Common PPARγ binding regions that are shared between NCI-H2347 and NCI-H1993 cells were used for secondary analyses. ChIP-seq data is available from NCBI GEO (www.ncbi.nlm.nih.gov/geo) under accession no. GSE58382.

Motif analysis

De novo motif discovery analysis for shared PPARγ-bound regions was performed with the Multiple EM for Motif Elicitation (MEME) software tool (Bailey et al., 2006) using 200 bp, i.e. 100 bp sequence 5' and 3' to the binding peak summit. High quality motifs were aligned against transcription factor motifs retrieved from JASPAR and TRANSFAC by using the TOMTOM software tool (Bailey et al., 2009) to identify known transcription factor motifs that match the MEME predicted motifs. Next, we analyzed the enrichment of known transcription factor motifs in JASPAR and ENCODE. For this analysis, we determined the frequency of the motifs in the 200 bp PPARγ-bound regions and for 75,000 random sets of the same sample size by using Motif Scanner (Aerts et al., 2003). The motif enrichment score is calculated as the ratio of the motif frequency in the PPARγ binding region set and the mean motif frequency in 75,000 random sets. The *Z* value and the statistical significance (*P* value) of the enrichment score are calculated based on the variance and the mean obtained from the 75,000 random simulations.

Target gene analysis

Potential protein-coding target genes associated with the identified PPARγ binding regions were identified based on the distance of their transcription start sites (TSSs) according to their RefSeq annotation in the hg19 assembly to PPARγ binding peak summits. Genes whose TSSs were within 100 kb or nearest to a PPARγ peak were called as target genes. We performed pathway enrichment analysis on shared PPARγ target genes in NCI-H2347 and NCI-H1993 cells. Gene sets used for this analysis were obtained from Merico et. al. (Merico et al., 2010). Enrichment p-values were calculated using hypergeometric test and significantly enriched pathways (FDR ≤ 1%) were used to construct an enrichment map.

Treatment of cultured lung adenocarcinoma and breast cancer cells with compounds
All cells grown in tissue culture were treated in the presented experiments with compounds at the following concentrations unless specifically stated otherwise: Pioglitazone (50 μM), Troglitazone (10 μM), NAC (5 mM), Vitamin C (30 μM), DCA (10 mM), Trimetazidine (50 μM), BPTES (10 μM), Rotenone (100 μM), Antimycin A (100 μM), Apocynin (100 μM), Okadaic acid (10 nM).

Quantitative PCR and microarray analysis
Total RNA was isolated using the RNeasy Mini Kit (Qiagen) including an on-column DNase digestion (Qiagen) step. For quantitative PCR cDNA was synthesized using the iScript™ Reverse Transcription Supermix (Bio-Rad). Expression levels were determined by quantitative PCR using iTaq™ SYBR® Green Supermix with ROX (Bio-Rad) on a StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). The following oligonucleotide primers were used: (PDK1, 5'-CCAAGGAGACCTGAAGCTGT-3', 5'-CGTGGTTGGTGTTGTAATGC-3'; PDK2, 5'-AGCTGGTGCAGAGCTGGTAT-3', 5'-TGTACTCAAGCACGCCTTGT-3'; PDK3, 5'-GCCAATTTCCCGTCTGTATG-3', 5'-CGCCATGCGGACTTATTAAA-3'; PDK4, 5'-AATGCTCCTTTGGCTGGTTT-3', 5'-GGCTGACTTGTTAAAAACTGGAA-3'; PPARG, 5'-CGACCAAGTAACTCTCCTCA-3', 5'-GTTCGTGACAATCTGTCTG-3'). B2M was used as a calibrator for the comparative C_T method ($\Delta\Delta C_T$) WITH the following oligonucleotide primers: 5'-TGACTTTGTCACAGCCCAAG-3' and 5'-AGCAAGCAAGCAGAATTTGG-3'. All experiments were performed in triplicate.

