Supplemental Material

MYC acetylated lysine residues drive oncogenic cell transformation and regulate select genetic programs for cell adhesion-independent growth and survival.

Matthew Hurd[1#], Jeffrey Pino[1#], Kay Jang[1], Michael M. Allevato[1], Marina Vorontchikhina[1], Wataru Ichikawa[1], Yifan Zhao[1], Ryan Gates[1], Emily Villalpando[1], Michael Hamilton[1], Francesco Faiola[1], Songqin Pan[2,3], Yue Qi[4], Yu-Wen Hung[4, 5], Thomas Girke[2,3], David Ann[4, 5], Victoria Seewaldt[5, 6], and Ernest Martinez[1,2] *

[1] Department of Biochemistry, [2] Institute for Integrative Genome Biology, [3] Department of Botany and Plant Sciences, University of California Riverside, 900 University Ave., Riverside, CA 92521, USA.
[4] Department of Diabetes Complications and Metabolism, [5] Irell and Manella Graduate School of Biological Sciences, [6] Department of Population Sciences, Beckman Research Institute, Comprehensive Cancer Center, City of Hope, Duarte, CA 91010, USA.

Supplemental material includes:

Supplemental Material and Methods

Supplemental References

Supplemental Figures (S1-S19)

Supplemental Table S1: excel spreadsheet of edgeR DEGs in Rat1a cells.

Supplemental Table S2: excel spreadsheet of edgeR DEGs in MCF10A cells.

Supplemental Material and Methods

DNA Plasmids

The pCbs expression vectors for Flag-tagged mouse and human MYC (pCbs-Flag-mMYC and pCbS-Flag-hMYC) WT and R-mutants were created with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) and verified by DNA sequencing; the pCbs vector and pCBS-Flag-mMYC 5R-mutant were described previously (McMahon et al., 1998; Faiola et al., 2005). The expression vector for HA-tagged human Max p22, pCbS-HA-Max was described previously (Faiola et al., 2007). Retroviral pMIG-Flag-mMYC WT, R-mutants and Q-mutants expression vectors were generated by digestion with Bam HI of pCbs-Flag-mMYC (WT or mutants) plasmids and cloning of the recovered Bam HI Flag-MYC DNA fragment into the Bgl II site of pMSCV-IRES-GFP (pMIG) retroviral vector (Addgene plasmid 9044, provided by Dr. William Hahn). All constructs were verified by DNA sequencing. The pBABE-puro vector (Addgene plasmid 1764) was a gift from Dr. Sergio Nasi. The retroviral packaging vector pCL-Eco (Naviaux et al., 1996) was obtained from Dr. Inder Verma and J.Michael Bishop. The expression vectors for mouse GCN5 (pcDNA3.1-mGCN5) and mouse GCN5 HAT mutant (pcDNA3.1-mGCN5[GYG→AYA/582Y584]) were previously described (Liu et al., 2003). The human p300 expression vectors pcDNA3.1-p300 (Chen et al., 2002) and pCMVβ-p300-CHA (Eckner et al., 1994) were gifts from, respectively, Dr. Warner Greene and Dr. Richard Goodman.

Cell lines and culture conditions

Human embryonic kidney 293 (HEK293) and HeLa cells were obtained from Dr. Frances M. Sladek. The Rat1a cell line was a gift from Dr. Michael Cole. The human mammary MCF10A and MDA-MB-231 cell lines were purchased from ATCC. The 293FT cell line was purchased from Invitrogen. The P493-6 cell line was provided by Drs. Bruno Amati and Dirk Eick. The Rat1a, HEK293, HeLa, and MDA-MB-231 cell lines were maintained in high glucose-containing Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The 293FT cell line was maintained in the same DMEM with 10%FBS medium containing 0.5mg/ml G418. The MCF10A cells were cultured in DMEM/F12 (Thermo Fisher Scientific, 11330-032) medium supplemented with 20 ng/mL EGF (Sigma), 10 μg/mL insulin (Sigma), 0.5 μg/mL hydrocortisone (Sigma), 100 ng/mL cholera toxin (Sigma), and 5% horse serum (Sigma). The P493-6 cell line was maintained in Roswell Park Memorial Institute (RPMI) medium

supplemented with 10% FBS; where indicated 0.2 μg/mL of doxycycline was added to the RPMI medium (for 12h) to inhibit MYC expression (Pajic et al., 2000). All cell lines were cultured at 37ºC in a humidified incubator with 5% CO_2. Where indicated, cells were incubated with either 2.5 μM or 30 μM C646 (Millipore), or 100 μg/mL cycloheximide (MP Biomedicals) for the indicated times, or with 20 μM MG132 (Millipore) for 2 h before lysis.

Generation of MYC-overexpressing cell lines

To generate stable Rat1a-MYC cell lines, 80% confluent Rat1a cells in 10-cm plates were transfected using Lipofectamine 2000 (Invitrogen) and 1.5 μg pBABE-puro (for antibiotic selection) and 15 μg of either pMIG-Flag-mMYC wild type (WT), pMIG-Flag-mMYC K149R (R149), pMIG-Flag-mMYC K158R (R158), pMIG-Flag-mMYC K323R (R323) or pMIG-empty vector (E). After 48 hours, the cells were re-plated into 15-cm plates, and 24 hours later selected in medium containing 5 μg/mL puromycin. The selection medium (DMEM + 10% FBS + 1% penicillin-streptomycin solution + 0.1% gentamicin sulfate + 5 μg/mL puromycin) was changed every 48 hours. All resistant clones obtained were pooled, and after 4 weeks in selection medium, the cells were further selected for GFP expression (encoded by the MYC-IRES-GFP bicistronic mRNA) by fluorescence-activated cell sorting (FACS). Cells were trypsinized and resuspended in filter-sterilized FACS sorting buffer (1X phosphate buffered saline (PBS), 1mM EDTA, 25mM HEPES pH 7.0, and 1% heat-inactivated fetal bovine serum). FACS sorting was performed with a BD Biosciences FACSAria III cell sorter at the UCR Genomics core facility. An independent transfection/selection procedure, as above, generated a second set of stable Rat1a-MYC polyclonal cell lines (Rat1a-E, Rat1a-MYC WT, R149, R158 and R323), which allowed confirmation of results, as described in the text.

Transiently transduced Rat1a-MYC cells were obtained with retroviral particles produced in 293FT cells. The 293FT cells were transfected in 6-well plates with Lipofectamine 2000 (Invitrogen, 11668019) and 2-3 μg pCL-Eco and 2-3 μg pMIG-Flag-mMYC (WT or mutants) or empty vector. After 48h, supernatant medium containing viral particles was collected and filtered using a 0.45 μM filter (Fisher Scientific). Rat1a cells at 60% confluency were transduced in 6-well plates with 1.0 mL of diluted viral supernatant (2 mL filtered viral supernatant diluted with 3.0 mL fresh DMEM/10%FBS) containing 5 μg/ml polybrene (Millipore). After 48 h, the transduced cells (~95% GFP-positive) were either directly used for serum-starvation and apoptosis analyses or further propagated for spheroid formation assays.

The polyclonal MCF10A-MYC cell lines were obtained by transduction of MCF10A cells with retroviral particles encoding pMIG-empty (E), pMIG-Flag-mMYC (WT), or the different pMIG-Flag-mMYC R-mutants (R149, R158 and R323) essentially as described above with the following modifications. MCF10A cells in 6-well plates were first treated with complete growth medium containing an Ecotropic Receptor Booster [Takara; Cat# 631471 (10μL)] for 4 hours, as recommended by the manufacturer, and then transduced with 1.5 mL of filtered retroviral medium containing 5 μg/ml polybrene. MCF10A cells were incubated at 37ºC with 5% CO_2 for 48 hours and the media was replaced with fresh complete growth medium. Cells were then seeded at low density in 15cm plates and nine GFP-expressing clones for each cell line were selected, expanded, and equal numbers of cells from each clonal line were pooled together to generate the polyclonal cell lines. Phase contrast and GFP/fluorescence microscopy images were obtained with a Keyence BZ-X710 at 20X magnification.

RNA interference and MYC stability assays

HeLa cells (80% confluent) in 10-cm plates were transiently transfected with Lipofectamine 2000 and 7.5 μg of either pCbS-Flag-mMyc WT or the indicated R-mutants and with 400 pmol of a specific p300 siRNA (Grönroos et al., 2002). A control siRNA (Dharmacon) was used as a negative control. After 24h the cells were transfected again as above; 48h after the first transfection the cells were collected for anti-FLAG (M2) immunoprecipitation and Western blot analyses. For MYC protein stability assays, cells at 80% confluence were treated for the indicated times with 100 μg/mL cycloheximide (MP Biomedicals) with or without HDAC inhibitors, as indicated, before cell lysis and protein analysis by immunoprecipitation and western blot with the indicated antibodies.

Cell extracts, immunoprecipitation, western blot, and mass spectrometry analyses

Preparation of whole cell extracts (WCE) and immunoprecipitation (IP) of endogenous MYC or ectopic Flag-tagged MYC proteins and western blot analyses were performed with the indicated antibodies essentially as previously described (Faiola et al., 2005). For IP of endogenous MYC or ectopic Flag-MYC in human mammary MDA-MB-231 and MCF10A cell lines, the cells in 15 cm plates were scraped in cold PBS and lysed with 200 μl modified E1A lysis buffer (150mM NaCl, 1% IGEPAL CA-630, 50mM HEPES pH 7.9 at 4ºC, 0.2 mM EDTA, 2 mM 2-Mercaptoethanol, 0.2 mM PMSF) supplemented with 1% SDS and inhibitors of proteases and HDACs (see below). Cell lysates were heated at 95ºC for 5 min, treated with 250U Benzonase

(Sigma) for 15 min at 37°C, and re-heated at 95°C for 5 min. The cell lysates were then diluted with 4 volumes of E1A lysis buffer (without SDS) and immunoprecipitated with 5 - 10 µg of MYC C-33 or AcK antibodies (or 20 µL of FLAG M2-agarose beads) and analyzed by western blot with the indicated antibodies, as above. Relative quantitation of the western blot signals was performed by using NIH ImageJ software. Alternatively, endogenous MYC:MAX complex was immunoprecipitated with MAX C-17 antibody from WCE of HeLa cells treated with MG132 for 2h, proteins were resolved by SDS-PAGE and the MYC protein band was in-gel trypsin digested and analyzed by LC-MS/MS as previously described (Faiola et al., 2005).

For nuclear/cytoplasmic fractionation, cells were lysed with hypotonic lysis buffer (50 mM HEPES pH 7.9, at 4°C, 20 mM NaCl, 0.1% IGEPAL CA-630, 0.2 mM EDTA, 0.2 mM PMSF, 2 mM 2-mercaptoethanol) on ice for 10 min with occasional mixing and then the lysate was spun in a microfuge for 1min at 5000rpm at 4°C. After removing the cytoplasmic supernatant, the nuclear pellet was resuspended in nuclear extraction buffer (50 mM HEPES pH 7.9, at 4°C, 400 mM NaCl, 20% glycerol, 0.1% IGEPAL CA-630, 0.2 mM EDTA, 0.2 mM PMSF, 2 mM 2-mercaptoethanol), incubated on ice for 30 min with occasional mixing and then centrifuged in a microfuge at 13,000 rpm for 10 min at 4°C to recover the supernatant soluble nuclear extract. Unless otherwise indicated, the HDAC inhibitors (HDACi) 10 mM sodium butyrate (Alfa Aesar), 10 mM nicotinamide (Sigma), and 2 µM trichostatin A (TCI) were added to the cell culture medium 2 h before cell lysis and to all cell lysis/extraction buffers, which also included protease inhibitors (Pierce™ Protease Inhibitor Mini Tablets, Thermo Fisher Scientific).

Chromatin immunoprecipitation (ChIP)

MCF10A-Empty and MCF10A-MYC WT, or R-mutant cell lines at 95% confluence in 15-cm plates were cross-linked 10 min with 1% formaldehyde. The crosslinking was stopped by incubating cells for 5 min in 125mM Glycine in final concentration. Cells were then washed with cold 1X PBS three times and collected in 1.5ml Eppendorf tubes. Cell pellets were resuspended in 1ml of cell lysis buffer (50mM HEPES-KOH pH 7.5, 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% triton X-100) with protease inhibitors tablet (Pierce, thermos scientific, A32955) and gently mixed at 4°C for 10 min. Cell lysate (nuclei) were centrifuged at 1,300g for 5 mins at 4°C and nuclei were resuspended with 1ml of TNEE-200 buffer (10mM Tris pH8, 200mM NaCl, 1mM EDTA, 0.5mM EGTA) containing protease inhibitors (tablet) and gently mixed 10 min at 4°C. Nuclei were spun down and resuspended in 1ml sonication buffer (100mM Tris pH8, 0.3% SDS 1.7% Triton X-100, 5mM EDTA) supplemented with protease inhibitors

and sonicated with a Covaris S220 sonicator (average 52.5Watts; peak incident: 175 Watts; duty factor: 30%; cycle: 200; duration: 160s) to obtain DNA fragments of 200-500bp. Chromatin was centrifuged at 16,000 g for 10 min at 4°C. Soluble chromatin was collected and diluted 1:5 ratio with IP-dilution buffer (50mM Tris pH8, 167mM NaCl, 1.1% triton X-100, 0.11% sodium deoxycholate) containing protease inhibitors.

For the Flag ChIP experiment, diluted soluble chromatin was incubated with either anti-Flag magnetic beads (Pierce, thermos scientific, A36797) or mouse IgG (Santa Cruz biotechnology) at 4^{o}C overnight. For the RNAPII ChIP experiments, 1ug of anti-Pol II (AB300575, Abcam), anti-pS2/RNAPII (AB238146, Abcam), or IgG rabbit serum (sc-2027, Santa Cruz biotechnology) was pre-bound with 20ul slurry of protein A/G magnetic beads (Pierce, Thermo Scientific, #88802) at 4^{o}C for at least 6 hours. Diluted soluble chromatin was incubated with pre-bound antibodies and beads at 4^{o}C overnight. Beads were washed sequentially twice with 1ml each of RIPA-150 buffer (50mM Tris pH8.0, 150mM NaCl, 1mM EDTA, 0.1% SDS, 1% TritonX-100, 0.1% Sodium deoxycholate) with protease inhibitors and with 1 ml RIPA-500 (50mM Tris pH8.0, 500mM NaCl, 1mM EDTA, 0.1% SDS, 1% TritonX-100, 0.1% Sodium deoxycholate) with protease inhibitors tablet twice; and further washed once with 1 ml each of RIPA-LiCl buffer (50mM Tris pH8.0, 250mM LiCl, 1mM EDTA, 1% NP-40, 0.7% Sodium deoxycholate) with protease inhibitors and TE buffer at 4^{o}C for 5 mins. Protein-DNA complexes were eluted and reverse cross-linked with SDS elution buffer (100mM NaHCO3, 1% SDS) and 0.3M NaCl final concentration at 65^{o}C overnight. DNA was purified with the PCR purification kit (PureLink Invitrogen, Thermo Scientific, K31001) and aliquots were analyzed by real-time qPCR along with an Input control with the following qPCR primers:

Chr6	Forward 5’-GGCATTGTCCTAATACTTCAGTGAT-3’	Reverse 5’- CATTGACGAGGGAAACGCAC-3’
SNAI1 prom	Forward 5’- CTGCTTTGCAAAAAGGCCGT-3’	Reverse 5’- CATTGACGAGGGAAACGCAC-3’
NOTCH3 prom	Forward 5- CAAGGCATGTACCCACGAGT-3’	Reverse 5’- TAGGGATGGCGATGTTGGAC-3’
TERT prom	Forward 5’-AGTGGATTCGCGGGCACAGA-3’	Reverse 5’-GCGGCGCGAGTTTCAG-3’
HK2 prom	Forward 5’-CCCTTTTTCCCTGTTACTGGAG-3’	Reverse 5’-ATTCTCTCCACGTGCTCTCG-3’
ESPN prom	Forward 5’-GACCTCAGCACGTCCAGC-3’	Reverse 5’-CGCAAGAACACGTGCATGG-3’
SLC05A1 prom	Forward 5’-CTATTGACTGAGCGCACCCC-3’	Reverse 5’-CGAGAGAGGTTCCAGTACGC-3’
KAT2A prom	Forward 5’-CCTGGGAAGGTTCCGCC-3’	Reverse 5’-GCGCGGGAAGACTACAACTC-3’
CLIC2 prom	Forward 5’-AAACTGCCTGGCTAGAAGGG-3’	Reverse 5’-GCATGTGTGTGTTTCTGTGCA-3’
DDB1 prom	Forward 5’-AGAAAGAGGGACACAAGCGA-3’	Reverse 5’-TGAGGCCGTCCCGTGAT-3’
SNAI1 3’UTR	Forward 5- TTCCCATGGCCATTTCTGTG-3’	Reverse 5’- ACAAAAACCCACGCAGACAG-3’
NOTCH3 3’UTR	Forward 5’- TGTTCCATAGCCTTGCTGGG-3’	Reverse 5’- CAGCCCCCTAGTTCCCAAAG-3’
TERT 3’UTR	Forward 5’-ATTCGCCATTGTTCACCCCT-3’	Reverse 5’-AGGGTCCTTCTCAGGGTCTC-3’

Immunofluorescence

Rat1a cell lines were plated in glass bottom 6 well plates. After 16 hours the cells were washed three times with PBS and fixed with 4% paraformaldehyde (Fisher Scientific) for 15 minutes. The cells were washed 3 times with PBS and permeabilized with 0.1% Triton X-100 (EMD Chemicals Inc.) in PBS for 10 minutes followed by 3 washes with PBS. The cells were blocked with 5% normal goat serum (Invitrogen) and 0.3% Triton X-100 in PBS for 10 minutes and then incubated overnight at 4^{o}C with MYC N-262x antibody diluted 1:200 in 1% BSA and 0.3% Triton X-100 in 1X PBS. The following day the cells were washed 3 times, 5 minutes each, with PBS and then incubated 2 hours in the dark at room temperature with Alexa Fluor ® 594 goat anti-rabbit IgG (Life Technologies) diluted 1:500 (as above). The cells were then washed 3 times, 5 minutes each, with PBS. VECTASHIELD ® Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA, USA) was then applied and cells were covered with coverslips. The cells were analyzed with an ECLIPSE TI inverted fluorescence microscope (Nikon Instruments) at 20X magnification. The images were obtained using NIS-Elements AR 4.13 software at the UCR Stem Cell core facility.

