Supplemental Information

MYC and MCL1 cooperatively promote chemotherapy-resistant breast cancer stem cells through regulation of mitochondrial oxidative phosphorylation

Kyung-min Lee, Jennifer M. Giltnane, Justin M. Balko, Luis J. Schwarz, Angel L. Guerrero-Zotano, Katherine E. Hutchinson, Mellissa J. Nixon, Mónica V. Estrada, Violeta Sánchez, Melinda E. Sanders, Taekyu Lee, Henry Gómez, Ana Lluch, J. Alejandro Pérez-Fidalgo, Melissa Magdalene Wolf, Gabriela Andrejeva, Jeffrey C. Rathmell, Stephen W. Fesik, Carlos L. Arteaga

Figure S1

A

-log$_{10}$IC50 for chemotheraphy reagents (nM)

Drug	MDA-MB-436		SUM159PT	
	ADH	MS	ADH	MS
Paclitaxel	4.663	6.417	4.655	7.19
Docetaxel	49.67	315	22.69	59.23
Doxorubicin	40.4	93.94	1.045	16.92

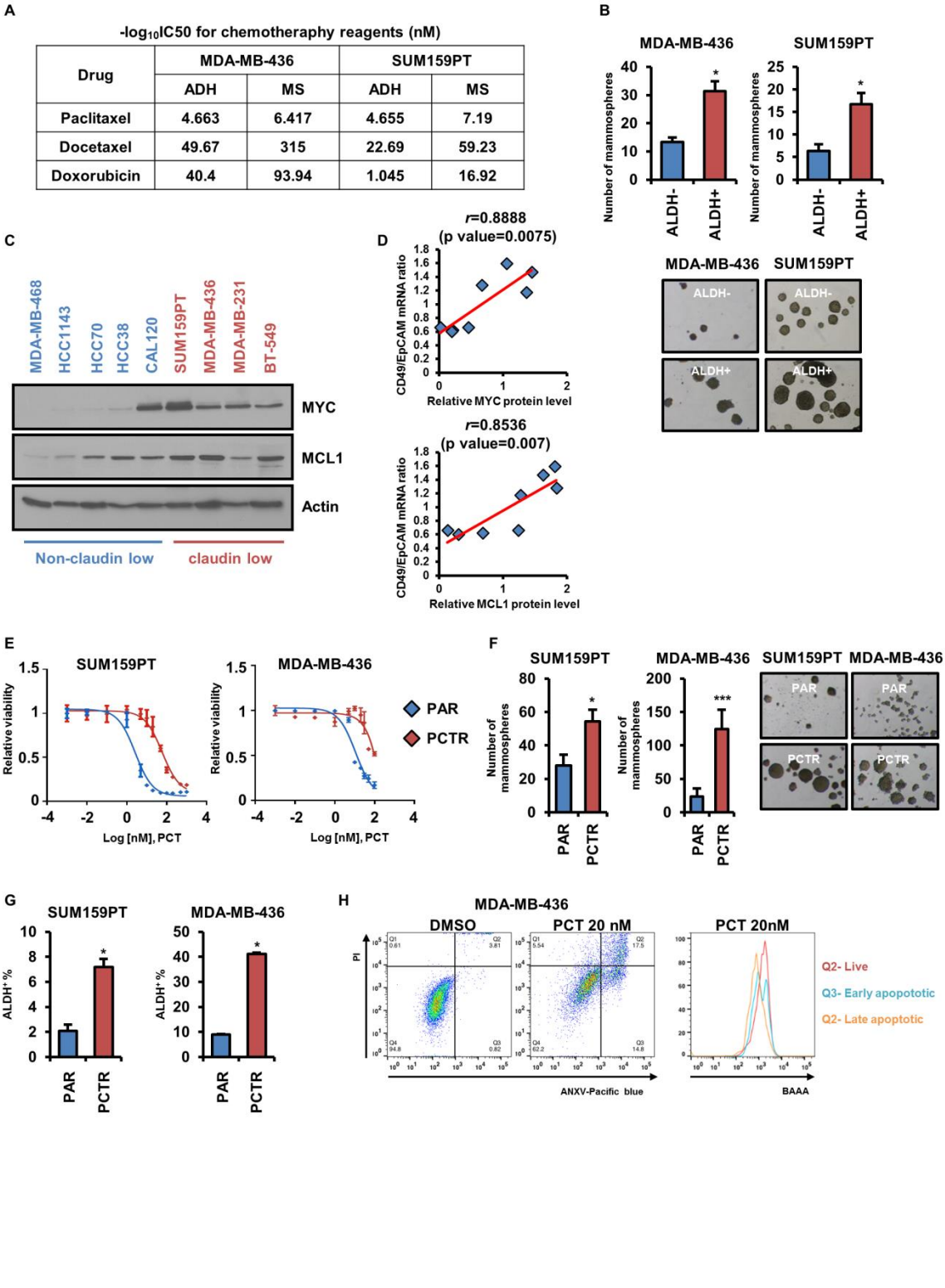


Figure S1, related to Figure 1. Expression of MYC and MCL1 in TNBC cell lines and establishment of paclitaxel resistant cells. (A) MDA-MB-436 and SUM159PT cells grown as mammospheres or as adherent monolayers were treated with the indicated dose range of paclitaxel, docetaxel, or doxorubicin for 72 h. Cell viability was determined with the SRB assay as described in Methods. IC_{50} values were calculated with the GraphPad Prism 7. (B) $ALDH^+$ MDA-MB-436 and SUM159PT cells sorted by flow cytometry were seeded in mammosphere assays