For microarray analyses, NCI-H2347 cells were treated with pioglitazone or vehicle (DMSO) for 12, 24 and 48 hours. Triplicate experiments were performed for each time point. The quality of the isolated RNA was analyzed using the Bio-Rad Experion System. RNA was then biotin labeled using the Illumina Total Prep kit (Ambion, AMIL-1791). The obtained cRNA was quality-checked again on the Experion System for labeling efficieny. Seven hundred and fifty ng cRNA was loaded onto the Illumina Human HT12v4.0 Expression Beadchip according to Illumina's protocol. The array was hybridized and washed according to Illumina's protocol, and subsequently scanned on the HiScan SQ (Illumina).

Following data extraction and normalization of the microarray data using GenomeStudio (Illumina), linear models for microarray data (LIMMA) (Smyth, 2004) was used to identify genes that showed significant expression changes between the pioglitazone treated and vehicle samples for each time point. We used a cut-off of adjusted $p < 0.05$ and log2 fold change of ≥ 1 and ≤ -1 to define significant transcript changes. Microarray data is available from NCBI GEO (www.ncbi.nlm.nih.gov/geo) under accession no. GSE59735.

Genes with significant expression change at any given time point and were used for clustering analysis. To identify gene clusters that have similar expression pattern we performed hierarchical clustering with euclidean distance metric and "agglomerative average" method, or centroid based clustering (k-means clustering).

To determine the enrichment of gene sets at the top or bottom of a ranked list of differentially regulated genes upon pioglitazone treatment, we performed gene set enrichment analysis (GSEA) using the time course expression data and *a priori* defined gene sets. Gene sets that were significantly enriched (FDR ≤ 5%) at any given time point were selected for clustering analysis. Hierarchical clustering was performed on the normalized enrichment scores obtained for all significant gene sets for all three time points.

Cell cycle analysis and BrdU incorporation assay

$1x10^6$ cells were plated for each experiment. 24 hours after plating complete media was replaced with 5% FBS RPMI (Phenol red free) medium containing pioglitazone (50 μM) or vehicle (DMSO). After 24 hours of treatment cells were fixed with 70% ethanol for 15 minutes on ice, and were then washed once with PBS. Following permeabilization with 0.05% Triton X-100 in PBS for 3 minutes, propidium iodide (10 μg/ml) concentration and RNase A (10 μg/ml) in PBS was used to stain the cells for 45 minutes at 37°C with gentle agitation. Finally, cells were washed twice with PBS and resuspended in 300 μl of PBS. DNA content of ~10,000 cells was measured by FACS (excitation at 488 nm, emission was measured with a 600 nm band pass filter, FL-2H) using a FACScan flow cytometer (BD Bioscience). The obtained DNA content profiles were quantified using CellQuest (BD Bioscience).

The BrdU incorporation assay was performed in 96 well plates with 24 replicates for each condition using the Chemiluminescent Cell Proliferation ELISA, BrdU Kit (Roche). Prior to two hours of incubation with BrdU cells were pre-treated with vehicle (DMSO) or pioglitazone (24 or 48 hours). The samples were then processed according to the instructions of the kit's manufacturer and luminescence was measured with the Glomax luminometer (Promega).

Analysis of ROS levels

Intracellular ROS and mitochondrial superoxide were detected with cell-permeable oxidation-sensitive fluorescent probes (DCFDA and MitoSOX). $1x10^6$ cells were plated for each experiment. Following 12 hours of treatment cells were harvested and washed twice with PBS, resuspended in 500 μl of 2,7-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (5 μM) or MitoSOX (5μM) and incubated at 37°C degree for 30 minutes in complete medium protected from light. The cells were then washed twice with ice-cold PBS and finally re-suspended in 200 μl of PBS. ROS levels of ~10,000 cells measured as DCF fluorescence was analyzed by FACS (excitation at 488 nm, emission was measured with a 530 nm band pass filter, FL-1H) or mitochondrial superoxide levels as MitoSOX fluorescence (FL-2) using a FACScan flow cytometer (BD Bioscience).

