High-throughput profiling of signaling networks identifies mechanism-based combination therapy to eliminate microenvironmental resistance in acute myeloid leukemia

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Received: December 15, 2016.

Accepted: June 27, 2017

Pre-published: June 28, 2017.

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Supplemental Materials and Methods

Agents and antibodies

Temsirolimus was provided by the National Cancer Institute (Rockville, MD). ABT737 and Nutlin-3a were purchased from Selleckchem (Houston, TX). The antibodies used in the reverse-phase protein array (RPPA) and immunoblotting analyses, along with their sources, are listed in Supplemental Table 2.

Cell lines and primary samples

The AML cell line OCI-AML3 was provided by Dr. M. Minden (Ontario Cancer Institute, Toronto, ON, Canada) and previously described.[1] U937 cells were purchased from ATCC (Manassas, VA). The murine stromal cell line MS-5 was provided by Dr. K. Itoh (Niigata University, Nishi-Ku, Niigata, Japan) [2] and previously described.[3] Authentication of each cell line was confirmed by short tandem repeat DNA profiling (PowerPlex 16 HS System, Promega, Madison, WI) within six months prior to the experiments. Bone marrow and peripheral blood samples were collected from patients who had been diagnosed with AML. Normal bone marrow samples were obtained from healthy volunteers. All samples were collected during routine diagnostic procedures in accordance with protocols approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center. Informed consent was obtained in accordance with the Declaration of Helsinki. Mononuclear cells were separated by Ficoll-Hypaque (Sigma Chemical, St. Louis, MO) density-gradient centrifugation. Cells were maintained either in RPMI 1640 medium or in α-minimum essential medium (Life Technologies Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 1 mmol/L L-glutamine (Life Technologies Laboratories), and 50 μg/mL penicillin/streptomycin (Life Technologies Laboratories) in a humidified incubator at 37°C in 5% CO_2. Isolation, expansion, and purification of normal mesenchymal stromal cells were performed according to a previously published protocol.[4]

Statistical analysis

In this study, we used a two-sided "fold-change-filtered" binomial test to identify the distinct protein alterations triggered by a single inhibitor or two-inhibitor combination with or without stromal co-culture. For each protein, we checked whether there was significant up- or downregulation for the contrasts of interest. We first calculated the fold change in expression of a protein between treated and untreated conditions for each sample using a cutoff fold change of 1.2 to determine whether the protein showed upregulation, no difference, or downregulation. For example, if the expression level of protein A in sample i after treatment was more than 1.2 times the corresponding level before treatment, then protein A was considered to be upregulated in sample i. If the fold change was −1.2 or less, the protein was considered to be downregulated. Samples with proteins flagged as "no difference" were considered uninformative and excluded

from the binomial analysis. For each protein, we then calculated the probability that the imbalance between the numbers of samples showing up- and downregulation would be the same as or more extreme than the imbalance actually observed under the null hypothesis that change direction is completely random (p = 0.5). These imbalance probabilities were taken as the p values of our binomial tests. We then used the Benjamini-Hochberg method [5] to adjust for multiple hypothesis testing and estimated adjusted p values. Imbalances for which the adjusted p value was below 0.05 were considered to be significant.

To identify the single-inhibitor-induced specific apoptosis that was significantly affected by stroma, we grouped the treated samples based on the difference in apoptosis between treated samples in co-culture and those in monoculture. In group I (inhibitor-insensitive samples), the difference in apoptosis in treated samples in co-culture and those in monoculture was less than or equal to −2%; in group II, the difference was between −2% and 2%; and in group III (inhibitor-sensitive samples), the difference was 2% or greater. We applied a two-tailed paired Student t test to compare apoptosis with and without stroma in each group. We also used a two-tailed unequal variance Student t test to compare the difference in protein expression between untreated samples in groups I and III with and without stroma and to compare baseline protein expression between untreated samples in groups I and III in co-culture. Statistical significance was defined as $p \leq 0.05$.

To identify the co-treatment-induced specific apoptosis that was significantly affected by stroma, we grouped the samples based on the difference in apoptosis between samples treated with single-inhibitor ABT737 or Nutlin-3a and those treated with the dual inhibitors of temsirolimus plus ABT737 or Nutlin-3a in co-culture. In group I, the difference in apoptosis between samples treated with ABT737 or Nutlin-3a alone and those treated with two inhibitors was less than or equal to -2%; in group II, the difference was between −2% and 2% (note: none of the samples fell within the range designated by group II); and in group III, the difference was 2% or greater. The difference in apoptosis between combined-treatment and single-treatment in groups I and III was calculated using a two-tailed paired Student t test. Statistical significance was defined as $p \leq 0.05$.