for 7 days (*p<0.005). Total number of the mammosphere was quantified using the Gelcount mammalian cell colony counter. Original magnification, x100. (C) Lysates obtained from TNBC cell lines were subjected to immunoblot analysis with MYC, MCL1, and actin antibodies. (D) Plots of MYC or MCL1 protein levels vs. CD49:EpCAM mRNA ratio in TNBC cell lines (Pearson's correlation). (E) Paclitaxel resistant (PCTR) and parental (PAR) MDA-MB-436 and SUM159PT cells were treated with paclitaxel for 72 h; cell viability was determined with SRB assay. (F) Paclitaxel resistant and parental SUM159PT cells were seeded as mammospheres for 7 days (*p<0.05). (G) Proportion of $ALDH^+$ cells was determined by flow cytometry (*p<0.0005). (H) MDA-MB-436 cells stained with BODIPY-aminoacetaldehyde (BAAA) were labeled with annexin V pacific blue™ and propidium iodide (PI). Proportion of each fraction was determined by flow cytometry. Data are represented as mean ± SD.

Figure S2

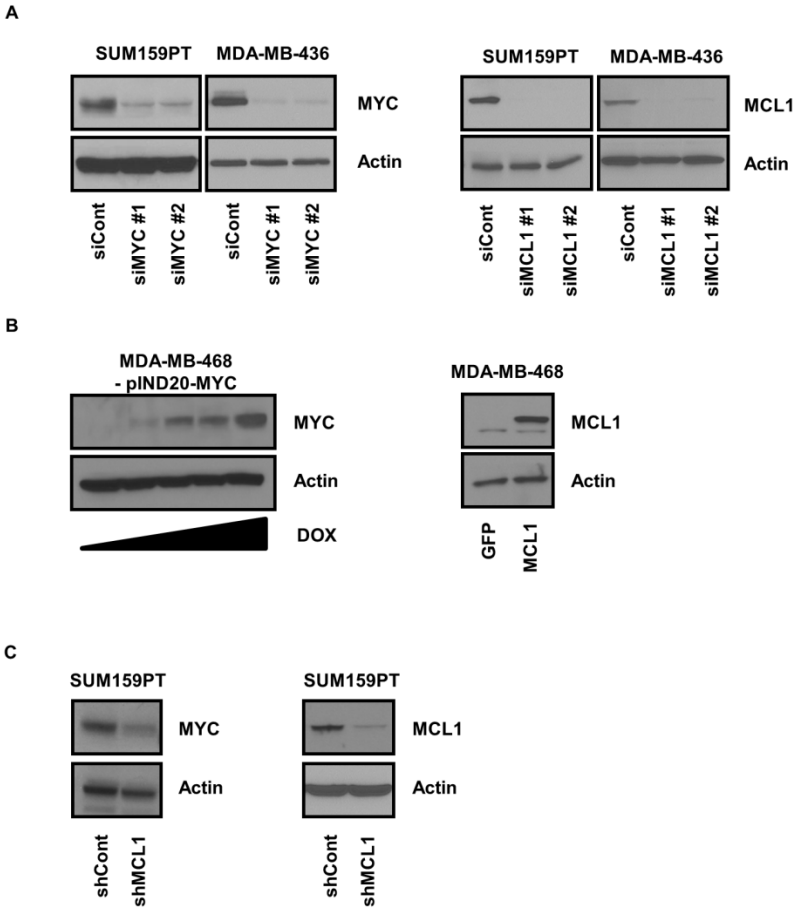

A
SUM159PT
MDA-MB-436
MYC
Actin
siCont
siMYC #1
siMYC #2
siCont
siMYC #1
siMYC #2
SUM159PT
MDA-MB-436
MCL1
Actin
siCont
siMCL1 #1
siMCL1 #2
siCont
siMCL1 #1
siMCL1 #2
B
MDA-MB-468
- pIND20-MYC
MYC
Actin
DOX
MDA-MB-468
MCL1
Actin
GFP
MCL1
C
SUM159PT
MYC
Actin
shCont
shMCL1
SUM159PT
MCL1
Actin
shCont
shMCL1

