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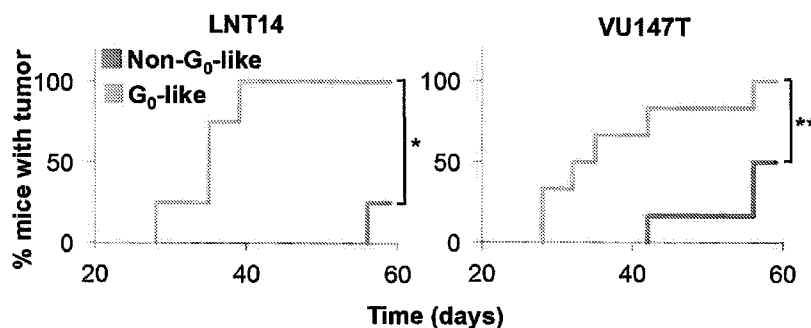


Figure 18G

(57) **Abstract:** Provided herein are methods and compositions related to modulation of the rate of asymmetric proliferation of cancer cells. In some embodiments, the methods described herein relate to the treatment of cancer, at least in part, via the modulation of the rate of asymmetric proliferation of cancer cells.

MODULATION OF ASYMMETRIC PROLIFERATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application Nos. 62/101,510 filed January 9, 2015, 62/213,334 filed September 2, 2015, and 62/247,389 filed October 28, 2015 and U.S. Application No. 14/603,866 filed January 23, 2015, which claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. Nos. 62/101,510 filed January 9, 2015 and which is a continuation-in-part application of International Patent Application No. PCT/US13/60842 filed September 20, 2013, which designates the United States and which claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application Nos. 61/704,033 filed September 21, 2012, the contents of which are incorporated herein by reference in their entirety.

GOVERNMENT SUPPORT

[0002] This invention was made with federal funding under Grant No. C06 CA059267 awarded by the National Cancer Institute. The U.S. government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on January 14, 2015, is named 030258-075393-US_SL.txt and is 782 bytes in size.

TECHNICAL FIELD

[0004] The technology described herein relates to the modulation of asymmetric cell division and proliferation in cancer cells.

BACKGROUND

[0005] Tumors can comprise both rapidly proliferating and slowly proliferating cells. Tumors comprising particularly rapidly proliferating cells clearly are capable of faster growth and progression. But these tumors also contain many slowly proliferating cancer cells that may complicate treatment by resisting cancer therapeutics which preferentially target fast proliferators. While clonal selection theory clearly explains how rapidly proliferating cancer cells evolve, it remains difficult to understand within this framework why even advanced tumors contain so many slowly proliferating cancer cells (P. C. Nowell, Science 194, 23 (Oct 1, 1976)). In culture, cancer cells have been observed to occasionally divide in such a manner that one daughter cell will have a markedly slower proliferative rate than the other, a phenomenon referred to as “asymmetric proliferation.” The occurrence of asymmetric proliferation is generally assumed to simply reflect random variation among individual cancer cells in the many genetic and non-genetic factors that influence transit through the cell cycle (J. Massague, Nature 432, 298 (Nov 18, 2004)).

BRIEF DESCRIPTION OF THE DRAWINGS

[0006] Figs. 1A-1P demonstrate that mTORC2-AKT1 signaling regulates asymmetric cancer cell division. Fig. 1A depicts a crystal structure of AKT1 protein with mutated sites noted. In Figs. 1B-1E and 1G-1P, the bar graphs depict percentages of H3K9me2^{low}/MCM2^{low}/HES1^{high} asymmetric cells and GO-like cells. Fig. 1B depicts the results of AKT1/2^{-/-} HCT116 cells replaced with AKT1 and AKT2 cDNAs, HCT116 wild type (WT). Fig. 1C depicts the results of mutation of AKT, e.g. with AKT1-T308A, AKT1-S473A, AKT1-T308A/S473A and AKT1-T450A cDNAs, HCT116 WT. Figs. 1D-1E depict the results of HCT116 and MCF7 cells, respectively treated with DMSO, control, TORIN1, AZD8055, INK128, Palomid 529, Rapamycin and RAD-001, for 72 h. Figs. 1G-1K depict the results of RICTOR knockdown in HCT116 (Fig. 1G), MCF7 (Fig. 1H), MDA-MB-231 (Fig. 1I), PC9 (Fig. 1J) and A375 (Fig. 1K) cells with control, non-silencing(NS) hairpin(hp) and RICTOR knockdown hp1,4. Figs. 1L-1M depict the results of HCT116 and MCF7 cells, respectively treated with DMSO, control and kinase inhibitors AZD5363, GDC0068, for 72 hours. Fig. 1N depicts the results of AKT1/2^{-/-} HCT116 cells replaced with AKT1-E17K cDNA, HCT116 WT. Figs. 1O-1P depicts the results of HCT116 and MCF7 cells, respectively treated with DMSO, control and AKT allosteric inhibitors AKT1/2, MK2206, for 72 h. Error bars indicate mean $\pm$ SEM. Fig. 1F depicts the results of a Western blot for RICTOR in HCT116 cells.

[0007] Figs. 2A-2F demonstrate that a TTC3-proteasome pathway is necessary for GO-like cells. Fig. 2A depicts the results of a Western blot for TTC3 in HCT116 cells. Figs. 2B-2F depict bar graphs of percentages of H3K9me2^{low}/MCM2^{low}/HES1^{high}. Figs. 2B-2C depict GO-like cells in HCT116 (Fig. 2B) and MCF7 (Fig. 2C) cells with control, NS hp, and TTC3 knockdown hp3-5. Fig. 2D depicts the results of AKT1/2^{-/-} HCT116 cells replaced with AKT1-K8R, AKT1-K14R and AKT1-K8R/K14R cDNAs, HCT116 WT. Figs. 2E-2F depict the results of HCT116 (Fig. 2E) and MCF7 (Fig. 2F) cells treated with DMSO, control, MG-132 and Bortezomib for 24hours. Error bars indicate mean $\pm$ SEM. Arrow indicates a GO-like TTC3⁺ cell.

[0008] Fig. 3 depicts a graph demonstrating that mTORC2 signaling induces slow proliferators. Percentage of sibling pairs with cell cycle times $< t$ (β curves) for HCT-116 cells transfected with either control, NS (lighter dots forming the right hand trend) vs. RICTOR knockdown, hp4 (darker dots forming the left-hand trend) shRNA. Each point is calculated at 20 minute intervals and only shown if there was at least one event occurring. N = 701 and 1295 for total number of cells counted for NS and RICTOR knockdown groups, respectively.

[0009] Figs. 4A-4N demonstrate that asymmetrically dividing cancer cells and slow proliferators promote tumorigenesis *in vivo*. Figs. 4A-4E depict the results of control, NS (squares) and RICTOR knockdown shRNAs hp1,4 (triangles and diamonds, respectively) of HCT116 (Fig. 4A), MCF7 (Fig. 4B), MDA-MB-231 (Fig. 4C), PC9 (Fig. 4D) and A375 (Fig. 4E) cells were injected in Nu/Nu mice and their tumor growth followed over a number of days. Figs. 4F-4J, 4L, and 4N demonstrate that tumor forming potential of cells treated with DMSO, control (squares) or AKT1/2 inhibitor for 72 h

(diamonds) in (Figs. 4F-4J) HCT116 and (Figs. 4L and 4M) MCF7 cells, respectively. Serial dilutions of (Figs. 4F,4L) 5×10^6 , (4G,4M) 5×10^5 , (4H) 5×10^4 , (4I) 5×10^3 , (4J) 5×10^2 cells were used to inject mice and the tumor formation and growth was monitored over several days. Error bars indicate mean $\pm$ SEM. Figs. 4K and 4N depict images of mice injected with (4K) 5×10^5 HCT116 cells, (4N) 5×10^6 MCF7 cells, C (control, DMSO treated), I (induced, AKT1/2 inhibitor treated).

[0010] Fig. 5 depicts a working model for asymmetric cancer cell division.

[0011] Figs. 6A-6E demonstrate that RICTOR knockdown does not alter proliferation *in vitro*. Proliferation assay were performed over 5 days for control, NS shRNA (squares) and RICTOR knockdown shRNAs hp1,4 (diamonds and triangles, respectively) in (6A) HCT116, (6B) MCF7, (6C) MDA-MB-231, (6D) PC9 and (6E) A375 cell lines, under normal conditions. Error bars indicate mean $\pm$ SEM.

[0012] Figs. 7A-7J demonstrate that $\beta 1$ -integrin-FAK-mTORC2-AKT1 signaling regulates the production of slow proliferators. Fig. 7A depicts a schematic model of AKT1 protein. Figs. 7B-7H depict graphical representation of percentage of change of H3K9me2low/MCM2low/HES1high asymmetrically dividing and GO-like cells compared to control in HCT116 and MCF7 cell lines. Error bars indicate mean $\pm$ SEM. Figs. 7I-7J depict plots for percentage of sibling pairs with cell cycle time difference $< t$. (K) HCT116 (L) MCF7 cells with control, NS (right-hand trend) or RICTOR, hp4 (left hand trend). N = 701 and 1295 for total number of cells counted for NS and RICTOR knockdown groups, respectively.

[0013] Fig. 8 demonstrates the interaction of FAK with RICTOR. In HCT116 and MCF7 cells, RICTOR was immunoprecipitated with anti-FAK and immunoblotted with anti-mTOR, anti-RICTOR and anti-RAPTOR antibody. Reciprocally, FAK was immunoprecipitated with anti-RICTOR and immunoblotted with anti-mTOR and anti-FAK, in G1 as well as M phase of the cell cycle.

[0014] Figs. 9A-9C demonstrate that slow proliferators promote tumorigenesis *in vivo*. Fig. 9A depicts the experimental procedure and results for mice with subcutaneous tumors treated with TS2/16 antibody once a week for 5 weeks or untreated (control). Fig. 9B depicts the experimental procedure and results when Inducible Non-Silencing shRNA (control) or RICTOR knockdown shRNAs hp1,4 of 5 different cell lines were injected into mice. Fig. 9C depicts the experimental procedure and results when serial dilutions of HCT116 and MCF7 cells incubated with DMSO (control) or AKT1/2i for 72 hours were injected into mice. Tumor volume was followed weekly. Error bars indicate mean $\pm$ SEM for five mice per group.

[0015] Fig. 10 depicts a working model for asymmetric cancer cell division.

[0016] Figs. 11A-11C demonstrate knockdown of proteins in HCT116 cells. Figs. 11A and 11B depict knockdown of FAK (Fig. 11A) and $\beta 1$ -integrin (Fig. 11B) in HCT116 cells with Non-Silencing (NS) as control shRNA. Fig. 11C depicts a graphical representation of percentage of change of

H3K9me2low/MCM2low/HES1high asymmetrically dividing and G0-like cells compared to control in RICTOR knockdown cell lines. Error bars indicate mean $\pm$ SEM.

[0017] Figs. 12A-12O demonstrate that RICTOR knockdown does not alter proliferation in vitro. Figs. 12A-12O depict graphs of the results of proliferation assay over 5 days for control, NS hp (squares) and RICTOR knockdown shRNAs hp1,4 (triangles and circles, respectively) in (Fig. 12A) HCT116, (Fig. 12B) MCF7, (Fig. 12C) MDA-MB-231, (Fig. 12D) PC9 and (Fig. 12E) A375 cell lines, under normal conditions; in (Fig. 12F) HCT116, (Fig. 12G) MCF7, (Fig. 12H) MDA-MB-231, (Fig. 12I) PC9 and (Fig. 12J) A375 cell lines, under hypoxia conditions; in (Fig. 12K) HCT116, (Fig. 12L) MCF7, (Fig. 12M) MDA-MB-231, (Fig. 12N) PC9 and (Fig. 12O) A375 cell lines, under low serum conditions. Error bars indicate mean $\pm$ SEM.

[0018] Figs. 13A-13J demonstrate that RICTOR knockdown does not alter proliferation in vitro. Figs. 13A-13E depict graphs of proliferation assay over 5 days for control, NS hp (squares) and RICTOR knockdown shRNAs hp1,4 (triangles and circles, respectively) in (Fig. 13A) HCT116, (Fig. 13B) MCF7, (Fig. 13C) MDA-MB-231, (Fig. 13D) PC9 and (Fig. 13E) A375 cell lines, under low glucose conditions. Figs. 13F-13J depict the results of clonogenic assay over 2 weeks after irradiation for control, NS hp and RICTOR knockdown shRNAs hp1,4 in (Fig. 13F) HCT116, (Fig. 13G) MCF7, (Fig. 13H) MDA-MB-231, (Fig. 13I) PC9 and (Fig. 13J) A375 cell lines. Error bars indicate mean $\pm$ SEM.

[0019] Figs. 14A-14D demonstrate that RICTOR knockdown does not alter invasion in vitro. Figs. 14A-14D depict the results of invasion assay over 24 hours for control, NS hp (first bar) and RICTOR knockdown shRNAs hp1,4 (second and third bars, respectively) in (Fig. 14A) HCT116, (Fig. 14B) MCF7, (Fig. 14C) PC9 and (Fig. 14D) A375 cell lines. Error bars indicate mean $\pm$ SEM.

[0020] Figs. 15A-15D depict a mechanism for AKT1^{low} slow proliferators: AKT1, TTC3, and proteasome. Fig. 15A depicts a bar graph of percentages of H3K9me2low/MCM2low/HES1high asymmetric mitoses and G0-like cells in AKT1/2^{-/-} HCT116 cells with cDNAs for AKT1 or AKT2 or AKT1-K179M or AKT1-D292A. Fig. 15B depicts a schematic model of AKT1 protein with C, catalytic; P, phosphorylation; Ub, ubiquitination; PH, pleckstrin homology; HD, hydrophobic domain. Fig. 15C depicts a graphical representation of percentage change in H3K9me2low/MCM2low/HES1high asymmetrically dividing and G0-like cells relative to control in HCT116 and MCF7 cell lines. Solid bars represent asymmetrically dividing and clear bars represent G0-like cancer cells. Error bars indicate mean $\pm$ SEM for 3 replicates. Fig. 15D depicts Western blot analysis of short hairpin TTC3 knockdown.

[0021] Figs. 16A-16C demonstrate a mechanism for AKT1low slow proliferators: FAK, mTORC2, and AKT1. Fig. 16A depicts a graphical representation of percentage change in H3K9me2low/MCM2low/HES1high asymmetrically dividing and G0-like cells relative to control in HCT116 and MCF7 cell lines. Solid bars represent asymmetrically dividing and clear bars represent

G0-like cancer cells. Error bars indicate mean $\pm$ SEM for 3 replicates. Fig. 16B depicts Western blot analysis of short hairpin RICTOR knockdown. Fig. 16C depicts HCT116 and MCF7 cells in M-phase of the cell cycle, FAK IP with anti-FAK and immunoblotted with anti-FAK, anti-mTOR, anti-RICTOR, and anti-RAPTOR antibody. Reciprocally, RICTOR IP with anti-RICTOR and immunoblotted with anti-RICTOR or anti-FAK antibody.

[0022] Figs. 17A-17E demonstrate a mechanism for AKT1low slow proliferators: β 1-integrin and FAK. Figs. 17A-17B depict graphical representation of percentage change in H3K9me2low/MCM2low/HES1high asymmetrically dividing and G0-like cells relative to control in HCT116 and MCF7 cell lines. Solid bars represent asymmetrically dividing and clear bars represent G0-like cancer cells. Error bars indicate mean $\pm$ SEM for 3 replicates. Fig. 17C depicts bar graphs of percentages of H3K9me2low/MCM2low/HES1high asymmetric mitoses and G0-like cells in MCF7 cells plated on control (random) or aligned type-I collagen fibrils (aligned). Figs. 17D and 17E depicts Western blots of short hairpin FAK and β 1-integrin knockdown in HCT116 cells with nonsilencing shRNA (NS) as control.

[0023] Figs. 18A-18G demonstrate that G0-like cells within OSCCs exhibit stem cell-like functional properties. Fig. 18A, FACS plot of PyroninY versus Hoechst-33342 illustrating the G0-like gate in representative LNT14 cell line (left). H2DCFDA profiles are compared between G0-like and non-G0-like fractions in two OSCC cell lines (right). * $p < 0.001$, ** $p < 0.0001$. Fig. 18B, Western blots (WBs) of total (unfractionated) and FACS-purified G0-like cells from two OSCC cell lines. Fig. 18C, H2DCFDA profiles of G0-like vs. non-G0-like fractions from a PDX and patient tumor. Fig. 18D, Quantitation by WB of Akt levels in G0-like vs. non-G0-like fractions within two primary tumors (LST34 and LST42). Fig. 18E, Confocal IF of LNT14 patient tumor and its derivative PDX stained for H3K9me2, Hes1, pan-Akt, and DAPI. By this staining method, the distribution of G0-like cell content among 9 patient tumors is shown. Fig. 18F, Primary and secondary sphere-forming frequency by G0-like vs. non-G0-like fractions of two OSCC cell lines. * $p < 0.05$, ** $p < 0.0001$. Fig. 18G, Xenograft tumor formation by G0-like vs. non-G0-like fractions from LNT14 (100 cells/mouse, $n = 4/\text{group}$) and VU147T (1000 cells/mouse, $n = 6/\text{group}$) cell lines. * $p < 0.05$, ** $p < 0.025$.

[0024] Figs. 19A-19F demonstrate that high JARID1B is a distinct basis for detecting OSCC stem cell-like function. Fig. 19A, The JARID1Bhigh gate is defined in LNT14_J1BpromEGFP cells (left). JARID1B expression in JARID1Bhigh cells isolated by FACS is shown by QRT-PCR (middle, * $p < 0.025$) and WB (right), where values indicate JARID1B normalized to HSP90. Fig. 19B, FC histogram shows PKH26 label distribution in JARID1Bhigh vs. total LNT14_J1BpromEGFP cells after 10 days. Fig. 19C, PyroninY vs. Hoechst-33342 FACS plots illustrate the cell cycle distribution of the G0-like and JARID1Bhigh fractions and quantitate of the percentage of G0-like cells in JARID1Bhigh vs. total fractions in two J1BpromEGFP-expressing cell lines. * $p < 0.001$, ** $p < 0.0001$. Fig. 19D, EGFP reporter profiles depicting the JARID1Bhigh gate are compared between

LNT14_J1BpromEGFP cells grown under monolayer versus sphere culture conditions. Parental LNT14 cells are used as negative control. * $p < 0.01$. Fig. 19E, Primary and secondary sphere-forming frequency of JARID1B^{high} vs. total cells from two J1BpromEGFP-expressing OSCC cell lines. * $p < 0.0005$, ** $p < 0.0001$. Fig. 19F, Xenograft tumor formation at limiting cell dose of G0-like, JARID1B^{high}, or bulk cells purified from the LNT14_J1BpromEGFP line (100 cells/mouse, $n = 6$ /group). * $p < 0.05$, ** $p < 0.0025$.

