Supplementary Materials and Methods

Cell lines and culturing conditions. Melanoma cell lines were kindly provided by Dr. Meenhard Herlyn at the Wistar Institute (Philadelphia, PA). Non-small cell lung cancer cell lines H23 and H838 were obtained from Dr. Charles Rudin's lab and H1395 from Dr. Craig Peacock's lab at Johns Hopkins University (JHU). Glioblastoma cell lines were obtained from Dr. Greg Riggins' lab at JHU. Melanoma and glioblastoma cells were maintained in Dulbecco's modified Eagle medium. NSCLCs were maintained in RPMI medium 1640. Both types of media were supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin (pen-strep), and L-glutamine. The media, pen-strep, and L-glutamine were purchased from Invitrogen. FBS was obtained from Gemini Bio-Products (#100106).

Cell treatment for ^{3}H-thymidine incorporation. Cells were plated in 96-well plates at sub-confluency and allowed to attach to the plate surface prior to treatment with 10 μM C646, 10 μM C37, or 0.01% DMSO for 24 h. Following treatment, ^{3}H-thymidine (1 mCi/ml stock) was added to media at 10 μCi/ml. Cells were incubated in this media for an additional 5 h and then trypsinized and collected onto a filter mat for radioactivity measurement.

XTT assay. The assay was performed using the Cell Proliferation Kit II (XTT) according to manufacturer's protocol (Roche). Cells were seeded in 96-well plates at low confluency and treated with molecules of interest for 9 days. Cell culture media was changed every other day with the addition of fresh compounds. Absorbance measurements were taken on days 1, 3, 6, 7 and 9.

Cell cycle analysis. Cells were treated with C646 or DMSO for 24 h. Equal numbers of cells ($1x10^6$) from each sample were then collected and fixed in 70% ethanol for at least 2 h at 4^oC and washed with PBS. The cells were then stained for ~20 min at 37^oC with a solution containing 0.02 mg/ml PI, 0.1% Triton X-100 and ~20 units/ml RNase A (Ambion RNase Cocktail #AM2286, 200 μl for 5 ml staining solution) in PBS. The stained cells were then immediately analyzed using flow cytometry.

Quantitative real time PCR. qPCR was performed using the Power SYBR Green PCR Master Mix (ABI) for both microarray validation and ChIP DNA analyses. PCR was run at 95^oC for 5 min, followed by 40 cycles at 95^oC for 15 sec, annealing for 30 sec, and 72^oC for 30 sec per cycle, followed by a melt curve stage. The annealing temperature for all the microarray validation PCRs was 60^oC. For ChIP-qPCR, the annealing temperatures were between 58^oC and 60^oC, depending on the primers. Each sample had four replicates. PCRs were performed with real time systems from ABI and data acquisition was done with ABI's proprietary softwares. Further data analysis was done in Excel. On the graphical representations of PCR quantification, the error bars represent the difference between the mean RQ (relative quantity) values and the maximum or the minimum values (+ error and - error, respectively).

Gene expression values were determined with the ΔΔCt method using ABI's softwares. To control for variations in DNA amount between wells, GAPDH was used as the internal control. For the microarray validation PCRs on gene transcripts, DMSO-treated samples were used as the reference for data normalization. The graphs represent mean RQ values after such normalization. For ChIP-qPCR, DMSO treated samples from total histone H3 pulldown was

used to normalize all DMSO ChIP data, and C646-treated samples from total H3 pulldown was used to normalize all C646 samples. This was done to account for any differences in the amount of chromatin pulled down by the H3 antibody between DMSO- and C646-treated cells. Therefore, data from ChIP-qPCR were represented as %total H3, which were equal to mean RQ*100.

Chromatin immunoprecipitation. ChIP was performed based on a published protocol (O'Geen, Frietze and Farnham, 2010). Formulations for all buffers are taken from the protocol. Briefly, the cells were cross-linked with 0.37% formaldehyde for 10 min at room temperature (RT) with agitation. Nuclei were initially released by lysing the cells on ice for 15-30min followed by centrifugation at 430xg for 5 min at 4^{o}C. The nuclei were then lysed on ice for an additional 30 min. Vigorous pipetting during the two lysis steps was sufficient to break up the cells. The chromatin was sheared using the Bioruptor (Diagenode) at the high power setting for 30 min with a 30-sec pulse followed by a 1.5-min cool down between cycles. The immunoprecipitation was conducted overnight at 4^{o}C with antibodies against acetylated H3, total H3, and p300. Approximately 10% of the total chromatin for one ChIP was saved as the input before the IP. The bound chromatin was collected with magnetic Protein G beads and washed. The cross-link was reversed in 0.54 M NaCl, and the DNA was then purified with a PCR clean up kit and re-dissolved in 50 μl H_2O. Each PCR reaction used 1 μl of the DNA.

Western blot. For the analysis of growth regulatory proteins, cells were treated with DMSO, 10 and 20 μM of C646, and 20 μM of C37 for 24 h. Whole cell lysate was then prepared by incubating cells in lysis buffer containing 50 mM Tris•Cl (pH 7.5), 250 mM NaCl, 5 mM EDTA and 0.1% IGEPAL, supplemented with protease inhibitors (Sigma #P8340) and PMSF, on ice

for ~20 min. Standard SDS-PAGE gel electrophoresis was used to separate the proteins, which were then transferred onto PVDF membranes. Ponceau S staining of the blots was done to verify complete transfer.

For the analysis of γH2AX, cells were treated for 24 h as above and the nuclei were first separated from the cytoplasm by incubating trypsinized cells with a buffer containing 10 mM Tris•Cl (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% IGEPAL for 10-15 min on ice. The nuclear fraction was then pelleted at 430xg and 4^{o}C for 5 min. The histones were extracted with 0.4 N sulfuric acid overnight at 4^{o}C, followed by precipitation with trichloroacetic acid, acetone washes, and resuspension in water.