Colony formation assay

MCF10A cell lines were seeded (in triplicate) at a density of 1000 cells per well in 6-well plates in complete growth medium and were incubated for seven days at 37^{o}C with 5% CO_2 with medium change every three days. After seven days, the colonies were stained with Crystal Violet (0.5%) and de-stained with 1.5 mL of methanol for 30 minutes (on an orbital shaker) and the absorbance (A590nm) was measure by spectrophotometry.

Adherent cell proliferation assays

For logarithmic cell growth, cell lines were seeded (in triplicate for each time point) at low density (1.0 x 10^6 Rat1a cells/well of 6-well plate; 0.5 x 10^4 MCF10A cells/well of 24-well plate) in complete growth medium and were incubated at 37^{o}C with 5% CO_2. Every 24 hours, a plate was trypsinized and cells were stained with Trypan Blue (Corning) and counted with a hemacytometer (or with a Nexcelom Bioscience Cellometer Mini). After 3 days, the remaining plates were replenished with fresh medium.

For post-confluency cell proliferation and contact inhibition analysis, cell lines were plated (in triplicates) at a high density so that MYC-overexpressing cells reached confluency after 16-20h and then were counted every 24h, as above. For p300 inhibition analyses, the

indicated MCF10A cell lines were seeded at high density (1.0 x 10^5 cells per well in 24-well plates) in complete growth medium. After 20 hours, 2.5 μM C646 (Millipore, 382113), or its solvent (DMSO), was added and cells were counted every 24 hours, as above.

Cell death and apoptosis assays

Rat1a cell lines were plated at high density in regular medium (in triplicates) to reach confluency after over-night incubation (16 hours) prior to the start of serum starvation. Cells were washed with PBS and then culture medium containing reduced serum (0.1% FBS) was added (time 0) and the cells were maintained in medium with reduced serum for the indicated times (0-24h). At each time point, both floating and attached cells were collected, stained with Trypan Blue solution (Corning) and counted with a Nexcelom Bioscience Cellometer Mini. The percent cell viability was averaged from 3 independent experiments, each in triplicates. To analyze apoptosis, the stable cell lines (or transiently transduced cells 48 h after viral infection) were serum-starved for 21-24h as above and cleaved caspase-3 was detected by western blot. Alternatively, cells were serum-starved for 12 h as above and analyzed by flow cytometry with the Annexin V-FITC Apoptosis Detection Kit (Calbiochem/Millipore-Sigma) according to the manufacturer's instructions. Flow cytometry was performed with a FACSAria (BD Biosciences) at UC Riverside genomics core facility.

Spheroid growth assays

For spontaneous spheroid formation, 6-well plates coated with agarose were prepared by adding 5 mL of sterile 1% agarose (KSE Scientific, BMKA1705) to each well to cover the bottom and sides of each well and then quickly removing most of the agarose to leave a thin layer to solidify at room temperature for 30 minutes. Cell lines were then seeded in triplicates (30,000 cells/well) in 2 mL regular growth medium (or in medium containing 0.5% methylcellulose). The cells were left at 37°C with 5% CO_2 for 7 days without further feeding or were fed on day 3 and day 6 by addition of 500 μL fresh medium, as indicated. The images of spheroids were obtained with a Leica DMi8 microscope at 50X total magnification. The spheroids were collected spun at 200 RCF for 3 minutes and the cells were trypsinized and counted with a Cellometer Mini cell counter (Nexcelom bioscience) or were lysed with 100 μL of ice-cold RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.0% NP-40/IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) for 10 minutes on ice with occasional mixing. The lysate was spun in a

microfuge at 13,000rpm for 5 minutes at 4°C and the supernatant was analyzed by western blot.

Facilitated spheroid formation assays were performed in 96-well non-adherent U-bottom plates (Nunclon™ Sphera™ 96-Well, Nunclon Sphera-Treated, U-Shaped-Bottom Microplate; Thermo Scientific) by seeding 100 cells/well of each Rat1a cell line in triplicates with 100 µL of regular growth medium. Plates were then spun down at 100 RCF for 3 minutes to gather the cells at the bottom of the wells and incubated at 37°C with 5% CO_2 for the indicated times. Images were taken every 24 hours using a Leica DMi8 microscope with an Andor Technologies Zyla 4.2 sCMOS camera at 50X total magnification.

Soft agar colony formation assays

Soft agar assays were performed in triplicates in 6-well plates. The bottom of each well was first coated with warm 1.5 mL of 0.5% Nobel Agar (Sigma-Aldrich, A5431) in complete growth medium. After solidification of the base layer, 1.5 mL of a cell suspension made with 0.35% Noble Agar in complete growth medium was added on top of the base agar layer (5×10^3 Rat1a cells/well; 6×10^4 MCF10A cells/well). Plates were incubated for about 3 weeks at 37°C in a humidified incubator with 5%CO_2 with addition every other day of 100 µL fresh complete growth medium to the top agar layer. Colonies were stained with 0.005% Crystal Violet, briefly destained with water, and imaged with a Bio-Rad ChemiDoc imager. Colonies larger than or equal to 200 µm (Rat1a) or 100 µm (MCF10A) were counted using NIH ImageJ software.

Mouse xenografts

Athymic nude male mice (Crl:NU(NCr)-Foxn1nu; strain code 490, homozygous, 6-week-old) were purchased from Charles River Laboratories (San Diego, CA, USA) and kept in a barrier vivarium facility at UCR, certified for Animal Welfare Assurance by the NIH Office of Laboratory Animal Welfare (OLAW) and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The Rat1a cells were verified pathogen-free before injection (IDEXX BioAnalytics). At 7-week-old the mice were anesthetized with isoflurane and injected in the right rear flank with 5.0×10^6 cells (harvested from healthy log proliferating cultures) in cold PBS containing 25% Matrigel ® (Corning) with a 1 mL syringe fitted to a 27-gauge sterile needle. The mice were continuously monitored, tumor dimensions - length (L) and height (H) - were measured using digital calipers, and tumor volumes were calculated using a modified ellipsoidal formula [$1/2(L \times H^2)$] (Euhus et al., 1986; Tomayko and

Reynolds,1989). At experimental endpoint mice were euthanized with carbon dioxide (CO_2) and tumors were dissected, photographed, and stored at -20^{o}C in RNAlater® (Sigma-Aldrich). All procedures were approved by the UCR Institutional Animal Care and Use Committee (IACUC).

Metabolic analyses

The Seahorse Bioscience XF96 Extracellular Flux Analyzer (Agilent) was used to measure glycolysis (Glycolysis Stress Test assay) and mitochondrial function (Mito Stress Test assay), as recommended by the manufacturer. Briefly, the day before the assays, sensor cartridges were hydrated overnight at 37°C. The experimental cell lines were sub-cultured and seeded to a density of 40,000 cells per well onto 96 well Seahorse XF96 Cell Culture Microplates (Agilent, 101085-004). On the day of the assay, the culture medium was replaced with Agilent Seahorse XF Assay Medium (175 µL) (Agilent; 102365) supplemented with glucose (25 mM) and pyruvate (1 mM) for measurement of oxygen consumption rate (OCR), or with Glutamine (4 mM) for measurement of extracellular medium acidification (ECAR) and incubated for 1 h before injection of nutrients or inhibitors at pre-optimized conditions for each cell line. During all the Seahorse analyses the cells were maintained at 37^{o}C with 5% CO2 and approximately 20% O_2.

For the Glycolysis Stress Test/ECAR assay, sequential injections of nutrients/inhibitors included for MCF10A cell lines: 25 mM Glucose, 1 µM Oligomycin, and 50 mM 2-deoxyglucose (2-DG), final concentrations; and for Rat1a cell lines: 10 mM Glucose, 1 µM Oligomycin, and 50 mM 2-DG. At the end of the recording period, OCR and ECAR values were normalized to cell numbers. Parameters were calculated according to the manufacturer's user guidelines (Agilent). The data were plotted as the mean ± s.e.m (MCF10A) or ± S.D. (Rat1a) for n=5-8 repeats.

For the Mito Stress Test/OCR assay, the following inhibitors were injected sequentially Oligomycin (ATP synthase inhibitor), carbonyl cyanide-p-trifluoromethoxy phenylhydrazone (FCCP; potent uncoupler of oxidative phosphorylation), and rotenone (mitochondrial complex I inhibitor). The final concentrations were: 1 µM oligomycin, 0.5 µM FCCP, and 2.5 µM rotenone (for both Rat1a and MCF10A cell lines). The following parameters were calculated: Basal respiration = the OCR measurement prior to oligomycin injection - the measurement after rotenone injection; Maximum respiration = the highest OCR measurement after FCCP injection - the measurement after rotenone injection.

For determination of mitochondrial fuel dependencies, Mito Fuel Flex Test assays were used to measure the oxidization of glucose, glutamine, and fatty acids (FA) in mitochondria using the Seahorse XF Mito Fuel Flex Test Kit (Agilent Technologies, 103260) according to the manufacturer's manual. Cells ($5x10^4$ per well) were seeded on Seahorse XF96 Cell Culture Microplates (Agilent, 101085-004) in cell growth medium overnight. On the day of the assay the cells were equilibrated in XF Base Medium (Agilent, 102353) supplemented with glucose (25 mM), sodium pyruvate (1 mM), and L-glutamine (2 mM). For this assay, BPTES (4 μM), Etomoxir (10 μM) or UK5099 (5 μM) were used to inhibit glutamine, FA or glucose oxidation. To calculate fuel dependence percentage, or the cell's reliance on a particular fuel pathway, this equation was used: (basal OCR – OCR after treatment with target inhibitor)/(basal OCR – OCR after treatment with all fuel inhibitors). To calculate fuel capacity percentage, or the cell's ability to oxidize a fuel when other pathways are inhibited, this equation was used: 1 – [(basal OCR – OCR after treatment with other two inhibitors)/(basal OCR – OCR after treatment with all fuel inhibitors)].

Glutamine starvation and UK5099 viability assays were performed by seeding cells ($7x10^3$ cells/well, 6 repeats) on 96-well plates. Upon attaching, the cells were exposed to treatment medium containing different concentrations of glutamine or UK5099, as indicated, for 72 hours. DMEM medium used in glutamine restriction survival assay contained 10mM glucose. Cell viability was measured using the acid phosphatase (ACP) assay (Yang et al., 1996).

RNA-seq analyses

Total RNA from log growing Rat1a and MCF10A cell lines (3 biological replicates) was isolated using, respectively, the RNeasy Mini Kit (Qiagen) and the Direct-zol RNA MiniPrep Plus kit (Zymo Research). RNA was quantified and quality controlled with a Nanodrop (Thermo Scientific) and 2100 Bioanalyzer (Agilent). Stranded Rat1a cDNA libraries were constructed using the TruSeq Stranded (dUTP) mRNA Library Prep Kit (Illumina). Stranded MCF10A cDNA libraries were prepared with the Kapa mRNA HyperPrep kit (Kapa Biosystems, Cat. KR1352). The libraries where sequenced on Illumina HiSeq 2000 (Rat1a libraries; single ends, 1x50 nts) and on NovaSeq 6000 (MCF10A libraries; paired ends, 2x101 nts) at the Core Genomic facilities of UCR and City of Hope, respectively.

Rat1a sequence reads were mapped to the rat genome (Rnor_6.0.82) using TopHat v.2.1.0 with default settings and strand-specific read counting was performed with GenomicFeatures v.1.22.4 and the summmarizeOverlaps function. Differential expression

analysis was performed with edgeR using default settings. The entire pipeline, including hierarchical clustering analysis, was performed within the systemPipeR package (Backman and Girke, 2016). MCF10A sequence reads/fragments were mapped to the human reference genome hg38 using STAR v.2.7.9a (Dobin et al., 2013). Strand-specific read counts were calculated by featureCounts v.1.6.4 (Liao et al., 2014). Only genes with counts per million > 1 in at least three samples were used for downstream analysis. Bioconductor package edgeR v.3.32.1 (Robinson et al., 2010) with quasi-likelihood pipeline was used for differential expression analysis. P-values were adjusted by Benjamini and Hochberg method. All sequencing data (fastq files) were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) under accession number GSE220920.

Gene set enrichment analyses

Gene ontology (GO) and pathway enrichment analyses of significantly deregulated genes (>2.0-fold or >1.5-fold; FDR<0.05, as indicated) were performed using Metascape (Zhou et al., 2019) and Enrichr (Chen et al., 2013; Xie et al., 2021). Gene set enrichment analysis (GSEA) was performed using the GSEA software development by the Broad Institute (Mootha et al., 2003; Subramanian et al., 2005). Rat genes were first converted to their human orthologues and genes with low expression were filtered out. Read counts were then normalized and transformed using DESeq2. Subsequently, genes were ranked according to the signal-to-noise ratio and GSEA was performed using a weighted analysis. All other settings were at default settings. For comparison of gene sets enriched in common in Rat1a and MCF10A cell lines, genes from edgeR results were ranked by the sign of log2FC in combination with -log10 of the P-value and pre-ranked GSEA was performed with gene sets obtained from MSigDB v7.4 (Liberzon et al., 2015). A list of significantly enriched gene sets (FDR q-value <25%) for Rat1a and MCF10A cell lines was created and overlapping gene sets were cross-referenced and plotted with a CRAN Package, ggplot2 v.3.3.6 (Wickham, 2016).

Reverse transcription and quantitative PCR (RT-qPCR)

Primers sequences were designed by using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) or the Primer3Plus web tool (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) using the default qPCR settings. All primers were synthesized by Integrated DNA Technologies (Riverside, CA). RNA was

extracted using the Thermoscientific RNA extraction Kit and were verified for quality and quantity using gel electrophoresis and the Thermoscientific Nanodrop2000 spectrophotometer. cDNA was synthesized using 1ug of total RNA using the iScript reverse transcription supermix (Biorad, Irvine, CA) according to the manufacturer's instructions. Quantitative PCR was performed using the Biorad iQ SYBR green supermix and a Biorad CFX Connect thermocylcer (Biorad, Irvine, CA) and analyzed using the CFX manager software (v3.1). Using a single threshold Cq determination, the Livak method was employed for all gene expression analyses. All qPCR analyses were normalized to *Rps13*. For endpoint PCR GoTaq® Green Master Mix (Promega, Madison, WI) was used as described by the manufacturer for 40 cycles. The primers used for PCR/qPCR are shown below:

Primer Name	**Primer Sequence 5'-3'**
Rat genes/mRNAs	
R.N-Dmrt3_LeftPrimer	CGCAGATTGCTAAGCCAGATTT
R.N-Dmrt3_RightPrimer	TTGTCACTGAAGGACTCCGC
R.N-Etnppl_LeftPrimer	GGACCATGAGGAAAGGACGC
R.N-Etnppl_RightPrimer	AGTATCCCTTTGCCTTTCATCTCAT
R.N-Grip1_LeftPrimer	CAAACAGCCCTCCGGCTATG
R.N-Grip1_RightPrimer	GGCTTCGAGGTAAAGTCCCC
R.N-Nectin1_LeftPrimer	TACATCTGTGAGGCCACCAAC
R.N-Nectin1_RightPrimer	GGGGTGTAGGGGAATTCTGTGA
R.N-Pdha1_LeftPrimer	CAAGCAGAGTGCTGGTAGCTT
R.N-Pdha1_RightPrimer	TAGACGATGAAGGTCACATTTCTTA
R.N-Rps13_LeftPrimer	ACTCCTTCCCAGATAGGTGTG
R.N-Rps13_RightPrimer	TTCCGGTCACAAAACGGACC
R.N-Scd1_LeftPrimer	GCTGGAGTACGTCTGGAGGA
R.N-Scd1_RightPrimer	AAATATCCCCCAGAGCAAGGTG
R.N-Sema3d_LeftPrimer	GTATTCAAGCACCCAACAGCC
R.N-Sema3d_RightPrimer	GTGCAAGGAGAGCTGAACCA
R.N-Sorbs2_LeftPrimer	CTTGACTGACGGAGGCACTT
R.N-Sorbs2_RightPrimer	AGGAGTCGCTGGCCTTTAAC

Human Genes/mRNAs	
CLIC2_LeftPrimer	CCAATCCTCCGTTCCTGGTGTA
CLIC2_RightPrimer	ACTTGGGACTCAGGTGAGGGTA
ESPN_LeftPrimer	TGCAGACCAAGAACAAACTCCGC
ESPN_RightPrimer	TTGCGGCTGGAGTCCTGCCTC
GBP2_LeftPrimer	GTTCCTACATCCTCAGCCATTCC
GBP2_RightPrimer	CCACTGCTGATGGCATTGACGT
HK2_LeftPrimer	GAGTTTGACCTGGATGTGGTTGC
HK2_RightPrimer	CCTCCATGTAGCAGGCATTGCT
KAT2A _LeftPrimer	CAGTTTCGGCAGAGGTCTCA
KAT2A _RightPrimer	ATGAGTGGTTTCGTAGCGGG
NOTCH3_LeftPrimer	TACTGGTAGCCACTGTGAGCAG
NOTCH3_RightPrimer	CAGTTATCACCATTGTAGCCAGG
SLCO5A1_LeftPrimer	CGAGTATGAGCCAGTCTGTGGA
SLCO5A1_RightPrimer	CTTGGCGACTTTGGACACAGGT
SNAI1_LeftPrimer	TGCCCTCAAGATGCACATCCGA
SNAI1_RightPrimer	GGGACAGGAGAAGGGCTTCTC
TERT_LeftPrimer	CCAAGTTCCTGCACTGGCTGA
TERT_RightPrimer	TTCCCGATGCTGCCTGAC
TOP1_LeftPrimer	TCGAAGCGGATTTCCGATTGA
TOP1_RightPrimer	CTTTGTGCCGGTGTTCTCGAT
UBC_LeftPrimer	CTGGAAGATGGTCGTACCCTG
UBC_RightPrimer	GGTCTTGCCAGTGAGTGTCT