Western blot

Cells were lysed with cell lysis buffer (10 mM Tris-cl, 100 mM Nacl, 1% Triton X-100, 0,5% NP 40, 1mM EDTA, 1mM EGTA, 1mM NaF, 1mM DTT, 1X protease inhibitor cocktail mix) for 15 minutes on ice and centrifuged at 14000 rpm for 10 minutes. The supernatant was collected and protein concentration was measured using the Bradford assay. 10 μg of protein was loaded per well of a PAGE gel after denaturation by boiling with 2X SDS loading dye 5 minutess. SDS PAGE was performed at 200 Volt for 30 minutess. The gel was then transferred to a nitrocellulose membrane using the Trans-Blot semidry blotting device (BioRad) according to the manufacturer's manual. The nitrocellulose membrane was then blocked with 5% milk (or 5% BSA) for 1 hour. The membrane was incubated with the primary antibody (β-Actin, 1:2000; Caspase-3, 1:1000; PARP, 1:1000; total Rb, 1:1000; Phospho-RB,1:1000; PPARG, 1:1000). After washing the membrane three times with 0.1% PBST, the membrane was incubated with 1:5,000 horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody or horseradish peroxidase (HRP)-conjugated anti-Rabbit IgG secondary antibody (Cell Signaling Technology) for 2 hours at room temperature. Protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Cell culture and transfection

Cells were cultured in RPMI with 5% FBS and incubated at 37°C with 5% CO_2. For all experiments, cells were seeded one day prior to treatment. After 24 hours the media was replaced with 5% RPMI (Phenol red free media) media. Experiments were performed at 60-70% confluency. For the glutamine withdrawal experiment, cells were cultured in glutamine free RPMI supplemented with 5% fetal bovine serum. siRNAs were transfected with Lipofectamine 2000 (Invitrogen). For the generation of NCI-H2347 cells stably expressing shRNAs were transfected at 80% confluency with shRNA expression vectors using Effectene transfection reagent (Qiagen). After 24 hours medium was replaced, and cells were cultured in the presence of 0.5 mg/ml Puromycin for one week and maintained in the presence of 0.25 µg/ml Puromycin.

Xenograft experiments

Female athymic NOD SCID were obtained from the UTSW transgenic core facility. NCI-H2347 cells were suspended in complete RPMI medium and mixed 1:1 (v/v) with Matrigel (BD Biosciences). One hundred microliter of NCI-H2347 cells ($2x10^6$ cells/site, 2 sites/animal) suspension were then injected subcutaneously into both flank regions of the mice. When tumors became visible (~5-10 mm^3), animals were divided into groups, and were treated (i.p.) with (for series 1) 100 µl of Pioglitazone (25 mg/kg), NAC (1000 mg/kg), Pioglitazone (25 mg/kg) and NAC (1000 mg/kg) or DMSO; (for series 2) Pioglitazone (25 mg/kg), Trimetazidine (10 mg/kg), DCA (100 mg/kg), BPTES (12.5 mg/kg), Trimetazidine (10 mg/kg) and Pioglitazone (25 mg/kg), DCA (100 mg/kg) and Pioglitazone (25 mg/kg), BPTES (12.5 mg/kg) and Pioglitazone (25 mg/kg), or DMSO, (for series 3, shPPARG-1/2 and shNT), Pioglitazone (25 mg/kg) or vehicle every other day for a total of four to five injections.