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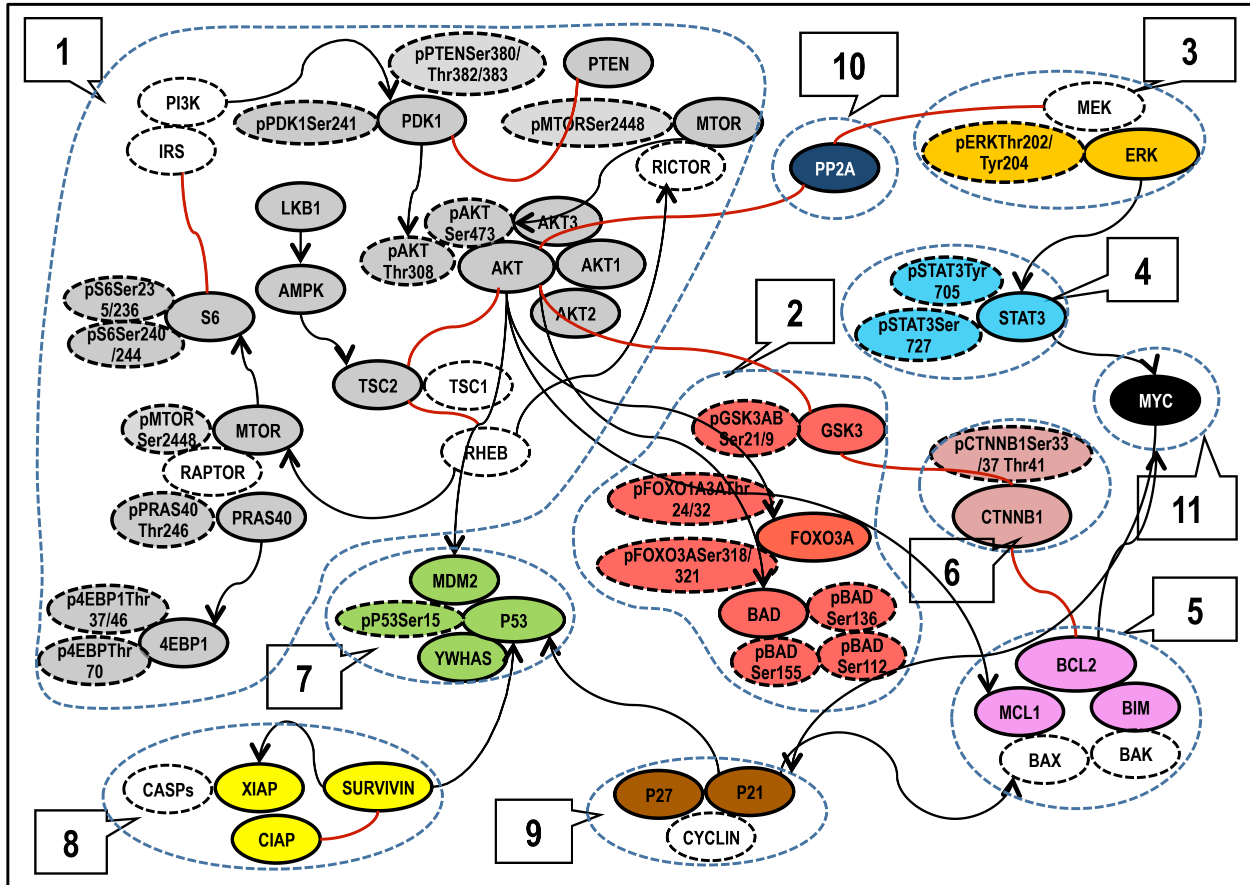
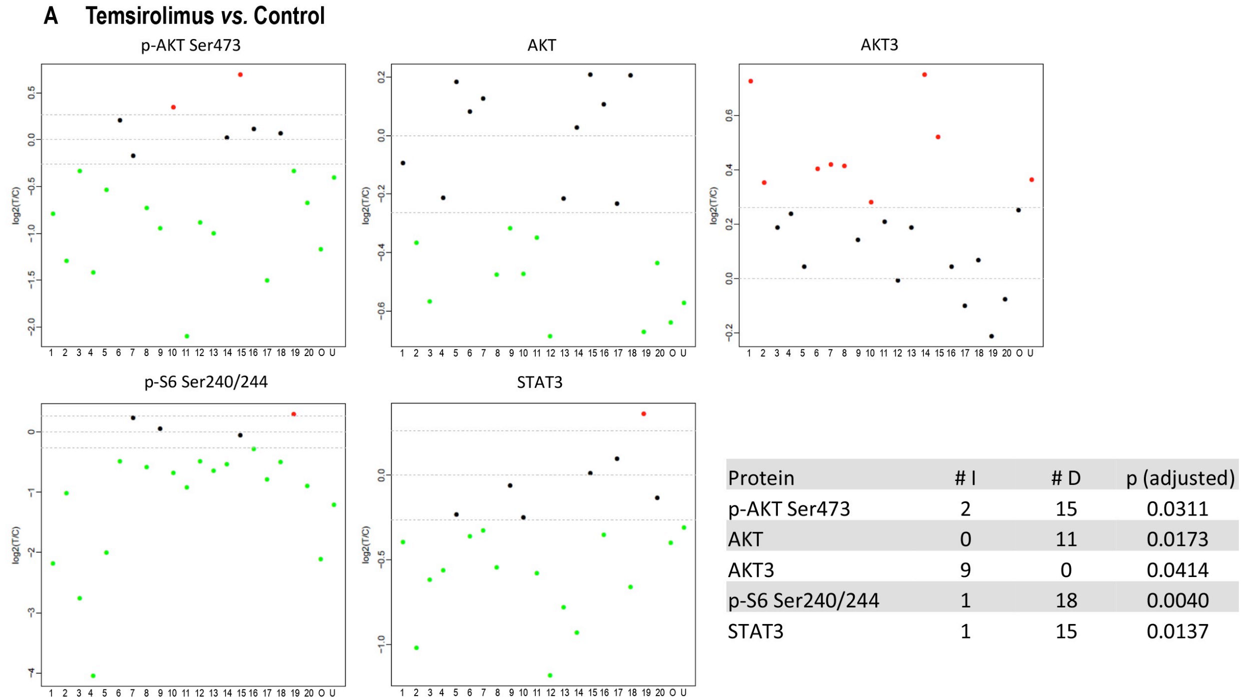
SUPPLEMENTAL FIGURES