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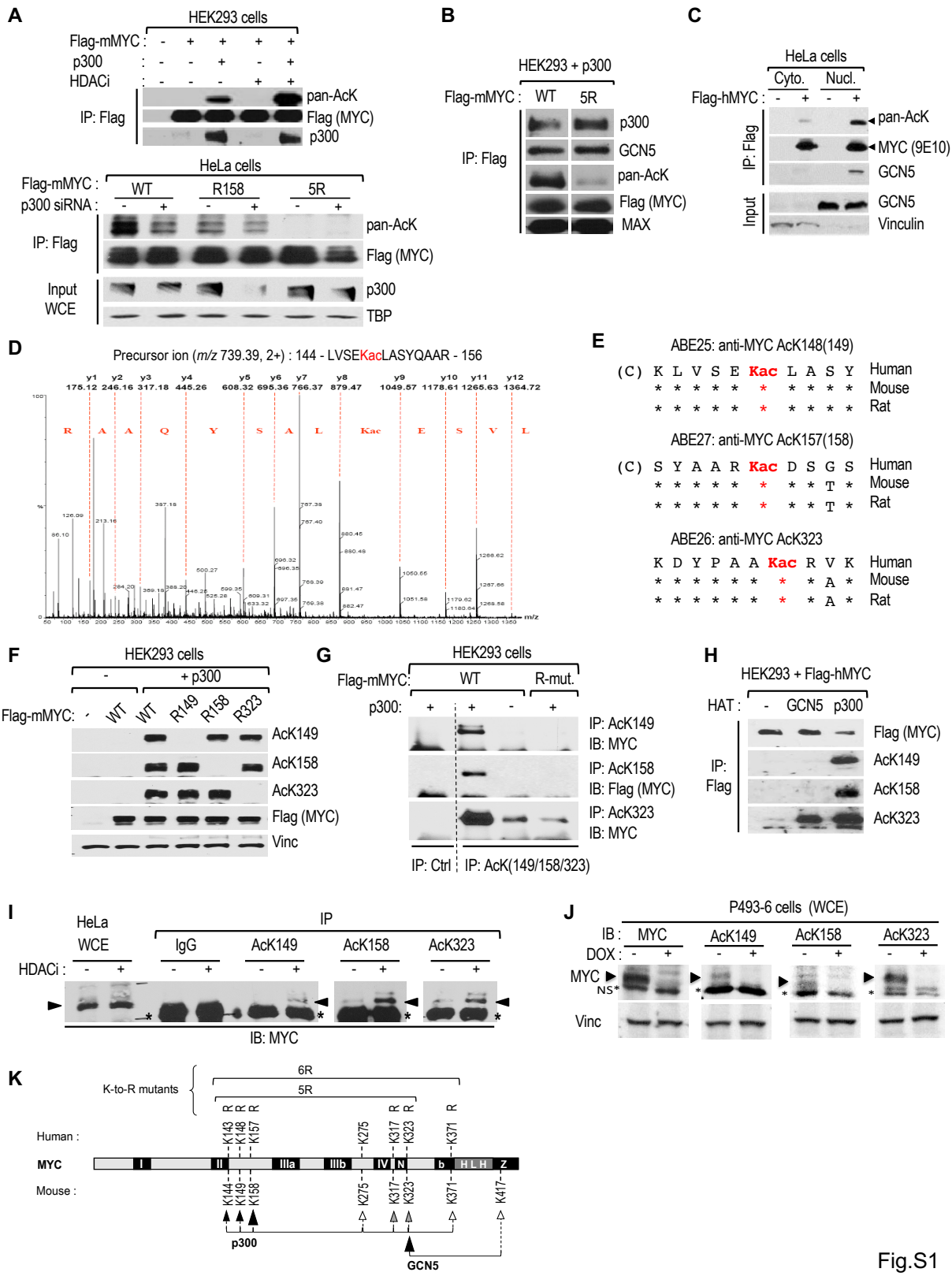
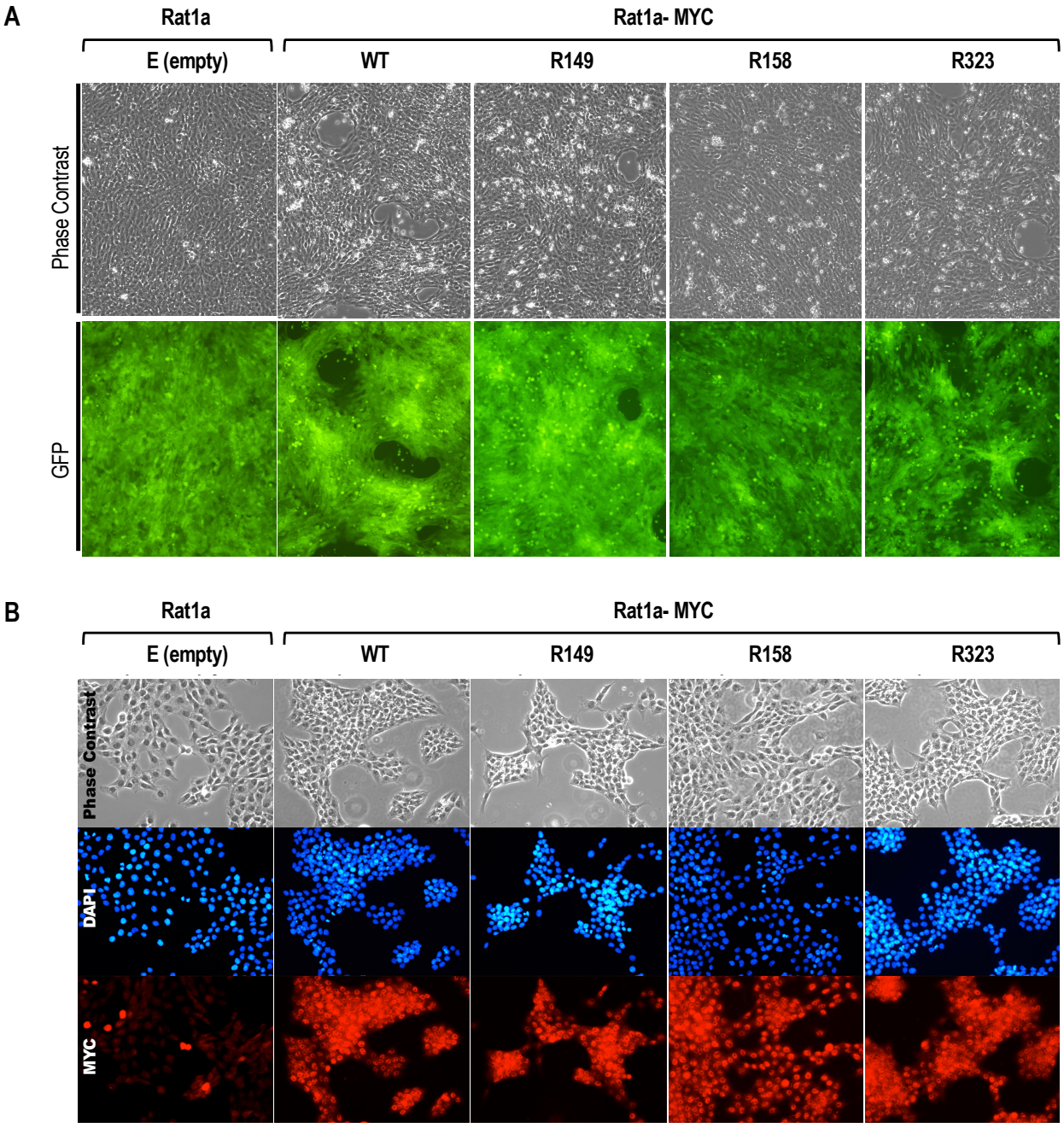


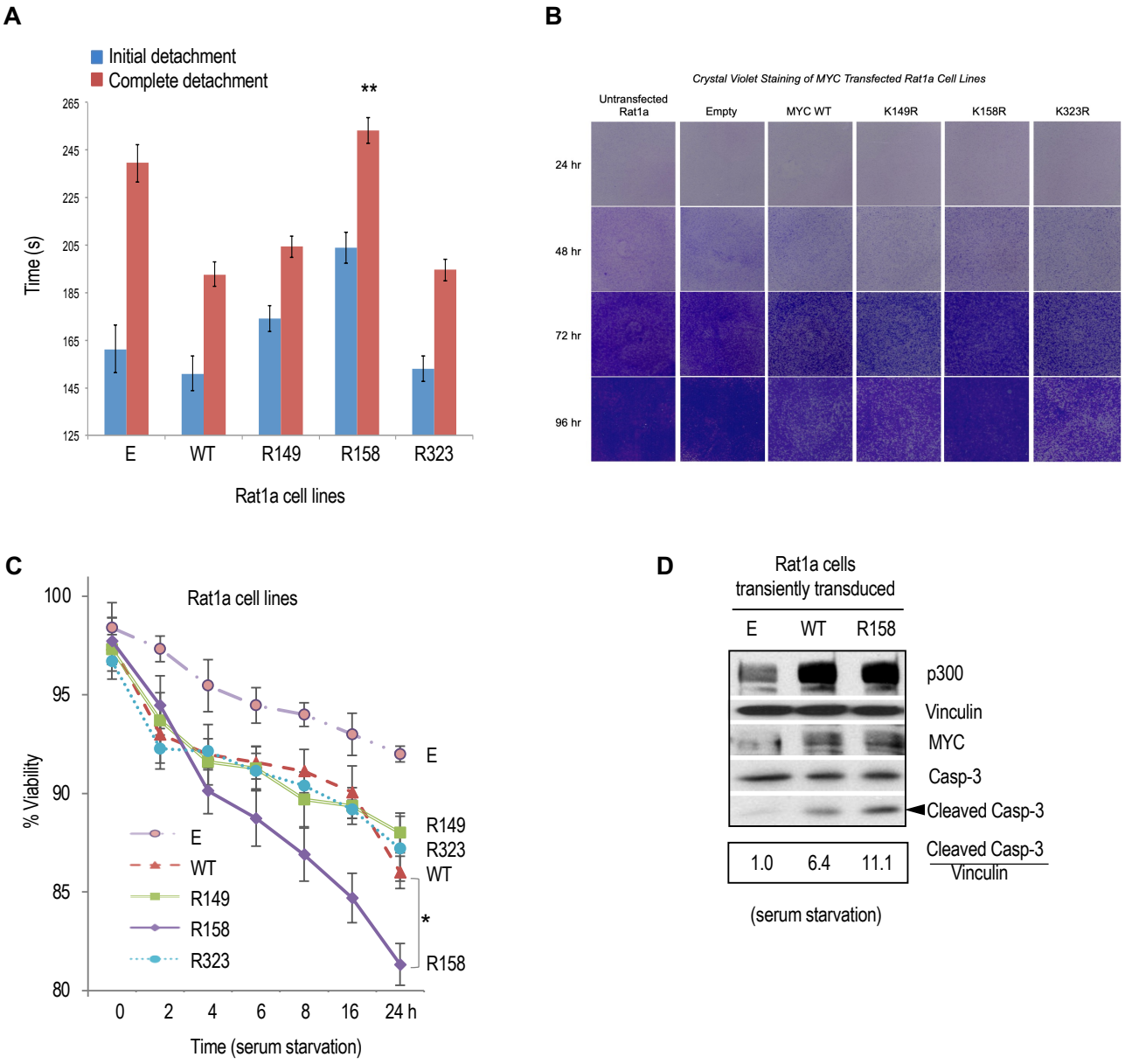
Fig.S1

Supplemental Figure S1. Characterization of site-specific MYC acetylation.
(A) Top: HEK293 cells were transfected with empty vector (-) or vectors (+) for Flag-mMYC and/or p300 and treated with/without (+/-) HDAC inhibitors (HDACi) for 2h before cell lysis,

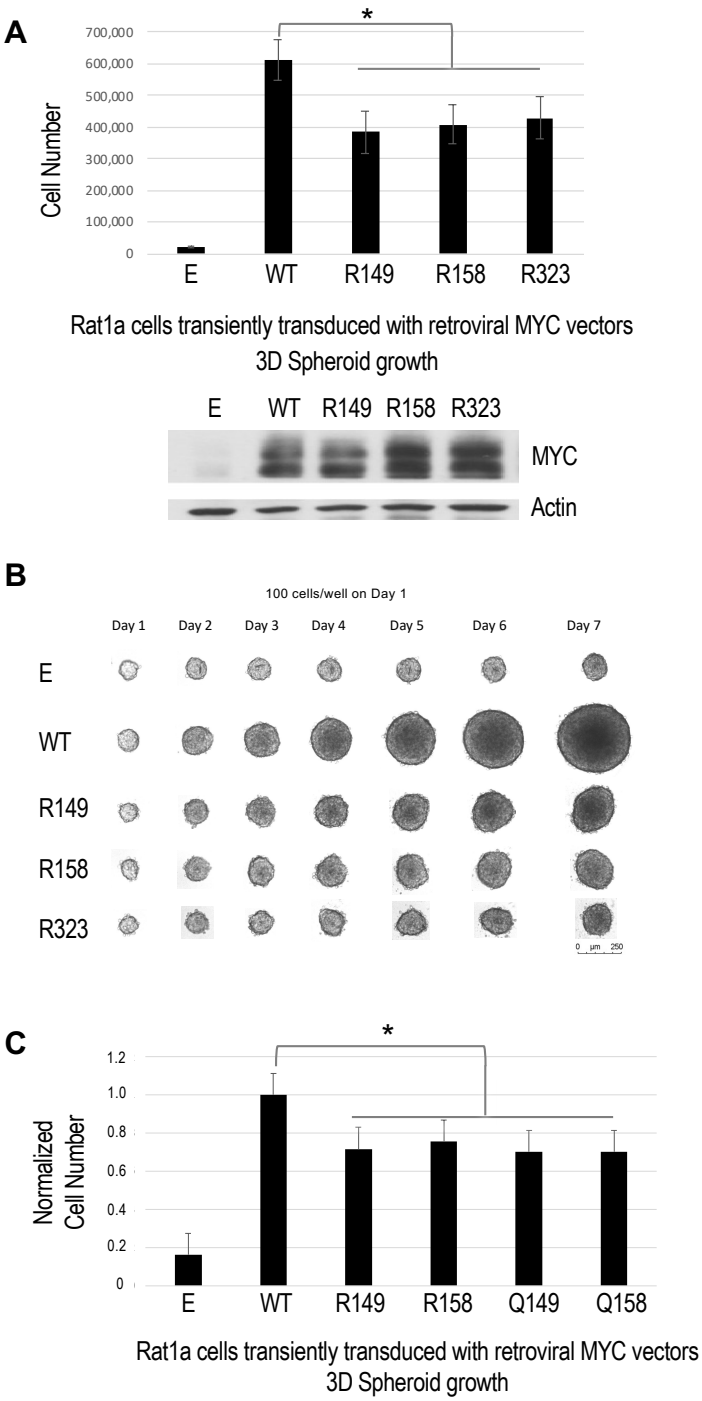
followed by Immunoprecipitation (IP) with anti-Flag antibody and western blot analysis. Bottom: HeLa cells were transfected with Flag-mMYC (WT or the indicated R-mutants) and either a control siRNA (-) or a specific p300 siRNA (+) and analyzed by anti-Flag IP and western blotting. Immunoblotting was performed with a pan-acetyl-lysine antibody (pan-AcK) and the indicated antibodies. WCE is whole cell extract used as input for the IP. **(B)** HEK293 cells transfected with p300 and Flag-mMYC WT or 5R mutant were analyzed by IP with anti-Flag antibody followed by western blot with the indicated antibodies. **(C)** Cytoplasmic and nuclear fractions of HeLa cells (Cyto. and Nucl. Input) transfected with empty vector (-) or with Flag-hMYC (+) were immunoprecipitated and analyzed by western blot with the indicated antibodies, as above. **(D)** LC-MS/MS analysis of endogenous MYC:MAX complexes in HeLa cells immunoprecipitated with a MAX (C-17) antibody. **(E)** Human MYC acetylated peptides used to generate site-specific MYC AcK antibodies and amino acid sequence conservation in mouse and rat MYC proteins (identical residues are marked with an asterisk*). **(F)** Specificity of MYC AcK antibodies was tested by western blot of WCE from HEK293 cells transfected with p300 and the indicated MYC WT or R-mutants. **(G)** Immunoprecipitation (IP) of acetylated MYC with site-specific AcK antibodies was tested in HEK293 cells transfected as indicated and immunoblotting (IB) was performed with a MYC antibody. **(H)** Analysis of acetylated MYC with site-specific AcK antibodies in HEK293 cells transfected with GCN5 or p300 HATs. WCE were immunoprecipitated with the Flag/M2 antibody. **(I)** Endogenous acetylated MYC was immunoprecipitated (IP) from HeLa WCE with the indicated control (IgG) or site-specific AcK antibodies; HDAC inhibitors (HDACi) were added (+) or not (-) to the culture medium 2 h before cell lysis. An asterisk indicates the IgG heavy chains. **(J)** Western blot analysis with the indicated antibodies (IB: MYC, AcK and vinculin antibodies) of WCE from P493-6 cells untreated (-) or treated (+) with 0.2μg/mL doxycycline (DOX) for 12 h before cell lysis. **(K)** Summary of all known mouse and human MYC lysine (K) residues acetylated by p300 and GCN5 in transfected HEK293 cells. Preferred/major sites of acetylation for each HAT are indicated with black arrowheads. The individual and combined K-to-R mutants are indicated.