[0025] Figs. 20A-20E demonstrate that JARID1B^{high} and G0-like cells exhibit disparate stem cell marker expression and PI3K pathway function. Fig. 20A, The percentage of CD44^{high} cells by FC in the G0-like, JARID1B^{high}, and total populations of two OSCC cell lines. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$. Fig. 20B, ALDH1A1 mRNA expression by QRT-PCR in G0-like, JARID1B^{high}, and bulk LNT14 cells. * $p < 0.05$. Fig. 20C, WBs for stem cell markers in total or FACS-purified G0-like, JARID1B^{high}, and bulk LNT14 cells. Fig. 20D, Venn diagram depicts genes upregulated relative to the bulk population in G0-like and JARID1B^{high} LNT14 cells. Fig. 20E, WBs of JARID1B^{high} vs. total cells from two OSCC cell lines.

[0026] Figs. 21A-21D demonstrate G0-like cells are primed to enter the JARID1B^{high} state. A, EGFP histograms illustrating JARID1B^{high} gate in G0-like, bulk, and total LNT14_J1BpromEGFP cells upon FACS-purification (day 0) and re-culture (days 4 and 7). Arrow highlights EGFP profile shift by G0-like population (top). Quantification of JARID1B^{high} cells (bottom). * $p < 0.0025$, ** $p < 0.0001$, n.s. not significant. B, Experimental schematic for CellTrace Violet labeling, re-culture, and analysis of G0-like vs. bulk LNT14_J1BpromEGFP cells (left) shows interpretation of four quadrants in the FC plot (right, top). Histogram compares size of the CellTrace label-retaining component of JARID1B^{high} cells arising from G0-like vs. bulk fractions (right, bottom). * $p < 0.0025$, ** $p < 0.0005$. C, Percentage of JARID1B^{high} cells by FC in spheres generated from G0-like vs. bulk LNT14_J1BpromEGFP cells. * $p < 0.0001$. D, EGFP fluorescence distribution within spheres arising from G0-like, JARID1B^{high}, and bulk fractions of LNT14_J1BpromEGFP cells. Gates define size of the more intense peak in the bimodal EGFP distribution.

[0027] Figs. 22A-22G demonstrate that the G0-like fraction exerts stem cell-like function by a JARID1B-dependent mechanism. Fig. 22A, JARID1B expression by QRT-PCR and WB (inset) in LNT14 cells expressing scramble (scr) or JARID1B (sh) shRNA. * $p < 5 \times 10^{-5}$. Fig. 22B, Primary and secondary clonal sphere formation by G0-like and bulk cells from sh vs. scr LNT14 cells. * $p < 0.0005$, ** $p < 0.0001$. Fig. 22C, Xenograft tumor formation by G0-like or bulk fractions from sh vs. scr LNT14 cell lines (100 cells/mouse, $n = 6$ /group). * $p < 0.025$. Fig. 22D, Percentage of cells in the G0-like gate of two sh vs. scr cell lines. * $p < 0.025$. Fig. 22E, Sphere formation by (left) and Oct4 and BMI1 expression (right) in EGFP^{high} and total cells from sh or scr LNT14_J1BpromEGFP lines. WB values are relative band densities normalized to GAPDH. * $p < 0.0001$. Fig. 22F, Cell surface CD44 expression by FC (left) and ALDH1A1 expression by QRT-PCR (right) in sh vs. scr LNT14 cells.

* $p < 0.0001$. Fig. 22G, Cell surface CD44 profile (left) and ALDH1A1 expression (middle) are defined by FC and QRT-PCR, respectively, in LNT14 cells after JARID1B cDNA or mock transfection. Sphere formation is shown in JARID1B- or mock-transfected LNT14 cells (right) * $p < 0.0001$.

[0028] Figs. 23A-23F demonstrate that the PI3-kinase pathway regulates the dynamics between G0-like and JARID1B^{high} states. Fig. 23A, Akt and pAkt levels are shown by WB in sh vs. scr LNT14 cells (left) and in EGFP^{high} vs. total LNT14_J1BpromEGFP_shJARID1B cells (right). Fig. 23B, The size of the JARID1B^{high} fraction is defined in LNT14_J1BpromEGFP_myrAktER cells treated with vehicle (ethanol) or 10 nM 4-hydroxytamoxifen (4-OHT) for 72 hours. * $p < 0.0001$. Fig. 23C, LNT14_J1BpromEGFP cells treated for 72 hrs. with 10 μ M LY294002 or DMSO were analyzed for EGFP by FC (left) and JARID1B by WB (right). * $p < 0.01$. Fig. 23D, LNT14_J1BpromEGFP cells treated with 10 μ M LY294002 or DMSO for 72 hours were analyzed for G0-like content; representative PyroninY vs. Hoechst-33342 FC plots shown (left). G0-like and bulk LNT14_J1BpromEGFP cells were purified after 72 hours of 250 nM GDC-0941 (GDC) and analyzed for sphere (middle) and xenograft tumor (right, $n = 6$ /group) formation. * $p < 0.05$. Fig. 23E, Tumor growth over time in LNT14_J1BpromEGFP xenograft-bearing mice ($n = 5$ /group) treated with 100mg/kg daily GDC or vehicle (left). Tumors from GDC- or vehicle-treated mice ($n = 4$ tumors/group) were analyzed for JARID1B^{high} and G0-like cells. Representative EGFP histograms (middle) and PyroninY vs. Hoechst-33342 FC plots (right) are shown. * $p < 0.025$. Fig. 23F, Confocal IF of tumor tissue from GDC- or vehicle-treated mice were stained for H3K9me2, Hes1, pan-Akt, and DAPI. Quantitation of G0-like cell frequency based on IF staining is shown for GDC vs. vehicle groups ($n = 4$ tumors/group). * $p < 0.05$.

[0029] Fig. 24 depicts a model of G0-like and JARID1B^{high} cells as distinct but related subsets within the oral CSC pool. The G0-like fraction exerts its stem cell-like function by efficient entry to a JARID1B^{high} state distinguished by upregulation of conventional stem cell-like molecular traits. PI3K activation acts proximally to increase JARID1B expression in G0-like cells and drives their transition into the JARID1B^{high} fraction. G0-like cells are shown to arise from rapid-cycling cells. Dotted grey arrows represent other cell state transitions that potentially impact homeostasis of the oral CSC pool but are not addressed experimentally in this study.

[0030] Figs. 25A-25J depict analysis of AKT^{low} cells treated with Akti-1/2. Figs. 25A-25B demonstrate tumorigenicity of Akti-1/2 treated cells. Plots depict tumor growth curves after injection of 500,000 HCT116 (Fig. 25A) or MCF7 (Fig. 25B) cells in two sets of mice. Indicated curves correspond to mice that were injected with Akti-1/2 treated cells, while other curves correspond to mice that were injected with DMSO treated cells. Five mice were initially used for each cell line and condition. Each data point represents the average of the replicates and error bars show the standard error of the mean (SEM). P-values correspond to the t-test statistical differences in tumor volume on the last day of follow-up. Figs. 25C-25E depict multi-scale genomics profiling of AKT^{low} cells.

Average M-A plots for GRO-Seq (Fig. 25C), RNA-Seq (Fig. 25D) and protein (Fig. 25E) datasets for both HCT116 and MCF7 cell lines. X-axis shows the average log₂ expression, and y-axis shows the average log₂ fold-change between both conditions (Akti-1/2 - DMSO). Positive log₂ fold-changes correspond to overexpression in Akti-1/2 treated cells compared to DMSO. Only genes or proteins displaying consistent changes after Akti-1/2 treatment (i.e. log₂ fold change either positive or negative in both cell lines) are shown. Numbers on the right side of the plots depict the number of genes or proteins with an average fold change larger than 2-fold (i.e. 1 in log₂ space) in absolute terms (grey dashed lines). Figs. 25F-25H depict correlation between changes in each data type. Scatterplots of log₂ fold changes for genes or proteins in common between the different data types: GRO-Seq vs RNA-Seq (Fig. 25F), GRO-Seq vs protein (Fig. 25G), RNA-Seq vs protein (Fig. 25H). For each plot, only genes or proteins that show consistent change in both cell lines for both data types are selected. Numbers in the corners correspond to the number of genes or proteins that show a log₂ fold change outside the 2-fold change region (grey dashed lines). Figs. 25I-25J depict gene-set enrichment analysis of proteomics results. Barplots depict the number of significantly down-regulated (Fig. 25I) or up-regulated (Fig. 25J) gene sets in AKTlow cells that fall in each functional category (FWER < 5%). Only canonical gene sets (i.e., KEGG, REACTOME, BIOCARTE, PID, GO) were included in these analyses.

[0031] Figs. 26A-26H depict cellular profiling. Fig. 26A depicts metabolic profiling of AKTlow cells. Barplot depicts the log₂ fold-changes (Akti-1/2 - DMSO) of 13 up-regulated metabolites with an average fold change > 2 (log₂ > 1). Bars correspond to lysolipid metabolic derivatives. Fig. 26B depicts secretory profiling of AKTlow cells. Barplot depicts the log₂ fold-changes (Akti-1/2 - DMSO) of 13 up-regulated secreted proteins with an average fold change > 2 (log₂ > 1). Bars correspond to proteins related to the TNF, VEGF and WNT families, respectively. Figs. 26C-26D depict Western blots of purified microvesicle fractions probed for CD63 and CD68 (i.e., exosome markers), Calnexin (i.e. ER vesicles marker), and GM130 (i.e., Golgi vesicles marker) in HCT116 and MCF7 (Fig. 26C), and HCT116 AKT1/2-/- knockout (Fig. 26D) cell lines after treatment either with DMSO or Akti-1/2 for 72h. A whole cell lysate (WCL) has been added as a control. For each cell line and condition microvesicle fractions were isolated from equivalent number of cells (1x10⁶). Fig. 26E depicts Western blots of purified microvesicle fractions probed for TNFSF10 in HCT116 (left panel) and MCF7 (right panel) after treatment either with DMSO or Akti-1/2. Microvesicle fractions used for the Western Blots were isolated from equivalent number of cells (1x10⁶). Figs. 26F-26G depict microvesicle (Fig. 26F) and cell (Fig. 26G) small RNA-Seq profiling. Average M-A plot of both HCT116 and MCF7 cell lines. X-axis shows the average log₂ small RNA abundance, and y-axis shows the average log₂ fold-change between both conditions (Akti-1/2 - DMSO). Positive log₂ fold-changes correspond to a small RNA abundance increase in Akti-1/2 treated cells compared to DMSO. Only small RNAs displaying consistent changes after Akti-1/2 treatment (i.e., log₂ fold change either

positive or negative in both cell lines) are shown. Numbers on the right side of the plots depict the number of small RNAs with an average fold change larger than 2-fold (i.e. 1 in log₂ space) in absolute terms (grey dashed lines). Fig. 26H depicts the correlation between changes in microvesicle and cell small RNA abundance. X-axis corresponds to log₂ fold change in cellular small RNA expression (Akti-1/2 - DMSO), while y-axis corresponds to log₂ fold change in microvesicle small RNA expression (Akti-1/2 - DMSO). Positive log₂ fold-changes correspond to a small RNA abundance increase in Akti-1/2 treated cells compared to DMSO-treated cells. Numbers in the corners correspond to the number of small RNAs that show a log₂ fold change outside the 2-fold change region (grey dashed lines).

[0032] Figs. 27A-27E depict the effect of microvesicles on growth rate. Different tumor cell lines (e.g., HCT116 (Fig. 27A), MCF7 (Fig. 27B), MDA-MB-231 (Fig. 27C), PC9 (Fig. 27D), and A375 (Fig. 27E)) were exposed to microvesicles derived from either Akti-1/2 or DMSO treated cells of the same type for 1 hour. Experiments were done in triplicate for each cell line and treatment. Error bars show the standard error of the mean (SEM). P-values shown at the top-left corner on each panel correspond to the model comparing the two slopes of the linear models fitted for each condition (i.e., DMSO or Akti-1/2 microvesicles). Non-significant p-values in all five cell lines support the finding that there is no evidence of any significant difference in cell growth based on the differential microvesicle treatment. Figs. 27F-27J depict Akti-1/2 microvesicles bioactivity in vitro. Barplots depict the log₂ fold change (Akti 1/2 - DMSO) in the total number of cells exposed to microvesicles derived from either Akti-1/2 or DMSO treated cells of the same type for 1 hour. Cells were then placed under three different stress conditions (e.g. 1% serum, 4% oxygen, standard chemotherapy agent) for 72h (grey bar). After pre-conditioning, cells were also passaged for two weeks before placing under three different stress conditions (e.g. 1% serum, 4% oxygen, standard chemotherapy agent) for 72h (open bar). Experiments were done in triplicates for each 5 different cells line (e.g., HCT116 (Fig. 27F), MCF7 (Fig. 27G), MDA-MB-231 (Fig. 27H), PC9 (Fig. 27I), and A375 (Fig. 27J)), treatment, and stress condition. Error bars show the standard error of the mean (SEM). Asterisks on top of bars designate statistically significant increases (i.e., $p < 0.05$, one-sided t-test) in cell count after exposing them to microvesicles derived from Akti-1/2 treated cells, compared to microvesicles derived from DMSO treated cells.

[0033] Figs. 28A-28E depict Akti-1/2 microvesicles bioactivity in vivo. Plots depict tumor growth curves after injection of tumorigenic cells exposed during 1 hour to microvesicles derived from equivalent (i.e., 1×10^6) numbers of either Akti-1/2 or DMSO treated cancer cells (e.g., HCT116 (Fig. 28A), MCF7 (Fig. 28B), MDA-MB-231 (Fig. 28C), PC9 (Fig. 28D), and A375 (Fig. 28E)). 500,000 cells were injected into each mice. Indicated curves correspond to mice that were injected with cells admixed with microvesicles derived from Akti-1/2 treated cells, while other curves correspond to mice that were injected with cells admixed with microvesicles derived from DMSO

treated cells. Six mice were initially used for each cell line and condition. Each data point is an average of all replicates and error bars show the standard error of the mean (SEM). P-values correspond to the t-test statistical differences in tumor volume on the last day of follow-up.

[0034] Figs. 29A-29B depict changes in RNA Pol II pausing after AKTi treatment. Comparison of the genome-wide log₂ Pausing Index between AKTi and DMSO treated cells (i.e., HCT116 (Fig. 29A), MCF7 (Fig. 29B)). Pearson correlation coefficient is shown on the bottom right corner of each plot. Only genes with a Pausing Index higher than 0.1 (log₂) in at least one of the samples were included in the plot, yielding a total of 12652 genes.

[0035] Figs. 30A-30E depict the effect of AKTi microvesicles on cell vitality (i.e., MTS assay). Different tumor cell lines (e.g., HCT116 (Fig. 30A), MCF7 (Fig. 30B), MDA-MB-231 (Fig. 30C), PC9 (Fig. 30D), and A375 (Fig. 30E)) were exposed to microvesicles derived from Akti-1/2, MK-2206, or DMSO treated cells of the same type for 1 hour, and a subsequent MTS assay was performed in triplicate for each cell line and treatment. Bars depict the log₂ fold-change in cell survival between AKTi-treated cells (i.e., Akti-1/2 or MK-2206) and DMSO-treated cells after 120h. Error bars show the standard error of the mean (SEM). Asterisks on top of bars designate statistically significant increases (i.e., $p < 0.05$, t-test). Figs. 30F-30J depict the effect of AKTi microvesicles on cell colony counts after exposure to stress conditions. Barplots depict the log₂ fold change (i.e., Akti-1/2 - DMSO, grey bar; MK-2206 - DMSO, open bar) in the number of colonies formed after exposure to microvesicles derived from either AKTi or DMSO treated cells of the same type for 1 hour. Exposed cells were placed under three different stress conditions (e.g., 1% serum, 4% oxygen, standard chemotherapy agent) for 72h. Experiments were done in triplicate for each different cells line (e.g., HCT116 (Fig. 30F), MCF7 (Fig. 30G), MDA-MB-231 (Fig. 30H), PC9 (Fig. 30I), and A375 (Fig. 30J)), treatment, and stress condition. Error bars show the standard error of the mean (SEM). Asterisks on top of bars designate statistically significant increases (i.e., $p < 0.05$, one-sided t-test) in colony counts after exposing cells to microvesicles derived from AKTi-treated cells, compared to microvesicles derived from DMSO-treated cells.

[0036] Figs. 31A-31-E depict the effect of microvesicles on growth rate using MK-2206, a different AKT1 allosteric inhibitor. Different tumor cell lines (e.g., HCT116 (Fig 31A), MCF7 (Fig. 31B), MDA-MB-231 (Fig. 31C), PC9 (Fig. 31D), and A375 (Fig. 31E)) were exposed to microvesicles derived from either MK-2206 or DMSO treated cells of the same type for 1 hour. Experiments were done in triplicate for each cell line and treatment. Error bars show the standard error of the mean (SEM). P-values shown at the top-left corner on each panel correspond to the model comparing the two slopes of the linear models fitted for each condition (i.e., DMSO or Akti-1/2 microvesicles). Non-significant p-values in all five cell lines support the finding that there is no evidence of any significant difference in cell growth based on the differential microvesicle treatment. Figs. 31F-31J depict MK-2206 microvesicles bioactivity in vitro. Barplots depict the log₂ fold change

(MK-2206 - DMSO) in the total number of cells exposed to microvesicles derived from either MK-2206 or DMSO treated cells of the same type for 1 hour. Cells were then placed under three different stress conditions (e.g. 1% serum, 4% oxygen, standard chemotherapy agent) for 72h. Experiments were done in triplicates for 5 different cells lines (e.g., HCT116 (Fig. 31F), MCF7 (Fig. 31G), MDA-MB-231 (Fig. 31H), PC9 (Fig. 31I), and A375 (Fig. 31J)), treatment, and stress condition. Error bars show the standard error of the mean (SEM). Asterisks on top of bars designate statistically significant increases (i.e., $p < 0.05$, one-sided t-test) in cell count after exposing them to microvesicles derived from MK-2206 treated cells, compared to microvesicles derived from DMSO treated cells.