Flow cytometry for annexin V/PI stained cells. Cells were treated with 20 μM C646 or DMSO for 5 days. Media and compounds were replaced every other day. Cells were collected on day 3 and day 5 for flow cytometry analysis. Approximately 10^5 cells per sample were stained with the annexin V/PI kit. Gating was done with cells stained with only one of the two fluorophores as well as un-stained cells. Early apoptotic cells were annexin V positive and PI negative. Late stage apoptotic cells, necrotic cells, or dead cells in general, were positive for both fluorophores.

SA-β-gal assay. Cells were plated in 12-well plates at a density of ~16,000 cells /well for the C646 treatment and ~4000 cells/well for the DMSO treatment. After 24 hours the cells were treated with 20 μM C646 or DMSO. C646 or DMSO was added every 24 h for 4 days. Cells were stain for β-galactosidase following the manufacturer protocol (#9860, Cell Signaling

Technology). In brief, the media were removed and the cells were washed with PBS. The cells were then fixed with 1X Fixative solution from the kit for 15 min at RT. After fixation, the cells were washed 2 X with PBS and stained with β-galactosidase Staining Solution for 48 h in a 37°C incubator without CO_2. Cells were observed under a light microscope and β-gal positive cells had a blue perinuclear staining pattern. Pictures were taken under an Olympus IX71 inverted wide-field epifluorescence microscope with a Zeiss Axiocam MRc5 color camera and Zeiss Axiovision imaging software.

PML immunofluorescence assay. WM35 and WM902B cells were plated on 2-well chamber slides at 1000 cells/well for the DMSO treatment and 4000 cells/well for the C646 treatment. The cells were treated with C646 (20 μM) or DMSO 24 h after plating. The media and the compounds were replaced every day for 4 days. Afterwards, the cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 10 min at 4°C. After washing with PBS briefly 3 times, the cells were permeabilized for 5 min at 4°C with 0.5% Triton X-100 in PBS. The cells were then washed in 3% bovine serum albumin (BSA) in PBS (BSA/PBS) and incubated with the anti-PML antibody (#sc-966, Santa Cruz Biotechnology) at 1:1000 in 3% BSA/PBS at 4°C for 4 h. Afterwards, the cells were washed 3 times in 3% BSA/PBS and incubated with the secondary antibody (1:2000 Alexa-Fluor 488 goat anti-rabbit) for 1 h at RT. Finally, the cells were rinsed 3 times with 0.5% BSA/PBS and once with PBS alone. The slides were mounted using VECTASHIELD Mounting Medium with DAPI (#H-1200, Vector Labs) and examined under 40× magnification using a Nikon Eclipse E400 microscope equipped with a RTKE SPOT digital camera (Diagnostic Instruments, Inc.). FITC and DAPI images were overlapped using the Advanced SPOT software.

C646/cisplatin combination treatment. WM35 cells were pre-treated with DMSO or C646 at 10 or 20 μM for 24 h, followed by a medium change and a 48 h recovery period. Then, the cells were treated with water or 20 μM cisplatin for an additional 48 h, collected, and fixed in 1% paraformaldehyde on ice. The cells were then washed in PBS, resuspended in 0.5 ml PBS plus 5 ml 70% ethanol, and stored at -20^{o}C overnight before the TUNEL assay. The assay was performed using the APO-BrdU TUNEL Assay Kit (Invitrogen A35125) following the manufacturer's protocol. After staining, the cells were incubated with the PI/RNase solution from the kit for 30 min before FACS analysis.

CCNA2 siRNA knockdown. WM35 cells were seeded in 6-well plates for 24 h before being transfected in duplicates. There were 4 conditions, including scrambled siRNA, siCCNA2 #1, siCCNA2 #2, and untransfected (medium only). Each transfection reaction was prepared as follows: (1) 7.5 μl siRNA was mixed with 192.5 μl OptiI-MEM Medium for 5 min at RT; (2) 5.0 μl lipofectamine was mixed with 195.0 μl OptiI-MEM Medium for 5 min at RT; (3) the two vials were combined and incubated for another 20 min at RT; (4) the siRNA/lipofectamine complex was then added to the cells drop wise. The total volume of the medium was 2 ml. The medium was changed 24 h after transfection and RNA was isolated after another 24 h using the RNeasy mini kit (#74104 Qiagen) according to the manufacturer's protocol.

To assess the efficiency of the knockdown, 2 μg of RNA was reverse transcribed with SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's protocol. qPCR analysis of CCNA2 expression was performed using SybrGreen at an annealing temperature of 57^{o}C for 45 cycles. Gene expression values were determined with the ΔΔCt method using β-actin as the internal control. The CCNA2 primers were as follows: (Forward) 5'-TGCATCTCTGGGCGTCTTTG-3'; (Reverse) 5'-ACCCGGCCAAAGAATAGTCG-3'.

The β-actin primers were as follows: (Forward) 5'-GCATTGTTACAGGAAGTCC-3'; (Reverse) 5'-CATTACATAATTTACACGAAGC-3'. To assess the effect of CCNA2 knockdown on cell proliferation, the MTT assay was performed on WM35 cells transfected with the siRNAs (CCNA2 siRNAs or scrambled siRNA) and the untransfected cells. The cells were seeded in triplicates in 24-well plates and analyzed after 96 h. Specifically, 50 μl of 5 mg/ml MTT was added to each well with a total medium volume of 500 μl, and the cells were incubated in the regular CO_2 incubator at 37^{o}C for 2 h. The medium was then carefully removed and the MTT solvent was added. The plate was covered with tinfoil and the cells were agitated on a shaker for 15-30 min. Absorbance was read at 590 nm with a reference filter at 620 nm.