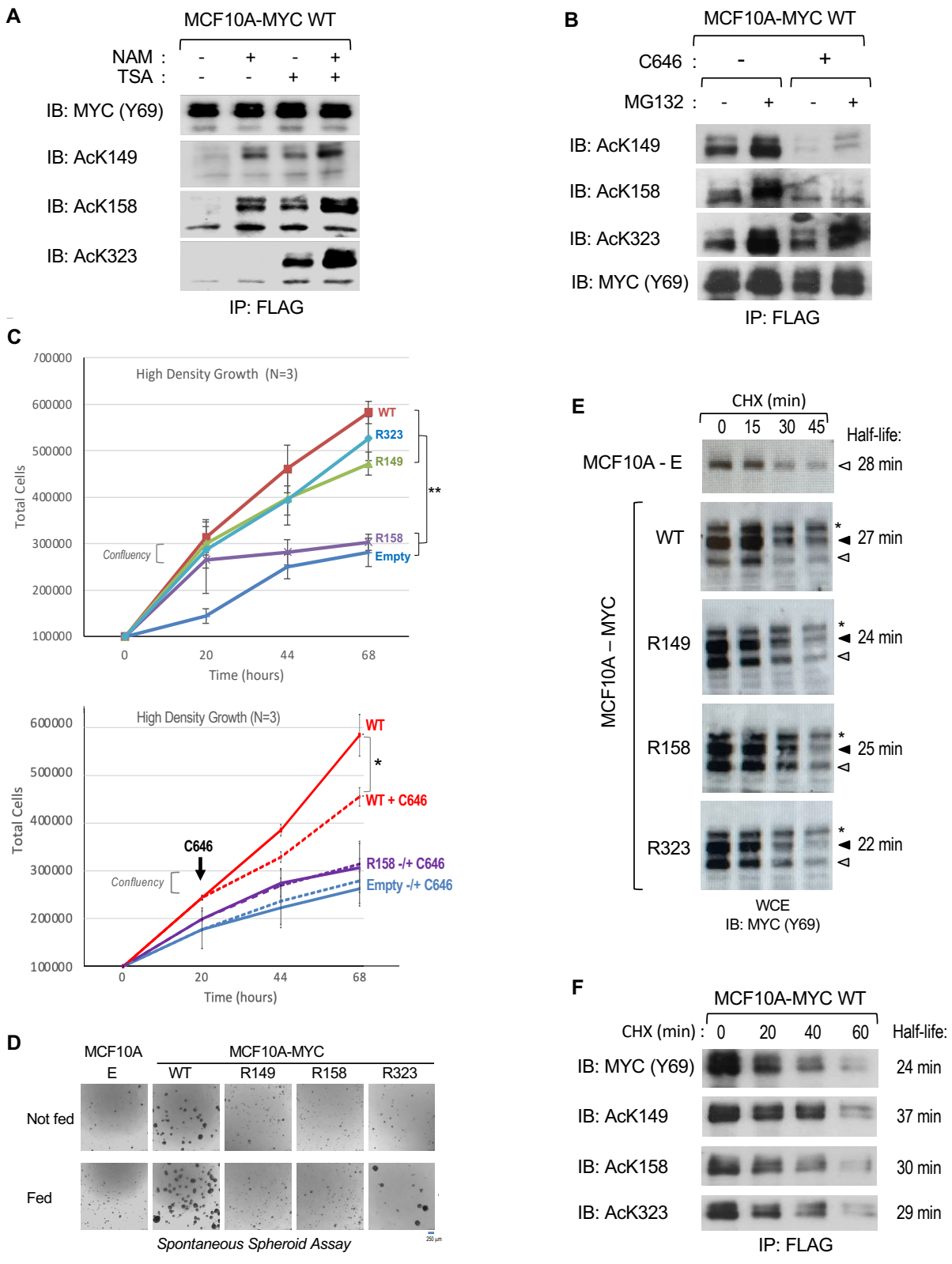
Supplemental Figure S2. Rat1a-E and Rat1a-MYC WT and R-mutant polyclonal cell lines. Cells were imaged by phase contrast microscopy or fluorescence microscopy (GFP, DAPI) and indirect immunofluorescence microscopy (anti-MYC N-262 antibody), as indicated at **(A)** 10X magnification or **(B)** 20X magnification.



Supplemental Figure S3. Cell adhesion and apoptosis of MYC-transformed Rat1a fibroblasts are regulated by the acetylated K158 residue of MYC. (A) Time to detachment assay with Trypsin-EDTA solution of the indicated cell lines (N=3, each in triplicates). **(B)** Representative detachment assay of logarithmic-growing cell lines at different times after seeding on regular cell culture plates. Cells were washed with PBS at different times of log growth (24h-96h) and cells remaining attached to the plates were stained with Crystal Violet. The control Rat1a-E (empty) cell line and the R158 (K158R) mutant cell line retained strong adhesions (stronger staining) compared to MYC WT, R149 (K149R), and R323 (k323R) cell lines. **(C)** The R158 cell line is more sensitive to cell death induced by serum starvation. The fraction of viable cells is plotted as a function of time in serum-deprived medium (N=3, each in triplicates). **(D)** The MYC R158 mutation increases MYC-dependent apoptosis-associated cleavage of caspase-3. Rat1a cells were transiently transduced (48h) with the indicated empty (E) or MYC-expressing WT or R158 mutant retroviral expression vectors, serum-starved for 24 h and then analyzed by western blot with the indicated antibodies. Cleaved caspase-3 signals are normalized to vinculin and are relative to control (E) cells.

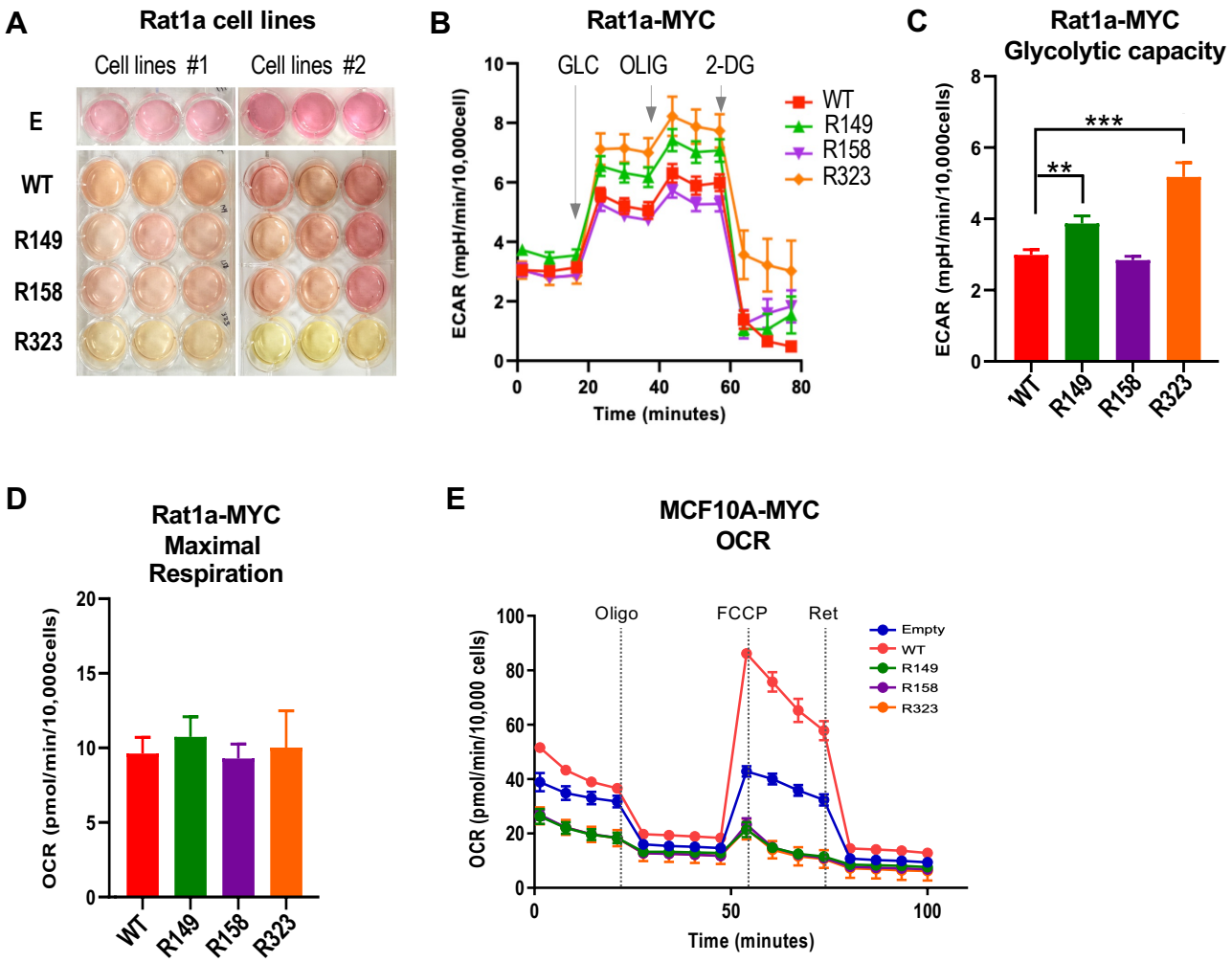


Supplemental Figure S4. MYC-driven tumor spheroid growth is dependent on individual acetylated K residues. (A) Spontaneous spheroid growth assay with Rat1a cells transiently transduced with empty (E) or the indicated MYC WT or R-mutant retroviral vectors. Top is a plot showing total cell number (N=3, in triplicates), and bottom is a western blot of expressed MYC proteins. **(B)** Facilitated spheroid formation assay with the indicated Rat1a-E and Rat1-MYC WT/mutant stable polyclonal cell lines seeded at 100 cells/well on day 1 in low-adherence round bottom wells (bar is 250 μm). **(C)** Spontaneous spheroid assay as in panel A, comparing the indicated MYC K-to-R and K-to-Q mutants.



Supplemental Figure S5. MYC reversible acetylation, MYC stability, and high-density growth properties of MYC-transformed human mammary MCF10A cell lines. (A) Different classes of HDACs deacetylate MYC with different AcK-site preferences. MCF10A-MYC/WT cells were treated for 2h with nicotinamide (NAM) and/or TSA and MYC was analyzed by IP

and western bot immunoblotting (IB) with the indicated antibodies. **(B)** The p300-specific inhibitor C646 selectively inhibits acetylation of MYC at K149 and K158 but not at K323 in MYC-transformed MCF10A cell line. Representative western blot of anti-Flag immunoprecipitates from WCE of cells treated (+) or not (-) with C646 (30 μM) and/or MG132 for 1 h just before cell lysis. **(C)** High density proliferation assays with the indicated MYC-transformed MCF10A cell lines. Cells were plated at high density and reached confluency at a density of about 250-300 x 10^3 cells (Confluency bracket). Bottom graph shows the effect of 2.5 μM C646 on high density proliferation (dashed lines) added to the indicated cell lines at 20 h after seeding (N=3, each in triplicates). **(D)** Phase contrast microscopy photographs of suspension spheroids generated by the indicated cell lines after 7-day culture without (Not fed) or with refeeding (Fed) by the addition of fresh complete medium at days 3 and 6 (bar is 250μm). **(E) and (F**) MYC turnover analyzed by cycloheximide (CHX) chase assays and western blot of WCE (C) or immunoprecipitated Flag-MYC (D) from the indicated MCF10A-E or MCF10A-MYC cell lines. In WCE (C), white arrowhead indicates endogenous MYC, black arrowheads indicate main ectopic Flag-MYC bands with their half-lives indicated; gray arrowhead indicates a MYC band that is a combination/overlap of endogenous MYC and a minor ectopic Flag-MYC band with a combined half-life of 24-34 min. Asterisk (*) in WCE indicates a minor band of unknown origin that is stable over the time course tested. Turnover of acetylated MYC was analyzed with the indicated site-specific AcK antibodies.



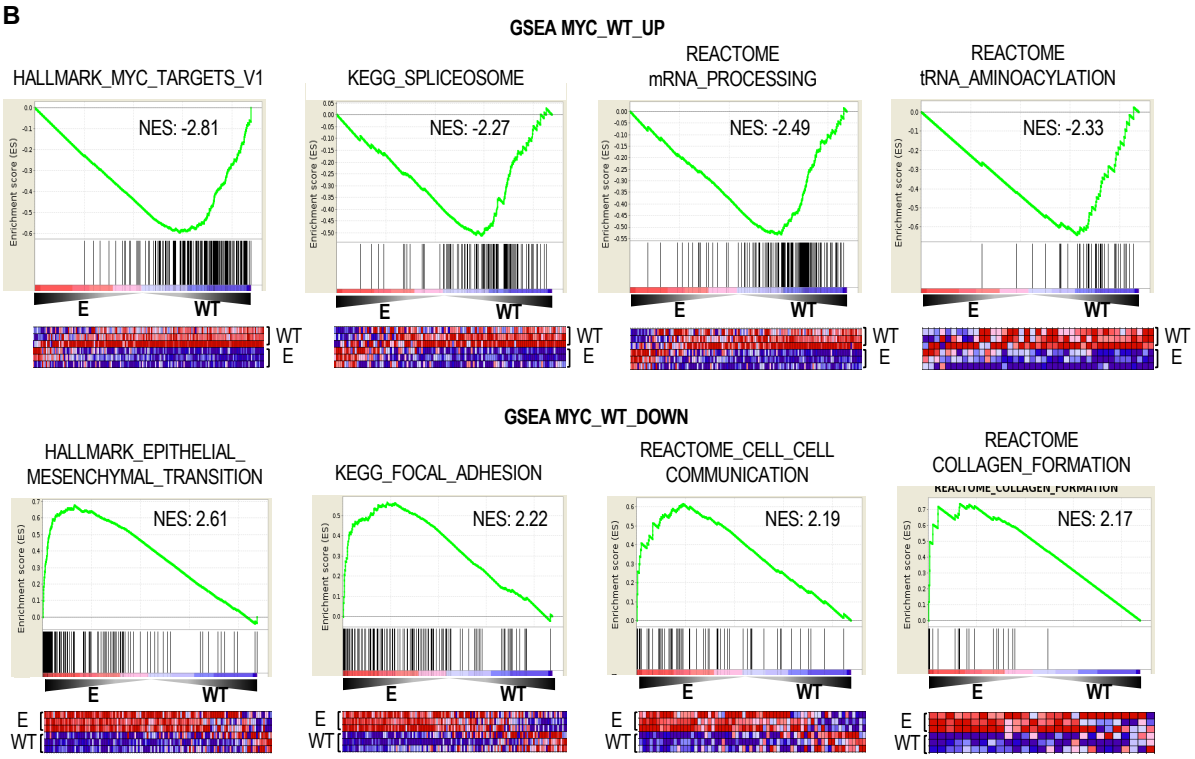
Supplemental Figure S6. Metabolic analyses of Rat1a and MCF10A cell lines overexpressing MYC WT or individual K-to-R mutants. (A) Extracellular medium acidification (visualized by medium color change) is increased in Rat1a-MYC R323 mutant cells. Two independent sets of polyclonal cell lines were tested. **(B) and (C)** Extracellular medium acidification rate (ECAR) measurement and determination of glycolytic capacity for the indicated Rat1a cell lines was performed with a Seahorse analyzer (Agilent); glucose (GLC), oligomycin (OLIG), and 2-deoxy-D-glucose (2-DG) were added at the indicated times (n=5). **(D) and (E)** Oxygen consumption rates (OCR) of the indicated cell lines were determined with a Seahorse analyzer after addition of oligomycin (Oligo), FCCP, and rotenone (Ret), as indicated.