[0037] Fig. 32 depicts Akti-1/2 microvesicles in vitro cross-cell line bioactivity assay. Five cancer cell lines (e.g., HCT116, MCF7, MDA-MB-231, PC9, and A375) and one human fibroblast cell line (e.g., AG11726), represented in the panel rows, were exposed to microvesicles secreted by each one of the five cancer cell lines (i.e., panel columns). Barplots depict the log₂ fold change (Akti1/2 - DMSO) in the total number of cells exposed to microvesicles derived from either Akti-1/2 or DMSO treated cells for 1 hour. Cells were then placed under three different stress conditions (e.g. 1% serum, 4% oxygen, standard chemotherapy agent) for 72h (grey bar). Experiments were done in triplicates for each cell line, treatment, and stress condition. Error bars show the standard error of the mean (SEM). Asterisks on top of bars designate statistically significant increases (i.e., $p < 0.05$, one-sided t-test) in cell count after exposing them to microvesicles derived from Akti-1/2 treated cells, compared to microvesicles derived from DMSO treated cells.

DETAILED DESCRIPTION

[0038] Embodiments of the technology described herein relate to the inventor's discovery of a signaling pathway controlling asymmetric cell division and proliferation of cancer cells. Briefly, asymmetric proliferation is induced by the degradation of AKT1 protein. Modulating the rate of degradation of AKT1 protein can thus increase or decrease the rate of asymmetric proliferation and therefore the level of slow proliferator cancer cells within a population of cancer cells. In some embodiments, the degradation of AKT1 can be asymmetric. Methods relating to this modulation are described herein.

[0039] For convenience, the meaning of some terms and phrases used in the specification, examples, and appended claims, are provided below. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. If there is an apparent discrepancy between the usage of a term in the art and its definition provided herein, the definition provided within the specification shall prevail.

[0040] For convenience, certain terms employed herein, in the specification, examples and appended claims are collected here.

[0041] The terms “decrease”, “reduced”, “reduction”, or “inhibit” are all used herein to mean a decrease by a statistically significant amount. In some embodiments, the terms “reduced”, “reduction”, “decrease”, or “inhibit” can mean a decrease by at least 10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or more or any decrease of at least 10% as compared to a reference level. In some embodiments, the terms can represent a 100% decrease, i.e. a non-detectable level as compared to a reference level. In the context of a marker or symptom, a “decrease” is a statistically significant decrease in such level. The decrease can be, for example, at least 10%, at least 20%, at least 30%, at least 40% or more, and is preferably down to a level accepted as within the range of normal for an individual without such disorder.

[0042] The terms “increased”, “increase”, “enhance”, or “activate” are all used herein to mean an increase by a statically significant amount. In some embodiments, the terms “increased”, “increase”, “enhance”, or “activate” can mean an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level. In the context of a marker or symptom, a “increase” is a statistically significant increase in such level.

[0043] As used herein, a "subject" means a human or animal. Usually the animal is a vertebrate such as a primate, rodent, domestic animal or game animal. Primates include chimpanzees, cynomologous monkeys, spider monkeys, and macaques, e.g., Rhesus. Rodents include mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. In some embodiments, the subject is a mammal, e.g., a primate, e.g., a human. The terms, “individual,” “patient” and “subject” are used interchangeably herein.

[0044] Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but is not limited to these examples. Mammals other than humans can be advantageously used as subjects that represent animal models of cancer. A subject can be male or female.

[0045] A subject can be one who has been previously diagnosed with or identified as suffering

from or having a condition in need of treatment (e.g. cancer) or one or more complications related to such a condition, and optionally, have already undergone treatment for cancer or the one or more complications related to cancer. Alternatively, a subject can also be one who has not been previously diagnosed as having cancer or one or more complications related to cancer. For example, a subject can be one who exhibits one or more risk factors for cancer or one or more complications related to cancer or a subject who does not exhibit risk factors.

[0046] A “subject in need” of treatment for a particular condition can be a subject having that condition, diagnosed as having that condition, or at risk of developing that condition.

[0047] A “cancer” or “tumor” as used herein refers to an uncontrolled growth of cells which interferes with the normal functioning of the bodily organs and systems. A subject that has a cancer or a tumor is a subject having objectively measurable cancer cells present in the subject's body. Included in this definition are benign and malignant cancers, as well as dormant tumors or micrometastases. Cancers which migrate from their original location and seed vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs. In some embodiments, a cancer cell can be a cell obtained from a tumor.

[0048] By “metastasis” is meant the spread of cancer from its primary site to other places in the body. Cancer cells can break away from a primary tumor, penetrate into lymphatic and blood vessels, circulate through the bloodstream, and grow in a distant focus (metastasize) in normal tissues elsewhere in the body. Metastasis can be local or distant. Metastasis is a sequential process, contingent on tumor cells breaking off from the primary tumor, traveling through the bloodstream, and stopping at a distant site. At the new site, the cells establish a blood supply and can grow to form a life-threatening mass. Both stimulatory and inhibitory molecular pathways within the tumor cell regulate this behavior, and interactions between the tumor cell and host cells in the distant site are also significant. Metastases are most often detected through the sole or combined use of magnetic resonance imaging (MRI) scans, computed tomography (CT) scans, blood and platelet counts, liver function studies, chest X-rays and bone scans in addition to the monitoring of specific symptoms.

[0049] Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include, but are not limited to, basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and CNS cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma (GBM); hepatic carcinoma; hepatoma; intra-epithelial neoplasm; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (e.g., small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); lymphoma including Hodgkin's and non-Hodgkin's lymphoma; melanoma; myeloma; neuroblastoma; oral cavity cancer (e.g., lip,

tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; as well as other carcinomas and sarcomas; as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome.

[0050] As used herein, the terms "protein" and "polypeptide" are used interchangeably herein to designate a series of amino acid residues, connected to each other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms "protein", and "polypeptide" refer to a polymer of amino acids, including modified amino acids (e.g., phosphorylated, glycosylated, glycosylated, etc.) and amino acid analogs, regardless of its size or function. "Protein" and "polypeptide" are often used in reference to relatively large polypeptides, whereas the term "peptide" is often used in reference to small polypeptides, but usage of these terms in the art overlaps. The terms "protein" and "polypeptide" are used interchangeably herein when referring to a gene product and fragments thereof. Thus, exemplary polypeptides or proteins include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, fragments, and analogs of the foregoing.

[0051] As used herein, the term "nucleic acid" or "nucleic acid sequence" refers to any molecule, preferably a polymeric molecule, incorporating units of ribonucleic acid, deoxyribonucleic acid or an analog thereof. The nucleic acid can be either single-stranded or double-stranded. A single-stranded nucleic acid can be one strand nucleic acid of a denatured double-stranded DNA. Alternatively, it can be a single-stranded nucleic acid not derived from any double-stranded DNA. In one aspect, the nucleic acid can be DNA. In another aspect, the nucleic acid can be RNA. Suitable nucleic acid molecules are DNA, including genomic DNA or cDNA. Other suitable nucleic acid molecules are RNA, including mRNA.

[0052] As used herein, the terms "treat," "treatment," "treating," or "amelioration" refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a condition associated with a disease or disorder, e.g. cancer. The term "treating" includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder associated with a cancer therapy. Treatment is generally "effective" if one or more

symptoms or clinical markers are reduced. Alternatively, treatment is "effective" if the progression of a disease is reduced or halted. That is, "treatment" includes not just the improvement of symptoms or markers, but also a cessation of, or at least slowing of, progress or worsening of symptoms compared to what would be expected in the absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, remission (whether partial or total), and/or decreased mortality, whether detectable or undetectable. The term "treatment" of a disease also includes providing relief from the symptoms or side-effects of the disease (including palliative treatment).

[0053] As used herein, the term "pharmaceutical composition" refers to the active agent in combination with a pharmaceutically acceptable carrier e.g. a carrier commonly used in the pharmaceutical industry. The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0054] As used herein, the term "administering," refers to the placement of a compound as disclosed herein into a subject by a method or route which results in at least partial delivery of the agent at a desired site. Pharmaceutical compositions comprising the compounds disclosed herein can be administered by any appropriate route which results in an effective treatment in the subject.

[0055] The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) or greater difference.

[0056] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term "about." The term "about" when used in connection with percentages can mean $\pm 1\%$.

[0057] As used herein the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the method or composition, yet open to the inclusion of unspecified elements, whether essential or not.

[0058] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[0059] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment.

[0060] The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g." is derived from the Latin *exempli gratia*, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[0061] As used herein, the term "AKT1" or "v-akt murine thymoma viral oncogene homolog 1" refers to a serine-threonine protein kinase activated by platelet-derived growth factor. The sequence of AKT1 for a number of species is well known in the art, e.g. human AKT1 (e.g. NCBI Ref Seq: NP_001014431; NCBI Gene ID: 207).

[0062] As used herein, the term "mTORC2" refers to mTOR complex 2, a multi-protein complex comprising RICTOR, mTOR, GβL, and MAPKAP1, and which phosphorylates Akt. The sequences of the components of mTORC2 are well known in the art, eg. human mTOR (e.g. NCBI Ref Seq: NP_004949; NCBI Gene ID: 2475), human GβL (e.g. NCBI Ref Seq: NP_001186102; NCBI Gene ID: 64223), and human MAPKAP1 (e.g. NCBI Ref Seq: NP_001006618; NCBI Gene ID: 79109).

[0063] As used herein, the term "RICTOR" or "RPTOR independent companion of MTOR, complex 2" refers to a subunit of the mTORC2 complex. The sequence of RICTOR for a number of species is well known in the art, e.g. human RICTOR (e.g. NCBI Ref Seq: NP_689969; NCBI Gene ID: 253260).

[0064] As used herein, the term "TTC3" or "tetratricopeptide repeat domain 3" refers to an E3 ligase that controls the ubiquitination of AKT1. The sequence of TTC3 for a number of species is well known in the art, e.g. human TTC3 (e.g. NCBI Ref Seq: NP_001001894; NCBI Gene ID: 7267).

[0065] As used herein, the term "focal adhesion kinase" or "FAK" (also known as PTK2 in humans) refers to a tyrosine kinase found at focal adhesions and which is phosphorylated in response to integrin engagement and growth factor perception, thereby regulating cell movement, growth, and survival. The sequence of FAK for a number of species is well known in the art, e.g. human FAK (e.g. NCBI Ref Seq: NP_005598; NCBI Gene ID: 5747).

[0066] As described herein, "integrin" refers to a class of transmembrane receptors that mediate the attachment of a cell to surrounding materials, e.g. extracellular matrix (ECM) or other cells, as well as transduce signals relating to the chemical and mechanical status of the surrounding materials and/or transduce signals from the cell to the surrounding materials. Integrins function as heterodimers, comprising an alpha chain and a beta chain. Mammalian genomes contain eighteen alpha subunits and eight beta subunits. In some embodiments of any of the aspects described herein, an integrin can be a β1- integrin. As described herein, "β1- integrin" refers to a complete integrin heterodimer comprising a β1 beta chain and any of the eighteen possible alpha chains (e.g. α1-α11, αD, αE, αL, αM, αV, αX or α2B). The sequence of the β1 beta chain (i.e. ITGB1) for a number of species is well known in the art,

e.g., human ITGB1 (e.g. NCBI Gene ID: 3688; (mRNA: NCBI Ref Seq: NM_002211) (polypeptide NCBI Ref Seq:NP_002202).

[0067] As used herein, the term "stem cell" refers to a cell in an undifferentiated or partially differentiated state that has the property of self-renewal and has the developmental potential to naturally differentiate into a more differentiated cell type, without a specific implied meaning regarding developmental potential (i.e. , totipotent, pluripotent, multipotent, etc.). By self-renewal is meant that a stem cell is capable of proliferation and giving rise to more such stem cells, while maintaining its developmental potential. Accordingly, the term "stem cell" refers to any subset of cells that have the developmental potential, under particular circumstances, to differentiate to a more specialized or differentiated phenotype, and which retain the capacity, under certain circumstances, to proliferate without substantially differentiating. The term "somatic stem cell" is used herein to refer to any stem cell derived from non-embryonic tissue, including fetal, juvenile, and adult tissue. Natural somatic stem cells have been isolated from a wide variety of adult tissues including blood, bone marrow, brain, olfactory epithelium, skin, pancreas, skeletal muscle, and cardiac muscle. Exemplary naturally occurring somatic stem cells include, but are not limited to, mesenchymal stem cells and hematopoietic stem cells. In some embodiments, the stem or progenitor cells can be embryonic stem cells. As used herein, "embryonic stem cells" refers to stem cells derived from tissue formed after fertilization but before the end of gestation, including pre-embryonic tissue (such as, for example, a blastocyst), embryonic tissue, or fetal tissue taken any time during gestation, typically but not necessarily before approximately 10-12 weeks gestation. Most frequently, embryonic stem cells are totipotent cells derived from the early embryo or blastocyst. Embryonic stem cells can be obtained directly from suitable tissue, including, but not limited to human tissue, or from established embryonic cell lines. In one embodiment, embryonic stem cells are obtained as described by Thomson et al. (U.S. Pat. Nos. 5,843,780 and 6,200,806; Science 282:1145, 1998; Curr. Top. Dev. Biol. 38:133 ff, 1998; Proc. Natl. Acad. Sci. U.S.A. 92:7844, 1995 which are incorporated by reference herein in their entirety).

[0068] Exemplary stem cells include embryonic stem cells, adult stem cells, pluripotent stem cells, neural stem cells, liver stem cells, muscle stem cells, muscle precursor stem cells, endothelial progenitor cells, bone marrow stem cells, chondrogenic stem cells, lymphoid stem cells, mesenchymal stem cells, hematopoietic stem cells, central nervous system stem cells, peripheral nervous system stem cells, and the like. Descriptions of stem cells, including method for isolating and culturing them, may be found in, among other places, Embryonic Stem Cells, Methods and Protocols, Turksen, ed., Humana Press, 2002; Weisman et al., Annu. Rev. Cell. Dev. Biol. 17:387 403; Pittinger et al., Science, 284:143 47, 1999; Animal Cell Culture, Masters, ed., Oxford University Press, 2000; Jackson et al., PNAS 96(25):14482 86, 1999; Zuk et al., Tissue Engineering, 7:211 228, 2001 ("Zuk et al."); Atala et al., particularly Chapters 33 41; and U.S. Pat. Nos. 5,559,022, 5,672,346 and

5,827,735. Descriptions of stromal cells, including methods for isolating them, may be found in, among other places, Prockop, *Science*, 276:71-74, 1997; Theise et al., *Hepatology*, 31:235-40, 2000; *Current Protocols in Cell Biology*, Bonifacino et al., eds., John Wiley & Sons, 2000 (including updates through March, 2002); and U.S. Pat. No. 4,963,489.

[0069] As used herein, “progenitor cells” refers to cells in an undifferentiated or partially differentiated state and that have the developmental potential to differentiate into at least one more differentiated phenotype, without a specific implied meaning regarding developmental potential (i.e., totipotent, pluripotent, multipotent, etc.) and that does not have the property of self-renewal. Accordingly, the term “progenitor cell” refers to any subset of cells that have the developmental potential, under particular circumstances, to differentiate to a more specialized or differentiated phenotype.

[0070] Definitions of common terms in cell biology and molecular biology can be found in “The Merck Manual of Diagnosis and Therapy”, 19th Edition, published by Merck Research Laboratories, 2006 (ISBN 0-911910-19-0); Robert S. Porter et al. (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9). Definitions of common terms in molecular biology can also be found in Benjamin Lewin, *Genes X*, published by Jones & Bartlett Publishing, 2009 (ISBN-10: 0763766321); Kendrew et al. (eds.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8) and *Current Protocols in Protein Sciences 2009*, Wiley Intersciences, Coligan et al., eds.

[0071] Unless otherwise stated, the present invention was performed using standard procedures, as described, for example in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (3 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2001); Davis et al., *Basic Methods in Molecular Biology*, Elsevier Science Publishing, Inc., New York, USA (1995); or *Methods in Enzymology: Guide to Molecular Cloning Techniques Vol.152*, S. L. Berger and A. R. Kimmel Eds., Academic Press Inc., San Diego, USA (1987); *Current Protocols in Protein Science (CPPS)* (John E. Coligan, et. al., ed., John Wiley and Sons, Inc.), *Current Protocols in Cell Biology (CPCB)* (Juan S. Bonifacino et. al. ed., John Wiley and Sons, Inc.), and *Culture of Animal Cells: A Manual of Basic Technique* by R. Ian Freshney, Publisher: Wiley-Liss; 5th edition (2005), *Animal Cell Culture Methods (Methods in Cell Biology, Vol. 57)*, Jennie P. Mather and David Barnes editors, Academic Press, 1st edition, 1998) which are all incorporated by reference herein in their entireties.

[0072] Other terms are defined herein within the description of the various aspects of the invention.

[0073] Described herein are methods of modulating asymmetric proliferation in a cancer cell. As used herein, “asymmetric proliferation” refers to a process of cell division in which one daughter cell proliferates at the same rate as the parent cell while the other daughter cell proliferates at a statistically

significantly slower rate. These slowly proliferating daughter cells are referred to herein as “slow proliferators.” As used herein, the terms “slow proliferator” or “G0-like cell”, which are used interchangeably herein, refer to a cancer cell which proliferates at a statistically significantly slower rate than the rate observed for at least 70% of cancer cells obtained from the same tumor. In some embodiments, slow proliferators can be cancer cells which have statistically significantly decreased levels of expression of Akt1, H3K9me2, and MCM2 and statistically significantly increased levels of expression of TTC3 and Hes1 as compared to the levels of expression found in at least 70% of cancer cells obtained from the same tumor. In some embodiments, the level of expression of these markers can be the level of polypeptide expression product. In some embodiments, a slow proliferator can revert to a normal, fast-proliferator phenotype, e.g. the slow proliferator phenotype can be reversible.

[0074] In one aspect, described herein is a method of modulating the rate of asymmetric proliferation in a cell, the method comprising: contacting the cell with a modulator of AKT1 degradation; wherein an increase in AKT1 degradation increases the rate of asymmetric proliferation in the cell; and wherein a decrease in AKT1 degradation decreases the rate of asymmetric proliferation in the cell. In some embodiments, the rate of asymmetric proliferation in a population of cells can be modulated. As referred to herein, “AKT1 degradation” refers to the ubiquitination and proteasome-mediated degradation of AKT1.

[0075] In some embodiments, the cell can be a cancer cell. In some embodiments, the cell can be a stem and/or progenitor cell. In some embodiments, the cell can be a cell engaged in wound repair, e.g. a cell located at a site of a wound and/or defect. In some embodiments, the cell can be a cell undergoing asymmetric division, e.g. cells whose daughter cells comprise slow proliferators. Methods of identifying slow proliferator cells are described elsewhere herein.