Intracellular signaling networks

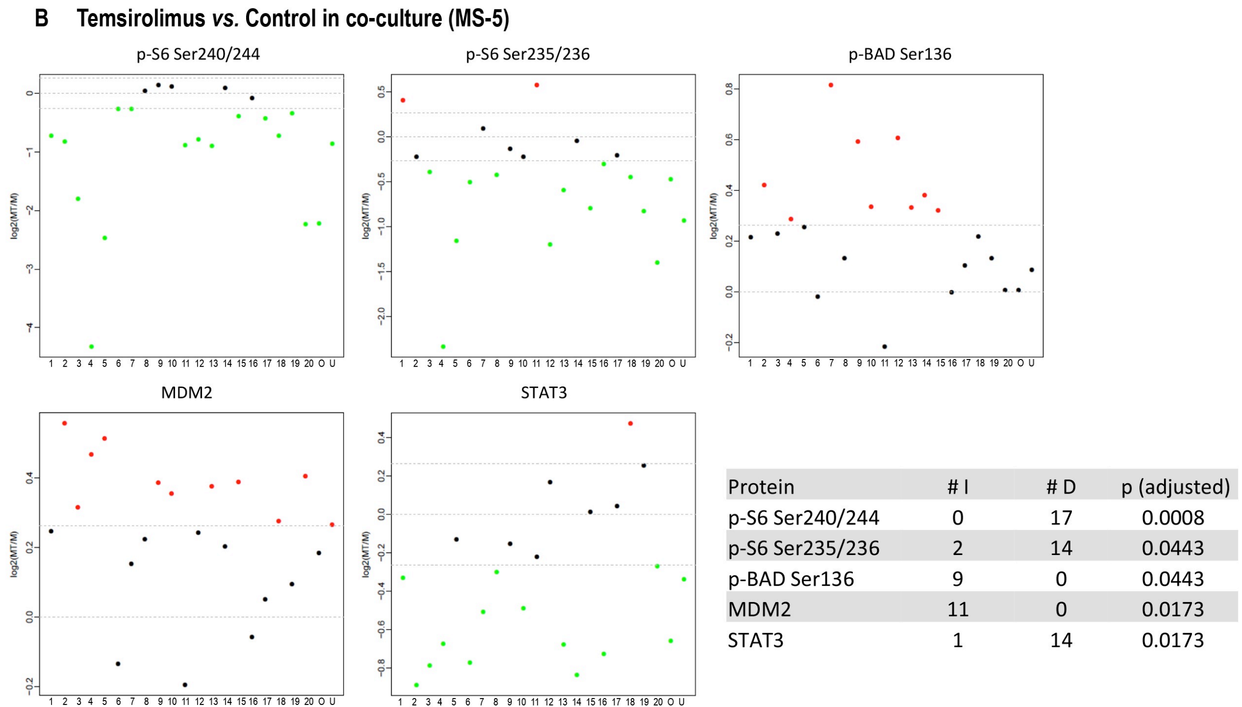


1. PI3K/AKT/MTOR signaling
2. AKT/MTOR major downstream signaling
3. MEK/ERK signaling
4. STAT3 signaling
5. BCL2 protein family
6. WNT CATENIN signaling
7. P53 family
8. IAP family
9. Cell cycle regulation
10. PP2A phosphatase family
11. MYC signaling

Supplemental Figure 1. RPPA profiling of key proteins in multiple signaling pathways in AML. RPPA was used to profile 53 proteins in 11 signaling pathways involved in cell survival. In the figure, each pathway is surrounded by a blue dashed line. The profiled protein is represented by a color-filled circle with a solid border (total protein) or a broken border (phosphorylated protein).



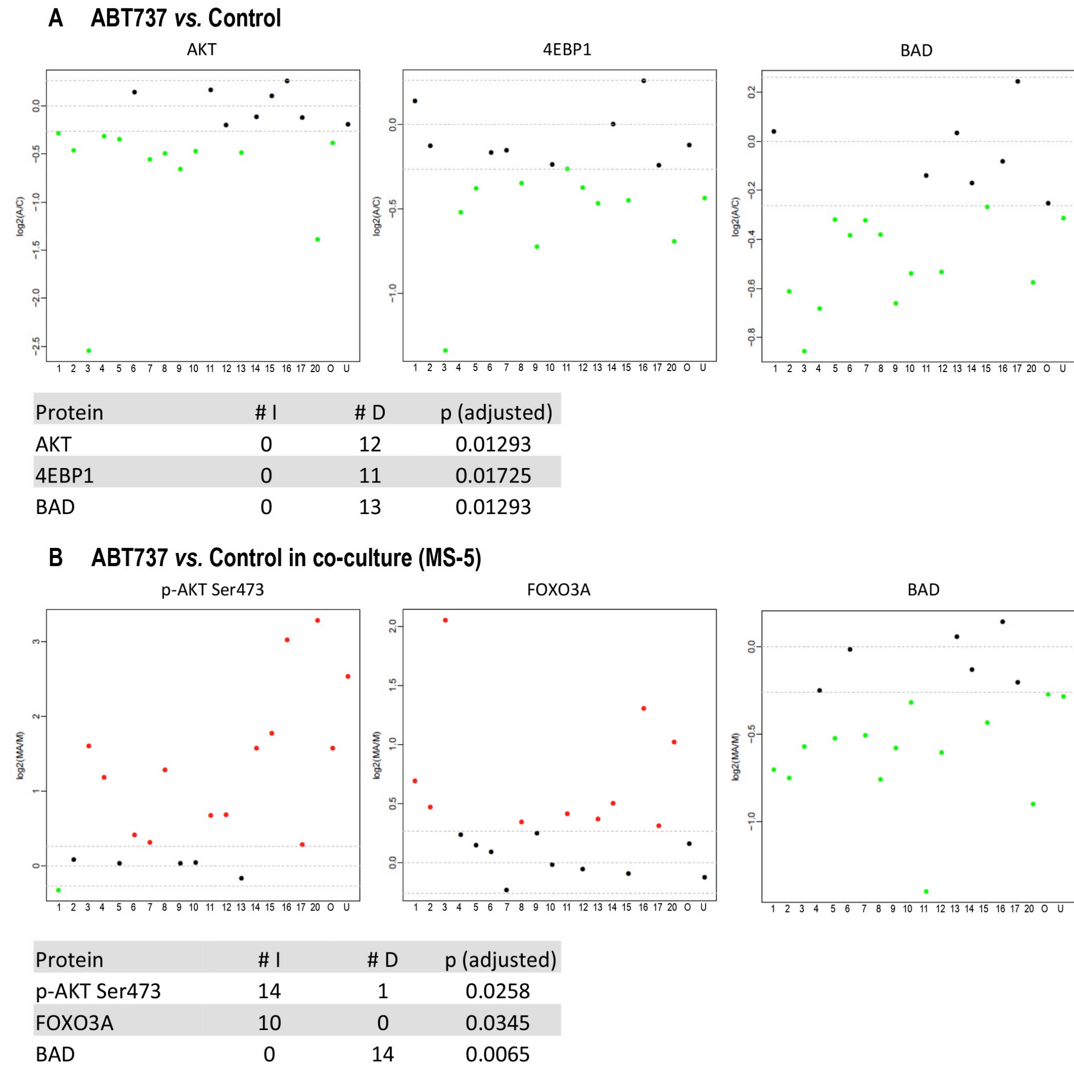
Protein	# I	# D	p (adjusted)
p-AKT Ser473	2	15	0.0311
AKT	0	11	0.0173
AKT3	9	0	0.0414
p-S6 Ser240/244	1	18	0.0040
STAT3	1	15	0.0137