Figure S2, related to Figure 2. Modulation of MYC and MCL1 expression in TNBC cell lines. (A) Lysates from SUM159PT and MDA-MB-436 cells transduced with *MYC* or *MCL1* siRNA were subjected to immunoblot analysis with MYC, MCL1, and actin antibodies. (B) MDA-MB-468 cells transduced by pINDUCER20-MYC were treated with 0, 1, 10, 100, 500 ng/ml doxycycline (DOX) for 48 h to induce MYC expression. MDA-MB-468 cells were also transduced with pLX302-MCL1. Cell lysates were subjected to immunoblot analysis with MYC, MCL1, and actin antibodies. (C) Lysates of SUM159PT cells stably transduced either by *MYC* or *MCL1* shRNA were subjected to immunoblot analyses with MYC, MCL1, and actin antibodies.

Figure S3

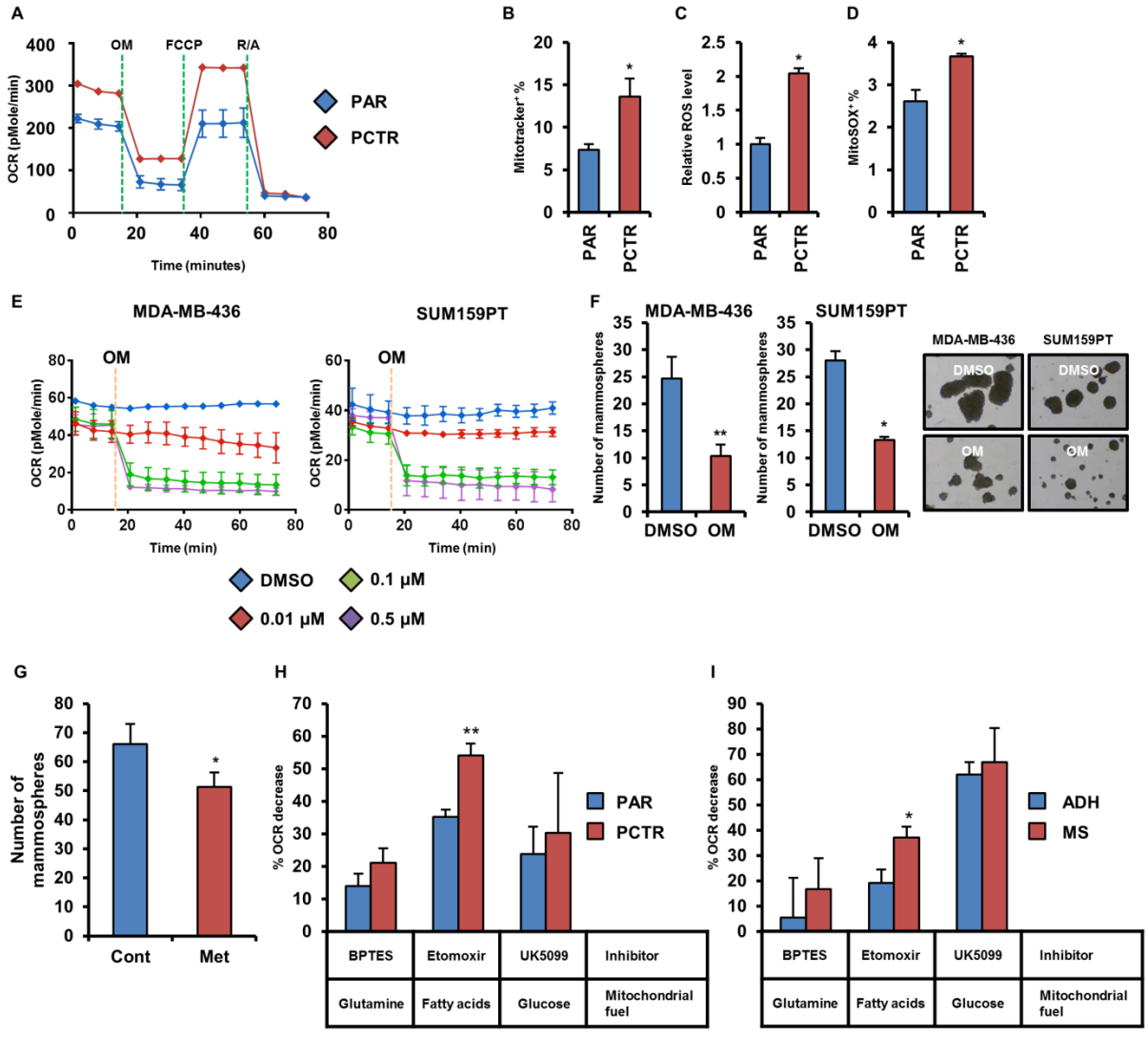

A
OM
FCCP
R/A
OCR (pMole/min)
Time (minutes)
PAR
PCTR
B
Mitotracker+ %
C
Relative ROS level
D
MitoSOX+ %
E
MDA-MB-436
SUM159PT
OM
OCR (pMole/min)
Time (min)
DMSO
0.1 µM
0.01 µM
0.5 µM
F
MDA-MB-436
SUM159PT
Number of mammospheres
DMSO
OM
G
Number of mammospheres
Cont
Met
H
% OCR decrease
PAR
PCTR
BPTES
Etomoxir
UK5099
Inhibitor
Glutamine
Fatty acids
Glucose
Mitochondrial fuel
I
% OCR decrease
ADH
MS
BPTES
Etomoxir
UK5099
Inhibitor
Glutamine
Fatty acids
Glucose
Mitochondrial fuel