[0076] A modulator of AKT1 can be an agonist of AKT1 degradation or an inhibitor of AKT1 degradation. Modulators can be agents of any type and/or structure. The term “agent” refers generally to any entity which is normally not present or not present at the levels being administered to a cell, tissue or subject. An agent can be selected from a group comprising: polynucleotides; polypeptides; small molecules; antibodies; or functional fragments thereof. A polynucleotide can be RNA or DNA, and can be single or double stranded, and can be selected from a group comprising: nucleic acids and nucleic acid analogues that encode a polypeptide. A polypeptide can be, but is not limited to, a naturally-occurring polypeptide, a mutated polypeptide or a fragment thereof that retains the function of interest. Further examples of agents include, but are not limited to a nucleic acid (DNA or RNA), small molecule, aptamer, protein, peptide, antibody, polypeptide comprising an epitope-binding fragment of an antibody, antibody fragment, peptide-nucleic acid (PNA), locked nucleic acid (LNA), small organic or inorganic molecules; saccharide; oligosaccharides; polysaccharides; biological macromolecules, e.g., peptides, proteins, and peptide analogs and derivatives; peptidomimetics; nucleic acids; nucleic acid analogs and derivatives; extracts made from

biological materials such as bacteria, plants, fungi, or mammalian cells or tissues; naturally occurring or synthetic compositions; peptides; aptamers; and antibodies, or fragments thereof. An agent can be applied to the media, where it contacts the cell and induces its effects. Alternatively, an agent can be intracellular as a result of introduction of a nucleic acid sequence encoding the agent into the cell and its transcription resulting in the production of the nucleic acid and/or protein environmental stimuli within the cell. In some embodiments, the agent is any chemical, entity or moiety, including without limitation synthetic and naturally-occurring non-proteinaceous entities. In certain embodiments the agent is a small molecule having a chemical moiety. For example, chemical moieties included unsubstituted or substituted alkyl, aromatic, or heterocyclyl moieties including macrolides, leptomycins and related natural products or analogues thereof. Agents can be known to have a desired activity and/or property, or can be selected from a library of diverse compounds.

[0077] In some embodiments, the modulator of AKT1 degradation can be an agonist and/or promoter of AKT1 degradation. An agonist of AKT1 degradation can be any agent that increases the level and/or rate of AKT1 degradation, either through direct or indirect action. As used herein, the term “agonist” refers to an agent which increases the expression and/or activity of the target by at least 10% or more, e.g. by 10% or more, 50% or more, 100% or more, 200% or more, 500% or more, or 1000 % or more.

[0078] Non-limiting examples of agonists of AKT1 degradation include allosteric inhibitors of AKT1 and clustered homology domain inhibitors of AKT1. In some embodiments, an agonist of AKT1 degradation can be a dual-specific (e.g. in inhibits AKT1 and AKT2) inhibitor or an AKT1-specific inhibitor. Allosteric inhibitors and clustered homology domain inhibitors of AKT1 are known in the art and include, by way of non-limiting example, AKT1/2; ARQ 092; and MK2206. Allosteric inhibitors of AKT1 are also described, e.g. in U.S. Patent No. 8,183,249; Cherrin et al. *Cancer Biol Ther* 2010 9:493-503; Calleja et al. *PLoS Biol* 2009 20: e17; Lindsley et al. *Bioorganic & Medicinal Chemistry Letters* 2005 15:761-764; Bilodeau et al. *Bioorganic and Medicinal Chemistry Letters* 2008 18:3178-3182; and Lindsley et al. *Current Cancer Drug Targets* 2008 8:7-18; which are incorporated by reference herein in their entireties. In some embodiments, an agonist of AKT1 degradation is not a catalytic inhibitor of AKT1.

[0079] In some embodiments, contacting a cancer cell with an agonist of AKT1 degradation leads to the production, or the increased production, of slow proliferator cancer cells.

[0080] As described herein, AKT1 degradation is negatively regulated by FAK activity. Accordingly, an agonist of AKT1 degradation can include, by way of non-limiting example, an inhibitor of FAK expression and/or activity. Inhibitors of FAK are known in the art, e.g., inhibitory nucleic acids, inhibitor antibody reagents, or small molecules, e.g., PF-562271; NVP-TAE226; PF-573228; Y15; and PND-1186.

[0081] As described herein, AKT1 degradation is negatively regulated by β 1-integrin activity. Accordingly, an agonist of AKT1 degradation can include, by way of non-limiting example, an inhibitor of β 1-integrin expression and/or activity. Non-limiting examples of β 1-integrin inhibitors can include inhibitory antibody reagents, e.g., A2B2 and P4C10 antibodies.

[0082] In some embodiments, a modulator of AKT1 degradation can be an inhibitor of AKT1 degradation. An inhibitor of AKT1 degradation can be any agent that decreases the level and/or rate of AKT1 degradation, whether by direct or indirect action. As used herein, the term “inhibitor” refers to an agent which reduces the expression and/or activity of the target by at least 10%, e.g. by 10% or more, 20% or more, 30% or more, 50% or more, 75% or more, 90% or more, 95% or more, 98% or more, or 99% or more.

[0083] As described herein, AKT1 degradation is positively regulated by mTORC2, RICTOR, and TTC3. Accordingly, inhibiting these proteins and/or expression of these proteins can inhibit AKT1 degradation. Thus, inhibitors of AKT1 degradation include inhibitors of mTORC2 signaling, inhibitors of mTORC2, inhibitors of mTORC2 expression, inhibitors of RICTOR, inhibitors of RICTOR expression, inhibitors of TTC3, and inhibitors of TTC3 expression. AKT1 degradation is negatively regulated by β -integrin activity. Accordingly, activating or increasing β -integrin expression or activity can inhibit AKT1 degradation. Thus, inhibitors of AKT1 degradation include activators of β -integrin activity and activators of β -integrin expression.

[0084] Further, irregular concentrations of collagen in the extracellular environment can create polar activation of β -integrin by the collagen, which can increase AKT1 degradation. Accordingly, providing a substrate or growth medium for a cell such that the individual cell is exposed to a homogeneous concentration of collagen can inhibit AKT1 degradation. In some embodiments, the substrate or growth medium with a homogeneous concentration of collagen can comprise a substrate or growth medium with a structural collagen matrix having a fibrillar pattern.

[0085] In some embodiments, inhibitors of AKT1 degradation can include an inhibitor of AKT1 expression, e.g. an inhibitory nucleic acid.

[0086] In some embodiments, inhibitors of AKT1 degradation can include an agonist of β 1-integrin, e.g. a nucleic acid encoding β 1-integrin or an activating antibody reagent. Such reagents are known in the art, e.g., the TS2/16 and 12G10 monoclonal antibodies.

[0087] Inhibitors of the expression of a given gene can be an inhibitory nucleic acid. For example, gene silencing or RNAi can be used. In certain embodiments, contacting a cell with the inhibitor results in a decrease in the target mRNA level in a cell of at least about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 99%, about 100% of the mRNA level found in the cell without the presence of the miRNA or RNA interference molecule. In one embodiment, the mRNA levels are decreased by at least about 70%, about 80%, about 90%, about 95%, about 99%, about 100%. In certain

embodiments, the inhibitor can comprise an expression vector or viral vector comprising the RNAi molecule.

[0088] As used herein, the term “RNAi” refers to any type of interfering RNA, including but are not limited to RNAi, siRNA, shRNA, endogenous microRNA and artificial microRNA. For instance, it includes sequences previously identified as siRNA, regardless of the mechanism of down-stream processing of the RNA (i.e. although siRNAs are believed to have a specific method of *in vivo* processing resulting in the cleavage of mRNA, such sequences can be incorporated into the vectors in the context of the flanking sequences described herein). The term “RNAi” and “RNA interfering” with respect to an agent of the technology described herein, are used interchangeably herein.

[0089] As used herein a “siRNA” refers to a nucleic acid that forms a double stranded RNA, which double stranded RNA has the ability to reduce or inhibit expression of a gene or target gene when the siRNA is present or expressed in the same cell as the target gene. The double stranded RNA siRNA can be formed by the complementary strands. In one embodiment, a siRNA refers to a nucleic acid that can form a double stranded siRNA. The sequence of the siRNA can correspond to the full length target gene, or a subsequence thereof. Typically, the siRNA is at least about 15-50 nucleotides in length (e.g., each complementary sequence of the double stranded siRNA is about 15-50 nucleotides in length, and the double stranded siRNA is about 15-50 base pairs in length, preferably about 19-30 base nucleotides, preferably about 20-25 nucleotides in length, e.g., 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length).

[0090] As used herein “shRNA” or “small hairpin RNA” (also called stem loop) is a type of siRNA. In one embodiment, these shRNAs are composed of a short, e.g. about 19 to about 25 nucleotide, antisense strand, followed by a nucleotide loop of about 5 to about 9 nucleotides, and the analogous sense strand. Alternatively, the sense strand can precede the nucleotide loop structure and the antisense strand can follow.

[0091] RNAi may be delivered with the help of nanoparticles as described for example in Schiffelers and Storm, Expert Opin Drug Deliv. 2006 May;3(3):445-54 or liposomes (e.g. Hughes et al., Methods Mol Biol. 2010;605:445-59).

[0092] Inhibitors of mTORC2 are known in the art and include, by way of non-limiting example, TORIN1, AZD8055, INK128, and Palomid-529. Further examples of mTORC2 inhibitors include OSI-027; MK8669; TOP216; TORISEL; CERTICAN; ABI-009; KU-0063794; AZD2014; NVP-BGT226; PF-04691502; PP242; XL765; EXEL-2044; EXEL-3885; EXEL-4431; EXEL-7518 and those described, e.g. in US Patent Publication 2012/0165334; 2011/0224223; 2012/0114739; 2010/0184760; 2012/0178715; Bhagwat and Crew. Curr Opin Investig Drugs 2010 11:638-645; which are incorporated by reference herein in their entireties. In some embodiments, an inhibitor of mTORC2 can be an inhibitor of mTORC1 and mTORC2. In some embodiments, an inhibitor of mTORC2 can be specific for inhibition of mTORC2.

[0093] Inhibitors of RICTOR are known in the art and include, by way of non-limiting example, NVP-BEZ235.

[0094] Inhibitors of TTC3 are known in the art and include, by way of non-limiting example, MG-132 and bortezomib.

[0095] Inhibitors of FAK are known in the art and include, by way of non-limiting example, PF-562271 and NVP-TAE226.

[0096] Inhibitors of β -integrin activity are known in the art and include, by way of non-limiting example, the monoclonal antibodies A2B2 and P4C10.

[0097] Therapies which target fast proliferator cells can be ineffective in decreasing populations of slow proliferators (see, e.g. Dey-Guha et al. PNAS 2011 108:12845-12850; which is incorporated by reference herein in its entirety). Accordingly, in one aspect, described herein is a method of treating cancer in a subject in need thereof, the method comprising: administering an inhibitor of AKT1 degradation to the subject (i.e. decreasing the number of slow proliferators in the subject). In some embodiments, the method can further comprise administering a cancer therapy that targets fast proliferator cancer cells. In some embodiments, the inhibitor of AKT1 degradation can be administered before the administration of a cancer therapy that targets fast proliferator cancer cells. In some embodiments, the inhibitor of AKT1 degradation can be administered at least 1 day before the administration of a cancer therapy that targets fast proliferator cancer cells, e.g. 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days or further before. In some embodiments, the inhibitor of AKT1 degradation can be administered at least 3 days before administration of a cancer therapy that targets fast proliferator cancer cells. Cancer therapies that target fast proliferator cells are well known in the art and include, by way of non-limiting example, therapies that degrade or disrupt nucleic acids, e.g. doxorubicin, alkylating agents, nitrogen mustard alkylating agents, agents that intercalate DNA; cyclophosphamide, or therapies that inhibit cell division, e.g. mitotic inhibitors, paclitaxel.

[0098] In some embodiments, an inhibitor of AKT1 degradation can be administered to reduce and/or reverse the growth of a cancer. In some embodiments, an inhibitor of AKT1 degradation can be administered to reduce the rate of the growth of a cancer. In some embodiments, an inhibitor of AKT1 degradation can be administered to prevent the growth of a cancer. In some embodiments, an inhibitor of AKT1 degradation can be administered to prevent relapse and/or development of a cancer.

[0099] Further examples of agents that can be fast proliferator targeting agents include, but are not limited to chemotherapeutic agents, growth inhibitory agents, cytotoxic agents, agents used in radiation therapy, anti-angiogenesis agents, apoptotic agents, anti-tubulin agents, and other agents to treat cancer, such as anti-HER-2 antibodies (e.g., Herceptin®), anti-CD20 antibodies, an epidermal growth factor receptor (EGFR) antagonist (e.g., a tyrosine kinase inhibitor), HER1/EGFR inhibitor (e.g., erlotinib (Tarceva®)), platelet derived growth factor inhibitors (e.g., GleevecTM (Imatinib Mesylate)), a COX-2 inhibitor (e.g., celecoxib), interferons, cytokines, antagonists (e.g., neutralizing

antibodies) that bind to one or more of the following targets ErbB2, ErbB3, ErbB4, PDGFR-beta, BlyS, APRIL, BCMA or VEGF receptor(s), TRAIL/Apo2, and other bioactive and organic chemical agents, etc. Combinations thereof are also included in the invention. The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (*e.g.* At²¹¹, I¹³¹, I¹²⁵, Y⁹⁰, Re¹⁸⁶, Re¹⁸⁸, Sm¹⁵³, Bi²¹², P³² and radioactive isotopes of Lu), chemotherapeutic agents, and toxins, such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, including fragments and/or variants thereof. As used herein, the terms "chemotherapy" or "chemotherapeutic agent" refer to any chemical agent with therapeutic usefulness in the treatment of diseases characterized by abnormal cell growth. Such diseases include tumors, neoplasms and cancer as well as diseases characterized by hyperplastic growth. Chemotherapeutic agents as used herein encompass both chemical and biological agents. These agents function to inhibit a cellular activity upon which the cancer cell depends for continued survival. Categories of chemotherapeutic agents include alkylating/alkaloid agents, antimetabolites, hormones or hormone analogs, and miscellaneous antineoplastic drugs. Most if not all of these agents are directly toxic to cancer cells and do not require immune stimulation. In one embodiment, a chemotherapeutic agent is an agent of use in treating neoplasms such as solid tumors. In one embodiment, a chemotherapeutic agent is a radioactive molecule. One of skill in the art can readily identify a chemotherapeutic agent of use (*e.g.* see Slapak and Kufe, Principles of Cancer Therapy, Chapter 86 in Harrison's Principles of Internal Medicine, 14th edition; Perry *et al.*, Chemotherapy, Ch. 17 in Abeloff, Clinical Oncology 2.sup.nd ed., 2000 Churchill Livingstone, Inc; Baltzer L, Berkery R (eds): Oncology Pocket Guide to Chemotherapy, 2nd ed. St. Louis, Mosby-Year Book, 1995; Fischer D S, Knobf M F, Durivage H J (eds): The Cancer Chemotherapy Handbook, 4th ed. St. Louis, Mosby-Year Book, 1993). In some embodiments, the modulators of AKT1 degradation described herein can be used in conjunction with additional chemotherapeutic agents. By "radiation therapy" is meant the use of directed gamma rays or beta rays to induce sufficient damage to a cell so as to limit its ability to function normally or to destroy the cell altogether. It will be appreciated that there will be many ways known in the art to determine the dosage and duration of treatment. Typical treatments are given as a one-time administration and typical dosages range from 10 to 200 units (Grays) per day.

[00100] In some embodiments, the subject can be one who has been identified as having slow proliferator cells. In some embodiments, the subject has been determined to have a subpopulation of cancer cells expressing increased levels of one or more genes selected from the group consisting of: Hes1 and TTC3; and/or decreased levels of one or more genes selected from the group consisting of: AKT1; H3K9me2; and MCM2; wherein an increased level is a level statistically significantly higher than the level of expression found in at least 70% of cancer cells obtained from the same tumor and a decreased level is a level statistically significantly lower than the level of expression found in at least

70% of cancer cells obtained from the same tumor. In some embodiments, the subject can have been determined to have cancer cells expressing increased levels of TTC3; and, optionally, increased levels of Hes1 and/or decreased levels of one or more genes selected from the group consisting of: AKT1; H3K9me2; and MCM2. In some embodiments, the subject can have been determined to have cancer cells expressing increased levels of Hes1 and TTC3 and decreased levels AKT1; H3K9me2; and MCM2. In some embodiments, an increased level can be at least 2x higher than the level of expression found in at least 70% of cancer cells obtained from the same tumor, e.g. at least 2x, at least 3x, at least 4x, at least 5x, at least 10x, or higher. In some embodiments, an increased level can be at least 50% or less than the level of expression found in at least 70% of cancer cells obtained from the same tumor, e.g. 50% or less, 40% or less, 30% or less, 20% or less, 10% or less, or less.

[00101] The expression level of a gene can be the level of mRNA or polypeptide expression product. In some embodiments, the level of expression can be determined, e.g. by *in situ* hybridization and/or immunochemistry of biopsies or tissue samples. In some embodiments, the expression level of an mRNA expression product can be determined, e.g. by RT-PCR, quantitative RT-PCR, RNA-seq, Northern blot, or microarray based expression analysis. In some embodiments, the level of expression of a gene can be the level of polypeptide expression product. Methods for measuring polypeptide expression products are known in the art and include, by way of non-limiting example ELISA (enzyme linked immunosorbent assay), western blot, immunoprecipitation, immunohistochemistry, and immunofluorescence using detection reagents such as an antibody or protein binding agent. In some embodiments, the expression level can be determined by immunochemistry. Methods of detecting the expression level of slow proliferator markers have been described, e.g. in Dey-Guha et al. PNAS 2011 108:12845-12850; which is incorporated by reference herein in its entirety and in the Examples herein.

[00102] In some embodiments, immunohistochemistry (“IHC”) and immunocytochemistry (“ICC”) techniques can be used. IHC is the application of immunochemistry to tissue sections, whereas ICC is the application of immunochemistry to cells or tissue imprints after they have undergone specific cytological preparations such as, for example, liquid-based preparations. Immunochemistry is a family of techniques based on the use of an antibody, wherein the antibodies are used to specifically target molecules inside or on the surface of cells. The antibody typically contains a marker that will undergo a biochemical reaction, and thereby experience, e.g. a change in color, upon encountering the targeted molecules or upon treatment with a chemical agent. In some instances, signal amplification can be integrated into the particular protocol, wherein a secondary antibody, that includes the marker signal or marker activity (e.g. an enzyme activity), follows the application of a primary target-specific antibody.