Protein	# I	# D	p (adjusted)
p-S6 Ser240/244	0	17	0.0008
p-S6 Ser235/236	2	14	0.0443
p-BAD Ser136	9	0	0.0443
MDM2	11	0	0.0173
STAT3	1	14	0.0173

Supplemental Figure 2 (associated with Figure 1). Effects of temsirolimus treatment on protein expression in AML. RPPA analysis identified proteins whose expression was markedly affected by temsirolimus treatment. Dot plots display the level of alteration of the identified proteins in AML samples and cell lines cultured alone **(A)** and co-cultured with stroma **(B)**. Up- or downregulation of protein expression is indicated by red (upregulation) or green (downregulation) dots and separated by the top and bottom dashed lines. Black dots indicate no significant change in expression. The y-axis of the dot plots is the log2 of the ratio of the protein density of temsirolimus-treated samples to that of untreated samples. The x-axis displays sample number and cell lines, O: OCI-AML3, U: U937. Tables in

(A) and **(B)** display the identified proteins in samples treated in monoculture and in co-culture. First column: identified protein with a significant imbalance of alteration in response to treatment. Second column: number of samples showing increased expression of the indicated protein (I). Third column: number of samples showing decreased expression of the indicated protein (D). Fourth column: Adjusted *p* value for comparison of the numbers of samples with upregulation and downregulation of protein expression. Statistical analyses are described in the Supplemental Materials and Methods.

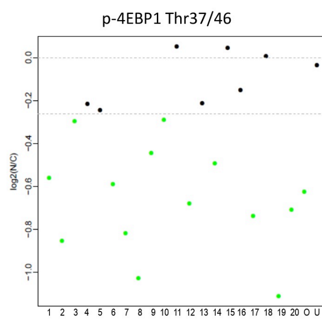


Protein	# I	# D	p (adjusted)
AKT	0	12	0.01293
4EBP1	0	11	0.01725
BAD	0	13	0.01293

Protein	# I	# D	p (adjusted)
p-AKT Ser473	14	1	0.0258
FOXO3A	10	0	0.0345
BAD	0	14	0.0065

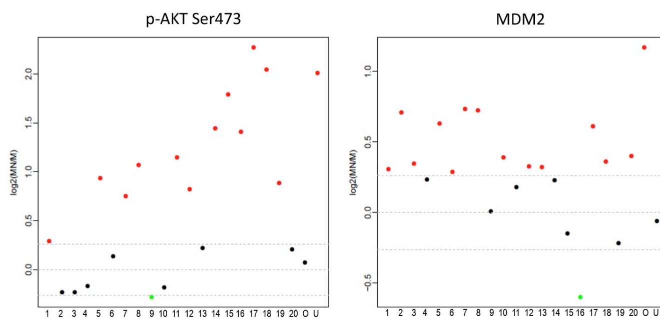
Supplemental Figure 3 (associated with Figure 2). Effects of ABT737 treatment on protein expression in AML. RPPA analysis identified proteins whose expression was markedly affected by ABT737 treatment. Dot plots display the level of alteration of the identified proteins in AML samples and cell lines cultured alone **(A)** and co-cultured with stroma **(B)**. The dot colors and the dashed lines are described in the Supplemental Figure 2 legend. The y-axis is the log2 of the ratio of the protein density of ABT737-treated samples to that of untreated samples. Tables in **(A)** and **(B)** display the identified proteins in samples treated with ABT737 in monoculture and in co-culture. The content of each column is described in the Supplemental Figure 2 legend.

A **Nutlin-3a *vs.* Control**



Protein	# I	# D	p (adjusted)
p-4EBP1 Thr37/46	0	14	0.0065

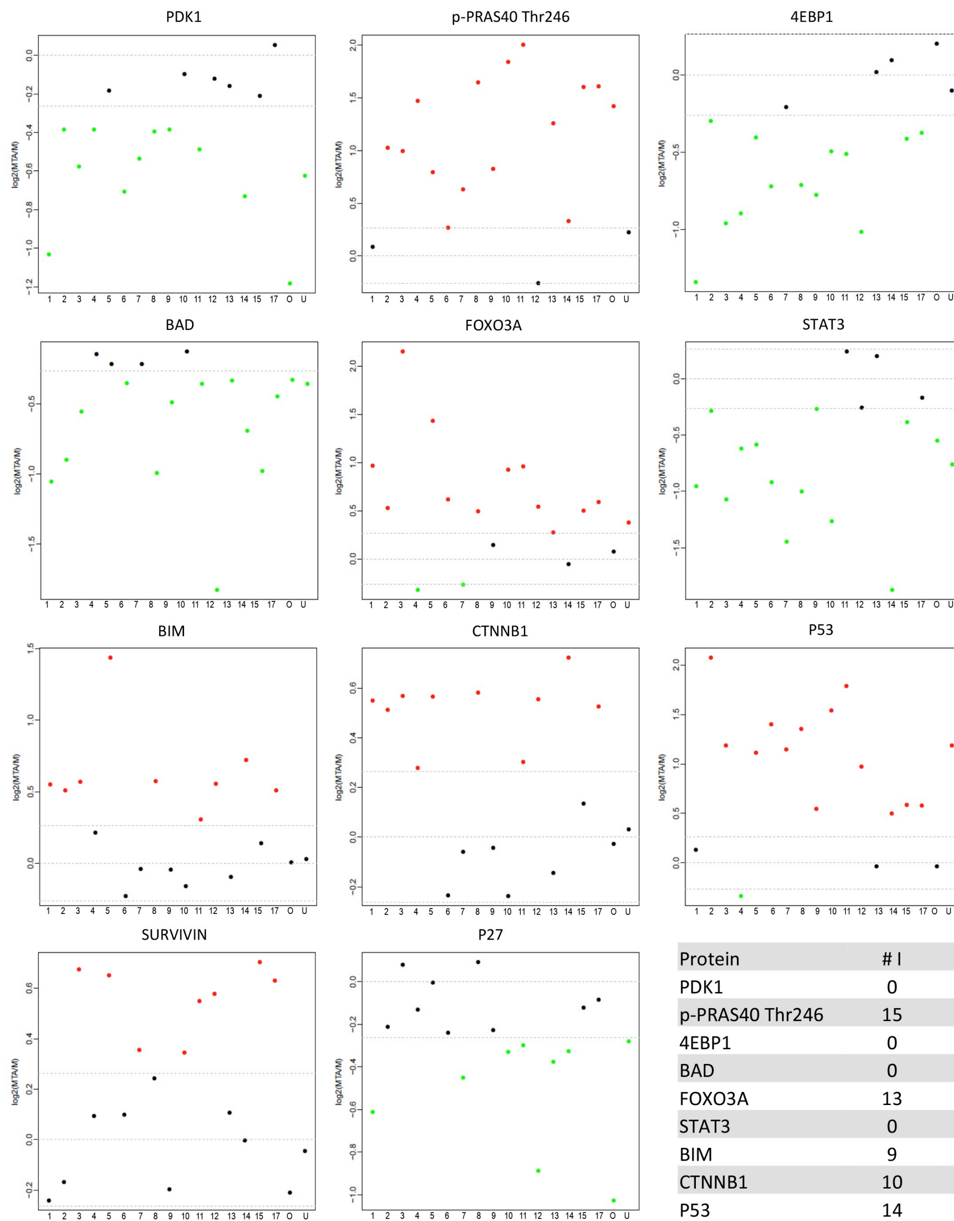
B **Nutlin-3a *vs.* Control in co-culture (MS-5)**