Figure S3, related to Figure 3. Inhibition of mtOXPHOS reduces enrichment of CSCs. (A) OCRs were measured by Seahorse XF^{e}96 extracellular flux analyzer in paclitaxel-resistant (PCTR) and parental SUM159PT cells (PAR). (B) Paclitaxel resistant and sensitive parental SUM159PT cells were stained with Mitotracker Red CMXRos and next analyzed by flow cytometry (*p<0.05). (C) ROS levels were determined by ROS-Glo in the paclitaxel resistant and parental SUM159PT cells (*p<0.0005). (D) Paclitaxel resistant and parental SUM159PT cells stained with MitoSOX™ Red were analyzed by flow cytometry (*p<0.005). (E) OCRs were determined by Seahorse XF^{e}96 extracellular flux analyzer in MDA-MB-436 and SUM159PT cells treated with 0.01, 0.1, and 0.5 μM oligomycin A (OM). (F) MDA-MB-436 and SUM159PT cells were seeded in mammosphere assays and treated with DMSO or 0.1 μM oligomycin A (OM) for 7 days (*p<0.005, **p<0.0005). (G) PCTR SUM159PT cells were seeded for mammosphere assays as described in Methods in presence of DMSO or 3 mM Metformin (Met, *p<0.005). (H,I) OCR in PCTR and PAR SUM159PT cells was measured by Seahorse XF^{e}96 extracellular flux analyzer in presence of 4 μM BPTES, 2 μM etomoxir or 3 μM UK5099 (H). OCR in SUM159PT cells grown as mammospheres (MS) or as adherent monolayers was measured as described in H (I). Y axis shows % decrease in OCR in cells treated with a specific fuel oxidation inhibitor (BPTES, etomoxir or UK5099) relative to the % decrease in OCR in cells treated with the other two inhibitors. PCTR cells and SUM159PT cells grown as mammospheres exhibited a statistically higher inhibition of OCR when treated with etomoxir, an inhibitor of fatty acid oxidation, compared to cells treated with BPTES and UK5099 (*p<0.005, **p<0.0005). Data are represented as mean ± SD.