Supplementary Figures

Figure S1. Stage one of NCI-60 screen on tumor cell sensitivity to 100 μM C646. For the methodology, refer to Shoemaker, 2006 and the NCI's Development Therapeutics Program website. The data are generated and graphed by the screening service. The numbers represent %cell growth relative to untreated control and to the cells seeded pre-treatment, which allows for detection of both growth inhibition and lethality. Negative numbers indicate cell death. The bar graph represents the difference between %growth of each cell line and the mean %growth of all cell lines. Note that the formula on the top of the graph is misprinted. It should state 'Growth Percent – Mean Growth Percent'.

Figure S2. Stage two of NCI-60 screen with 5 concentrations of C646. Each panel represents cell lines from one tumor type. Similar to stage one, each data point represents cell growth relative to control and starting number of cells.

Figure S3. Growth curve of the most C646-responsive cell line WM35 after p300 HAT inhibition as measured by the XTT assay. Cells were treated every other day with fresh media and compounds for 9 days.

Figure S4. Representative FACS histograms of cell cycle profiles after 24 h p300 HAT inhibition. Row 1, 2, 3, and 4 represent DMSO, 10 μM C646, 20 μM C646 and 20 μM C37, respectively. Each column corresponds to one cell line. M1: G1 phase; M2: S phase; M3: G2/M

phase. Note that there are 2 peaks after S phase in WM983B, which is most likely due to aneuploidy.

Figure S5. Summary of microarray results. (a) MvA plots of gene expression level comparison between C646 treated cells and DMSO treated cells at 6 and 24 h time points. Y-axes represent log2 difference of C646-DMSO for each gene. X-axes represent average of C646 and DMSO. Most genes do not show significant differences in expression and cluster near y=0. Data points above y=1 or under y=-1 indicate a greater than 2 fold (linear) change in expression. (b) Ontology analysis of genes upregulated by more than 2 fold at 24 h. The genes are grouped based on biological processes. p-values indicate the statistical significance of clustering. No statistically significant clustering was seen with the molecular functions GO database.

Figure S6. Western blots of additional growth regulatory proteins in WM35 cells after 24 h treatment with DMSO, C646 (10 and 20 μM) or C37. Expression of these proteins was not significantly affected by C646.

Figure S7. Cyclin A expression in non-small cell lung cancers after 24 h treatment with C646 or control. (a) Western blot analysis of cyclin A protein expression in H23, H838 and H1395 cells. Lane 1-4 represent DMSO, 10 μM C646, 20 μM C646 and 20 μM C37, respectively. (b) qRT-PCR analysis of cyclin A transcript level in the three cell lines. RQ: relative quantity normalized to DMSO; GAPDH is the internal gene control. N≥3.

Figure S8. Cyclin A expression in 6 glioblastoma cell lines after 24 h treatment with C646 or controls. Graphs represent qRT-PCRs of cyclin A transcript. Western blot data for each cell line are shown underneath each corresponding graph. Lanes match the 4 treatment conditions for PCR.

Supplementary Table

Table S3. Expression changes in genes associated with degrees of melanoma malignancy. The three categories of genes are taken from Ryu et al. Expression trend indicates the level of gene expression change as cells progress from less to more malignant stages. Note that most of the genes in the top two categories are repressed by more than 2 fold after p300 HAT inhibition in WM35 cells, whereas there is no significant change in the bottom category of genes.