A

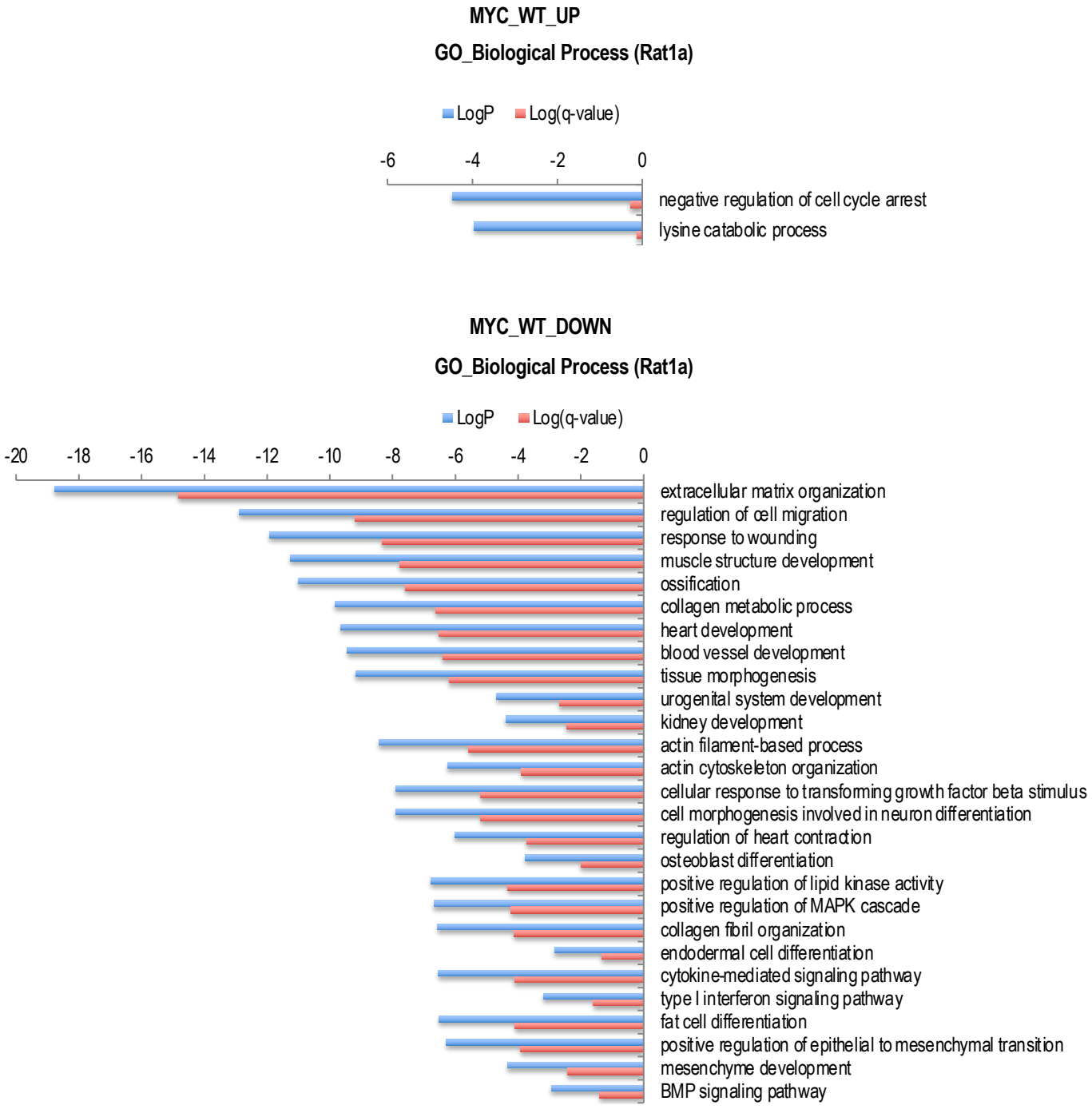
GSEA WT (MYC_WT_UP)	SIZE	FDR q-val	FWER p-val
HALL_MYC_TARGETS_V2	53	0	0
HALL_MYC_TARGETS_V1	178	0	0
HALL_E2F_TARGETS	189	0	0
HALL_G2M_CHECKPOINT	185	1.67E-04	0.001
KEGG_AMINOACYL_TRNA_BIOSYNTHESIS	40	0.0012	0.001
KEGG_SPLICEOSOME	112	0.0011	0.002
REACT_MRNA_PROCESSING	140	0	0
REACT_PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_MRNA	123	0	0
REACT_TRNA_AMINOACYLATION	41	0	0
REACT_METABOLISM_OF_NON_CODING_RNA	43	0	0
REACT_TRANSPORT_OF_MATURE_TRANSCRIPT_TO_CYTOPLASM	49	0	0
REACT_MITOCHONDRIAL_TRNA_AMINOACYLATION	20	3.86E-04	0.002

GSEA Empty (MYC_WT_DOWN)	SIZE	FDR q-val	FWER p-val
HALL_EPITHELIAL_MESENCHYMAL_TRANSITION	129	0	0
HALL_MYOGENESIS	122	0	0
HALL_TGF_BETA_SIGNALING	50	0	0
HALL_INFLAMMATORY_RESPONSE	104	0	0
HALL_APICAL_JUNCTION	119	0	0
HALL_APICAL_SURFACE	26	0	0
HALL_P53_PATHWAY	161	1.59E-04	0.001
HALL_TNFA_SIGNALING_VIA_NFKB	157	1.39E-04	0.001
KEGG_ECM_RECEPTOR_INTERACTION	46	0	0
KEGG_FOCAL_ADHESION	139	0	0
KEGG_DILATED_CARDIOMYOPATHY	43	0	0
KEGG_HYPERTROPHIC_CARDIOMYOPATHY_HCM	42	0	0
REACT_CELL_CELL_COMMUNICATION	66	0	0
REACT_COLLAGEN_FORMATION	27	0	0

B

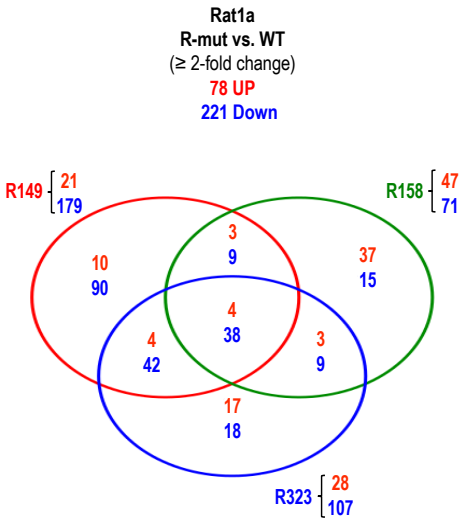


Supplemental Figure S7. Gene set enrichment analyses (GSEA). MYC-upregulated (UP) and MYC-downregulated (DOWN) genes in transformed Rat1a-MYC WT versus control Rat1a-E cell lines were analyzed by GSEA. **(A)** Top deregulated gene sets. **(B)** Selected enrichment plots with normalized enrichment scores (NES) and gene expression heatmaps of for the triplicates.



Supplemental Figure S8. Gene ontology (GO) analyses of MYC-upregulated and MYC-downregulated genes in Rat1a cells. Metascape online tool (https://metascape.org/) was used with default settings to analyze genes deregulated by MYC in Rat1a-MYC WT cell line vs. control Rat1a-E cells. Most significant biological processes are shown for genes deregulated by 2-fold or more (FDR ≤ 0.07).

A



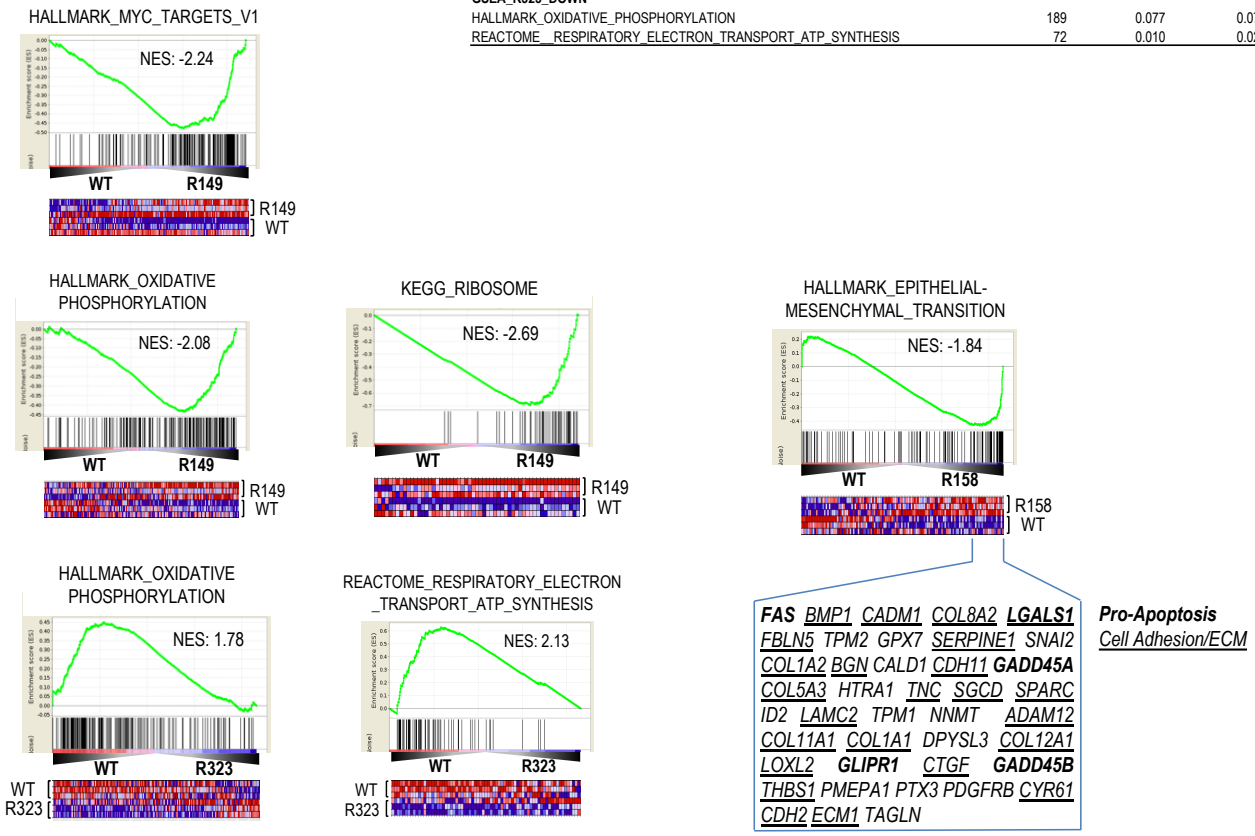
B

GSEA_R149_UP	SIZE	FDR q-val	FWER p-val
HALLMARK_MYC_TARGETS_V1	178	0	0
HALLMARK_OXIDATIVE_PHOSPHORYLATION	190	0	0
KEGG_OXIDATIVE_PHOSPHORYLATION	97	0	0
KEGG_RIBOSOME	57	0	0
REACTOME_INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION	71	0	0
REACTOME_PEPTIDE_CHAIN_ELONGATION	56	0	0
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	75	0	0
REACTOME_3_UTR_MEDIATED_TRANSLATIONAL_REGULATION	74	0	0
REACTOME_TRANSLATION	108	0	0
GSEA_R149_DOWN			
KEGG_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	33	0.00113	0.001

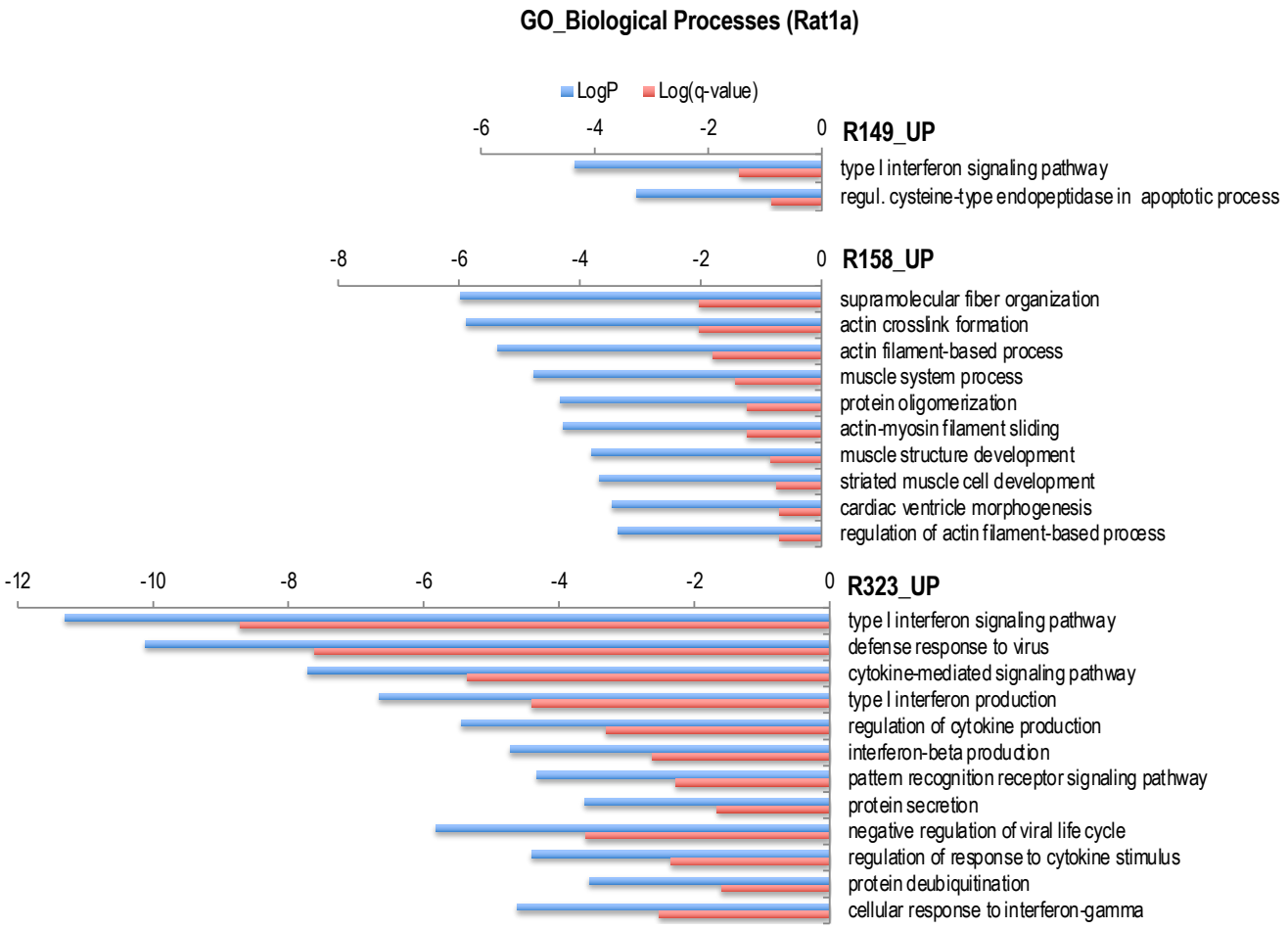
GSEA_R158_UP	SIZE	FDR q-val	FWER p-val
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	126	0.011	0.011
KEGG_RIBOSOME	57	0	0
REACTOME_INFLUENZA_VIRAL_RNA_TRANSCRIPTION_AND_REPLICATION	71	0	0
REACTOME_PEPTIDE_CHAIN_ELONGATION	56	0	0
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	75	0	0
REACTOME_3_UTR_MEDIATED_TRANSLATIONAL_REGULATION	74	2.03E-04	0.001
REACTOME_MUSCLE_CONTRACTION	22	2.54E-04	0.001
GSEA_R158_DOWN			
HALLMARK_MITOTIC_SPINDLE	183	0.015	0.017

GSEA_R323_UP	SIZE	FDR q-val	FWER p-val
HALLMARK_INTERFERON_ALPHA_RESPONSE	80	0	0
HALLMARK_INTERFERON_GAMMA_RESPONSE	137	0	0
KEGG_CYTOSOLIC_DNA_SENSING_PATHWAY	30	0	0
KEGG_RIG_I_LIKE_RECEPTOR_SIGNALING_PATHWAY	44	0.004	0.008
REACTOME_INTERFERON_ALPHA_BETA_SIGNALING	39	0	0
REACTOME_INTERFERON_SIGNALING	110	0	0
REACTOME_RIG_I_MDA5_MEDIATED_INDUCTION_OF_IFN_ALPHA_BETA_PATHWAYS	49	0	0
GSEA_R323_DOWN			
HALLMARK_OXIDATIVE_PHOSPHORYLATION	189	0.077	0.074
REACTOME__RESPIRATORY_ELECTRON_TRANSPORT_ATP_SYNTHESIS	72	0.010	0.021