Protein	# I	# D	p (adjusted)
p-AKT Ser473	13	1	0.0485
MDM2	14	1	0.0485

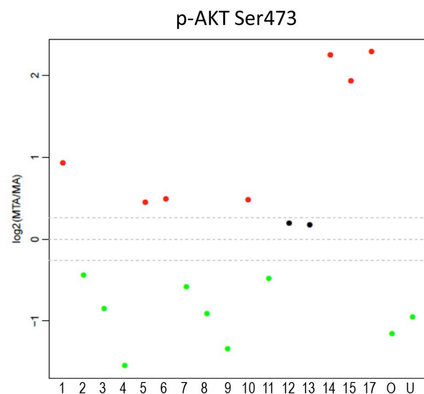
Supplemental Figure 4 (associated with Figure 3). Effects of Nutlin-3a treatment on protein expression in AML. RPPA analysis identified proteins whose expression was markedly affected by Nutlin-3a treatment. Dot plots display the level of alteration of the identified proteins in AML samples and cell lines cultured alone **(A)** and co-cultured with stroma **(B)**. The dot colors and dashed lines are described in the Supplemental Figure 2 legend. The y-axis is the log2 of the ratio of the protein density of Nutlin-3a-treated samples to that of untreated samples. Tables in **(A)** and **(B)** display the identified proteins in samples treated with Nutlin-3a in monoculture and in co-culture. The content of each column is described in the Supplemental Figure 2 legend.

A Temsirolimus + ABT737 *vs.* Control in co-culture (MS-5)



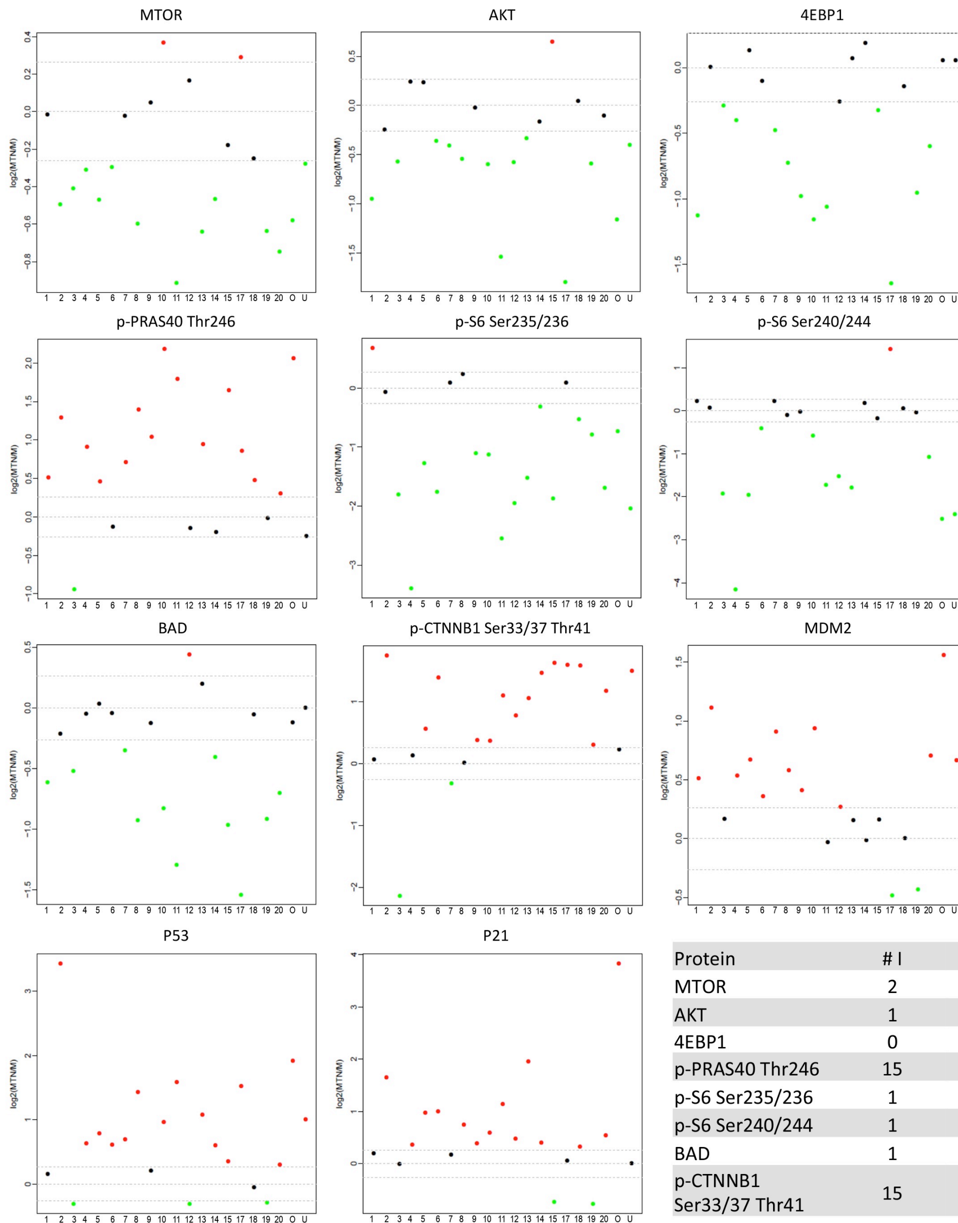
Protein	# I	# D	p (adjusted)
PDK1	0	12	0.0052
p-PRAS40 Thr246	15	0	0.0022
4EBP1	0	13	0.0032
BAD	0	14	0.0022
FOXO3A	13	2	0.0376
STAT3	0	14	0.0021
BIM	9	0	0.0230
CTNNB1	10	0	0.0148
P53	14	1	0.0086
SURVIVIN	8	0	0.0376
P27	0	9	0.0230