Immunohistochemistry

Tumors were harvested from anesthetized mice following fixation via *transcardial perfusion with 4% paraformaldehyde*. Subsequent paraffin processing, embedding, sectioning and hematoxylin and eosin staining were performed by standard procedures. Mouse anti-sera used for Ki-67 immunolabeling of paraffin sections was obtained from Vector Laboratories (Burlingame, CA, Cat# VP-K452). Following deparaffinization and pH 6.0 citra based heat antigen-retrieval, serial sections were quenched of autofluorescence with 100mM glycine and blocked against endogenous mouse IgG and secondary antibody host-serum affinity utilizing commercially available blocking reagents (Vector Mouse on Mouse Kit, Cat# BMK-2202). Sections were subjected to either primary antibody (1:300 dilution of commercially supplied stock) or normal mouse serum and incubated overnight at 4°C. Subsequent biotin/streptavidin-fluorescein detection of bound primary was conducted the following day according to MOM kit instructions and the sections were counterstained with propidium iodide. Terminal deoxynucleotidyltransferase-mediated UTP end label (TUNEL) staining for apoptotic cells was done according to the protocol supplied with DeadEnd Fluorometric TUNEL System (Promega). Apoptotic cells were labeled with fluorescein, and the sections were counterstained with propidium iodide. Image acquisition was performed with a DM5500B fluorescent microscope (Leica). For quantification of Ki-67 and TUNEL positivity two fields each containing at least 100 cells were manually analyzed.

Metabolic assays

Cells were plated onto 100-mm dishes so that on the day of the experiment they would be approximately 70% confluent. Cells were incubated in medium containing 5% charcoal stripped serum for 12 hours followed by incubation with compounds (50 μM) for 6, 12 or 24 hours:

To initiate studies on glucose metabolism, the cells were rinsed with PBS and medium was replaced with glucose-free RPMI (Sigma-Aldrich, R1383) containing 5% charcoal stripped serum supplemented with 25mM [U-^{13}C]glucose (Cambridge Isotope Labs). After 6 hours of labeling, medium was removed for glucose and lactate analysis using an automated electrochemical analyzer (BioProfile Basic-4 analyzer; NOVA Biomedical), with rates calculated as previously described (Yang et al., 2009). For analysis by mass spectrometry, the cells were processed as described (Cheng et al., 2011). Briefly, the cells were rinsed in cold normal saline, then lysed by scraping into 1.0 ml of cold 50% methanol, transferred to Eppendorf tubes and frozen in liquid nitrogen. After three freeze-thaw cycles, debris was removed by centrifugation and the supernatants were evaporated and derivatized with a trimethylsilyl donor. Three microliters of the derivatized sample were injected onto an Agilent 6890 gas chromatograph networked to an Agilent 5975 Mass Selective Detector, and informative mass fragments were monitored using MSDChem Data Analysis software (Agilent), followed by correction for natural isotopic abundance.

To quantify the production of ^{13}C-glutamate from ^{13}C-glutamine, cells were cultured in glutamine-free RPMI (Sigma-Aldrich, R0883) supplemented with 5% charcoal serum and 2 mM [U-^{13}C]glutamine (Cambridge Isotope Labs), and lysed at several time points over 60 minutes. Each sample was split into two aliquots. One aliquot was used to calculate the fraction of the glutamate pool labeled with five carbons from [U-^{13}C]glutamine. Into the second aliquot, 50 nmoles of unlabeled glutamate was added, and the resulting drop in fractional enrichment was used to calculate the total glutamate abundance.

Glutathione quantitation was performed using a Shimadzu UPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) coupled with an ABSCIEX QTRAP 5500 mass spectrometry detector (AB SCIEX, Framingham, MA, USA) on a Phenomenex® (Torrance, CA, USA) Synergi^{TM} Hydro-RP column (150 mm x 2.1 mm, 2.5μm d_p, 100 Å pore size). Mobile phases included an aqueous mobile phase A (0.03% formic acid) and an organic mobile phase B (acetonitrile, 0.03% formic acid). The initial elution condition was held at 0% B for 5 minutes, followed by a linear gradient from 0% B to 100% B over 5 minutes and further held at the last ratio for 5 minutes. The percentage of mobile phase B was then reduced to the initial condition and held for 5 minutes for column equilibration. The flow rate was 0.5 ml/minute and the injection volume was 3 μl. Mass spectrometry (MS) was operated in the positive mode with multiple reaction monitoring (MRM). All dependent MS parameters were optimized, based on infusion experiments. Data were acquired and analyzed using Analyst software version 1.6. For definitive quantitation of GSH and GSSG, stock solutions were prepared in water at a concentration of 10 mM. Standards were then prepared from 0.1 μM to 100 μM to construct calibration curves. These curves were used to determine the abundance of each analyte in cell extract samples.