[00103] In one aspect, described herein is a kit for determining if a subject has slow proliferator cells. In some embodiments, the kit can comprise a detection agent specific for an expression product

of TTC3. In some embodiments, the kit can comprise a detection agent specific for an expression product of at least one of the genes selected from the group consisting of: TTC3; Hes1; AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac. A detection agent can be any agent which can specifically detect the presence of the target (e.g. bind specifically to the target) according to an assay described herein, e.g. a detection reagent can be a nucleic acid probe or primer specific for the target or an agent which specifically binds to a target polypeptide. In some embodiments, the detection reagent can comprise a detectable signal or be capable of generating a detectable signal. In some embodiments, the detection agent can be an antibody reagent. In some embodiments, the detection agent can be a monoclonal antibody and/or comprise CDRs of a monoclonal antibody. Non-limiting examples of antibody reagents specific for the described slow proliferator markers are described in the Examples herein. In some embodiments, the kit can further comprise reagents necessary for performing the assay, e.g. buffers and/or reagents for generating and/or detecting a detectable signal. In some embodiments, the kit can further comprise instructions.

[00104] As used herein, the term "antibody reagent" refers to a polypeptide that includes at least one immunoglobulin variable domain or immunoglobulin variable domain sequence and which specifically binds a given antigen. An antibody reagent can comprise an antibody or a polypeptide comprising an antigen-binding domain of an antibody. In some embodiments, an antibody reagent can comprise a monoclonal antibody or a polypeptide comprising an antigen-binding domain of a monoclonal antibody. For example, an antibody can include a heavy (H) chain variable region (abbreviated herein as VH), and a light (L) chain variable region (abbreviated herein as VL). In another example, an antibody includes two heavy (H) chain variable regions and two light (L) chain variable regions. The term "antibody reagent" encompasses antigen-binding fragments of antibodies (e.g., single chain antibodies, Fab and sFab fragments, F(ab')₂, Fd fragments, Fv fragments, scFv, and domain antibodies (dAb) fragments (see, e.g. de Wildt et al., Eur J. Immunol. 1996; 26(3):629-39; which is incorporated by reference herein in its entirety)) as well as complete antibodies. An antibody can have the structural features of IgA, IgG, IgE, IgD, IgM (as well as subtypes and combinations thereof). Antibodies can be from any source, including mouse, rabbit, pig, rat, and primate (human and non-human primate) and primatized antibodies. Antibodies also include midibodies, humanized antibodies, chimeric antibodies, and the like.

[00105] The VH and VL regions can be further subdivided into regions of hypervariability, termed "complementarity determining regions" ("CDR"), interspersed with regions that are more conserved, termed "framework regions" ("FR"). The extent of the framework region and CDRs has been precisely defined (see, Kabat, E. A., et al. (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. et al. (1987) J. Mol. Biol. 196:901-917; which are incorporated by reference

herein in their entirety). Each VH and VL is typically composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

[00106] The terms "antigen-binding fragment" or "antigen-binding domain", which are used interchangeably herein are used herein to refer to one or more fragments of a full length antibody that retain the ability to specifically bind to a target of interest. Examples of binding fragments encompassed within the term "antigen-binding fragment" of a full length antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment including two Fab fragments linked by a disulfide bridge at the hinge region; (iii) an Fd fragment consisting of the VH and CH1 domains; (iv) an Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) *Nature* 341:544-546; which is incorporated by reference herein in its entirety), which consists of a VH or VL domain; and (vi) an isolated complementarity determining region (CDR) that retains specific antigen-binding functionality. Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules known as single chain Fv (scFv). See e.g., U.S. Pat. Nos. 5,260,203, 4,946,778, and 4,881,175; Bird et al. (1988) *Science* 242:423-426; and Huston et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883. Antibody fragments can be obtained using any appropriate technique including conventional techniques known to those of skill in the art. The term "monospecific antibody" refers to an antibody that displays a single binding specificity and affinity for a particular target, e.g., epitope. This term includes a "monoclonal antibody" or "monoclonal antibody composition," which as used herein refer to a preparation of antibodies or fragments thereof of single molecular composition, irrespective of how the antibody was generated.

[00107] As used herein, the term "specific binding" refers to a chemical interaction between two molecules, compounds, cells and/or particles wherein the first entity binds to the second, target entity with greater specificity and affinity than it binds to a third entity which is a non-target. In some embodiments, specific binding can refer to an affinity of the first entity for the second target entity which is at least 10 times, at least 50 times, at least 100 times, at least 500 times, at least 1000 times or greater than the affinity for the third nontarget entity.

[00108] The term "label" refers to a composition capable of producing a detectable signal indicative of the presence of an antibody reagent (e.g. a bound antibody reagent). Suitable labels include radioisotopes, nucleotide chromophores, enzymes, substrates, fluorescent molecules, chemiluminescent moieties, magnetic particles, bioluminescent moieties, and the like. As such, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means.

[00109] In some cases, the rate of growth of a cancer (e.g. a tumor) can be reduced by increasing the percentage of the cells which are slow proliferators. Accordingly, in one aspect, described herein is a method of treating cancer in a subject in need thereof, the method comprising: administering an agonist of AKT1 degradation to the subject. Administration of an agonist of AKT1 degradation can increase the number of slow proliferators present in a tumor, causing the overall growth rate of the tumor to decrease. In some embodiments, the subject can be a subject selected from the group consisting of: a subject with early stage cancer; a subject who is in remission or is likely to be in remission; a subject at risk of developing cancer and/or a subject at risk of having a cancer and/or tumor grow to the extent that it is clinically dangerous.

[00110] Described herein are treatments for cancer. The cancer to be treated can be any type of cancer in any location. In some embodiments, the cancer can be breast cancer, lung cancer, prostate cancer, colorectal cancer, lung cancer, and/or melanoma. In some embodiments, the cancer can comprise a metastasis

[00111] In one aspect, described herein is a method of producing slow proliferator cancer cells, the method comprising: (i) contacting a cancer cell with an agonist of AKT1 degradation; (ii) maintaining the cancer cells treated in step (i). The cells can be maintained in vivo or in vitro. Conditions suitable for maintaining cells in culture are well known in the art and can vary depending on the precise identity of the cells. In some embodiments, slow proliferators can be maintained under the same cell culture conditions as the cancer cells from which they originated. Examples of suitable cell culture conditions are described in the Examples herein. In some embodiments, the method can further comprise the step of enriching the cells of step (ii) for slow proliferators by selecting for cells having increased levels of expression of TTC3 and optionally, increased levels of expression of Hes1 or decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac. In some embodiments, the method can further comprise the step of enriching the cells of step (ii) for slow proliferators by selecting for cells having increased levels of expression of TTC3 and Hes1 and decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac. Enriching can encompass selecting for slow proliferators (e.g. treating with an agent specific for fast proliferators) or sorting slow proliferators from other cells, e.g. by FACS sorting.

[00112] In one aspect, described herein is a method of screening for an anti-slow proliferator agent, the method comprising: (i) contacting a cancer cell with an agonist of AKT1 degradation; (ii) contacting the cell obtained from step (i) with a test agent; (iii) determining the anti-tumor effect of the test agent; (iv) identifying a test agent as an anti-slow proliferator agent when a statistically significant anti-tumor effect is observed. As described herein, an “anti-slow proliferator agent” is any agent which can either 1) cause slow proliferators to convert to a fast proliferator phenotype (e.g.

proliferate at a fast proliferator rate) or 2) selectively kill slow proliferators. As described herein, an anti-tumor effect can comprise a reduction in the growth of a tumor, a reduction in signs or symptoms of cancer, a reduction in mortality, cytotoxic activity, cytotoxicity specific for slow proliferators, reduction in relapse after remission, a reduction in invasiveness, and/or a reduction of metastasis. One of ordinary skill in the art readily appreciates how to measure such phenotypes, e.g. by cell viability assays, or by measuring the size of tumors over time.

[00113] As described herein, the term “test agent” refers to a compound or agent and/or compositions thereof that are to be screened to determine whether they possess anti-tumor and/or anti-slow proliferator activity, as identified herein. In the context of the screening methods described herein, a “test agent” can be a nucleic acid (DNA or RNA), small molecule, aptamer, protein, peptide, antibody, polypeptide comprising an epitope-binding fragment of an antibody, antibody fragment, peptide-nucleic acid (PNA), locked nucleic acid (LNA), small organic or inorganic molecules; saccharide; oligosaccharides; polysaccharides; biological macromolecules, e.g., peptides, proteins, and peptide analogs and derivatives; peptidomimetics; nucleic acids; nucleic acid analogs and derivatives; extracts made from biological materials such as bacteria, plants, fungi, or mammalian cells or tissues; naturally occurring or synthetic compositions; peptides; aptamers; and antibodies, or fragments thereof.

[00114] The methods of screening described herein can be performed *in vitro* or *in vivo*. In some embodiments, the cancer cell contacted with the agonist of AKT1 degradation is located and/or maintained *in vivo*, e.g. in an animal model of cancer. In some embodiments, the cancer cell contacted with the agonist of AKT1 degradation is located and/or maintained *in vitro*, e.g. in cell culture. In some embodiments, the method can further comprise selecting for slow proliferator cells after step (i), e.g. sorting cells by FACS using the slow proliferator markers described herein (e.g. TTC3).

[00115] In some embodiments, contacting a population of cancer cells with a agonist of AKT1 degradation can increase the number and/or proportion of slow proliferators in the population by a statistically significant amount. In some embodiments, contacting a population of cancer cells with a agonist of AKT1 degradation can increase the number and/or proportion of slow proliferators in the population by at least 2x, e.g. 2x or more, 3x or more, 4x or more, 5x or more, 6x or more, 7x or more, 8x or more, 9x or more, 10x or more, 20x or more, 50x or more, or 100x or more.

[00116] Further, the methods of screening described herein can also be adapted to screen for agents which cause slow proliferators to retain a slow proliferator phenotype (i.e. cause a lower rate of reversion to a fast proliferator phenotype as compared to untreated cells) or agents which cause slow proliferators to enter a dormant or quiescent state. One of skill in the art readily appreciates how to screen for such phenotypes, e.g. by measuring proliferation rates and/or metabolic rates.

[00117] In one aspect, described herein is a method comprising: (i) obtaining a tumor biopsy from a subject; (ii) determining the expression level of TTC3 in cells obtained from the subject; (iii) identifying the presence of slow proliferators in the tumor when cells with increased levels of expression of TTC3 are detected. In some embodiments, the expression level of TTC3 and optionally Hes1, AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac can be determined; wherein the presence of slow proliferators in the tumor is indicated when cells with increased levels of expression of TTC3 and optionally increased levels of expression of Hes1 or decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac are detected. In some embodiments, the method can further comprise administering an inhibitor of AKT1 degradation to the subject. In some embodiments, the method can further comprise treating the cancer with an inhibitor of AKT1 degradation according to any of the embodiments described herein. The determination of the expression level of the expression products foregoing genes (excepting TTC3) can be performed as described in, e.g. Dey-Guha et al. PNAS 2011108:12845-12850; which is incorporated by reference herein in its entirety.

[00118] In one aspect, described herein is a method of screening for a biomarker of anti-slow proliferator cells, the method comprising: (i) contacting a cancer cell with an agonist of AKT1 degradation; (ii) measuring the expression of one or more genes in the cell of (i) and comparing that to the level of expression to a reference level (e.g. the level in the cell prior to step (i) or to a cell not treated according to step (i)), wherein a gene having expression after step (i) which varies by a statistically significant amount is identified as a biomarker of slow proliferator status. One of ordinary skill in the art readily appreciates how to measure such gene expression levels, e.g. by microarray.

[00119] In some embodiments of any of the aspect of the methods described herein, the method can further comprise administering a PI3K signaling inhibitor. In some embodiments of any of the aspect of the methods described herein, the method can comprise administering a PI3K signaling inhibitor in combination with a AKT1 degradation inhibitor. In some embodiments of any of the aspect of the methods described herein, the method comprising administering an inhibitor of AKT1 degradation can further comprise administering a PI3K signaling inhibitor. In some embodiments, the PI3K signaling inhibitor can be an inhibitor of PI3K. In some embodiments, PI3K signaling inhibitor is LY294002 or GDC-0941.

[00120] Phosphoinositide 3-kinases are a family of related enzymes that are capable of phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol. They are also known as phosphatidylinositol-3-kinases. PI3Ks interact with the IRS (Insulin receptor substrate) in order to regulate glucose uptake through a series of phosphorylation events. The phosphoinositide-3-kinase family is composed of Class I, II and Class III, with Class I the only ones able to convert PI(4,5)P2 to PI(3,4,5)P3 on the inner leaflet of the plasma membrane. As used herein, a "PI3K

inhibitor" refers to an agent that inhibits the activity of PI3K, as measured by the level of phosphorylation of the 3 position hydroxyl group of the inositol ring of phosphatidylinositol, or as measured by the activity and/or phosphorylation (where increased phosphorylation indicates PI3K activity) of molecules downstream of PI3K. Examples of such downstream molecules are known in the art and can include, but are not limited to AKT, SGK, mTOR, GSK3 β , PSD-95, S6, and 4EBP1. Methods of measuring the activity of PI3K, directly or indirectly are well known in the art, and include, by way of non-limiting example determining the level of phosphorylation of a molecule downstream of PI3K using phospho-isoform specific antibodies, which are commercially available (e.g. anti-phospho-AKT antibody, Cat No. ab66138 Abcam, Cambridge, MA). Non-limiting examples of PI3K inhibitors can include LY294002; BGT226; BEZ235; PI103, PI828, wortmannin, demethoxyviridin, IC486068, IC87114, GDC-0941, perifosine, CAL101, PX-866, IPI-145, BAY 80-6946, P6503, TGR1202, SF1126, INK1117, BKM120, IL147, XL765, Palomid 529, GSK1059615, ZSTK474, PWT33597, TG100-115, CAL263, GNE-447, CUDC-907, and AEZS-136.

[00121] *Administration*

[00122] In some embodiments, the methods described herein relate to treating a subject having or diagnosed as having cancer with a modulator of AKT1 degradation. Subjects having cancer can be identified by a physician using current methods of diagnosing cancer. Symptoms and/or complications of cancer which characterize these conditions and aid in diagnosis are well known in the art and include but are not limited to, growth of a tumor, impaired function of the organ or tissue harboring cancer cells, etc. Tests that may aid in a diagnosis of, e.g. cancer include, but are not limited to, tissue biopsies and histological examination. A family history of cancer or exposure to risk factors for cancer (e.g. smoking or radiation) can also aid in determining if a subject is likely to have cancer or in making a diagnosis of cancer.

[00123] The compositions and methods described herein can be administered to a subject having or diagnosed as having cancer. In some embodiments, the methods described herein comprise administering an effective amount of compositions described herein, e.g. modulators of AKT1 degradation to a subject in order to alleviate a symptom of a cancer. As used herein, "alleviating a symptom of a cancer" is ameliorating any condition or symptom associated with the cancer. As compared with an equivalent untreated control, such reduction is by at least 5%, 10%, 20%, 40%, 50%, 60%, 80%, 90%, 95%, 99% or more as measured by any standard technique. A variety of means for administering the compositions described herein to subjects are known to those of skill in the art. Such methods can include, but are not limited to oral, parenteral, intravenous, intramuscular, subcutaneous, transdermal, airway (aerosol), pulmonary, cutaneous, topical, injection, or intratumoral administration. Administration can be local or systemic.

[00124] The term "effective amount" as used herein refers to the amount of a modulator of AKT1 degradation needed to alleviate at least one or more symptom of the disease or disorder, and relates to

a sufficient amount of pharmacological composition to provide the desired effect. The term "therapeutically effective amount" therefore refers to an amount of a modulator of ATK1 degradation that is sufficient to effect a particular anti-tumor effect when administered to a typical subject. An effective amount as used herein, in various contexts, would also include an amount sufficient to delay the development of a symptom of the disease, alter the course of a symptom disease (for example but not limited to, slowing the progression of a symptom of the disease), or reverse a symptom of the disease. Thus, it is not generally practicable to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using only routine experimentation.

[00125] Effective amounts, toxicity, and therapeutic efficacy can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dosage can vary depending upon the dosage form employed and the route of administration utilized. The dose ratio between toxic and therapeutic effects is the therapeutic index and can be expressed as the ratio LD50/ED50. Compositions and methods that exhibit large therapeutic indices are preferred. A therapeutically effective dose can be estimated initially from cell culture assays. Also, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of a modulator of AKT1 degradation, which achieves a half-maximal inhibition of symptoms) as determined in cell culture, or in an appropriate animal model. Levels in plasma can be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay, *e.g.*, assay for tumor growth, among others. The dosage can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment.

[00126] In some embodiments, the technology described herein relates to a pharmaceutical composition comprising a modulator of ATK1 degradation as described herein, and optionally a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers and diluents include saline, aqueous buffer solutions, solvents and/or dispersion media. The use of such carriers and diluents is well known in the art. Some non-limiting examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, methylcellulose, ethyl cellulose, microcrystalline cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium stearate, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol (PEG); (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium

hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates and/or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; (22) C₂-C₁₂ alcohols, such as ethanol; and (23) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, coloring agents, release agents, coating agents, sweetening agents, flavoring agents, perfuming agents, preservative and antioxidants can also be present in the formulation. The terms such as "excipient", "carrier", "pharmaceutically acceptable carrier" or the like are used interchangeably herein. In some embodiments, the carrier inhibits the degradation of the active agent, e.g. a modulator of ATK1 degradation as described herein.

[00127] In some embodiments, the pharmaceutical composition comprising a modulator of ATK1 degradation as described herein can be a parenteral dose form. Since administration of parenteral dosage forms typically bypasses the patient's natural defenses against contaminants, parenteral dosage forms are preferably sterile or capable of being sterilized prior to administration to a patient. Examples of parenteral dosage forms include, but are not limited to, solutions ready for injection, dry products ready to be dissolved or suspended in a pharmaceutically acceptable vehicle for injection, suspensions ready for injection, and emulsions. In addition, controlled-release parenteral dosage forms can be prepared for administration of a patient, including, but not limited to, administration DUROS[®]-type dosage forms, and dose-dumping.