B Temsirolimus + ABT737 *vs.* ABT737 in co-culture (MS-5)



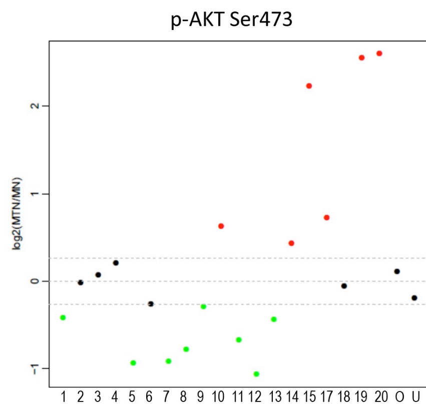
Supplemental Figure 5 (associated with Figure 4). Effects of treatment with the combination of temsirolimus and ABT737 on protein expression in co-cultured AML. RPPA analysis identified proteins whose expression was markedly affected by combination treatment with temsirolimus and ABT737 in co-culture. **(A)** Dot plots display the level of alteration of the identified proteins in AML samples and cell lines co-cultured with stroma. The dot colors and dashed lines are described in the Supplemental Figure 2 legend. The y-axis is the log2 of the ratio of the protein density of samples treated with temsirolimus and ABT737 to that of untreated samples in co-culture. The table in **(A)** displays the identified proteins in co-cultured samples treated with temsirolimus and ABT737. The content of each column is described in the Supplemental Figure 2 legend. **(B)** Dot plot of p-AKT (Ser473) alteration in treated samples in co-culture. The dot colors and dashed lines are described in the Supplemental Figure 2 legend. The y-axis is the log2 of the ratio of the protein density of combination-treated samples to that of ABT737-treated samples in co-culture.

A Temsirolimus + Nutlin-3a *vs.* Control in co-culture (MS-5)