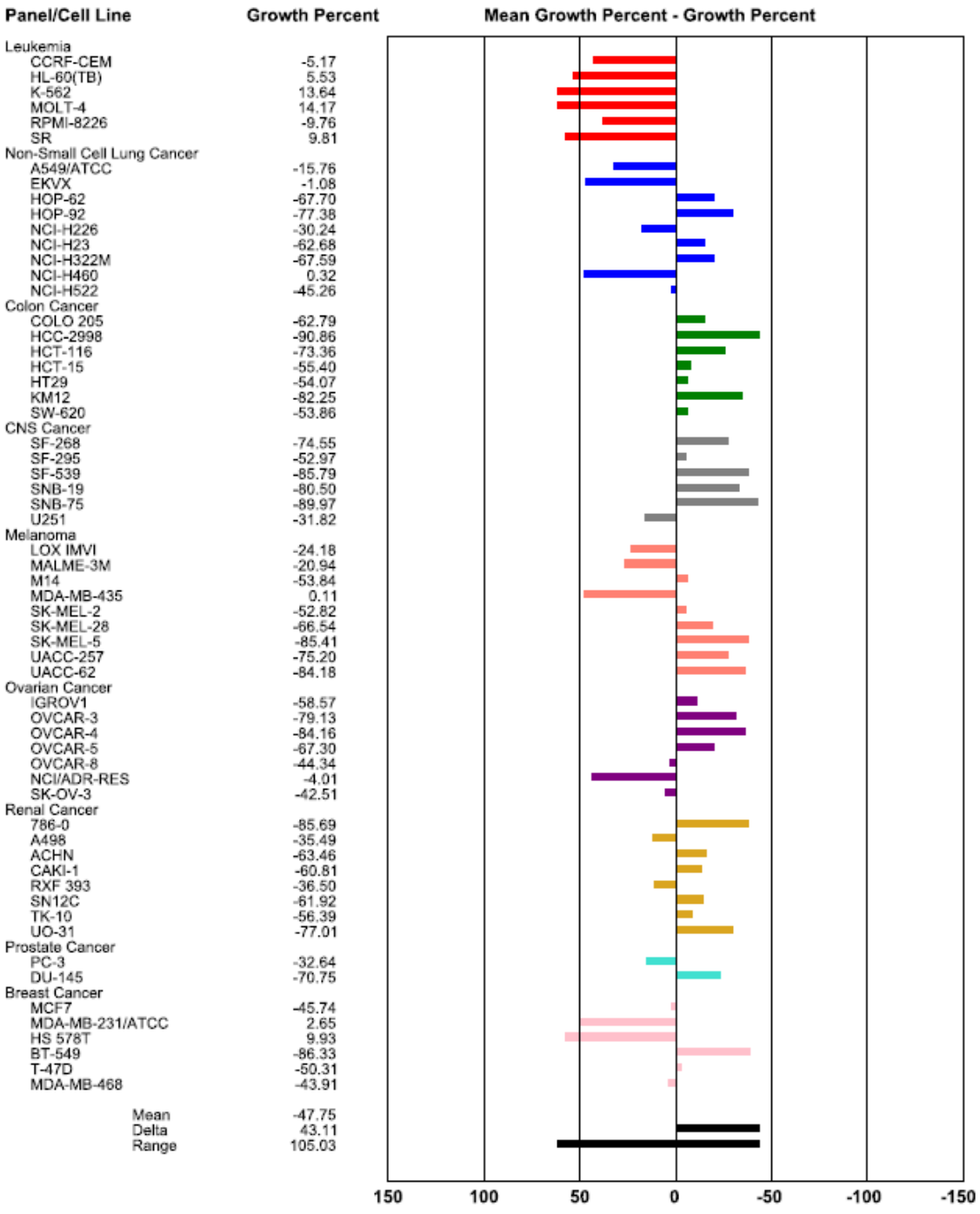
Panel/Cell Line
Growth Percent
Mean Growth Percent - Growth Percent
Leukemia
CCRF-CEM -5.17
HL-60(TB) 5.53
K-562 13.64
MOLT-4 14.17
RPMI-8226 -9.76
SR 9.81
Non-Small Cell Lung Cancer
A549/ATCC -15.76
EKVX -1.08
HOP-62 -67.70
HOP-92 -77.38
NCI-H226 -30.24
NCI-H23 -62.68
NCI-H322M -67.59
NCI-H460 0.32
NCI-H522 -45.26
Colon Cancer
COLO 205 -62.79
HCC-2998 -90.86
HCT-116 -73.36
HCT-15 -55.40
HT29 -54.07
KM12 -82.25
SW-620 -53.86
CNS Cancer
SF-268 -74.55
SF-295 -52.97
SF-539 -85.79
SNB-19 -80.50
SNB-75 -89.97
U251 -31.82
Melanoma
LOX IMVI -24.18
MALME-3M -20.94
M14 -53.84
MDA-MB-435 0.11
SK-MEL-2 -52.82
SK-MEL-28 -66.54
SK-MEL-5 -85.41
UACC-257 -75.20
UACC-62 -84.18
Ovarian Cancer
IGROV1 -58.57
OVCAR-3 -79.13
OVCAR-4 -84.16
OVCAR-5 -67.30
OVCAR-8 -44.34
NCI/ADR-RES -4.01
SK-OV-3 -42.51
Renal Cancer
786-0 -85.69
A498 -35.49
ACHN -63.46
CAKI-1 -60.81
RXF 393 -36.50
SN12C -61.92
TK-10 -56.39
UO-31 -77.01
Prostate Cancer
PC-3 -32.64
DU-145 -70.75
Breast Cancer
MCF7 -45.74
MDA-MB-231/ATCC 2.65
HS 578T 9.93
BT-549 -86.33
T-47D -50.31
MDA-MB-468 -43.91
Mean -47.75
Delta 43.11
Range 105.03
150
100
50
0
-50
-100
-150