For measuring flux of extracellular glutamine to GSH, cells were cultured in glutamine-free RPMI (Sigma-Aldrich, R0883) supplemented with 5% charcoal serum and 2 mM [U-^{13}C]glutamine for one hour after drug treatment washed twice with ice cold saline, then overlaid with 500 μl of cold methanol/water (80/20, v/v). Cells were transferred to an Eppendorf tube and subjected to three freeze-thaw cycles. After vigorous vortexing, the debris was pelleted by

centrifugation at 16,000 × *g* and 4°C for 15 min. Pellets were used for protein quantitation (BCA Protein Assay, Thermo). The supernatant was transferred to a new tube and evaporated to dryness using a SpeedVac concentrator (Thermo Savant, Holbrook, NY). Metabolites were reconstituted in 50 µL of 0.03% formic acid in analytical-grade water, vortex-mixed and centrifuged to remove debris. Thereafter, the supernatant was transferred to an HPLC vial for the analysis of both unlabeled (m+0) and isotope labeled (m+5) GSH. The measurement of GSH was performed using a liquid chromatography-tandem mass spectrometry (LC/MS/MS) approach. Separation was achieved on a Phenomenex Synergi Polar-RP HPLC column (150 × 2 mm, 4 µm, 80 Å) using a Nexera Ultra High Performance Liquid Chromatograph (UHPLC) system (Shimadzu Corporation, Kyoto, Japan). The mobile phases employed were 0.03% formic acid in water (A) and 0.03% formic acid in acetonitrile (B). The gradient program was as follows: 0-3 min, 100% A; 3-5 min, 100% - 0% A; 5-6 min, 0% A; 6-6.1 min, 0% - 100% A; 6.1-8min, 100% A. The column was maintained at 35°C and the samples kept in the autosampler at 4°C. The flow rate was 0.5 mL/min, and injection volume 10 µL. The mass spectrometer was an AB QTRAP 5500 (Applied Biosystems SCIEX, Foster City, CA) with electrospray ionization (ESI) source in multiple reaction monitoring (MRM) mode. Sample analysis was performed in negative mode. Declustering potential (DP) and collision energy (CE) were optimized by direct infusion of reference standards by syringe pump prior to sample analysis. The MRM MS/MS detector conditions were set as follows: curtain gas 30 psi; ion spray voltages 5000 V; temperature 650°C; ion source gas 1 50 psi; ion source gas 2 50 psi; interface heater on; entrance potential 10 V. The MRM transitions (*m/z*), DPs (V) and CEs (V) for unlabeled (m+0) and isotope labeled (m+5) GSH are 306 > 272, -60 V, -16 V, and 311 > 277, -60 V, -16 V, respectively. Dwell time for each transition was set at 100 msec. Cell samples were analyzed in a randomized order, and MRM data was acquired using Analyst 1.6.1 software (Applied Biosystems SCIEX, Foster City, CA). Chromatogram review and peak area integration were performed using MultiQuant software version 2.1 (Applied Biosystems SCIEX, Foster City, CA). Although the numbers of cells were very similar and each sample was processed identically and randomly, the peak area for each detected metabolite was normalized against the cell number of that sample to correct for any variations introduced from sample handling through instrument analysis. Although the numbers of cells were very similar and each sample was processed identically and randomly, the peak area for each detected metabolite was normalized against the protein content of that sample to correct for any variations introduced from sample handling through instrument analysis. The normalized area values were used as variables for data analysis.

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