Figure S4

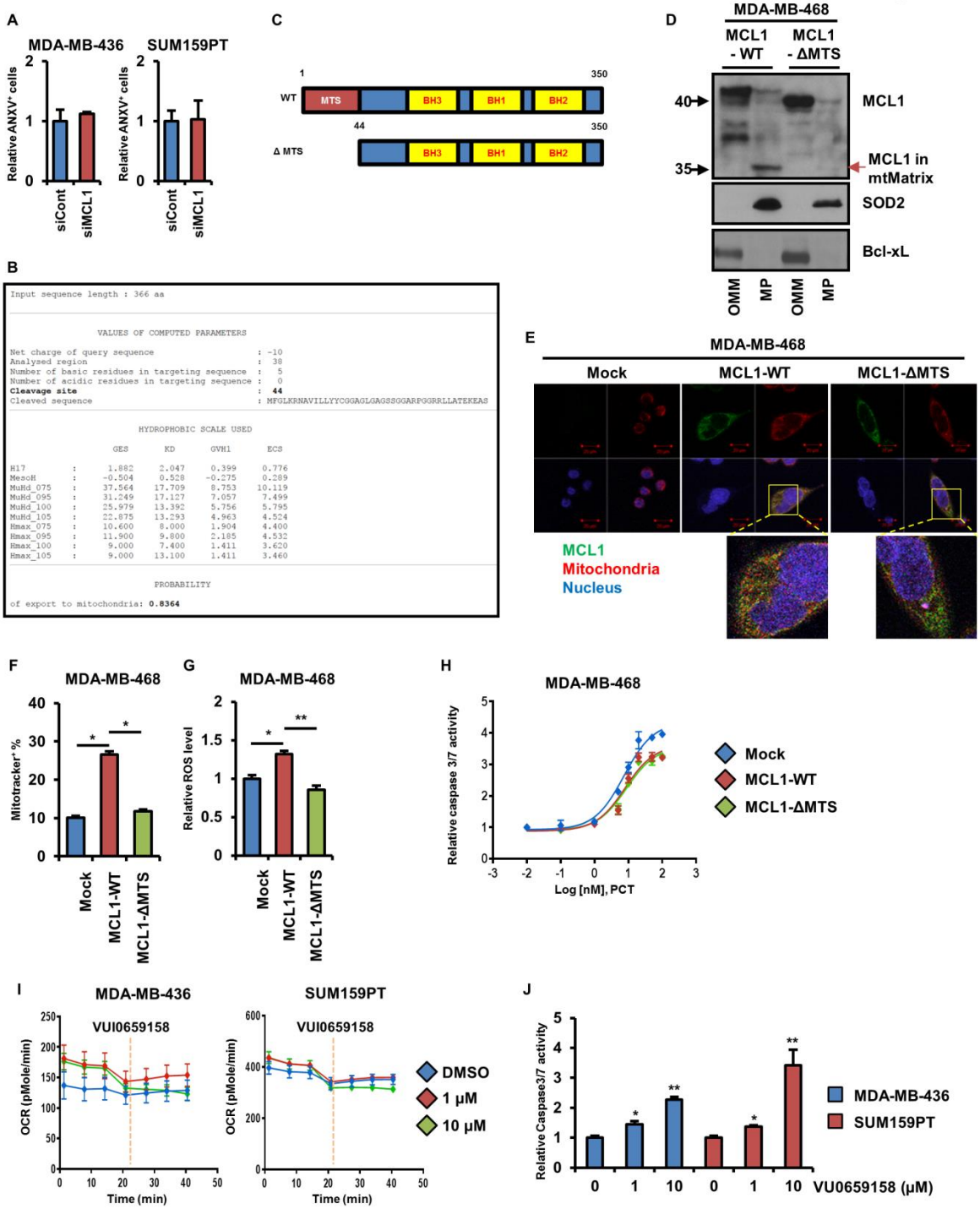

A
MDA-MB-436
SUM159PT
Relative ANXV+ cells
siCont
siMCL1
C
WT
MTS
BH3
BH1
BH2
Δ MTS
D
MDA-MB-468
MCL1 - WT
MCL1 - ΔMTS
MCL1
MCL1 in mtMatrix
SOD2
Bcl-xL
OMM
MP
B
Input sequence length : 366 aa
VALUES OF COMPUTED PARAMETERS
Net charge of query sequence : -10
Analysed region : 38
Number of basic residues in targeting sequence : 5
Number of acidic residues in targeting sequence : 0
Cleavage site : 44
Cleaved sequence : MFGLKRNAVILLYYCGGAGLGAGSSGGARPGGRRLLATEKEAS
HYDROPHOBIC SCALE USED
GES KD GVH1 ECS
H17 : 1.882 2.047 0.399 0.776
MesoH : -0.504 0.528 -0.275 0.289
MuHd_075 : 37.564 17.709 8.753 10.119
MuHd_095 : 31.249 17.127 7.057 7.499
MuHd_100 : 25.979 13.392 5.756 5.795
MuHd_105 : 22.875 13.293 4.963 4.524
Hmax_075 : 10.600 8.000 1.904 4.400
Hmax_095 : 11.900 9.800 2.185 4.532
Hmax_100 : 9.000 7.400 1.411 3.620
Hmax_105 : 9.000 13.100 1.411 3.460
PROBABILITY
of export to mitochondria: 0.8364
E
MDA-MB-468
Mock
MCL1-WT
MCL1-ΔMTS
MCL1
Mitochondria
Nucleus
F
MDA-MB-468
Mitotracker+ %
Mock
MCL1-WT
MCL1-ΔMTS
G
MDA-MB-468
Relative ROS level
H
MDA-MB-468
Relative caspase 3/7 activity
Log [nM], PCT
Mock
MCL1-WT
MCL1-ΔMTS
I
MDA-MB-436
SUM159PT
VUI0659158
OCR (pMole/min)
Time (min)
DMSO
1 μM
10 μM
J
Relative Caspase3/7 activity
MDA-MB-436
SUM159PT
VU0659158 (μM)

Figure S4, related to Figure 5. Deletion of MTS in MCL1 reduces MCL1-mediated mtOXPHOS. (A) MDA-MB-436 and SUM159PT cells were transfected with MCL1 siRNA for 48 h. At this time, apoptotic cells were determined by annexin V staining. (B) MTS in human MCL1 was determined by *in silico* analysis. (C) Schema of wild type (WT) MCL1 and mutant MCL1 lacking its N-terminal MTS (ΔMTS). (D) Fractions from outer mitochondrial membrane (OMM) and mitoplast (MP) were obtained by mitochondrial sub-fractionation as described in Experimental Procedures. SOD2 and Bcl-xL were used as markers for MP and OMM, respectively. (E) MDA-MB-468 cells stably expressing MCL1-WT or MCL-ΔMTS were stained with the nuclear marker TOPRO3 (blue), MCL1 antibody (green) and Mitotracker Red CMXRos (red). Absence of yellow dots in cells transfected with MCL-ΔMTS denotes inability of MCL1 to localize in mitochondria. (F) Proportion of Mitotracker Red $CMXRos^{+}$ cells were determined by flow cytometry (*p<0.0005). (G) ROS levels were measured by ROS-Glo (*p<0.005, **p<0.0005). (H) Caspase 3/7 activities were determined by Caspase-Glo 3/7 Assay in presence increasing doses of paclitaxel. (I) OCRs were determined by Seahorse $XF^{e}96$ extracellular flux analyzer in MDA-MB-436 and SUM159PT cells treated with VU0659158. (J) Caspase 3/7 activities were determined by Caspase-Glo 3/7 Assay in MDA-MB-436 and SUM159PT cells treated with VU0659158 for 24 h ((*p<0.05, **p<0.005). Data are represented as mean ± SD.