Figure S1

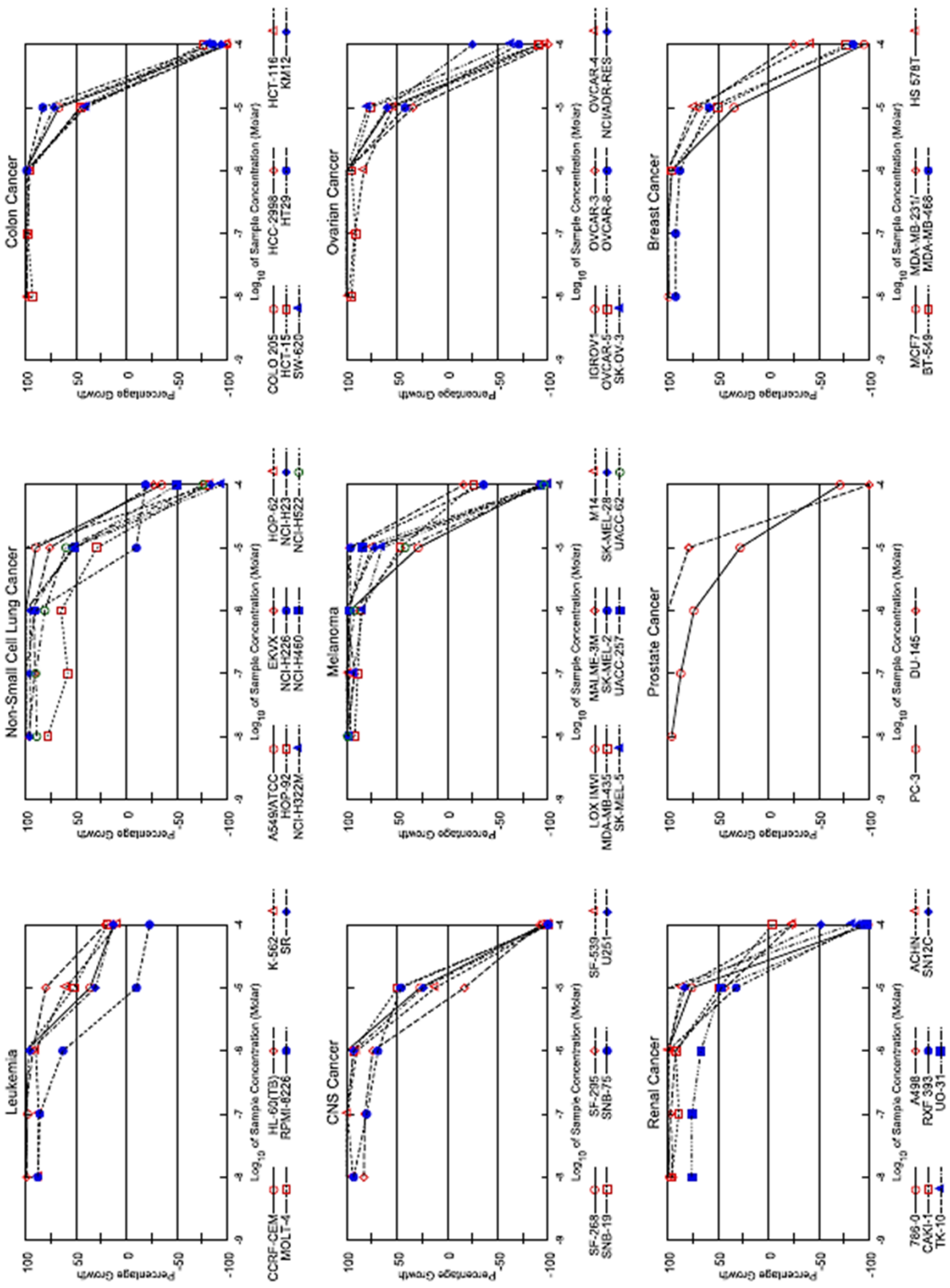
Leukemia
CCRF-CEM
HL-60(TB)
K-562
MOLT-4
RPMI-8226
SR
Non-Small Cell Lung Cancer
A549/ATCC
EKVX
HOP-62
HOP-92
NCI-H226
NCI-H23
NCI-H322M
NCI-H460
NCI-H522
Colon Cancer
COLO 205
HCC-2998
HCT-116
HCT-15
HT29
KM12
SW-620
CNS Cancer
SF-268
SF-295
SF-539
SNB-19
SNB-75
U251
Melanoma
LOX IMVI
MALME-3M
M14
MDA-MB-435
SK-MEL-2
SK-MEL-28
SK-MEL-5
UACC-257
UACC-62
Ovarian Cancer
IGROV1
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
NCI/ADR-RES
SK-OV-3
Renal Cancer
786-0
A498
ACHN
CAKI-1
RXF 393
SN12C
TK-10
UO-31
Prostate Cancer
PC-3
DU-145
Breast Cancer
MCF7
MDA-MB-231/
HS 578T
BT-549
MDA-MB-468
Percentage Growth
Log10 of Sample Concentration (Molar)

Figure S2

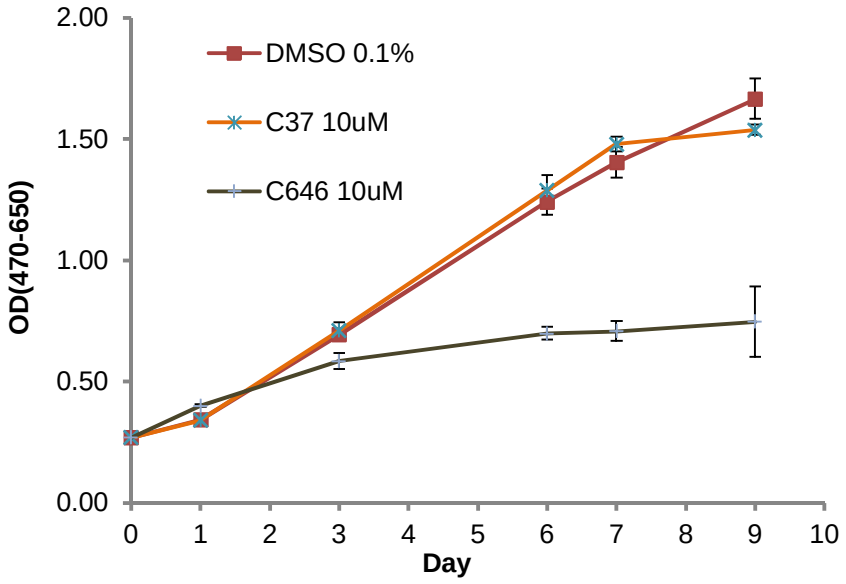
2.00
1.50
1.00
0.50
0.00
OD(470-650)
DMSO 0.1%
C37 10uM
C646 10uM
0
1
2
3
4
5
6
7
8
9
10
Day