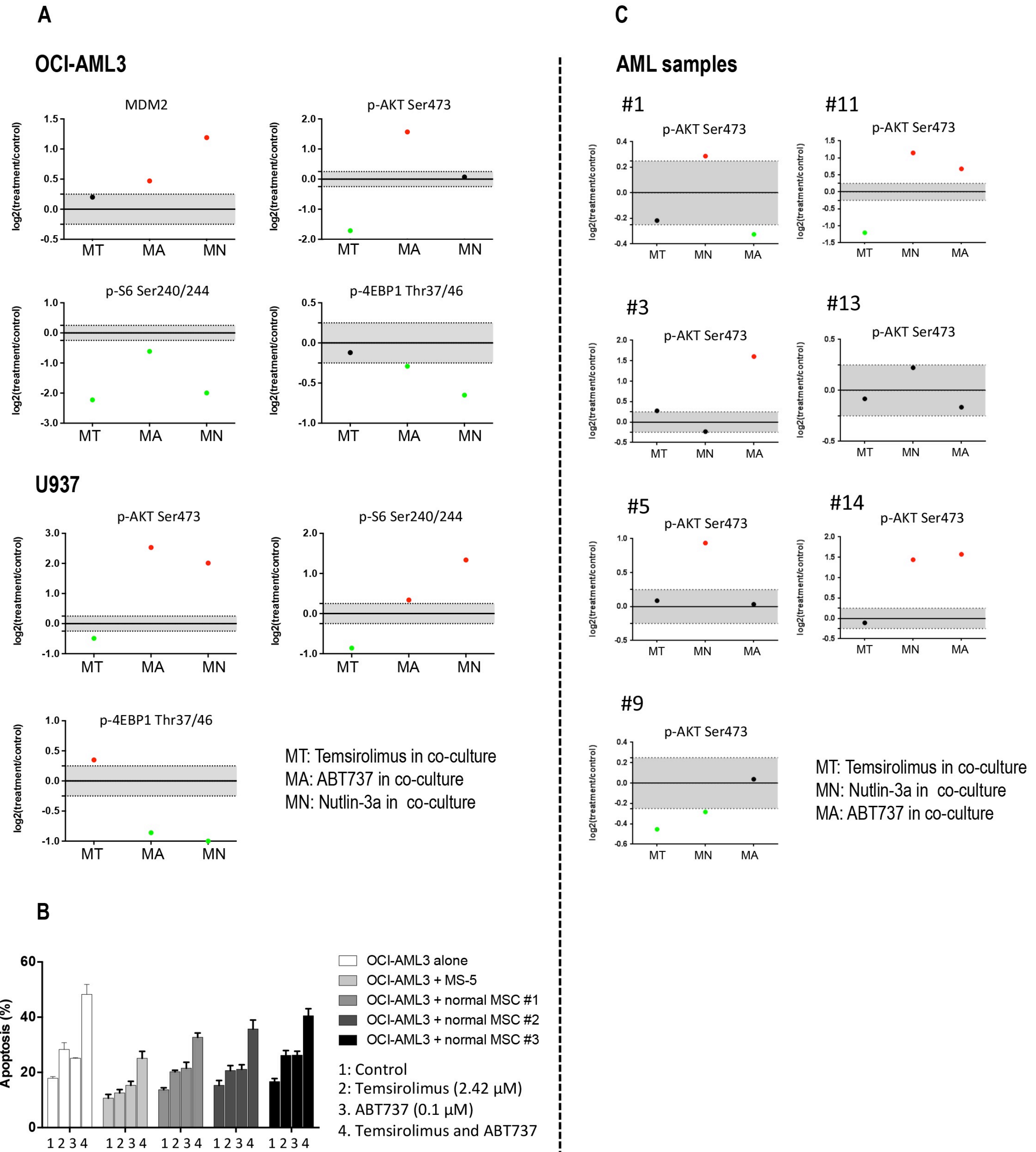


Protein	# I	# D	p (adjusted)
MTOR	2	13	0.0363
AKT	1	13	0.0242
4EBP1	0	12	0.0092
p-PRAS40 Thr246	15	1	0.0092
p-S6 Ser235/236	1	16	0.0092
p-S6 Ser240/244	1	11	0.0363
BAD	1	11	0.0363
p-CTNNB1 Ser33/37 Thr41	15	2	0.0249
MDM2	13	2	0.0363
P53	15	3	0.0363
P21	14	2	0.0363

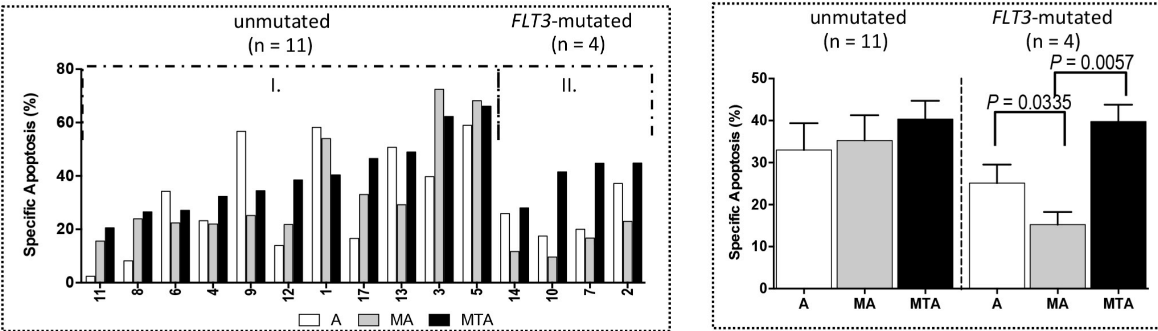
B Temsirolimus + Nutlin-3a *vs.* Nutlin-3a in co-culture (MS-5)

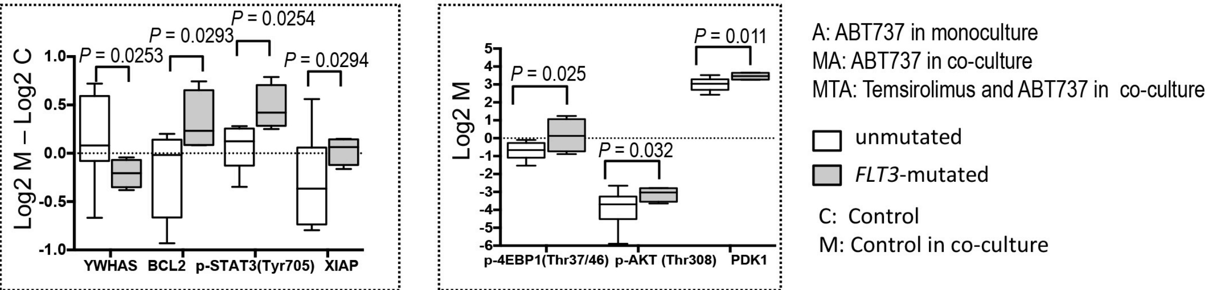


Supplemental Figure 6 (associated with Figure 5). Effects of treatment with the combination of temsirolimus and Nutlin-3a on protein expression in AML. RPPA analysis identified proteins whose expression was markedly affected by combination treatment with temsirolimus and Nutlin-3a in co-culture. **(A)** Dot plots display the level of alteration of the identified proteins in AML samples and cell lines co-cultured with stroma. The dot colors and dashed lines are described in the Supplemental Figure 2 legend. The y-axis is the log2 of the ratio of the protein density of samples treated with temsirolimus and Nutlin-3a to that of untreated samples in co-culture. The table in **(A)** displays the identified proteins in co-cultured samples treated with temsirolimus and Nutlin-3a. The content of each column is described in the Supplemental Figure 2 legend. **(B)** Dot plot of p-AKT (Ser473) alteration in treated samples in co-culture. The dot colors and dashed lines are described in the Supplemental Figure 2 legend. The y-axis is the log2 of the ratio of the protein density of combination-treated samples to that of Nutlin-3a-treated samples in co-culture.



Supplemental Figure 7 (associated with Figure 6). RPPA analysis of AML cell lines and primary AML samples. The dot plots display the indicated proteins whose expression was significantly altered by treatment with single-agent temsirolimus, ABT737, or Nutlin-3a in co-cultured AML cell lines **(A)** and selected primary AML samples **(C).** The y-axis is the log2 of the ratio of the protein density of treated samples to that of untreated samples. The dot colors and dashed lines are described in the Supplemental Figure 2 legend. **(B)** Bar graph displays apoptosis induction in treated OCI-AML3 cells cultured alone or co-cultured with MS-5 and normal BM MSCs for 72 hours. Values are presented as mean ± standard deviation of the mean.