Figure S5

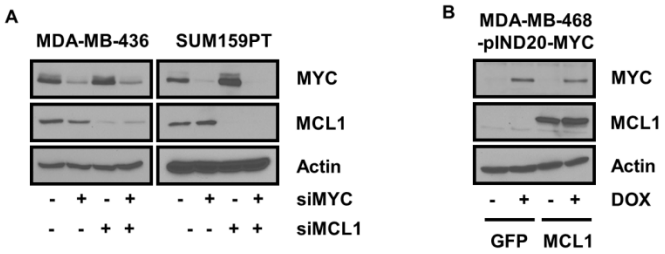

A
MDA-MB-436
SUM159PT
MYC
MCL1
Actin
- + - + - + - +
siMYC
- - + + - - + +
siMCL1
B
MDA-MB-468
-pIND20-MYC
MYC
MCL1
Actin
- + - +
DOX
GFP
MCL1

Figure S5, related with Figure 6. Modulation of MYC and MCL1 expression in TNBC cell lines. (A) Lysates from MDA-MB-436 and SUM159PT cells transduced with *MYC* and/or *MCL1* siRNA were subjected to immunoblot analysis to confirm knockdown. (B) Lysates from MDA-MB-468 cells stably transduced with both pINDUCER20-MYC and pLX302-MCL1 were subjected to immunoblot analysis to confirm modulation of MYC and/or MCL1 expression.