Figure S3

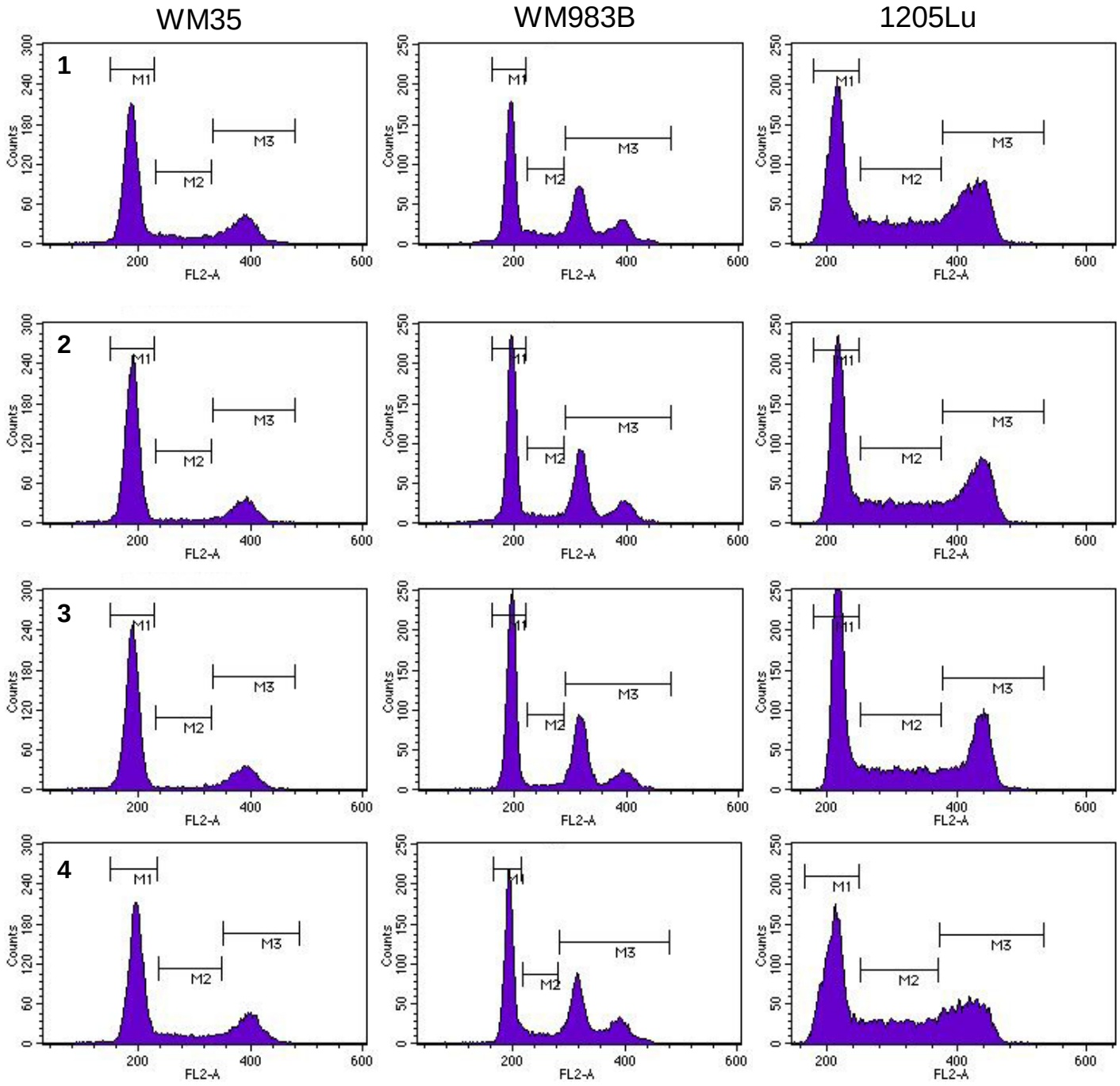

Figure S4

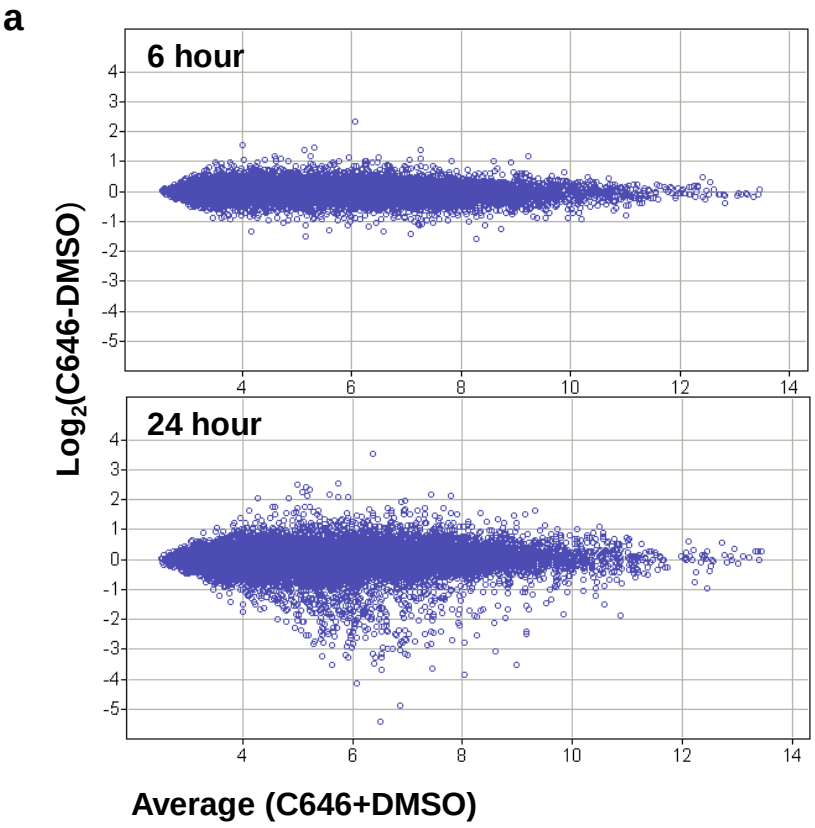
a
6 hour
24 hour
Log2(C646-DMSO)
Average (C646+DMSO)
b
p-value
1.0E-03
1.0E-02
1.0E-01
1.0E+00
Positive regulation of proteasomal ubiquitin-dependent protein catabolic process
Anti-apoptosis
Positive regulation of cell death