[00128] Suitable vehicles that can be used to provide parenteral dosage forms of a modulator of ATK1 degradation as disclosed within are well known to those skilled in the art. Examples include, without limitation: sterile water; water for injection USP; saline solution; glucose solution; aqueous vehicles such as but not limited to, sodium chloride injection, Ringer's injection, dextrose Injection, dextrose and sodium chloride injection, and lactated Ringer's injection; water-miscible vehicles such as, but not limited to, ethyl alcohol, polyethylene glycol, and propylene glycol; and non-aqueous vehicles such as, but not limited to, corn oil, cottonseed oil, peanut oil, sesame oil, ethyl oleate, isopropyl myristate, and benzyl benzoate. Compounds that alter or modify the solubility of a pharmaceutically acceptable salt of a modulator of ATK1 degradation as disclosed herein can also be incorporated into the parenteral dosage forms of the disclosure, including conventional and controlled-release parenteral dosage forms.

[00129] Pharmaceutical compositions comprising a modulator of ATK1 degradation can also be formulated to be suitable for oral administration, for example as discrete dosage forms, such as, but not limited to, tablets (including without limitation scored or coated tablets), pills, caplets, capsules, chewable tablets, powder packets, cachets, troches, wafers, aerosol sprays, or liquids, such as but not limited to, syrups, elixirs, solutions or suspensions in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion, or a water-in-oil emulsion. Such compositions contain a predetermined amount of

the pharmaceutically acceptable salt of the disclosed compounds, and may be prepared by methods of pharmacy well known to those skilled in the art. See generally, Remington: The Science and Practice of Pharmacy, 21st Ed., Lippincott, Williams, and Wilkins, Philadelphia PA. (2005); which is incorporated by reference herein in its entirety.

[00130] Conventional dosage forms generally provide rapid or immediate drug release from the formulation. Depending on the pharmacology and pharmacokinetics of the drug, use of conventional dosage forms can lead to wide fluctuations in the concentrations of the drug in a patient's blood and other tissues. These fluctuations can impact a number of parameters, such as dose frequency, onset of action, duration of efficacy, maintenance of therapeutic blood levels, toxicity, side effects, and the like. Advantageously, controlled-release formulations can be used to control a drug's onset of action, duration of action, plasma levels within the therapeutic window, and peak blood levels. In particular, controlled- or extended-release dosage forms or formulations can be used to ensure that the maximum effectiveness of a drug is achieved while minimizing potential adverse effects and safety concerns, which can occur both from under-dosing a drug (i.e., going below the minimum therapeutic levels) as well as exceeding the toxicity level for the drug. In some embodiments, the agent can be administered in a sustained release formulation.

[00131] Controlled-release pharmaceutical products have a common goal of improving drug therapy over that achieved by their non-controlled release counterparts. Ideally, the use of an optimally designed controlled-release preparation in medical treatment is characterized by a minimum of drug substance being employed to cure or control the condition in a minimum amount of time. Advantages of controlled-release formulations include: 1) extended activity of the drug; 2) reduced dosage frequency; 3) increased patient compliance; 4) usage of less total drug; 5) reduction in local or systemic side effects; 6) minimization of drug accumulation; 7) reduction in blood level fluctuations; 8) improvement in efficacy of treatment; 9) reduction of potentiation or loss of drug activity; and 10) improvement in speed of control of diseases or conditions. Kim, Cherng-ju, Controlled Release Dosage Form Design, 2 (Technomic Publishing, Lancaster, Pa.: 2000); which is incorporated by reference herein in its entirety.

[00132] Most controlled-release formulations are designed to initially release an amount of drug (active ingredient) that promptly produces the desired therapeutic effect, and gradually and continually release other amounts of drug to maintain this level of therapeutic or prophylactic effect over an extended period of time. In order to maintain this constant level of drug in the body, the drug must be released from the dosage form at a rate that will replace the amount of drug being metabolized and excreted from the body. Controlled-release of an active ingredient can be stimulated by various conditions including, but not limited to, pH, ionic strength, osmotic pressure, temperature, enzymes, water, and other physiological conditions or compounds.

[00133] A variety of known controlled- or extended-release dosage forms, formulations, and devices can be adapted for use with the salts and compositions of the disclosure. Examples include, but are not limited to, those described in U.S. Pat. Nos.: 3,845,770; 3,916,899; 3,536,809; 3,598,123; 4,008,719; 5,674,533; 5,059,595; 5,591,767; 5,120,548; 5,073,543; 5,639,476; 5,354,556; 5,733,566; and 6,365,185 B1; each of which is incorporated herein by reference. These dosage forms can be used to provide slow or controlled-release of one or more active ingredients using, for example, hydroxypropylmethyl cellulose, other polymer matrices, gels, permeable membranes, osmotic systems (such as OROS[®] (Alza Corporation, Mountain View, Calif. USA)), or a combination thereof to provide the desired release profile in varying proportions.

[00134] The methods described herein can further comprise administering a second agent and/or treatment to the subject, e.g. as part of a combinatorial therapy. Non-limiting examples of a second agent and/or treatment can include radiation therapy, surgery, gemcitabine, cisplatin, paclitaxel, carboplatin, bortezomib, AMG479, vorinostat, rituximab, temozolomide, rapamycin, ABT-737, PI-103; alkylating agents such as thiotepa and CYTOXAN[®] cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratiastatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammaII and calicheamicin omegaII (see, *e.g.*, Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994))); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN[®] doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as

methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanol, testolactone; anti-adrenals such as aminogluthethimide, mitotane, trilostane; folic acid replenisher such as frolic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*, TAXOL® paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE® Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumburg, Ill.), and TAXOTERE® doxetaxel (Rhone-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR® gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE™, vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib (Tykerb™); inhibitors of PKC- α , Raf, H-Ras, EGFR (*e.g.*, erlotinib (Tarceva®)) and VEGF-A that reduce cell proliferation and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[00135] In addition, the methods of treatment can further include the use of radiation or radiation therapy. Further, the methods of treatment can further include the use of surgical treatments.

[00136] In certain embodiments, an effective dose of a composition comprising a modulator of ATK1 degradation as described herein can be administered to a patient once. In certain embodiments, an effective dose of a composition comprising a modulator of ATK1 degradation can be administered to a patient repeatedly. For systemic administration, subjects can be administered a therapeutic amount of a composition comprising a modulator of ATK1 degradation such as, *e.g.* 0.1 mg/kg, 0.5 mg/kg, 1.0 mg/kg, 2.0 mg/kg, 2.5 mg/kg, 5 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30

mg/kg, 40 mg/kg, 50 mg/kg, or more. A composition comprising a modulator of ATK1 degradation can be administered over a period of time, such as over a 5 minute, 10 minute, 15 minute, 20 minute, or 25 minute period. The administration can be repeated, for example, on a regular basis, such as hourly for 3 hours, 6 hours, 12 hours or longer or such as biweekly (i.e., every two weeks) for one month, two months, three months, four months or longer.

[00137] In some embodiments, after an initial treatment regimen, the treatments can be administered on a less frequent basis. For example, after treatment biweekly for three months, treatment can be repeated once per month, for six months or a year or longer. Treatment according to the methods described herein can reduce levels of a marker or symptom of a condition, e.g. cancer by at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80 % or at least 90% or more.

[00138] The dosage of a composition as described herein can be determined by a physician and adjusted, as necessary, to suit observed effects of the treatment. With respect to duration and frequency of treatment, it is typical for skilled clinicians to monitor subjects in order to determine when the treatment is providing therapeutic benefit, and to determine whether to increase or decrease dosage, increase or decrease administration frequency, discontinue treatment, resume treatment, or make other alterations to the treatment regimen. The dosing schedule can vary from once a week to daily depending on a number of clinical factors, such as the subject's sensitivity to the active agent. The desired dose or amount of activation can be administered at one time or divided into subdoses, e.g., 2-4 subdoses and administered over a period of time, e.g., at appropriate intervals through the day or other appropriate schedule. In some embodiments, administration can be chronic, e.g., one or more doses and/or treatments daily over a period of weeks or months. Examples of dosing and/or treatment schedules are administration daily, twice daily, three times daily or four or more times daily over a period of 1 week, 2 weeks, 3 weeks, 4 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, or 6 months, or more.

[00139] The dosage ranges for the administration of a modulator of ATK1 degradation according to the methods described herein depend upon, for example, the form of a modulator of ATK1 degradation, its potency, and the extent to which symptoms, markers, or indicators of a condition described herein are desired to be reduced, for example the percentage reduction desired for cancer growth or the extent to which, for example, tumor size are desired to be induced. The dosage should not be so large as to cause adverse side effects. Generally, the dosage will vary with the age, condition, and sex of the patient and can be determined by one of skill in the art. The dosage can also be adjusted by the individual physician in the event of any complication.

[00140] The efficacy of a modulator of ATK1 degradation in, e.g. the treatment of a condition described herein, or to induce a response as described herein (e.g. reduction in tumor growth) can be determined by the skilled clinician. However, a treatment is considered "effective treatment," as the

term is used herein, if any one or all of the signs or symptoms of a condition described herein are altered in a beneficial manner, other clinically accepted symptoms are improved, or even ameliorated, or a desired response is induced e.g., by at least 10% following treatment according to the methods described herein. Efficacy can be assessed, for example, by measuring a marker, indicator, symptom, and/or the incidence of a condition treated according to the methods described herein or any other measurable parameter appropriate, e.g. tumor size. Efficacy can also be measured by a failure of an individual to worsen as assessed by hospitalization, or need for medical interventions (i.e., progression of the disease is halted). Methods of measuring these indicators are known to those of skill in the art and/or are described herein. Treatment includes any treatment of a disease in an individual or an animal (some non-limiting examples include a human or an animal) and includes: (1) inhibiting the disease, e.g., preventing a worsening of symptoms (e.g. pain or inflammation); or (2) relieving the disease, e.g., causing regression of symptoms. An effective amount for the treatment of a disease means that amount which, when administered to a subject in need thereof, is sufficient to result in effective treatment as that term is defined herein, for that disease. Efficacy of an agent can be determined by assessing physical indicators of a condition or desired response, (e.g. reduction in tumor growth rate). It is well within the ability of one skilled in the art to monitor efficacy of administration and/or treatment by measuring any one of such parameters, or any combination of parameters. Efficacy can be assessed in animal models of a condition described herein, for example treatment of cancer. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant change in a marker is observed, e.g. tumor size.

[00141] *In vitro* and animal model assays are provided herein which allow the assessment of a given dose of a modulator of ATK1 degradation. By way of non-limiting example, the effects of a dose of a modulator of ATK1 degradation can be assessed by monitoring the growth of a xenograft of cancer cells in mice as described herein.

[00142] All patents and other publications; including literature references, issued patents, published patent applications, and co-pending patent applications; cited throughout this application are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the technology described herein. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00143] The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for,

the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and application to provide yet further embodiments of the disclosure. These and other changes can be made to the disclosure in light of the detailed description. All such modifications are intended to be included within the scope of the appended claims.

[00144] Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated with certain embodiments of the disclosure have been described in the context of these embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure.

[00145] The technology described herein is further illustrated by the following examples which in no way should be construed as being further limiting.

[00146] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. A method of modulating the rate of asymmetric proliferation in a cell, the method comprising:
contacting the cell with a modulator of AKT1 degradation;
wherein an increase in AKT1 degradation increases the rate of asymmetric proliferation in the cell; and
wherein a decrease in AKT1 degradation decreases the rate of asymmetric proliferation in the cell.
2. The method of paragraph 1, wherein the cell is selected from the group consisting of:
a cancer cell; a stem cell; a progenitor cell; and a cell engaged in wound repair.
3. The method of any of paragraphs 1-2, wherein the modulator of AKT1 degradation is an agonist of AKT1 degradation selected from the group consisting of:
an allosteric inhibitor of AKT1; AKT1/2; MK2206; an inhibitor of β -integrin expression; an inhibitor of β -integrin activity; A2B2; P4C10; an inhibitor of focal adhesion kinase (FAK) expression; an inhibitor of FAK activity; PF-562271; and NVP-TAE226.
4. The method of paragraph 3, whereby slow proliferator cancer cells are produced.

5. The method of any of paragraphs 1-2, wherein the modulator of AKT1 degradation is an inhibitor of AKT1 degradation selected from the group consisting of:
 - inhibitors of mTOR complex 2 (mTORC2) signaling; inhibitors of mTORC2; TORIN1; AZD8055; INK128; Palomid-529; inhibitors of mTORC2 expression; inhibitors of RPTOR independent companion of MTOR, complex 2 (RICTOR); inhibitors of RICTOR expression; an inhibitor of tetratricopeptide repeat domain 3 (TTC3); MG-132; bortezomib; activators of β -integrin activity; activators of β -integrin expression.
6. The method of any of paragraphs 1-2, wherein the modulator of AKT1 degradation is a substrate or growth medium which provides a homogeneous concentration of collagen to an individual cell;
 - wherein AKT1 degradation is inhibited by the symmetric activation of β -integrin by the homogeneous concentrations of collagen.
7. A method of treating cancer in a subject in need thereof, the method comprising:
 - administering an inhibitor of AKT1 degradation to the subject.
8. The method of paragraph 7, wherein the inhibitor of AKT1 degradation is selected from the group consisting of:
 - inhibitors of mTOR complex 2 (mTORC2) signaling; inhibitors of mTORC2; TORIN1; AZD8055; INK128; Palomid-529; inhibitors of mTORC2 expression; inhibitors of RPTOR independent companion of MTOR, complex 2 (RICTOR); inhibitors of RICTOR expression; an inhibitor of tetratricopeptide repeat domain 3 (TTC3); MG-132; bortezomib; activators of β -integrin activity; activators of β -integrin expression.
9. The method of any of paragraphs 7-8, wherein the cancer is selected from the group consisting of:
 - melanoma; lung cancer; colorectal cancer; and breast cancer.
10. The method of any of paragraphs 7-9, wherein the method further comprises administering a cancer therapy that targets fast proliferator cancer cells.
11. The method of paragraph 10, wherein the inhibitor of AKT1 degradation is administered before the administration of a cancer therapy that targets fast proliferator cancer cells.
12. The method of paragraph 11, wherein the inhibitor of AKT1 degradation is administered at least 1 day before the administration of a cancer therapy that targets fast proliferator cancer cells.
13. The method of paragraph 12, wherein the inhibitor of AKT1 degradation is administered at least 3 days before the administration of a cancer therapy that targets fast proliferator cancer cells.

14. The method of any of paragraphs 7-13, wherein the subject has been determined to have a subpopulation of cancer cells expressing increased levels of one or more genes selected from the group consisting of:
 - Hes1 and TTC3;
 - or decreased levels of one or more genes selected from the group consisting of:
AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph;
H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
 - wherein an increased level is a level statistically significantly higher than the level of expression found in at least 70% of cancer cells obtained from the same tumor and a decreased level is a level statistically significantly lower than the level of expression found in at least 70% of cancer cells obtained from the same tumor.
15. The method of paragraph 14, wherein the subject has been determined to have cancer cells expressing increased levels of TTC3; and optionally,
 - increased levels of Hes1;
 - or decreased levels of one or more genes selected from the group consisting of:
AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph;
H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
16. The method of paragraph 15, wherein the subject has been determined to have cancer cells expressing increased levels of Hes1 and TTC3 and decreased levels AKT1; H3K9me2; and MCM2.
17. The method of any of paragraphs 14-16, wherein the expression level of the one or more genes is the level of polypeptide expression product.
18. The method of any of paragraphs 14-17, wherein the expression level is determined by immunochemistry.
19. A method of treating cancer in a subject in need thereof, the method comprising:
 - administering an agonist of AKT1 degradation to the subject.
20. The method of paragraph 19, wherein agonist of AKT1 degradation is selected from the group consisting of:
 - an allosteric inhibitor of AKT1; AKT1/2; MK2206; an inhibitor of β -integrin expression; an inhibitor of β -integrin activity; A2B2; P4C10; an inhibitor of focal adhesion kinase (FAK) expression; an inhibitor of FAK activity; PF-562271; and NVP-TAE226.
21. The method of paragraph 20, wherein the subject is a subject selected from the group consisting of:
 - a subject with early stage cancer; a subject who is in remission or is likely to be in remission; and a subject at risk of developing cancer.

22. A method of screening for an anti-slow proliferator agent, the method comprising:
- (i) contacting a cancer cell with an agonist of AKT1 degradation;
 - (ii) contacting the cell obtained from step (i) with a test agent;
 - (iii) determining the anti-tumor effect of the test agent;
 - (iv) identifying a test agent as an anti-slow proliferator agent when a statistically significant anti-tumor effect is observed.
23. The method of paragraph 22, wherein the cancer cell is maintained *in vitro*.
24. The method of paragraph 22, wherein the cancer cell is maintained *in vivo*.
25. A method comprising:
- (i) obtaining a tumor biopsy from a subject;
 - (ii) determining the expression level of TTC3 in cells obtained from the subject;
 - (iii) identifying the presence of slow proliferators in the tumor when cells with increased levels of expression of TTC3 are detected.
26. The method of paragraph 25, wherein the expression level of TTC3 and optionally Hes1, AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac are determined; and wherein the presence of slow proliferators in the tumor is indicated when cells with increased levels of expression of TTC3 and optionally, increased levels of expression of Hes1 or decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac are detected.
27. The method of any of paragraphs 25-26, wherein the method further comprises administering an inhibitor of AKT1 degradation to the subject to a subject identified as having cancer cells with increased levels of expression of TTC3.
28. A method of producing slow proliferator cancer cells, the method comprising:
- (i) contacting a cancer cell with an agonist of AKT1 degradation;
 - (ii) maintaining the cancer cells treated in step (i).
29. The method of paragraph 28, wherein the method further comprises the step of enriching the cells of step (ii) for slow proliferators by selecting for cells having increased levels of expression of TTC3 and optionally, increased levels of expression of Hes1 or decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac.
30. The method of paragraph 29, wherein the method further comprises the step of enriching the cells of step (ii) for slow proliferators by selecting for cells having increased levels of expression of TTC3 and Hes1 and decreased levels of expression of AKT1; H3K9me2;

MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.