Figure S6

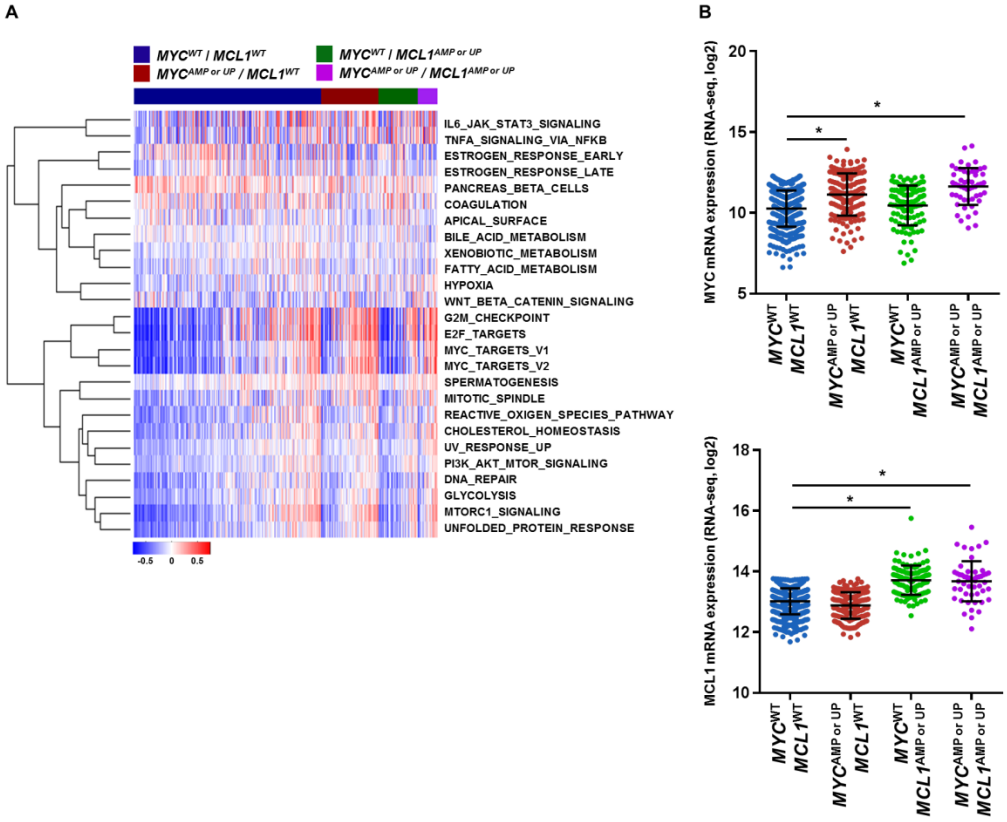
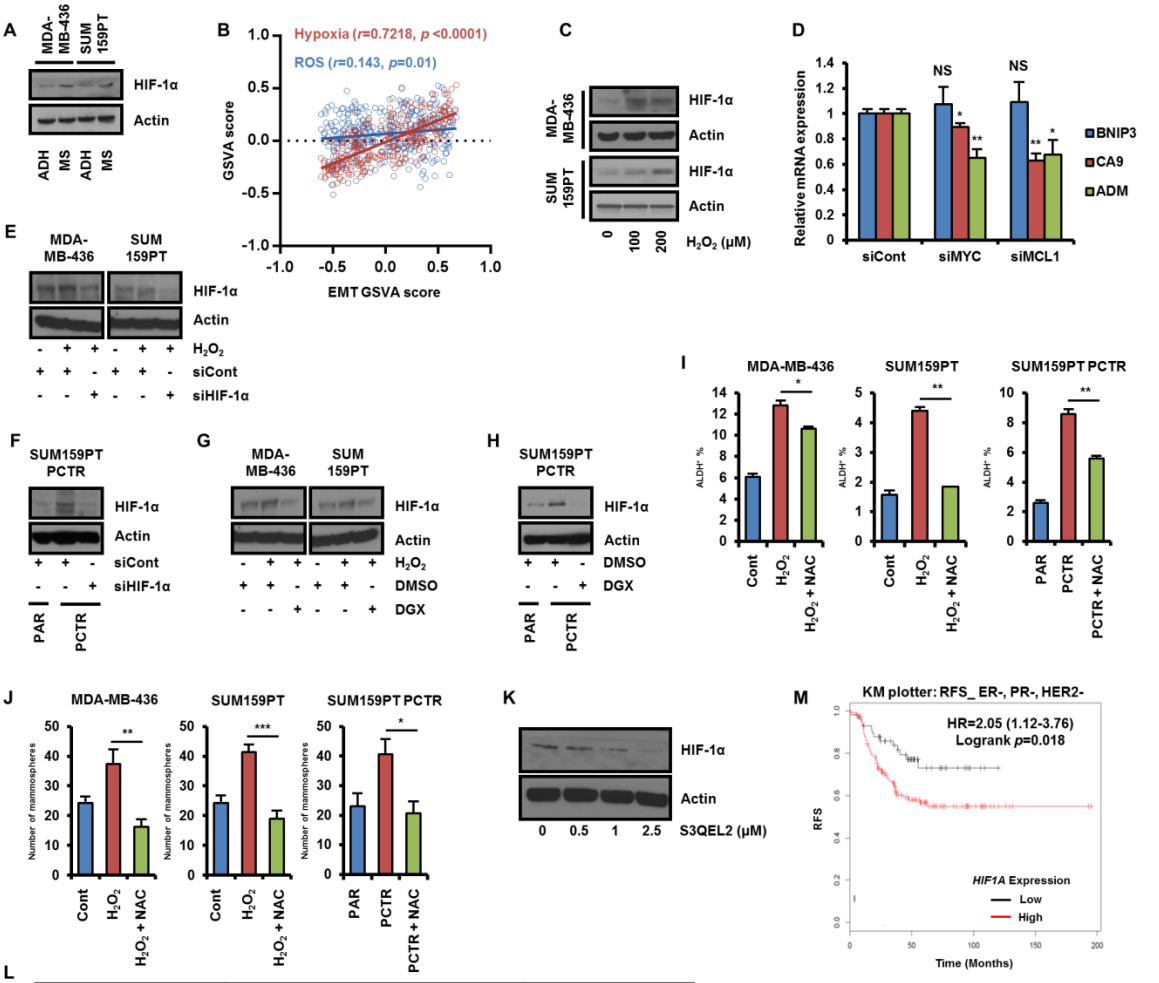

A
MYC^WT / MCL1^WT
MYC^AMP or UP / MCL1^WT
MYC^WT / MCL1^AMP or UP
MYC^AMP or UP / MCL1^AMP or UP
IL6_JAK_STAT3_SIGNALING
TNFA_SIGNALING_VIA_NFKB
ESTROGEN_RESPONSE_EARLY
ESTROGEN_RESPONSE_LATE
PANCREAS_BETA_CELLS
COAGULATION
APICAL_SURFACE
BILE_ACID_METABOLISM
XENOBIOTIC_METABOLISM
FATTY_ACID_METABOLISM
HYPOXIA
WNT_BETA_CATENIN_SIGNALING
G2M_CHECKPOINT
E2F_TARGETS
MYC_TARGETS_V1
MYC_TARGETS_V2
SPERMATOGENESIS
MITOTIC_SPINDLE
REACTIVE_OXIGEN_SPECIES_PATHWAY
CHOLESTEROL_HOMEOSTASIS
UV_RESPONSE_UP
PI3K_AKT_MTOR_SIGNALING
DNA_REPAIR
GLYCOLYSIS
MTORC1_SIGNALING
UNFOLDED_PROTEIN_RESPONSE
-0.5
0
0.5
B
MYC mRNA expression (RNA-seq, log2)
20
15
10
5
*
*
MYC^WT MCL1^WT
MYC^AMP or UP MCL1^WT
MYC^WT MCL1^AMP or UP
MYC^AMP or UP MCL1^AMP or UP
MCL1 mRNA expression (RNA-seq, log2)
18
16
14
12
10
*
*
MYC^WT MCL1^WT
MYC^AMP or UP MCL1^WT
MYC^WT MCL1^AMP or UP
MYC^AMP or UP MCL1^AMP or UP