Figure S5

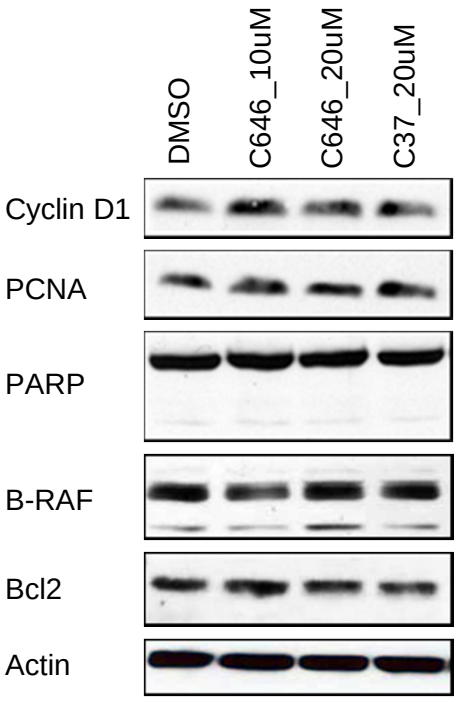
DMSO
C646_10uM
C646_20uM
C37_20uM
Cyclin D1
PCNA
PARP
B-RAF
Bcl2
Actin

Figure S6

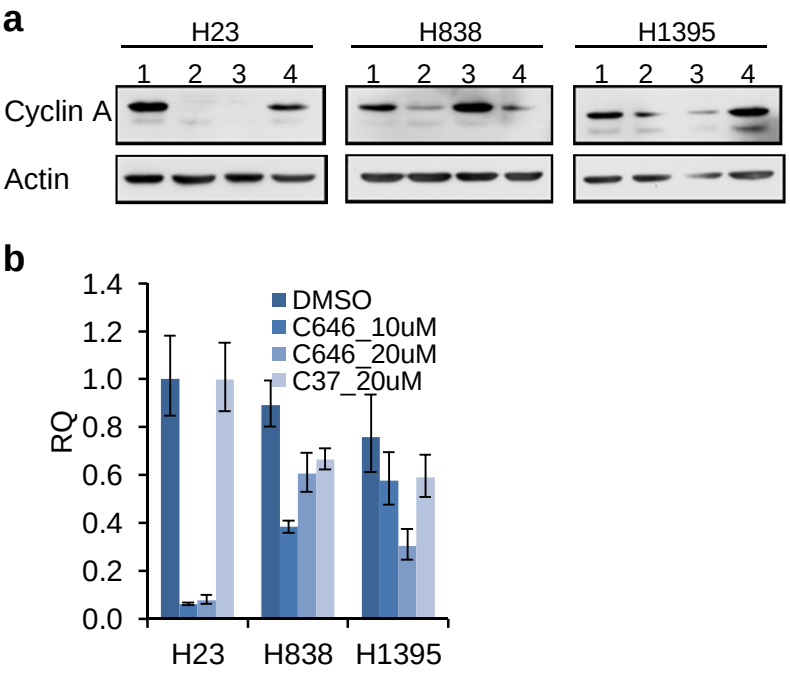
a
H23
H838
H1395
1 2 3 4
1 2 3 4
1 2 3 4
Cyclin A
Actin
b
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RQ
DMSO
C646_10uM
C646_20uM
C37_20uM
H23
H838
H1395

Figure S7

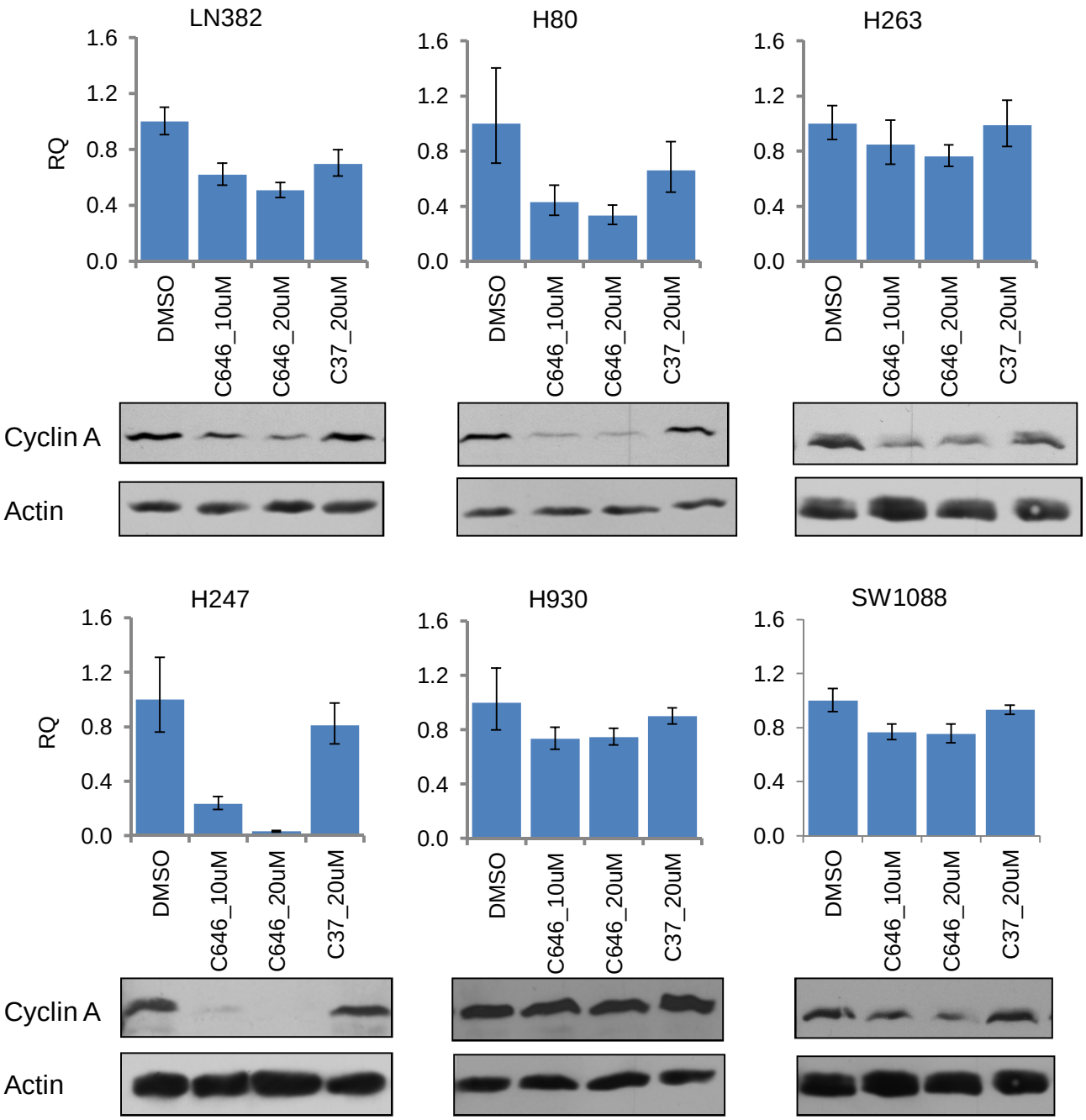
LN382
H80
H263
H247
H930
SW1088
RQ
1.6
1.2
0.8
0.4
0.0
DMSO
C646_10uM
C646_20uM
C37_20uM
Cyclin A
Actin