31. A kit for performing the method of any of paragraphs 25-30.
32. The kit of paragraph 31, wherein the kit comprises a detection agent specific for an expression product of TTC3.
33. The kit of any of paragraphs 31-32, wherein the kit comprises a detection agent specific for an expression product of at least one of the genes selected from the group consisting of: TTC3; Hes1; AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
34. The kit of any of paragraphs 31-33, wherein the detection agent is an antibody reagent.
35. The use of an inhibitor of AKT1 degradation to treat cancer, the use comprising administering an inhibitor of AKT1 degradation to a subject in need of treatment for cancer.
36. The use of paragraph 35, wherein the an inhibitor of AKT1 degradation is selected from the group consisting of:
 - inhibitors of mTOR complex 2 (mTORC2) signaling; inhibitors of mTORC2; TORIN1; AZD8055; INK128; Palomid-529; inhibitors of mTORC2 expression; inhibitors of RPTOR independent companion of MTOR, complex 2 (RICTOR); inhibitors of RICTOR expression; an inhibitor of tetratricopeptide repeat domain 3 (TTC3); MG-132; bortezomib; activators of β -integrin activity; activators of β -integrin expression.
37. The use of any of paragraphs 35-36, wherein the cancer is selected from the group consisting of:
 - melanoma; lung cancer; colorectal cancer; and breast cancer.
38. The use of any of paragraphs 35-37, wherein the subject is further administered a cancer therapy that targets fast proliferator cancer cells.
39. The use of paragraph 38, wherein the inhibitor of AKT1 degradation is administered before the administration of a cancer therapy that targets fast proliferator cancer cells.
40. The use of paragraph 39, wherein the inhibitor of AKT1 degradation is administered at least 1 day before the administration of a cancer therapy that targets fast proliferator cancer cells.
41. The use of paragraph 39, wherein the inhibitor of AKT1 degradation is administered at least 3 days before the administration of a cancer therapy that targets fast proliferator cancer cells.
42. The use of any of paragraphs 35-41, wherein the subject has been determined to have a subpopulation of cancer cells expressing increased levels of one or more genes selected from the group consisting of:
 - Hes1 and TTC3;
 - or decreased levels of one or more genes selected from the group consisting of:

AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.

wherein an increased level is a level statistically significantly higher than the level of expression found in at least 70% of cancer cells obtained from the same tumor and a decreased level is a level statistically significantly lower than the level of expression found in at least 70% of cancer cells obtained from the same tumor.

43. The use of paragraph 42, wherein the subject has been determined to have cancer cells expressing increased levels of TTC3; and optionally,
 - increased levels of Hes1;
 - or decreased levels of one or more genes selected from the group consisting of: AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
44. The use of paragraph 43, wherein the subject has been determined to have cancer cells expressing increased levels of Hes1 and TTC3 and decreased levels AKT1; H3K9me2; and MCM2.
45. The use of any of paragraphs 42-44, wherein the expression level of the one or more genes is the level of polypeptide expression product.
46. The use of any of paragraphs 42-45, wherein the expression level is determined by immunochemistry.
47. The use of an agonist of AKT1 degradation to treat cancer, the use comprising administering an agonist of AKT1 degradation to a subject in need of treatment for cancer.
48. The use of paragraph 47, wherein the agonist of AKT1 degradation is selected from the group consisting of:
 - an allosteric inhibitor of AKT1; AKT1/2; MK2206; an inhibitor of β -integrin expression; an inhibitor of β -integrin activity; A2B2; P4C10; an inhibitor of focal adhesion kinase (FAK) expression; an inhibitor of FAK activity; PF-562271; and NVP-TAE226.
49. The use of paragraph 48, wherein the subject is a subject selected from the group consisting of:
 - a subject with early stage cancer; a subject who is in remission or is likely to be in remission; and a subject at risk of developing cancer.

[00147] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. A method of modulating the rate of asymmetric proliferation in a cancer cell, the method comprising:
 - contacting the cancer cell with a modulator of AKT1 degradation;

wherein an increase in AKT1 degradation increases the rate of asymmetric proliferation in the cancer cell; and

wherein a decrease in AKT1 degradation decreases the rate of asymmetric proliferation in the cancer cell.

2. The method of paragraph 1, wherein the modulator of AKT1 degradation is an agonist of AKT1 degradation selected from the group consisting of:
 - an allosteric inhibitor of AKT1; an allosteric inhibitor of AKT1/2; MK2206; an inhibitor of FAK; an inhibitor of β 1-integrin; PF-562271; and NVP-TAE226.
3. The method of paragraph 2, whereby slow proliferator cancer cells are produced.
4. The method of paragraph 1, wherein the modulator of AKT1 degradation is an inhibitor of AKT1 degradation selected from the group consisting of:
 - inhibitors of mTORC2 signaling; inhibitors of mTORC2; TORIN1; AZD8055; INK128; Palomid-529; inhibitors of mTORC2 expression; inhibitors of RICTOR; inhibitors of RICTOR expression; an inhibitor of TTC3; MG-132; bortezomib; an inhibitor of ATK1 expression; an agonist of β 1-integrin; and a cell medium comprising a fibrillar pattern of collagen.
5. A method of treating cancer in a subject in need thereof, the method comprising:
 - administering an inhibitor of AKT1 degradation to the subject.
6. The method of paragraph 5, wherein the method further comprises administering a cancer therapy that targets fast proliferator cancer cells.
7. The method of paragraph 6, wherein the inhibitor of AKT1 degradation is administered before the administration of a cancer therapy that targets fast proliferator cancer cells.
8. The method of paragraph 7, wherein the inhibitor of AKT1 degradation is administered at least 1 day before the administration of a cancer therapy that targets fast proliferator cancer cells.
9. The method of paragraph 7, wherein the inhibitor of AKT1 degradation is administered at least 3 days before the administration of a cancer therapy that targets fast proliferator cancer cells.
10. The method of any of paragraphs 5-9, wherein the subject has been determined to have a subpopulation of cancer cells expressing increased levels of one or more genes selected from the group consisting of:
 - Hes1 and TTC3;
 - or decreased levels of one or more genes selected from the group consisting of: AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.

wherein an increased level is a level statistically significantly higher than the level of expression found in at least 70% of cancer cells obtained from the same tumor and a decreased level is a level statistically significantly lower than the level of expression found in at least 70% of cancer cells obtained from the same tumor.

11. The method of paragraph 10, wherein the subject has been determined to have cancer cells expressing increased levels of TTC3; and optionally,
 - increased levels of Hes1;
 - or decreased levels of one or more genes selected from the group consisting of:
AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph;
H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
12. The method of paragraph 10, wherein the subject has been determined to have cancer cells expressing increased levels of Hes1 and TTC3 and decreased levels AKT1; H3K9me2; and MCM2.
13. The method of any of paragraphs 10-12, wherein the expression level of the one or more genes is the level of polypeptide expression product.
14. The method of any of paragraphs 10-13, wherein the expression level is determined by immunochemistry.
15. A method of treating cancer in a subject in need thereof, the method comprising:
 - administering an agonist of AKT1 degradation to the subject.
16. The method of paragraph 15, wherein the subject is a subject selected from the group consisting of:
 - a subject with early stage cancer; a subject who is in remission or is likely to be in remission; and a subject at risk of developing cancer.
17. A method of screening for an anti-slow proliferator agent, the method comprising:
 - (i) contacting a cancer cell with an agonist of AKT1 degradation;
 - (ii) contacting the cell obtained from step (i) with a test agent;
 - (iii) determining the anti-tumor effect of the test agent;
 - (iv) identifying a test agent as an anti-slow proliferator agent when a statistically significant anti-tumor effect is observed.
18. The method of paragraph 17, wherein the cancer cell is maintained *in vitro*.
19. The method of paragraph 17, wherein the cancer cell is maintained *in vivo*.
20. A method comprising:
 - (i) obtaining a tumor biopsy from a subject;
 - (ii) determining the expression level of TTC3 in cells obtained from the subject;
 - (iii) identifying the presence of slow proliferators in the tumor when cells with increased levels of expression of TTC3 are detected.

21. The method of paragraph 20, wherein the expression level of TTC3 and optionally Hes1, AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac are determined; and wherein the presence of slow proliferators in the tumor is indicated when cells with increased levels of expression of TTC3 and optionally, increased levels of expression of Hes1 or decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac are detected.
 22. The method of any of paragraphs 20-21, wherein the method further comprises administering an inhibitor of AKT1 degradation to the subject to a subject identified as having cancer cells with increased levels of expression of TTC3.
 23. A method of producing slow proliferator cancer cells, the method comprising:
 - (i) contacting a cancer cell with an agonist of AKT1 degradation;
 - (ii) maintaining the cancer cells treated in step (i).
 24. The method of paragraph 23, wherein the method further comprises the step of enriching the cells of step (ii) for slow proliferators by selecting for cells having increased levels of expression of TTC3 and optionally, increased levels of expression of Hes1 or decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac.
 25. The method of paragraph 23, wherein the method further comprises the step of enriching the cells of step (ii) for slow proliferators by selecting for cells having increased levels of expression of TTC3 and Hes1 and decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
 26. A kit for performing the method of any of paragraphs 20-22.
 27. The kit of paragraph 26, wherein the kit comprises a detection agent specific for an expression product of TTC3.
 28. The kit of any of paragraphs 26-27, wherein the kit comprises a detection agent specific for an expression product of at least one of the genes selected from the group consisting of: TTC3; Hes1; AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
 29. The kit of any of paragraphs 27-28, wherein the detection agent is an antibody reagent.
- [00148]** Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. A method of modulating the rate of asymmetric proliferation in a cancer cell, the method comprising:

- contacting the cancer cell with a modulator of AKT1 degradation;
 wherein an increase in AKT1 degradation increases the rate of asymmetric proliferation in the cancer cell; and
 wherein a decrease in AKT1 degradation decreases the rate of asymmetric proliferation in the cancer cell.
2. The method of paragraph 1, wherein the modulator of AKT1 degradation is an agonist of AKT1 degradation selected from the group consisting of:
 an allosteric inhibitor of AKT1; an allosteric inhibitor of AKT1/2; MK2206; an inhibitor of FAK; an inhibitor of β 1-integrin; PF-562271; and NVP-TAE226.
 3. The method of paragraph 2, whereby slow proliferator cancer cells are produced.
 4. The method of paragraph 1, wherein the modulator of AKT1 degradation is an inhibitor of AKT1 degradation selected from the group consisting of:
 inhibitors of mTORC2 signaling; inhibitors of mTORC2; TORIN1; AZD8055; INK128; Palomid-529; inhibitors of mTORC2 expression; inhibitors of RICTOR; inhibitors of RICTOR expression; an inhibitor of TTC3; MG-132; bortezomib; an inhibitor of AKT1 expression; an agonist of β 1-integrin; and a cell medium comprising a fibrillar pattern of collagen.
 5. A method of treating cancer in a subject in need thereof, the method comprising:
 administering an inhibitor of AKT1 degradation to the subject.
 6. The method of paragraph 5, wherein the method further comprises administering a cancer therapy that targets fast proliferator cancer cells.
 7. The method of paragraph 6, wherein the inhibitor of AKT1 degradation is administered before the administration of a cancer therapy that targets fast proliferator cancer cells.
 8. The method of paragraph 7, wherein the inhibitor of AKT1 degradation is administered at least 1 day before the administration of a cancer therapy that targets fast proliferator cancer cells.
 9. The method of paragraph 7, wherein the inhibitor of AKT1 degradation is administered at least 3 days before the administration of a cancer therapy that targets fast proliferator cancer cells.
 10. The method of paragraph 5, wherein the subject has been determined to have a subpopulation of cancer cells expressing increased levels of one or more genes selected from the group consisting of:
 Hes1 and TTC3;
 or decreased levels of one or more genes selected from the group consisting of:
 AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph;
 H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.

wherein an increased level is a level statistically significantly higher than the level of expression found in at least 70% of cancer cells obtained from the same tumor and a decreased level is a level statistically significantly lower than the level of expression found in at least 70% of cancer cells obtained from the same tumor.

11. The method of paragraph 10, wherein the subject has been determined to have cancer cells expressing increased levels of TTC3; and optionally,
 - increased levels of Hes1;
 - or decreased levels of one or more genes selected from the group consisting of: AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
12. The method of paragraph 10, wherein the subject has been determined to have cancer cells expressing increased levels of Hes1 and TTC3 and decreased levels AKT1; H3K9me2; and MCM2.
13. The method of paragraph 10, wherein the expression level of the one or more genes is the level of polypeptide expression product.
14. The method of paragraph 10, wherein the expression level is determined by immunochemistry.
15. A method comprising:
 - (i) obtaining a tumor biopsy from a subject;
 - (ii) determining the expression level of TTC3 in cells obtained from the subject;
 - (iii) identifying the presence of slow proliferators in the tumor when cells with increased levels of expression of TTC3 are detected.
16. The method of paragraph 15, wherein the expression level of TTC3 and optionally Hes1, AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac are determined; and wherein the presence of slow proliferators in the tumor is indicated when cells with increased levels of expression of TTC3 and optionally, increased levels of expression of Hes1 or decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac are detected.
17. The method of paragraph 15, wherein the method further comprises administering an inhibitor of AKT1 degradation to the subject to a subject identified as having cancer cells with increased levels of expression of TTC3.
18. A method of producing slow proliferator cancer cells, the method comprising:
 - (i) contacting a cancer cell with an agonist of AKT1 degradation;
 - (ii) maintaining the cancer cells treated in step (i).

19. The method of paragraph 18, wherein the method further comprises the step of enriching the cells of step (ii) for slow proliferators by selecting for cells having increased levels of expression of TTC3 and optionally, increased levels of expression of Hes1 or decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; or H4K16ac.
20. The method of paragraph 18, wherein the method further comprises the step of enriching the cells of step (ii) for slow proliferators by selecting for cells having increased levels of expression of TTC3 and Hes1 and decreased levels of expression of AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.

[00149] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. A method of increasing the rate of asymmetric proliferation in a cancer cell, the method comprising:
 - contacting the cancer cell with an agonist of AKT1 degradation selected from the group consisting of:
 - an inhibitor of FAK; an inhibitor of β 1-integrin; PF-562271; and NVP-TAE226.
 - wherein an increase in AKT1 degradation increases the rate of asymmetric proliferation in the cancer cell.
2. The method of paragraph 1, whereby slow proliferator cancer cells are produced.
3. A method of decreasing the rate of asymmetric proliferation in a cancer cell, the method comprising:
 - contacting the cancer cell with an inhibitor of AKT1 degradation selected from the group consisting of:
 - an inhibitor of AKT1 expression; an agonist of β 1-integrin; and a cell medium comprising a fibrillar pattern of collagen.
 - wherein a decrease in AKT1 degradation decreases the rate of asymmetric proliferation in the cancer cell.
4. A method of treating cancer in a subject in need thereof, the method comprising:
 - administering an inhibitor of AKT1 degradation to the subject, wherein the inhibitor of AKT1 degradation is selected from the group consisting of:
 - an inhibitor of AKT1 expression and an agonist of β 1-integrin.
5. The method of paragraph 4, wherein the method further comprises administering a cancer therapy that targets fast proliferator cancer cells.

6. The method of paragraph 5, wherein the inhibitor of AKT1 degradation is administered before the administration of a cancer therapy that targets fast proliferator cancer cells.
7. The method of paragraph 6, wherein the inhibitor of AKT1 degradation is administered at least 1 day before the administration of a cancer therapy that targets fast proliferator cancer cells.
8. The method of paragraph 6, wherein the inhibitor of AKT1 degradation is administered at least 3 days before the administration of a cancer therapy that targets fast proliferator cancer cells.
9. The method of any of paragraphs 4-8, wherein the subject has been determined to have a subpopulation of cancer cells expressing increased levels of one or more genes selected from the group consisting of:
Hes1 and TTC3;
or decreased levels of one or more genes selected from the group consisting of:
AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph;
H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
wherein an increased level is a level statistically significantly higher than the level of expression found in at least 70% of cancer cells obtained from the same tumor and a decreased level is a level statistically significantly lower than the level of expression found in at least 70% of cancer cells obtained from the same tumor.
10. The method of paragraph 9, wherein the subject has been determined to have cancer cells expressing increased levels of TTC3; and optionally,
increased levels of Hes1;
or decreased levels of one or more genes selected from the group consisting of:
AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph;
H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
11. The method of paragraph 9, wherein the subject has been determined to have cancer cells expressing increased levels of Hes1 and TTC3 and decreased levels AKT1; H3K9me2; and MCM2.
12. The method of paragraph 9, wherein the expression level of the one or more genes is the level of polypeptide expression product.
13. The method of paragraph 9, wherein the expression level is determined by immunochemistry.
14. The method of any of paragraphs 1-13, wherein the agonist of β 1-integrin is a β 1-integrin activating antibody reagent.
15. The method of paragraph 14, wherein the β 1-integrin activating antibody reagent is TS2/16 monoclonal antibody or 12G10 monoclonal antibody.

16. The method of any of paragraphs 1-14, wherein the method comprises administering an inhibitor of AKT1 degradation, the method further comprising administering a PI3K inhibitor.
17. The method of paragraph 16, wherein the PI3K inhibitor is LY294002 or GDC-0941.

EXAMPLES

EXAMPLE 1

[00150] Human tumors contain rapidly proliferating cancer cells that dictate rate of growth, progression, and response to treatment. However, tumors also have many slowly proliferating cells whose origin and significance are poorly understood. Described herein is the discovery that cancer cells in culture occasionally trigger a fundamental mechanism to divide asymmetrically and produce slow proliferators at low frequency. This mechanism involves mTORC2 signaling during mitosis inducing asymmetric degradation of the AKT1 protein kinase via a TTC3 / proteasome-mediated pathway. Inhibiting this mechanism selectively reduces asymmetrically dividing cancer cells and slow proliferators in the population and significantly retards tumor growth *in vivo*. Conversely, inducing cancer cells to divide asymmetrically and produce slow proliferators markedly increases their tumorigenic potential. These results indicate that asymmetrically dividing cancer cells spawning a small fraction of slow proliferators provide a fundamental advantage for tumorigenesis.

[00151] Human tumors are heterogeneous with respect to the fraction of proliferating cancer cells that they contain (1, 2). Tumors with more rapidly proliferating cells clearly grow faster, progress further, and are more difficult to treat (2). But these tumors also contain many slowly proliferating cancer cells that may complicate treatment by resisting cancer therapeutics which preferentially target fast proliferators (3-5). While clonal selection theory clearly explains how rapidly proliferating cancer cells evolve, it remains difficult to understand within this framework why even advanced tumors contain so many slowly proliferating cancer cells (6). Interestingly, slow proliferators can also be found in established human cancer cell lines (7). Cancer cells in culture usually divide to produce two daughters that will divide again in relative synchrony, but occasionally these cells will divide to produce one daughter cell with a markedly slower proliferative rate than the other. Since established cell lines have been grown for many years under experimental conditions that ought to favor purifying selection for a rapidly and uniformly dividing population, this asynchronicity in cell culture is quite puzzling and remains poorly understood. It is generally assumed to simply reflect random variation among individual cancer cells in the many genetic and non-genetic factors that influence transit through the cell cycle (8).