Figure S6, related to Figure 6. Pathway analysis in breast tumors with *MYC* and/or *MCL1* alterations. (A) Hallmark gene sets enriched in breast tumor with *MYC* and/or *MCL1* amplifications and/or mRNA upregulations were determined using Gene Set Variation Analysis (GSVA). Heatmap shows significantly enriched gene sets ($q<0.05$). (B) *MYC* and *MCL1* mRNA levels from TCGA breast tumors were plotted ($*p<0.0005$). Data are represented as mean ± SD.

Figure S7



L

	Number of cells used for inoculation			CSC frequency (per 1 in.)
Group (*n*=10)	1 x 10^5	1 x 10^4	1 x 10^3	
Parental	8/10	5/10	4/10	28529
PCTR + DMSO	9/10	8/10	4/10	13545
PCTR + Digoxin	6/10	4/10	2/10	58953
PCTR + Metformin	5/10	4/10	2/10	71068
PCTR + S3QEL2	6/10	3/10	1/10	72750

p=0.000215

Figure S7, related to Figure 7. Role of HIF-1α in CSC enrichment and chemotherapy resistance in TNBC. (A) MDA-MB-436 and SUM159PT cells were grown as mammospheres (MS) or in adherent condition (ADH). Lysates of these cells were subjected to immunoblot analysis. (B) Plots of EMT GSVA scores vs. Hypoxia or ROS GSVA scores in TNBCs from METABRIC (Pearson's correlation). (C) Lysates of MDA-MB-436 and SUM159PT cells treated with 0, 100 or 200 μM hydrogen peroxide (H_2O_2) for 72 h were subjected to immunoblot analysis with the indicated antibodies. (D) mRNA expressions of *BNIP3*, *CA9*, and *ADM* were determined by RT-qPCR in SUM159PT cells transfected with *MYC* siRNA or *MCL1* siRNA for 4 days (**p*<0.05, ***p*<0.005). (E,F) MDA-MB-436 and SUM159PT cells were transduced with *HIF1A* siRNA ± 200 μM H_2O_2 for 48 h (E) and PCTR SUM159PT cells were transduced with *HIF1A* siRNA (F). Lysates of these cells were prepared and subjected to immunoblot analysis. (G,H) MDA-MB-436 and SUM159PT cells were treated with 100 nM digoxin ± 200 μM H_2O_2 for 48 h (G) and PCTR SUM159PT cells were treated with 100 nM digoxin for 48 h (H). Lysates of these cells were prepared and subjected to immunoblot analysis with the indicated antibodies. (I) MDA-MB-436 and SUM159PT cells were treated with 200 μM H_2O_2 ± 1 mM N-AC for 4 days and then were subjected to ALDH assay. PCTR SUM159PT cells were treated with 1 mM N-AC for 4 days and then subjected to ALDH assay (**p*<0.005, ***p*<0.0005). (J) MDA-MB-436 and SUM159PT were cells treated with 200 μM H_2O_2 ± 1 mM NAC for 4 days and then seeded in a mammosphere assay for 7 days. PCTR SUM159PT cells were treated with 1 mM NAC for 4 days and then seeded in a mammosphere assay for 7 days (**p*<0.05, ***p*<0.005, ****p*<0.0005). (K) Lysates of PCTR SUM159PT cells that had been treated with S3QEL2 for 48 h were prepared and subjected to immunoblot analysis with HIF-1α and actin antibodies. (L) Parental SUM159PT and PCTR SUM159PT cells treated with DMSO, digoxin, metformin, or S3QEL2 for 7 days were serially diluted and then injected subcutaneously (s.c.) in the lateral dorsum of athymic female mice for ELDA. (M) Kaplan-Meier plot curve assessing RFS of patients with TNBC as a function of *HIF1A* mRNA expression. (N) *Nanog* mRNA expression was determined by RT-qPCR in MDA-MB-436 cells treated with 200 μM H_2O_2 or transfected with *MYC* siRNA or *MCL1* siRNA for 4 days. Data are represented as mean ± SD.