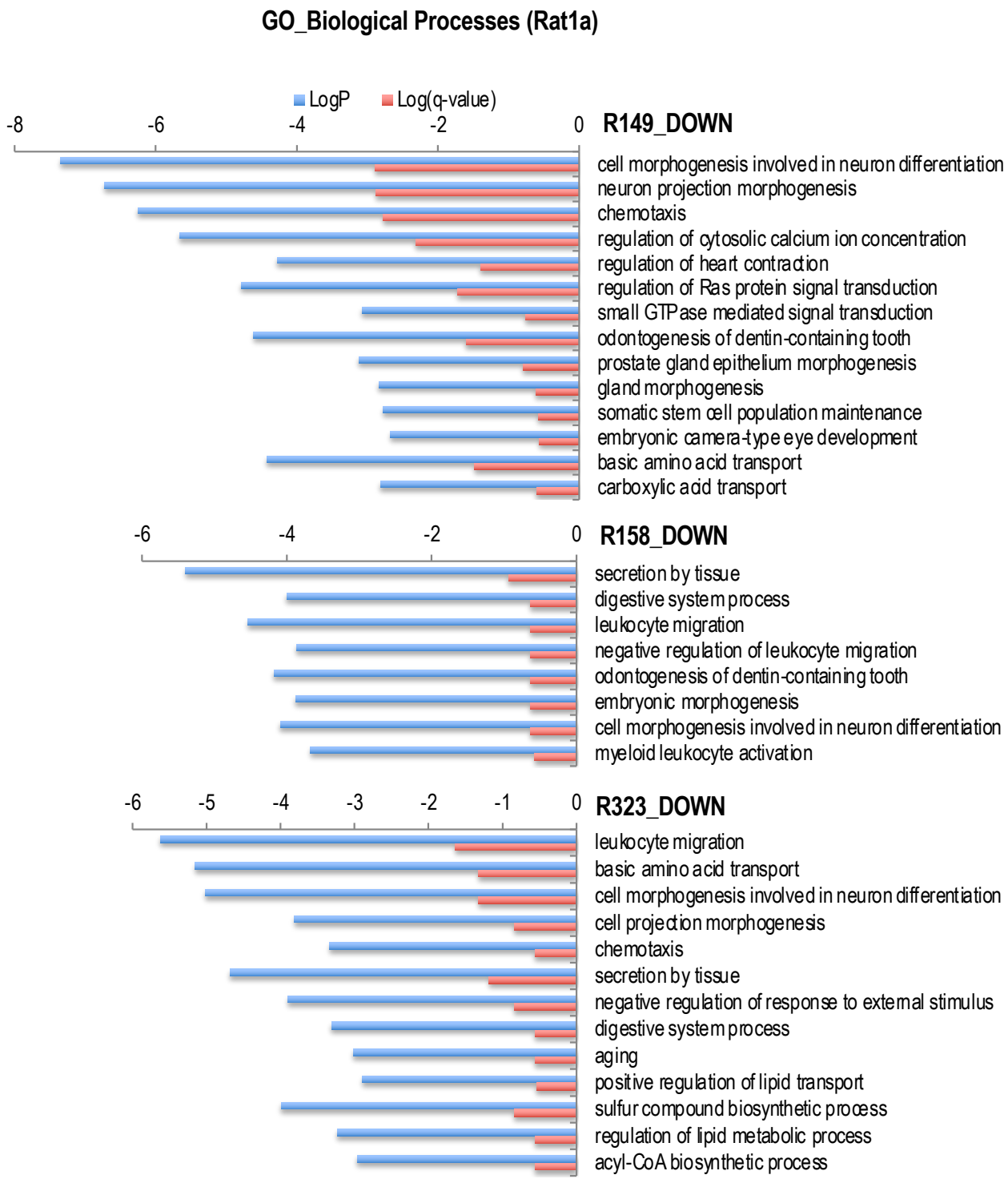
C



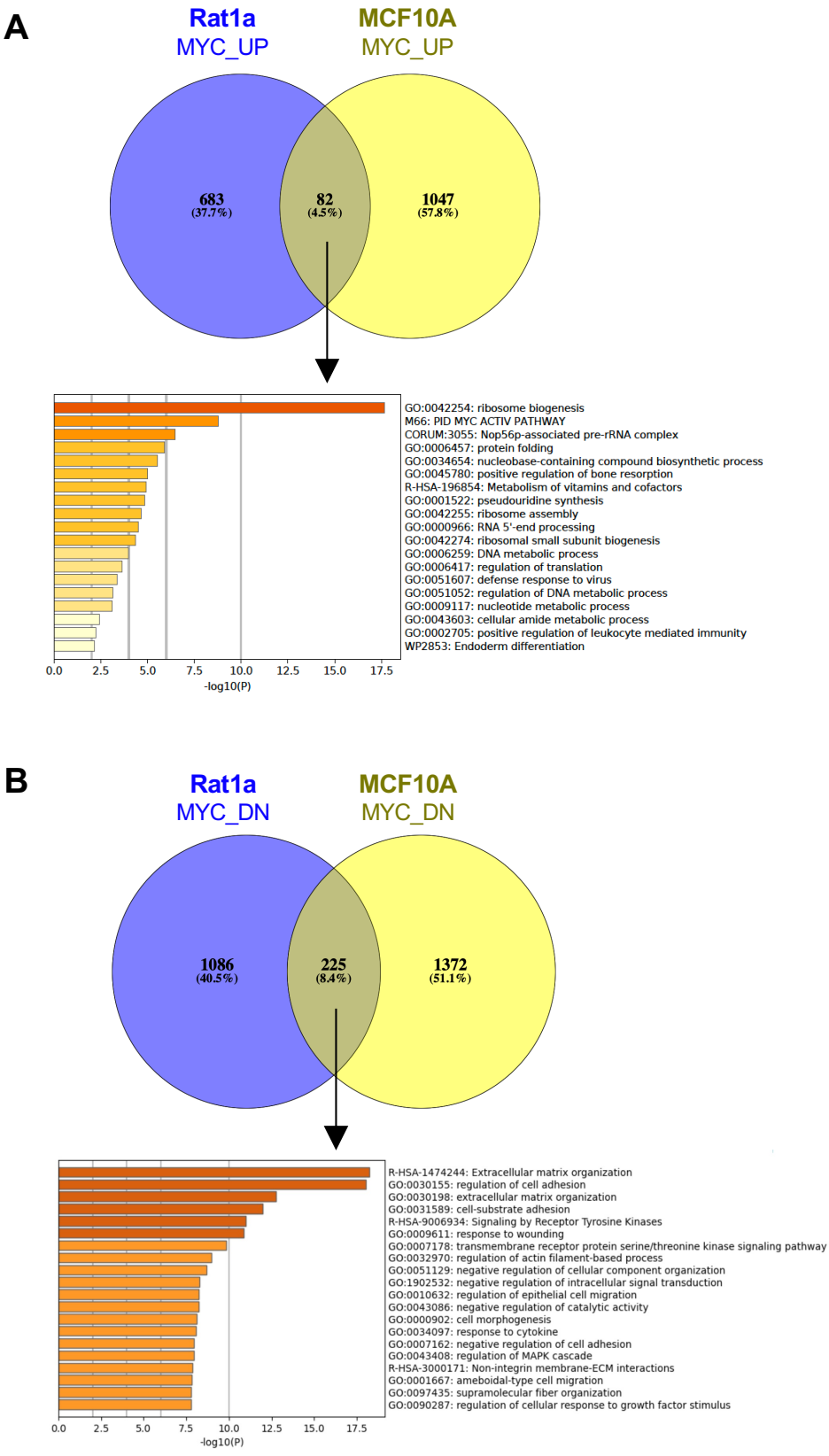
Supplemental Figure S9. Gene sets deregulated by MYC AcK-to-R mutations in Rat1a cell lines. (A) Venn diagram of genes deregulated by at least 2-fold (FDR 0.07) in Rat1a-MYC/R-mutant cell lines relative to Rat1a-MYC/WT cell line. The number of DEGs is indicated in red for genes upregulated, or blue for genes downregulated. **(B) and (C)** GSEA identified top deregulated gene sets and select enrichment plots, respectively. Pro-apoptotic genes and genes associated with cell adhesion/extracellular matrix (ECM) are indicated for most upregulated genes of the EMT gene set in the R158 cell line.



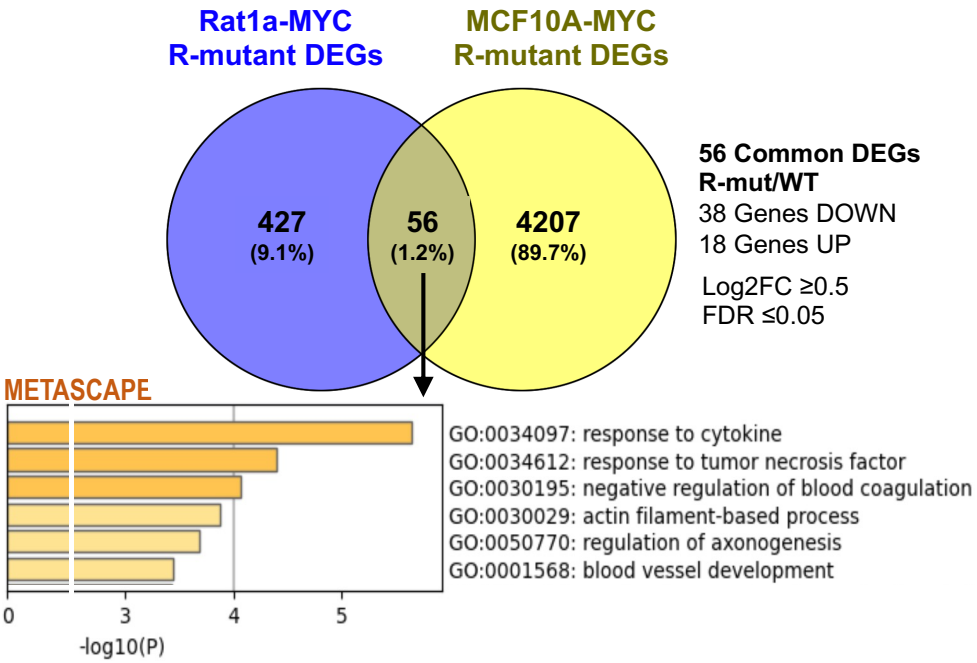
Supplemental Figure S10. Gene ontology (GO) analyses of genes upregulated by MYC AcK-to-R mutations in Rat1a cells. Metascape online tool (https://metascape.org/) was used with default settings to analyze genes upregulated in Rat1a-MYC/R-mutant cell lines (fold change ≥ 2-fold; FDR ≤ 0.07) relative to Rat1a-MYC/WT cell line. Only a selection of most significantly deregulated biological processes are shown (log q-values ≤ -0.5).



Supplemental Figure S11. Gene ontology (GO) analyses of genes downregulated by MYC AcK-to-R mutations in Rat1a cells. Metascape online tool (https://metascape.org/) was used with default settings to analyze genes downregulated in Rat1a-MYC/R-mutant cell lines (fold change ≥ 2-fold; FDR ≤ 0.07) relative to Rat1a-MYC/WT cell line. Only a selection of most significantly deregulated biological processes are shown (log q-values ≤ -0.5).



Supplemental Figure S12. Gene ontology (GO) analyses of common MYC-regulated genes in MYC-transformed Rat1a and MCF10A cell lines. Metascape online tool (https://metascape.org/) was used with default settings to analyze genes upregulated (A) and downregulated (B) in common in MYC-transformed Rat1a and MCF10A cell lines (log2FC ≥0.5; FDR 0.05). Only top significantly deregulated processes are shown.



Enrichr-MSigDB Hallmark 2020

Index	Name	P-value	Adjusted p-value	Odds Ratio	Combined score
1	TNF-alpha Signaling via NF-kB	0.002534	0.02788	7.60	45.45
2	Interferon Gamma Response	0.002534	0.02788	7.60	45.45
3	Cholesterol Homeostasis	0.01890	0.1068	10.04	39.83
4	Interferon Alpha Response	0.03126	0.1116	7.60	26.33
5	Androgen Response	0.03306	0.1116	7.36	25.11
6	Hedgehog Signaling	0.09773	0.2039	10.16	23.62

Enrichr-GO Biological Process 2021

Table of top 10 significant p-values and q-values for GO Biological Process 2021

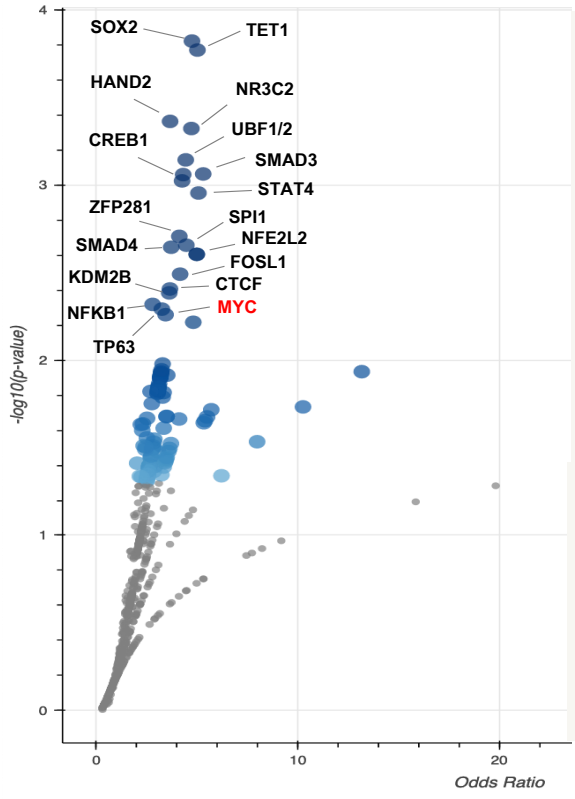
term	p-value	q-value	overlap_genes
actin filament bundle assembly (GO:0051017)	0.000107	0.025758	[DPYSL3, LCP1, ESPN]
actin filament bundle organization (GO:0061572)	0.000107	0.025758	[DPYSL3, LCP1, ESPN]
negative regulation of blood coagulation (GO:0030195)	0.000191	0.030667	[C1QTNF1, ADTRP, TFPI]
cytokine-mediated signaling pathway (GO:0019221)	0.000315	0.037962	[IKBKB, SOCS3, IFNLR1, LCP1, GBP2, EDA2R, BIRC3, IFIT2]
actin crosslink formation (GO:0051764)	0.000499	0.040098	[DPYSL3, LCP1]
negative regulation of hemostasis (GO:1900047)	0.000499	0.040098	[ADTRP, TFPI]
negative regulation of coagulation (GO:0050819)	0.000686	0.042145	[ADTRP, TFPI]
I-kappaB kinase/NF-kappaB signaling (GO:0007249)	0.000699	0.042145	[IKBKB, TBKBP1, BIRC3]
response to cytokine (GO:0034097)	0.000825	0.044165	[MME, DPYSL3, IFNLR1, CXCL16]
regulation of cytokine-mediated signaling pathway (GO:0001959)	0.001171	0.056434	[IKBKB, SOCS3, BIRC3]

Supplemental Figure S13. Gene ontology (GO) and pathway enrichment analyses of genes deregulated by MYC AcK-to-R mutations in both Rat1a and MCF10A cell lines. Venn diagram of genes deregulated by at least one MYC AcK-to-R mutation (log2FC≥0.5, FDR ≤ 5%). The 56 genes deregulated by at least one MYC R-mutation in both Rat1a and MCF10A cells were analyzed with the online tools Metascape (https://metascape.org/) and Enrichr (https://maayanlab.cloud/Enrichr/) using default settings.

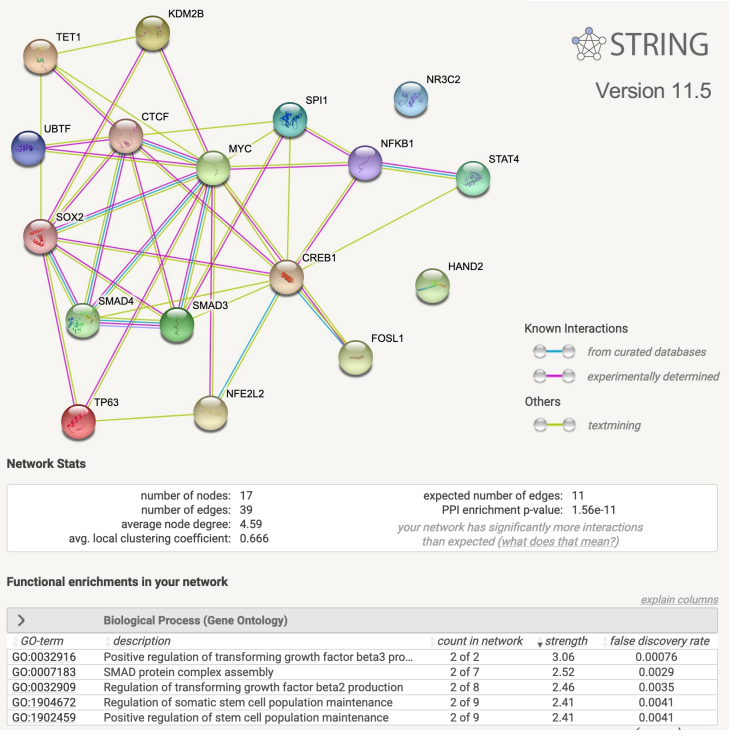
A

GENE (UP, DOWN)	Affected by MYC mutant(s) vs WT	Full Gene Name	Promoter bound by MYC in MCF10A and/or MCF7 cells (ENCODE ChIP-Seq)
BCL11B	R149	BAF Chromatin Remodeling Complex Subunit BCL11B	YES
CDR2L	R149	Cerebellar Degeneration Related Protein 2 Like	YES
CLIC2	R158	Chloride Intracellular Channel 2	YES
CRABP2	R149	Cellular Retinoic Acid Binding Protein 2	YES
ESPN	R158	Espin	YES
FAM161A	R323	FAM161 Centrosomal Protein A	YES
GSDMA	R149	Gasdermin A	YES
IQGAP2	R158	IQ Motif Containing GTPase Activating Protein 2	YES
ISOC1	R149	Isochorismatase Domain-Containing Protein 1	YES
LRRC20	R149	Leucine Rich Repeat Containing 20	YES
MACC1	R323	MET Transcriptional Regulator MACC1	YES
MAP2	R149, R323	Microtubule Associated Protein 2	YES
MFSD12	R149	Major Facilitator Superfamily Domain Containing 12	YES
PWWP3B	R149, R323	PWWP Domain Containing 3B	YES
PLCD4	R158	Phospholipase C Delta 4	No/Low
SCD5 (Scd1)	R149, R323	Stearoyl-CoA Desaturases	YES
SH3BP5	R158	SH3 Domain Binding Protein 5	YES
SLC4A11	R158	Solute Carrier Family 4 Member 11	YES
SLCO5A1	R149	Solute Carrier Organic Anion Transporter Family Member 5A1	YES
STAC	R149, R323	SH3 And Cysteine Rich Domain	YES
STEAP1	R149	STEAP Family Member 1	YES
TBKBP1	R158	TBK1 Binding Protein 1	YES
TMC6	R158	Transmembrane Channel Like 6	YES
EDA2R	R149	Ectodysplasin A2 Receptor	YES
EIF4EBP1	R323	Eukaryotic Translation Initiation Factor 4E Binding Protein 1	YES
FAM167A	R323	Family With Sequence Similarity 167 Member A	YES
GBP2	R149	Guanylate Binding Protein 2	No/Low
LCP1	R158	Lymphocyte Cytosolic Protein 1	No/Low
MCAM	R158	Melanoma Cell Adhesion Molecule	YES
MME	R158	Membrane Metalloendopeptidase	YES
PLXNA2	R158	Plexin A2	YES
RASSF8	R158	Ras Association Domain Family Member 8	YES
TFPI	R149	Tissue Factor Pathway Inhibitor	YES
ZFHX2	R323	Zinc Finger Homeobox 2	YES