Supplemental Figure 8. Stroma altered the sensitivity of *FLT3*-mutated AML to ABT737. **(A)** The bar graph on the left displays specific apoptosis for samples treated with ABT737 in monoculture and stromal co-culture and treated with temsirolimus plus ABT737 in co-culture. Samples were grouped based on mutation status: group I, unmutated samples; group II, *FLT3*-mutated samples. Difference in apoptosis between ABT737-treated samples in monoculture and co-culture and between ABT737- and temsirolimus plus ABT737-treated samples in co-culture was calculated using a two-tailed paired Student *t* test. Results are displayed in the bar graph on the right (mean ± standard error of the mean). Statistical significance was defined as $p \leq 0.05$. **(B)** Box and whisker plots display difference in protein expression between untreated samples in groups I and II with and without stroma (left panel) and baseline protein expression of untreated samples in groups I and II in co-culture (right panel). Significance was calculated using a two-tailed unequal variance Student *t* test. Statistical significance was defined as $p \leq 0.05$. Whiskers indicate the range from minimum to maximum values. The line in the middle of the box is plotted at the median.

SUPPLEMENTAL TABLES

Patient	Source	Diagnosis	Disease Status	FAB	Age	Sex	Blast %	Molecular Mutation	CG Karyotype
1	PB	AMoL	New Dx	M5A	55	F	96	Neg	t(9;11)
2	PB	AML	Rel	M2	57	M	87	FLT3-ITD	Diploid
3	PB	AML	Rel	M1	40	M	94	Neg	Complex
4	PB	AML	New Dx	Unk	60	F	48	Neg	Complex
5	BM	AML	New Dx	M2	63	M	65	Neg	Diploid
6	BM	AML	Rel	Unk	72	M	34	Neg	47,XY,+8
7	PB	AMML	New Dx	M4	66	F	79	FLT3-ITD	Diploid
8	PB	AML	Rel	M2	75	F	44	Neg	del(12)
9	PB	AML	Rel	Unk	60	M	62	Neg	Complex
10	PB	AML	New Dx	M2	55	F	61	FLT3-D835	Diploid
11	PB	AMoL	New Dx	M5A	28	F	92	Neg	Complex
12	PB	AEL	Rel	M6	52	F	60	Neg	Complex
13	PB	AML	New Dx	M0	45	F	86	Neg	45XX,-7
14	PB	AMML	New Dx	M4	35	M	60	FLT3-D835 + NRAS	inv(16)
15	PB	AML	New Dx	Unk	77	F	66	JAK2	Complex
16	BM	AMoL	Rel	M5A	30	M	97	Neg	Complex
17	PB	AML	Rel	M2	67	F	78	Neg	Complex
18	PB	AML	New Dx	M2	76	M	64	IDH1	45,X,-Y
19	PB	AML	Rel	Unk	28	M	71	Neg	Complex
20	BM	AML	Rel	M1	56	F	46	FLT3-D835	Complex

PB: Peripheral blood
BM: Bone marrow
Dx: Diagnosis
Rel: Relapse
AMoL: Acute monocytic leukemia
AMML: Acute myelomonocytic leukemia
AEL: Acute erythroid leukemia

FAB: French-American-British classification
Unk: Unknown
Neg: Negative
CG: Cytogenetic
t: translocation
del: deletion
inv: inversion

Supplemental Table 1. Clinical information for 20 primary AML samples.

	Protein	Vendor	Catalog Number
1	YWHAS (14-3-3 Sigma)	Cell signaling	632
2	4EBP1	Cell signaling	9452
3	p-4EBP1 Thr37/46	Cell signaling	9459
4	p-4EBP1 Thr70	Cell signaling	9455
5	AKT	Cell signaling	9272
6	AKT1	Cell signaling	2967
7	AKT2	Cell signaling	2962
8	AKT3	Cell signaling	4059
9	p-AKT Thr308	Cell signaling	9275
10	p-AKT Ser473	Cell signaling	9271
11	AMPK	Cell signaling	2532
12	p-BAD Ser112	Cell signaling	9291
13	p-BAD Ser136	Cell signaling	9295
14	p-BAD Ser155	Cell signaling	9297
15	BAD	Cell signaling	9292
16	BCL2	DAKO	M0887
17	BIM	Epitomics	EP1036
18	CTNNB1	Cell signaling	9562
19	p-CTNNB1 Ser33/37Thr41	Cell signaling	9561
20	CIAP	Millipore	07_759
21	p-ERK44/42 Thr202Tyr204	Cell signaling	9101
22	ERK2	Santa cruz	sc154
23	p-FOXO1A3AThr24Thr32	Cell signaling	9464
24	p-FOXO3A Ser318/321	Cell signaling	9465
25	FOXO3A	Cell signaling	9467
26	GSK3	Cell signaling	sc7291
27	p-GSK3AB Ser21/9	Cell signaling	9331
28	LKB1	Cell signaling	3050
29	MCL1	BD pharmingen	559027
30	MDM2	Santa cruz	sc813
31	MTOR	Cell signaling	2983
32	p-MTOR Ser2448	Cell signaling	2971
33	MYC	Cell signaling	9402
34	P21	Cell signaling	2946
35	P27	Cell signaling	sc528
36	p-P53 Ser15	Cell signaling	9284
37	P53	BD bioscience	554294
38	p-PDK1 Ser241	Cell signaling	3061
39	PDK1	Cell signaling	3062
40	PP2A	Santa cruz	sc18330
41	PRAS40	Invitrogen	AHO1031
42	p-PRAS40 Thr246	Cell signaling	2997
43	p-PTEN Ser380/Thr382/Thr383	Cell signaling	9554
44	PTEN	Cell signaling	9552
45	S6	Cell signaling	2217
46	p-S6 Ser235/236	Cell signaling	2211
47	p-S6 Ser240/244	Cell signaling	2215
48	p-STAT3 Tyr705	Cell signaling	9131
49	p-STAT3 Ser727	Cell signaling	9134
50	STAT3	Upstate	596
51	SURVIVIN	Cell signaling	2802
52	TSC2	Epitomics	EP1613.1
53	XIAP	Cell signaling	2042

Supplemental Table 2. Antibodies used in RPPA and immunoblotting.