Figure S8

Table S1 Primers for real time quantitative PCR validation of microarray results.

Gene	Forward	Reverse
FAM111B	CAAATCCAGAGCGGCGGGCA	ACAGGTGTGTCAGCATGTGTCTGC
HIST1H2BB	TTCTCGCCTGGCTCACTACA	GCACAGCCGTCTGAATCTCC
UHRF1	AGACAAGCTGTTCGCGGCGA	TGGCCGTCCTCCATCTGTTTGC
CCNE2	GGTAGCTGGTCTGGCGAGGT	ATTCCGTCTGGCTGGGCTGG
CCNA2	GTCACCACATACTATGGACATG	AAGTTTTCCTCTCAGCACTGAC
DEPDC1	TGCCTAGCAAATTGGCCAAGAAGCA	ACTGTGATGTAGCCACAAACAACCA
XRCC2	GAATGGCGTTGGTGGCGGGA	AGTCGGGCAAGGAGCTCGGT
BRCA2	GCAGACCCAGCTTACCTTGAGGGTT	ACGCAACTTCCACACGGTTGTGA
RAD51	GGCGGGAATTCTGAAAGCCGC	TGCCATTACTCGGTCCGCAGCG
BLM	AGCACATTGTGTCAGTCAGTGGGG	GTGGCCGTAAGAGCCATCACCG
RPA2	AGTAGCCAGAGCGGCGCAGT	GCTGGGCTCGGGCTCTTGATTTC
TIMP3	AAAGGAGGGGCCCTTCGGCA	GACGCGACCTGTCAGCAGGT
TRIM38	TGCGAAGACGAGGGGCAGCT	GGAGCTTTTCCTTGTAGCCCTGGC
PRSS35	ACCAAACAAGCCTGGCAGGACA	TGCCTCAAATGCGGGGCTGG
MIR34A	GCCAGCTGTGAGTGTTTCTTTGGC	GCCCCACAACGTGCAGCACT
TP53	TGCCTTCCGGGTCACTGCCAT	CGGCAAGGGGGACAGAACGTT
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT

Table S2 Primers for real time quantitative PCR analysis of ChIP DNA.

Gene	Forward	Reverse
BLM	GTGTGCCCACTTTCCCGGTTCA	CCAGGCGTCTGCACCCCATT
RPA2	AGGGGCTGGCAGAGCGGTAT	TGACGGCAGAAGTCGCGCAC
RAD51	CAAGCTCTCGAGCTCCCGTCTTG	GCCCGCGTCGACGTAACGTATC
XRCC2	ACCGGCGGCCTTGTTCCCATCT	TCGGGGCGATGTGTAGTGCCT
BRCA2	AGGAAACGGGCTCGGAGGTCT	TGGTAGTGGGCGGGGCTGTTA
CCNE2	GGAACCCATGACCCCCAGTCGT	GGTGAGGAGTTGCTGCGCGTA
CCNA2	GGAGAAACAAACTGGCTGGGGCG	TCGCTCACTAGGTGGCTCAGCTT
TP53	TGAAAATACACGGAGCCGAGAGCC	CGTGCTTTCCACGACGGTGACA
GAPDH	AGGTCGGAGTCAACGGGTGAGT	CAGCTACCCTGCCCCCATACGA

Table S3 Expression changes in genes associated with degrees of melanoma malignancy

Gene	Expression trend in melanoma	Fold Change in WM35 after C646
Activators of cell cycle progression, DNA replication and repair		
CDCA2		-6.65
NCAPH		-5.61
NCAPG		-7.88
NCAPG2		-3.08
PBK		-4.50
NUSAP1		-5.76
BIRC5		-2.52
ESCO2	Up	-3.60
HELLS		-4.93
MELK		-5.17
GINS1		-3.49
GINS4		-1.80
RAD54L		-3.34
TYMS		-2.00
DHFR		-2.45
Apoptosis resistance		
BIRC5	Up	-2.52
Cellular adhesion and melanocyte differentiation		
CDH3		1.20
CDH1		-1.34
KIT		-1.03
PAX3		1.20
CITED1	Down	1.34
TYR		-1.55
MLANA		1.32
MC1R		1.11
OCA2		1.25