B

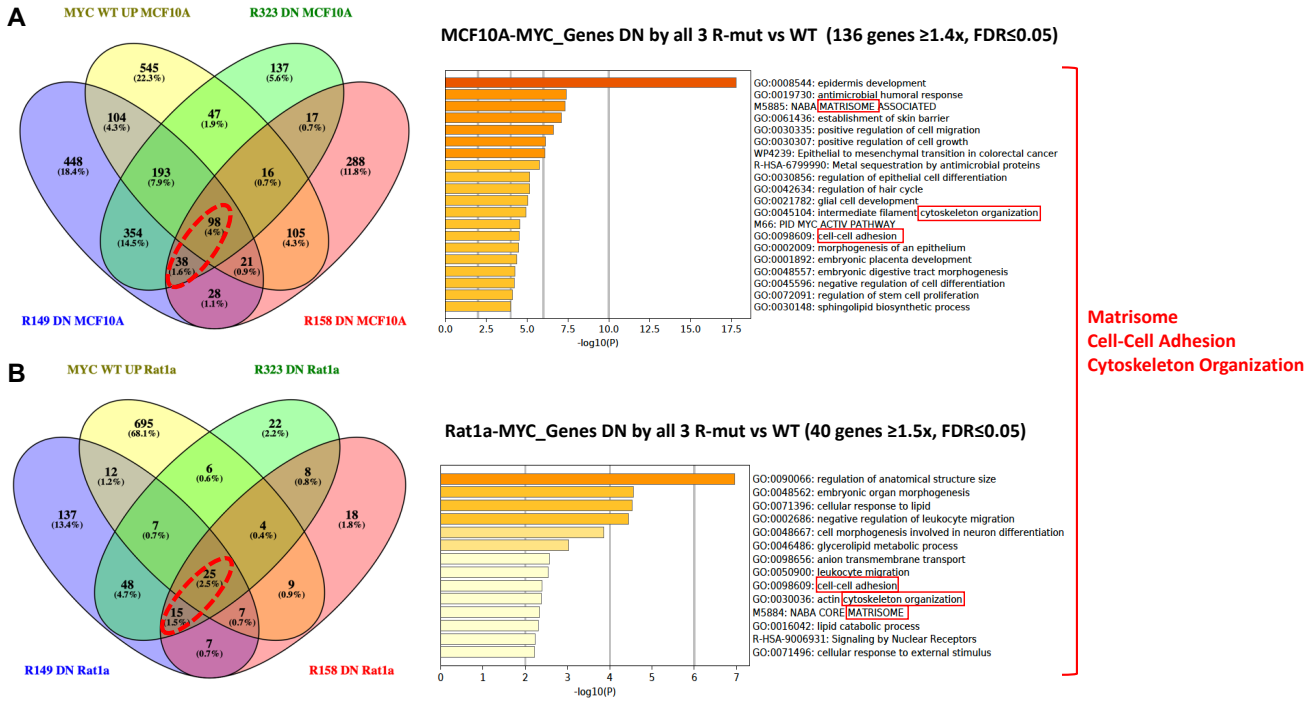


C



Supplemental Figure S14. (A) List of the common 34 genes deregulated (log2FC≥0.5, FDR ≤ 5%) by the same MYC R-mutants in Rat1a and MCF10A cells. Human *SCD5* is not found in rat

but the *Scd1* gene was found deregulated in Rat1a cells. **(B)** Results of Enrichr analyses of the 34 common deregulated genes of panel A (above) against the ChEA_2022 gene set database of ChIP-seq transcription factor binding data. The Volcano plot (created with an Appyter) shows the enriched terms/transcription factors; each point represents a single term, plotted by the corresponding odds ratio (x-position) and -log10(p-value) (y-position) from the enrichment results of the 34-gene input query. The larger and darker-colored the point, the more significantly enriched the input gene set is for the term. The indicated transcription factors passed the threshold of $P \leq 10^{-2}$ and were further analyzed. **(C)** The identified transcription factors in panel B ($P \leq 10^{-2}$) were further analyzed using STRING tool for potential protein-protein interaction (PPI) networks.



C

MCF10A all 3 R-mut Down

MSigDB Hallmark 2020 — Bar Graph | **Table** | Clustergram | Appyter

Hover each row to see the overlapping genes.

10 entries per page — Search:

Index	Name	P-value	Adjusted p-value	Odds Ratio	Combined score
1	KRAS Signaling Dn	0.00006859	0.002058	6.40	61.39
2	Estrogen Response Late	0.0004430	0.006645	5.53	42.71
3	KRAS Signaling Up	0.002487	0.02487	4.68	28.06
4	Apical Surface	0.03605	0.1435	7.04	23.41
5	Pperoxisome	0.03407	0.1435	4.41	14.92
6	TNF-alpha Signaling via NF-kB	0.04783	0.1435	3.04	9.24
7	Hypoxia	0.04783	0.1435	3.04	9.24
8	Estrogen Response Early	0.04783	0.1435	3.04	9.24
9	Interferon Gamma Response	0.04783	0.1435	3.04	9.24
10	Epithelial Mesenchymal Transition	0.04783	0.1435	3.04	9.24

Rat1a all 3 R-mut Down

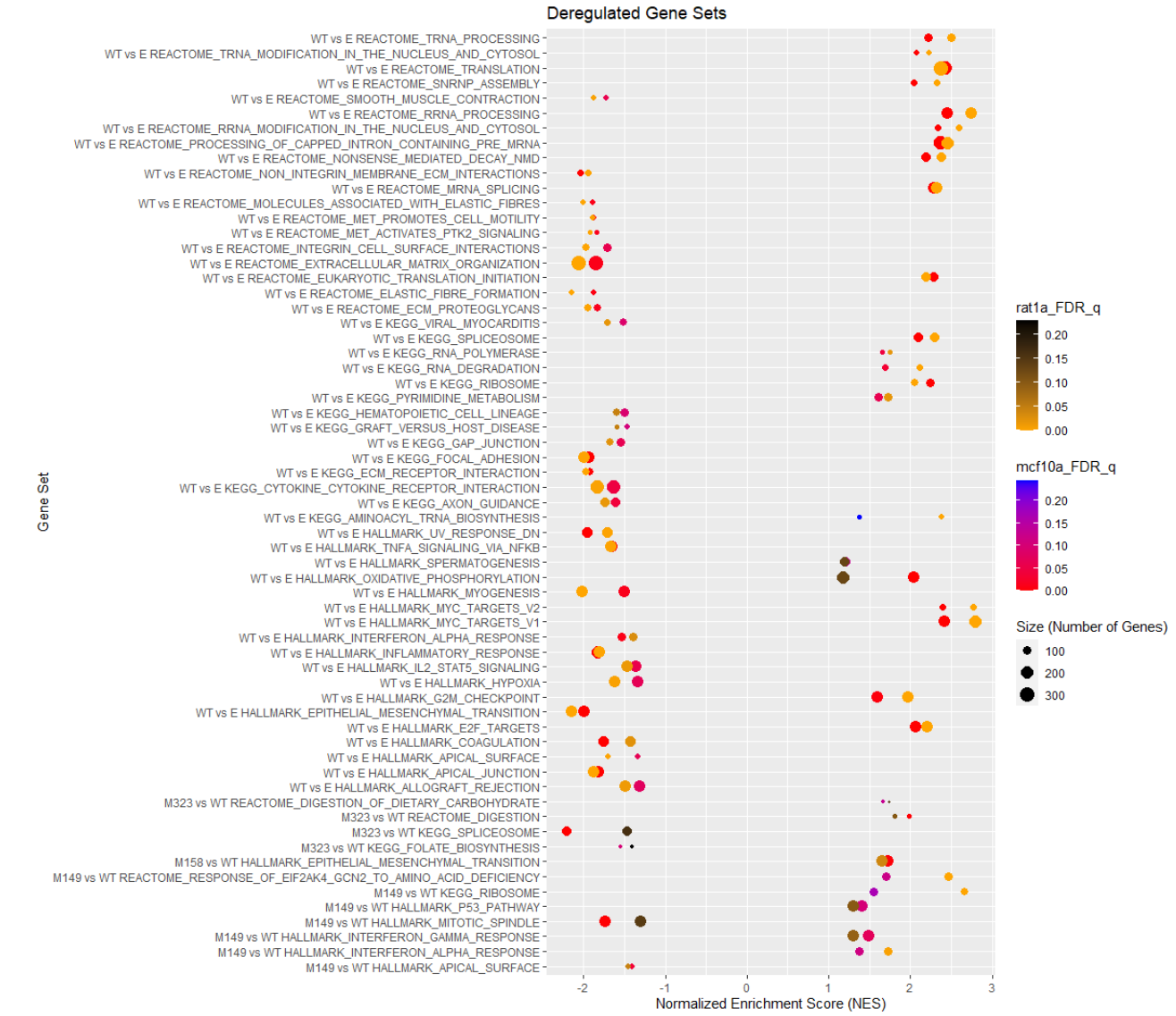
MSigDB Hallmark 2020 — Bar Graph | **Table** | Clustergram | Appyter

Hover each row to see the overlapping genes.

10 entries per page — Search:

Index	Name	P-value	Adjusted p-value	Odds Ratio	Combined score
1	KRAS Signaling Up	0.006909	0.05527	8.36	41.59
2	Epithelial Mesenchymal Transition	0.006909	0.05527	8.36	41.59
3	Angiogenesis	0.06791	0.2173	14.98	40.29
4	Coagulation	0.02965	0.1581	7.88	27.72
5	Myogenesis	0.05792	0.2173	5.40	15.37
6	Cholesterol Homeostasis	0.1347	0.3245	7.17	14.37
7	Androgen Response	0.1777	0.3245	5.28	9.12
8	Apoptosis	0.2706	0.3245	3.26	4.26
9	Mitotic Spindle	0.3232	0.3245	2.63	2.97
10	IL-2/STAT5 Signaling	0.3232	0.3245	2.63	2.97

Supplemental Figure S15. (A) and (B) Gene ontology (GO) analyses of the set of genes downregulated by all three MYC R-mutants (relative to MYC WT) in MCF10A cells (A) and Rat1a cells (B). Common enriched terms are indicated in red in the plots. **(C)** Molecular signature enrichment analysis of the above genes using Enrichr tool and the MSigDB Hallmark 2020 database. Red arrows point to signaling pathways enriched in both cell types.


Deregulated Gene Sets
WT vs E REACTOME_TRNA_PROCESSING
WT vs E REACTOME_TRNA_MODIFICATION_IN_THE_NUCLEUS_AND_CYTOSOL
WT vs E REACTOME_TRANSLATION
WT vs E REACTOME_SNRNP_ASSEMBLY
WT vs E REACTOME_SMOOTH_MUSCLE_CONTRACTION
WT vs E REACTOME_RRNA_PROCESSING
WT vs E REACTOME_RRNA_MODIFICATION_IN_THE_NUCLEUS_AND_CYTOSOL
WT vs E REACTOME_PROCESSING_OF_CAPPED_INTRON_CONTAINING_PRE_MRNA
WT vs E REACTOME_NONSENSE_MEDIATED_DECAY_NMD
WT vs E REACTOME_NON_INTEGRIN_MEMBRANE_ECM_INTERACTIONS
WT vs E REACTOME_MRNA_SPLICING
WT vs E REACTOME_MOLECULES_ASSOCIATED_WITH_ELASTIC_FIBRES
WT vs E REACTOME_MET_PROMOTES_CELL_MOTILITY
WT vs E REACTOME_MET_ACTIVATES_PTK2_SIGNALING
WT vs E REACTOME_INTEGRIN_CELL_SURFACE_INTERACTIONS
WT vs E REACTOME_EXTRACELLULAR_MATRIX_ORGANIZATION
WT vs E REACTOME_EUKARYOTIC_TRANSLATION_INITIATION
WT vs E REACTOME_ELASTIC_FIBRE_FORMATION
WT vs E REACTOME_ECM_PROTEOGLYCANS
WT vs E KEGG_VIRAL_MYOCARDITIS
WT vs E KEGG_SPLICEOSOME
WT vs E KEGG_RNA_POLYMERASE
WT vs E KEGG_RNA_DEGRADATION
WT vs E KEGG_RIBOSOME
WT vs E KEGG_PYRIMIDINE_METABOLISM
WT vs E KEGG_HEMATOPOIETIC_CELL_LINEAGE
WT vs E KEGG_GRAFT_VERSUS_HOST_DISEASE
WT vs E KEGG_GAP_JUNCTION
WT vs E KEGG_FOCAL_ADHESION
WT vs E KEGG_ECM_RECEPTOR_INTERACTION
WT vs E KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION
WT vs E KEGG_AXON_GUIDANCE
WT vs E KEGG_AMINOACYL_TRNA_BIOSYNTHESIS
WT vs E HALLMARK_UV_RESPONSE_DN
WT vs E HALLMARK_TNFA_SIGNALING_VIA_NFKB
WT vs E HALLMARK_SPERMATOGENESIS
WT vs E HALLMARK_OXIDATIVE_PHOSPHORYLATION
WT vs E HALLMARK_MYOGENESIS
WT vs E HALLMARK_MYC_TARGETS_V2
WT vs E HALLMARK_MYC_TARGETS_V1
WT vs E HALLMARK_INTERFERON_ALPHA_RESPONSE
WT vs E HALLMARK_INFLAMMATORY_RESPONSE
WT vs E HALLMARK_IL2_STAT5_SIGNALING
WT vs E HALLMARK_HYPOXIA
WT vs E HALLMARK_G2M_CHECKPOINT
WT vs E HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
WT vs E HALLMARK_E2F_TARGETS
WT vs E HALLMARK_COAGULATION
WT vs E HALLMARK_APICAL_SURFACE
WT vs E HALLMARK_APICAL_JUNCTION
WT vs E HALLMARK_ALLOGRAFT_REJECTION
M323 vs WT REACTOME_DIGESTION_OF_DIETARY_CARBOHYDRATE
M323 vs WT REACTOME_DIGESTION
M323 vs WT KEGG_SPLICEOSOME
M323 vs WT KEGG_FOLATE_BIOSYNTHESIS
M158 vs WT HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
M149 vs WT REACTOME_RESPONSE_OF_EIF2AK4_GCN2_TO_AMINO_ACID_DEFICIENCY
M149 vs WT KEGG_RIBOSOME
M149 vs WT HALLMARK_P53_PATHWAY
M149 vs WT HALLMARK_MITOTIC_SPINDLE
M149 vs WT HALLMARK_INTERFERON_GAMMA_RESPONSE
M149 vs WT HALLMARK_INTERFERON_ALPHA_RESPONSE
M149 vs WT HALLMARK_APICAL_SURFACE
Gene Set
-2
-1
0
1
2
3
Normalized Enrichment Score (NES)
rat1a_FDR_q
0.20
0.15
0.10
0.05
0.00
mcf10a_FDR_q
0.20
0.15
0.10
0.05
0.00
Size (Number of Genes)
100
200
300

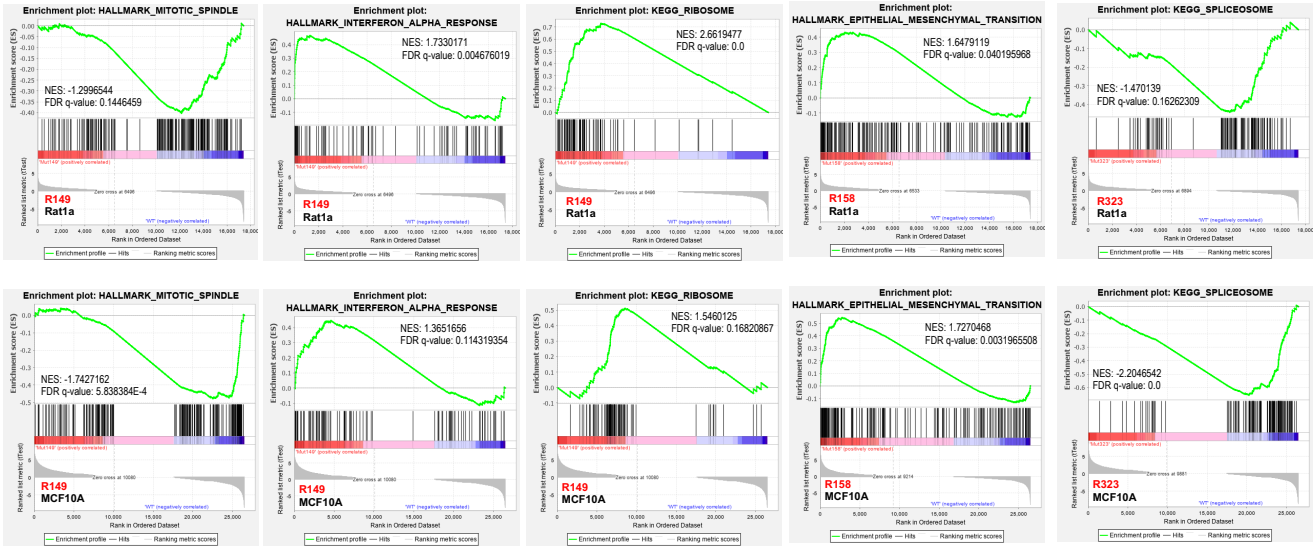
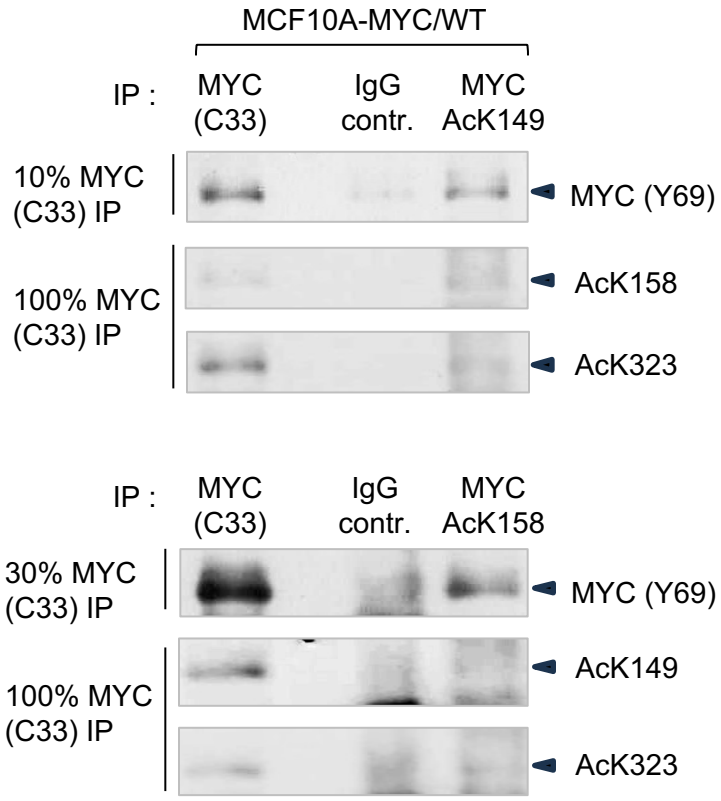
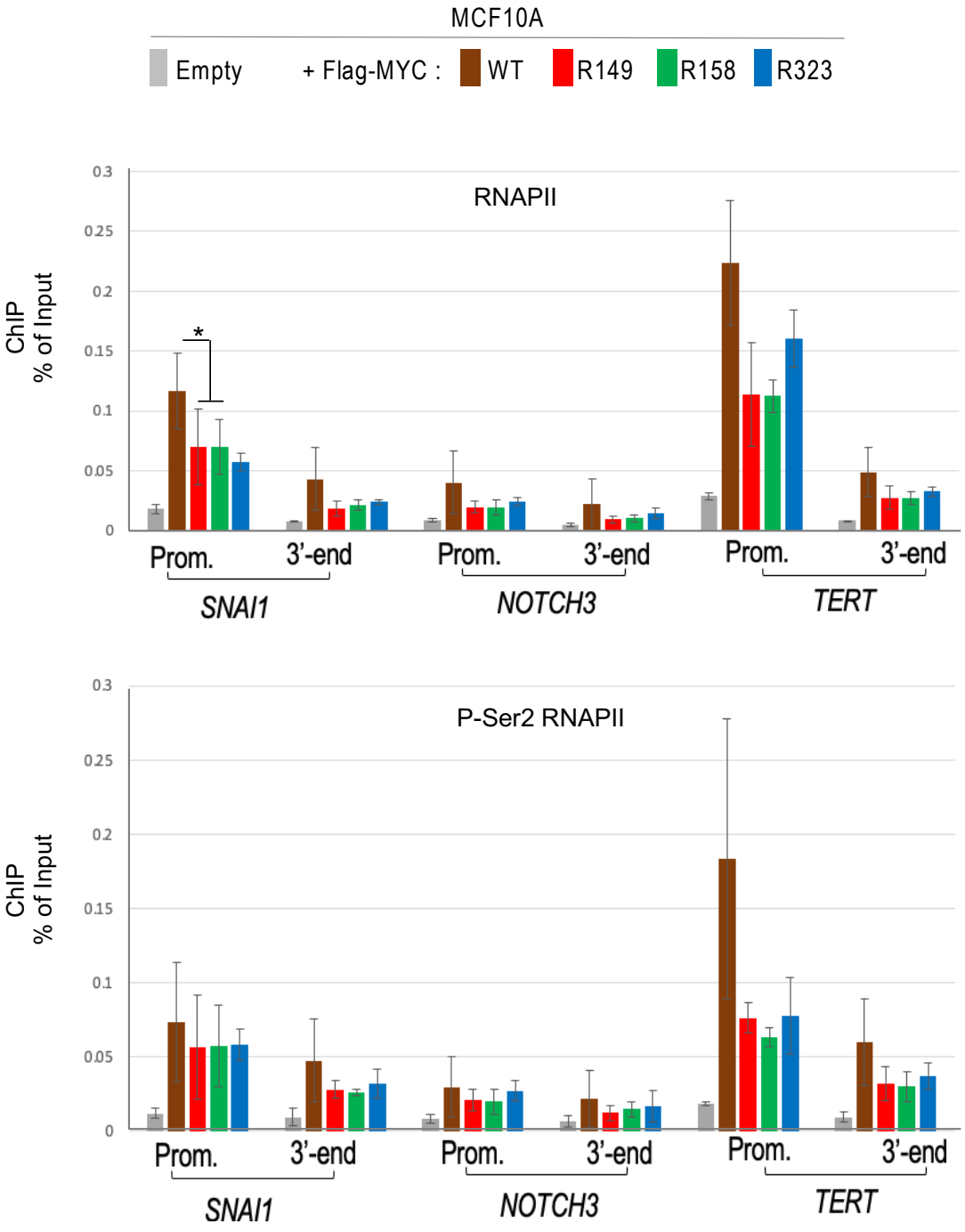

Enrichment plot: HALLMARK_MITOTIC_SPINDLE
NES: -1.2996544
FDR q-value: 0.1446459
R149
Rat1a
Enrichment plot:
HALLMARK_INTERFERON_ALPHA_RESPONSE
NES: 1.7330171
FDR q-value: 0.004676019
R149
Rat1a
Enrichment plot: KEGG_RIBOSOME
NES: 2.6619477
FDR q-value: 0.0
R149
Rat1a
Enrichment plot:
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
NES: 1.6479119
FDR q-value: 0.040195968
R158
Rat1a
Enrichment plot: KEGG_SPLICEOSOME
NES: -1.470139
FDR q-value: 0.16262309
R323
Rat1a
Enrichment plot: HALLMARK_MITOTIC_SPINDLE
NES: -1.7427162
FDR q-value: 5.838384E-4
R149
MCF10A
Enrichment plot:
HALLMARK_INTERFERON_ALPHA_RESPONSE
NES: 1.3651656
FDR q-value: 0.114319354
R149
MCF10A
Enrichment plot: KEGG_RIBOSOME
NES: 1.5460125
FDR q-value: 0.16820867
R149
MCF10A
Enrichment plot:
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
NES: 1.7270468
FDR q-value: 0.0031965508
R158
MCF10A
Enrichment plot: KEGG_SPLICEOSOME
NES: -2.2046542
FDR q-value: 0.0
R323
MCF10A

Fig.S16

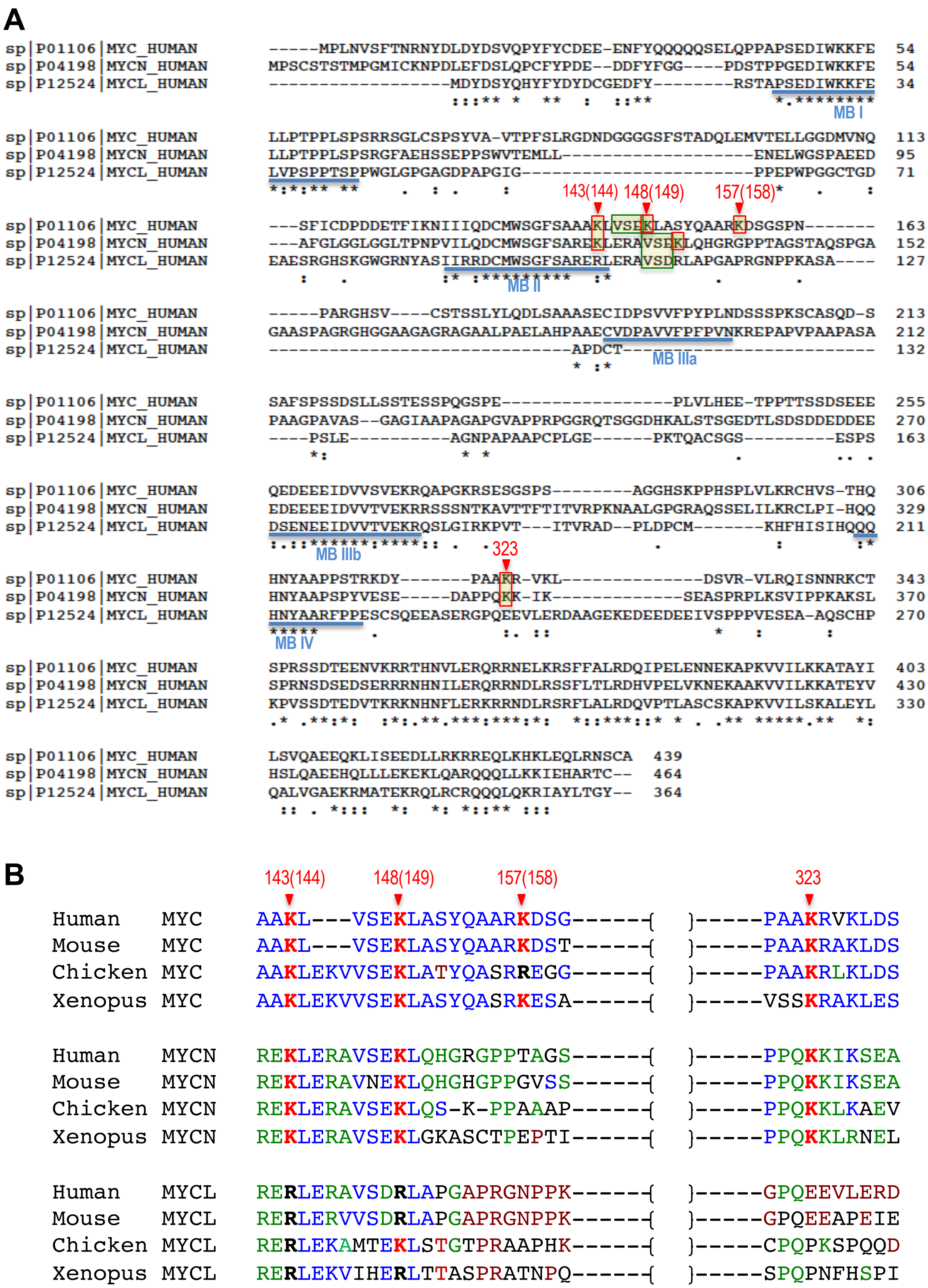
Supplemental Figure S16. GSEA analyses identified gene sets deregulated similarly by MYC and MYC R-mutants in both Rat1a and MCF10A cell lines. Top plot shows the enrichment scores for significantly deregulated gene sets (FDR q-value ≤0.25). Bottom are examples of enrichment plots for conserved deregulated gene sets.



Supplemental Figure S17. A fraction of MYC is doubly acetylated at K149/K158, K149/K323 and K158/K323. Total MYC in the MCF10A-MYC/WT cell line was immunoprecipitated (IP) with the C33 antibody and acetylated MYC was immunoprecipitated with either the AcK149 (top) or the AcK158 (bottom) antibodies. Rabbit IgG was used in control IP. As indicated, either 10%, 30%, or 100% of total MYC IP with C33 antibody (first lane) was analyzed by western blot in parallel with 100% of the IP with the AcK (and control IgG) antibodies. Immunoblotting was performed with the indicated antibodies (arrowheads).



Supplemental Figure S18. Analysis of RNAPII binding to select MYC AcK-dependent target genes. ChIP-qPCR analyses of total RNAPII and P-Ser2 RNAPII bound to the promoters and 3'-end (3'-UTR) of the indicated MYC-activated target genes in MCF10A-E, MCF10A-MYC WT or the indicated AcK-to-R mutant cell lines (R149, R158, R323). Data are the means and S.D. of the chromatin immunoprecipitated (% of the input chromatin) for 3 experiments each performed in triplicates (asterisk indicates $p<0.05$).


A
sp|P01106|MYC_HUMAN -----MPLNVSFTNRNYDLDYDSVQPYFYCDEE-ENFYQQQQQSELQPPAPSEDIWKKFE 54
sp|P04198|MYCN_HUMAN MPSCSTSTMPGMICKNPDLEFDSLQPCFYPDE--DDFYFGG----PDSTPPGEDIWKKFE 54
sp|P12524|MYCL_HUMAN -----------------MDYDSYQHYFYDYDCGEDFY--------RSTAPSEDIWKKFE 34
MB I
sp|P01106|MYC_HUMAN LLPTPPLSPSRRSGLCSPSYVA-VTPFSLRGDNDGGGGSFSTADQLEMVTELLGGDMVNQ 113
sp|P04198|MYCN_HUMAN LLPTPPLSPSRGFAEHSSEPPSWVTEMLL-------------------ENELWGSPAEED 95
sp|P12524|MYCL_HUMAN LVPSPPTSPPWGLGPGAGDPAPGIG-----------------------PPEPWPGGCTGD 71
143(144) 148(149) 157(158)*
sp|P01106|MYC_HUMAN ---SFICDPDDETFIKNIIIQDCMWSGFSAAAKLVSEKLASYQAARKDSGSPN------- 163
sp|P04198|MYCN_HUMAN ---AFGLGGLGGLTPNPVILQDCMWSGFSAREKLERAVSEKLQHGRGPPTAGSTAQSPGA 152
sp|P12524|MYCL_HUMAN EAESRGHSKGWGRNYASIIRRDCMWSGFSARERLERAVSDRLAPGAPRGNPPKASA---- 127
MB II
sp|P01106|MYC_HUMAN -----PARGHSV----CSTSSLYLQDLSAAASECIDPSVVFPYPLNDSSSPKSCASQD-S 213
sp|P04198|MYCN_HUMAN GAASPAGRGHGGAAGAGRAGAALPAELAHPAAECVDPAVVFPFPVNKREPAPVPAAPASA 212
sp|P12524|MYCL_HUMAN ------------------------------APDCT------------------------- 132
MB IIIa
sp|P01106|MYC_HUMAN SAFSPSSDSLLSSTESSPQGSPE-----------------PLVLHEE-TPPTTSSDSEEE 255
sp|P04198|MYCN_HUMAN PAAGPAVAS--GAGIAAPAGAPGVAPPRPGGRQTSGGDHKALSTSGEDTLSDSDDEDDEE 270
sp|P12524|MYCL_HUMAN ----PSLE----------AGNPAPAAPCPLGE------PKTQACSGS---------ESPS 163
sp|P01106|MYC_HUMAN QEDEEEIDVVSVEKRQAPGKRSESGSPS--------AGGHSKPPHSPLVLKRCHVS-THQ 306
sp|P04198|MYCN_HUMAN EDEEEEIDVVTVEKRRSSSNTKAVTTFTITVRPKNAALGPGRAQSSELILKRCLPI-HQQ 329
sp|P12524|MYCL_HUMAN DSENEEIDVVTVEKRQSLGIRKPVT---ITVRAD--PLDPCM-------KHFHISIHQQQ 211
MB IIIb
323
sp|P01106|MYC_HUMAN HNYAAPPSTRKDY-------PAAKR-VKL--------------DSVR-VLRQISNNRKCT 343
sp|P04198|MYCN_HUMAN HNYAAPSPYVESE-----DAPPQKK-IK-------------SEASPRPLKSVIPPKAKSL 370
sp|P12524|MYCL_HUMAN HNYAARFPPESCSQEEASERGPQEEVLERDAAGEKEDEEDEEIVSPPPVESEA-AQSCHP 270
MB IV
sp|P01106|MYC_HUMAN SPRSSDTEENVKRRTHNVLERQRRNELKRSFFALRDQIPELENNEKAPKVVILKKATAYI 403
sp|P04198|MYCN_HUMAN SPRNSDSEDSERRRNHNILERQRRNDLRSSFLTLRDHVPELVKNEKAAKVVILKKATEYV 430
sp|P12524|MYCL_HUMAN KPVSSDTEDVTKRKNHNFLERKRRNDLRSRFLALRDQVPTLASCSKAPKVVILSKALEYL 330
sp|P01106|MYC_HUMAN LSVQAEEQKLISEEDLLRKRREQLKHKLEQLRNSCA 439
sp|P04198|MYCN_HUMAN HSLQAEEHQLLLEKEKLQARQQQLLKKIEHARTC-- 464
sp|P12524|MYCL_HUMAN QALVGAEKRMATEKRQLRCRQQQLQKRIAYLTGY-- 364
B
143(144) 148(149) 157(158) 323
Human MYC AAKL---VSEKLASYQAARKDSG------{ }-----PAAKRVKLDS
Mouse MYC AAKL---VSEKLASYQAARKDST------{ }-----PAAKRAKLDS
Chicken MYC AAKLEKVVSEKLATYQASRREGG------{ }-----PAAKRLKLDS
Xenopus MYC AAKLEKVVSEKLASYQASRKESA------{ }-----VSSKRAKLES
Human MYCN REKLERAVSEKLQHGRGPPTAGS------{ }-----PPQKKIKSEA
Mouse MYCN REKLERAVNEKLQHGHGPPGVSS------{ }-----PPQKKIKSEA
Chicken MYCN REKLERAVSEKLQS-K-PPAAAP------{ }-----PPQKKLKAEV
Xenopus MYCN REKLERAVSEKLGKASCTPEPTI------{ }-----PPQKKLRNEL
Human MYCL RERLERAVSDRLAPGAPRGNPPK------{ }-----GPQEEVLERD
Mouse MYCL RERLERVVSDRLAPGAPRGNPPK------{ }-----GPQEEAPEIE
Chicken MYCL RERLEKAMTEKLSTGTPRAAPHK------{ }-----CPQPKSPQQD
Xenopus MYCL RERLEKVIHERLTTASPRATNPQ------{ }-----SPQPNFHSPI

Supplemental Figure S19. **(A)** Amino acid sequence comparisons of human MYC, MYCN and MYCL proteins using Clustal Omega Multiple Sequence Alignment tool from EMBL-EBI with default settings (https://www.ebi.ac.uk/Tools/msa/clustalo/). Homologous AcK residues are indicated in red and MYC boxes are underlined in blue. **(B)** Sequence context conservation around the AcK residues in MYC family proteins from different species. The AcK residues are generally absent and often replaced by R amino acids in MYCL proteins.