[00152] The inventors have discovered that cancer cells divide asymmetrically at low frequency (i.e. < 5% of cell divisions) in established lines. These asymmetrically dividing cancer cells produce one rapidly proliferating AKT^{high} daughter cell and another AKT^{low} daughter that down-regulates

multiple proliferation proteins and is very slowly cycling (e.g. MKI67^{low}, MCM2^{low}, CDC6^{low}, GMNN^{low}) (7). AKT^{low} cells also suppress multiple nuclear histone marks associated with both transcriptional activation and repression, mimicking an epigenomic profile that has been observed in quiescent cell populations (e.g. H3S10ph^{low}, H3K4me2^{low}, H3K9me2^{low}, H3K27me3^{low}). Furthermore, AKT^{low} cells up-regulate HES1, a transcription factor that marks cells that have exited the cell cycle into a G0 state. Since AKT^{low} cells do eventually divide, reverting to an AKT^{high} proliferative phenotype over time, the term “G0-like” is used herein to emphasize the temporary and reversible nature of this cell state. Cancer cells dividing asymmetrically in this way can produce symmetrically dividing progeny and vice versa, suggesting that asymmetric division is not the unique property of a specialized cancer cell subpopulation but rather can be found in any dividing population at equilibrium. Importantly, the inventors have also found AKT^{low} cancer cells within actual human tumors at low frequency where they preferentially survive exposure to combination chemotherapy, suggesting that these slow proliferators may represent an important but unappreciated reservoir of treatment resistance in patients. These intriguing observations prompted led to the question of how this asymmetric cancer cell division is regulated.

[00153] Since AKT^{low} cancer cells partially suppress AKT protein levels (by about 90%), it was first asked whether asymmetric cancer cell division occurs in the complete absence of AKT protein (Fig. 1A) (7). HCT116 colorectal cancer cells with adeno-associated virus (AAV)-mediated disruption of the AKT1 and AKT2 gene loci (i.e. AKT1/2^{-/-} cells) were obtained (9). Importantly, AKT1/2^{-/-} cells do not have AKT1 or AKT2, nor do they express AKT3, but they are able to survive and proliferate in the complete absence of AKT signaling, presumably through compensatory changes that arose during their initial selection. Confocal microscopy was used to score the AKT1/2^{-/-} cell line for rare, asymmetrically dividing and G0-like cancer cells that express the previously validated MCM2^{low} / H3K9me2^{low} / HES1^{high} marker profile. Interestingly, it was found that the AKT1/2^{-/-} line had virtually no asymmetrically dividing or G0-like cells compared to wild type HCT116 (Fig. 1B). In addition, lentiviral-mediated overexpression of an AKT1 cDNA in AKT1/2^{-/-} cells completely rescued production of both asymmetrically dividing and G0-like cells, while overexpression of AKT2 did not (Fig. 1B). These intriguing results indicated that AKT1 is both necessary and sufficient for asymmetric cancer cell division and the production of G0-like cells.

[00154] The identification of an upstream pathway that might suppress AKT1 protein levels during cell division was undertaken. Site-directed mutagenesis was used to create a series of AKT1 cDNA constructs with mutations in critical amino acids known to be important for different aspects of AKT1 signaling (Fig. 1A). Each mutant AKT1 construct was then overexpressed in AKT1/2^{-/-} cells and these engineered cells scored for both asymmetrically dividing and G0-like cancer cells. Two different upstream signaling pathways are known to activate the AKT1 protein kinase through phosphorylation: the PDPK1 kinase phosphorylates AKT1 at the T308 residue while the mTORC2

kinase complex phosphorylates the AKT1-S473 and AKT1-T450 sites (10, 11). It was asked whether either of these canonical AKT1 residues were necessary for asymmetric cancer cell division. Similar to wild-type AKT1, overexpression of the AKT1-T308A mutant (which cannot be phosphorylated by PDPK1) in AKT1/2^{-/-} cells completely restored the production of asymmetrically dividing and G0-like cells (Fig. 1C). However, overexpression of AKT1-S473A or AKT1-T450A (which cannot be phosphorylated by mTORC2), or an AKT1-T308A / AKT1-S473A double mutant, did not produce this phenotypic rescue (Fig. 1C). These results indicated that mTORC2 signaling can induce asymmetric cancer cell division.

[00155] To test this hypothesis, two complementary approaches were used to disrupt mTORC2 signaling and changes in the frequency of asymmetrically dividing and G0-like cells were then scored. First, it was found that four structurally-different small molecules that inhibit both mTORC1 and mTORC2 signaling significantly reduced the frequency of asymmetrically dividing and G0-like cells in both HCT116 and MCF7 breast cancer cells (i.e. TORIN1, AZD8055, INK-128, Palomid-529) (Fig. 1D,1E). In contrast, two different inhibitors that preferentially target the TORC1 signaling complex alone did not suppress production of these cells (i.e. Rapamycin, RAD-001) (Fig. 1D,1E). In addition, inducible shRNA knockdown of RICTOR (an obligate member of the mTORC2 signaling complex) with two different short hairpin RNAs suppressed both asymmetrically dividing and G0-like cells in a panel of human epithelial cancer cell lines with diverse oncogenomic profiles, including those with a functional dependency on driver mutations in the PI3K signaling pathway (i.e. HCT116 (*PIK3CA^{mutant}*), MCF7 (*PIK3CA^{mutant}*), MDA-MB-231 breast, PC9 lung, and A375 melanoma) (Fig. 1F-1K) (10, 12). These findings indicated an important and general role for mTORC2 signaling in triggering rare cancer cells to divide asymmetrically and produce slow proliferators.

[00156] It was also asked whether increases in AKT1 signaling would induce asymmetric cancer cell division. However, it was found that two different small-molecules that inhibit AKT1 kinase catalytic activity did not reduce the frequency of asymmetrically dividing or G0-like cells in HCT116 or MCF7 (i.e. AZD5363, GDC0068) (Fig. 1L,1M). Furthermore, AKT1-E17K (a variant oncogenic protein with constitutive enzymatic activity resulting from a somatic point mutation in the kinase domain) partially rescued but did not significantly increase asymmetrically dividing or G0-like cells compared with AKT1 in the AKT1/2^{-/-} line (Fig. 1N) (13). This indicated that AKT1 kinase activity itself most likely did not induce asymmetric cancer cell division. In striking contrast, it was found that two different allosteric (rather than catalytic) inhibitors of AKT1 at low doses dramatically increased the frequency of both asymmetrically dividing and G0-like cells in HCT116 and MCF7 (i.e. AKT1/2, MK2206) (Fig. 1O,1P). Unlike catalytic inhibitors, these allosteric inhibitors bind to the AKT1 pleckstrin homology domain, displacing the protein from the cell membrane, and inducing its ubiquitination and proteasome-mediated degradation (14). These results indicate that asymmetric cancer cell division depends on the targeted degradation of AKT1 protein.

[00157] TTC3 is a RING-type E3 protein-ligase known to ubiquitinate AKT1 at the lysine-8 and lysine-14 residues to trigger its destruction by the proteasome (15). Interestingly, it was found that G0-like cells express high levels of TTC3 protein compared to proliferating cells, suggesting that this E3 ligase might play a special role in the production of these slowly cycling cells (data not shown). Consistent with this hypothesis, inducible shRNA knockdown of TTC3 with three different short hairpin RNAs dramatically suppressed the frequency of G0-like cells in both HCT116 and MCF7 (Figs. 2A-2C). In addition, AKT1-K8R, AKT1-K14R, and AKT1-K8R / K14R double mutant proteins (which cannot be ubiquitinated by TTC3) did not rescue G0-like cells in the AKT1/2^{-/-} line (Fig. 2D). Furthermore, two different small molecules that inhibit proteasome function significantly reduced the frequency of G0-like cells in both HCT116 and MCF7 (i.e. MG-132, Bortezomib) (Figs. 2E,2F). These results indicated that the production of G0-like cells depends on the TTC3 / proteasome-mediated degradation of AKT1.

[00158] Live-cell imaging experiments were performed to further define the role that mTORC2 signaling plays in regulating asymmetric cancer cell division. Serial images of HCT116 cells dividing over seven days in culture were obtained, either with or without RICTOR knockdown. These images were analyzed to identify individual dividing cells, creating lineage traces of these cells and their progeny to identify sibling pairs, and differences in mitotic times between sister cells arising from the same precursor were plotted. At baseline, ninety percent of dividing cells produced two siblings that divided again within ten hours of each other (Fig. 3). However, approximately ten percent of cells divided more asymmetrically to produce daughters with larger differences in time to mitosis that were greater than ten hours. Remarkably, inducible shRNA knockdown of RICTOR abrogated this minority fraction of most asymmetrically dividing cells, dramatically reducing inter-sibling asynchronicity and proliferative heterogeneity in the population (Fig. 3). Single cell imaging thus confirmed that mTORC2 signaling specifically regulates asymmetric cancer cell division.

[00159] This mechanistic insight provided a unique opportunity to determine the physiological relevance of asymmetric cancer cell division and slow proliferators in growing tumors. Inhibition of mTORC2 signaling through shRNA knockdown of RICTOR clearly inhibited the production of rare asymmetrically dividing and G0-like cells in several human epithelial cancer cell lines (i.e. HCT116, MCF7, MDA-MB-231, PC9, A375) (see Figs. 1F-1K). However, it was found that disrupting RICTOR did not significantly alter the overall proliferation of these cell lines *in vitro* (Figs. 6A-6E). Whether asymmetrically dividing cancer cells might play a special role in tumor formation *in vivo* was next investigated. These five different cell lines were implanted subcutaneously into immune compromised nude mice. The growth of these lines as xenografts was assessed over several months with or without inducible shRNA knockdown of RICTOR. Remarkably, RICTOR (-) cancer cells with reduced frequency of asymmetric division were markedly less tumorigenic compared to RICTOR (+) cells across the cell line panel, resulting in tumors that were 50 to 80% smaller in

size (Figs. 4A-4E). These results suggested that the ability of cancer cells to divide asymmetrically and produce small numbers of slowly proliferating progeny significantly enhanced tumorigenesis.

[00160] Conversely, it was also asked whether experimentally inducing asymmetric division would increase the tumorigenic potential of cancer cell lines. HCT116 and MCF7 cells were pre-treated for 72 hours with low doses of an allosteric AKT inhibitor (i.e. AKT1/2), which had been found to induce a large fraction of asymmetrically dividing and G0-like cancer cells (see Figs. 1O,1P). Variable numbers of these pre-treated cells were implanted into nude mice and grown without further manipulation *in vivo*. Importantly, inducing asymmetric cancer cell division and slow proliferators in this way significantly increased the engraftment of both HCT116 and MCF7 cells over a wide range of initial cell number *in vivo*, producing tumors that were 50% larger on average than those produced by untreated cell line populations (Figs. 4F-4N). This was particularly interesting since this AKT inhibitor generally kills cancer cells when used at higher doses (7). This striking finding thus provided further support that G0-like slow proliferators can confer on cancer cell populations a significant selective advantage for growth *in vivo*.

[00161] The data presented herein indicate that rapidly dividing cancer cells in culture occasionally trigger an mTORC2 signaling event during mitosis to induce the asymmetric degradation of AKT1 via a TTC3-proteasome-mediated mechanism (Fig. 5). This mechanism continually produces a small fraction of AKT1^{low} slow proliferators in the population that, remarkably, appears to promote tumor formation *in vivo*. The mTORC2 signaling complex has been studied extensively over the past decade and is known to be required for tumorigenesis in certain contexts although the exact reasons are unclear (10, 16). The findings described herein suggest that this highly conserved complex may actually regulate a fundamental balance between symmetric and asymmetric cancer cell division that dictates the production of slow proliferators that influence the rate of tumor growth. Without wishing to be bound by theory, it seems unlikely that cancer cells divide asymmetrically in this way simply to promote tumorigenesis. First, cultured cells have a small probability of dividing asymmetrically without deriving obvious advantage *in vitro*. Second, slow proliferators arising through asymmetric division assume a different, G0-like cell state with global changes in transcription, epigenomic profile, signaling, and metabolic activity compared to their highly proliferative siblings (7). Without wishing to be bound by theory, it is possible that asymmetric cancer cell division may actually represent the execution of a novel cell cycle decision that involves mTORC2 signaling at a very specific point in late mitosis. In this view, the newborn cancer cell with suppressed AKT1 signaling assumes special characteristics (including a slowed cell cycle and G0-like features) that enable it to withstand harsh negative selective pressures during tumor formation, upon transplantation, or on exposure to cytotoxic insult. This model will naturally evoke comparisons to prior work describing putative cancer stem cell populations that might be interesting to pursue using the theoretical, experimental, and mechanistic framework described herein (17-22).

[00162] REFERENCES

1. M. Loddo *et al.*, *Br J Cancer* **100**, 959 (Mar 24, 2009).
2. J. F. Holland, W. K. Hong, American Association for Cancer Research., *Holland-Frei cancer medicine* 8. (People's Medical Pub. House, Shelton, Conn., ed. 8th), pp. xxv, 2021 p.
3. S. V. Sharma *et al.*, *Cell* **141**, 69 (Apr 2).
4. K. R. Coser *et al.*, *Proc Natl Acad Sci U S A* **106**, 14536 (Aug 25, 2009).
5. A. Roesch *et al.*, *Cell* **141**, 583 (May 14).
6. P. C. Nowell, *Science* **194**, 23 (Oct 1, 1976).
7. I. Dey-Guha *et al.*, *Proc Natl Acad Sci U S A* **108**, 12845 (Aug 2).
8. J. Massague, *Nature* **432**, 298 (Nov 18, 2004).
9. K. Ericson *et al.*, *Proc Natl Acad Sci U S A* **107**, 2598 (Feb 9).
10. D. D. Sarbassov, D. A. Guertin, S. M. Ali, D. M. Sabatini, *Science* **307**, 1098 (Feb 18, 2005).
11. B. D. Manning, L. C. Cantley, *Cell* **129**, 1261 (Jun 29, 2007).
12. M. J. Garnett *et al.*, *Nature* **483**, 570 (Mar 29).
13. J. D. Carpten *et al.*, *Nature* **448**, 439 (Jul 26, 2007).
14. H. Jo *et al.*, *Proc Natl Acad Sci U S A* **108**, 6486 (Apr 19).
15. F. Suizu *et al.*, *Dev Cell* **17**, 800 (Dec, 2009).
16. D. A. Guertin *et al.*, *Cancer Cell* **15**, 148 (Feb 3, 2009).
17. M. Al-Hajj, M. S. Wicha, A. Benito-Hernandez, S. J. Morrison, M. F. Clarke, *Proc Natl Acad Sci U S A* **100**, 3983 (Apr 1, 2003).
18. M. Diehn *et al.*, *Nature* **458**, 780 (Apr 9, 2009).
19. J. M. Rosen, C. T. Jordan, *Science* **324**, 1670 (Jun 26, 2009).
20. P. B. Gupta *et al.*, *Cell* **146**, 633 (Aug 19).
21. T. Reya, S. J. Morrison, M. F. Clarke, I. L. Weissman, *Nature* **414**, 105 (Nov 1, 2001).
22. J. A. Magee, E. Piskounova, S. J. Morrison, *Cancer Cell* **21**, 283 (Mar 20).

EXAMPLE 2 – Materials & Methods

[00163] *Cell culture.* HCT116 colon, MCF7 breast, MDA-MB-231 breast, A375 melanoma, and PC9 lung cancer cells were purchased from the American Type Culture Collection (ATCC). HCT116 AKT1/2^{-/-} cells were purchased from Horizon Discovery (Cambridge, UK). MCF7 and MDA-MB-231 cells were maintained in DMEM, 10% FCS, 40mMglutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. HCT116 and HCT116 AKT1-/AKT2- cells were maintained in McCoy's 5α medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. PC9 cells were maintained in RPMI, 25% glucose, 1% sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin. A375 cells were maintained in DMEM supplemented with high glucose HEPES buffer, 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. All the cells were grown in a humidified atmosphere at 37°C and 5% CO₂.

[00164] *Drug treatment in vitro.* Cells were seeded onto collagen IV-coated coverslips, allowed to attach overnight, and treated with vehicle (DMSO) or AKT1/2 inhibitor (HCT116: 20 μ M; MCF7: 2 μ M) (Sigma), MK2206 (HCT116: 10 μ M; MCF7: 3 μ M) (Selleck Chemicals), TORIN1 (HCT116: 0.5 μ M; MCF7: 0.25 μ M) (Tocris Bioscience), AZD8055 (HCT116: 0.7 μ M; MCF7: 0.1 μ M) (Selleck Chemicals), INK128 (HCT116: 0.05 μ M; MCF7: 0.01 μ M) (Active Biochem), Palomid 529 (HCT116: 10 μ M; MCF7: 20 μ M) (Selleck Chemicals), (Rapamycin (HCT116: 20 μ M; MCF7: 20 μ M)(Sigma), RAD-001 (HCT116: 10 μ M; MCF7: 5 μ M) (Selleck Chemicals), (AZD5363 (HCT116: 50 μ M; MCF7: 5 μ M) (Active Biochem), GDC0068 (HCT116: 50 μ M; MCF7: 5 μ M) (Active Biochem) for 72 h and Bortezomib (HCT116: 1 μ M; MCF7: 4 μ M) (Selleck Chemicals) MG-132 (vehicle: ethanol) (HCT116: 1 μ M; MCF7: 10 μ M), for 24 h.

[00165] *shRNA constructs.* Human TRIPZ lentiviral inducible shRNAmirs for Rictor (Clone ID: V2THS_120392, V2THS_120389, V2THS_38014, V2THS_225915), non-silencing, and empty vector were purchased from Open Biosystems and virus was generated using our standard protocol. Infection was performed 24 h later in MCF7, HCT116, A375, PC9 and MDA-MB-231 cell lines with the lentiviral particles followed by selection with 2 μ M puromycin. Following selection, cells were allowed to grow to confluency. The shRNAs were induced using 2 μ g/ml doxycycline for 72 h. The TTC3 virus was purchased from Sigma-Aldrich and infected in HCT116 and MCF7 cells and the standard protocol for selection was followed.

[00166] *Generation of AKT1 mutant cell lines.* AKT1(WT) cDNA was purified using PCR after cutting PDD AKT1(WT) with restriction enzymes BamHI and XhoI. Following purification, the product was ligated into pMSCVpuro-C-tag-mCherry cut with BglII and SalI. All the AKT1mutants were generated using the QuikChange site directed mutagenesis kit (Agilent technologies) and the product was ligated into pMSCVpuro- C-tag-mCherry. The resulting vector pMSCV-puro-AKT1-mCherry was sub-cloned into DH5 α competent cells (Invitrogen). Sequencing verification of the fusion product was performed by the MGH DNA Core Facility with primers pMSCV 5'-CCCTTGAACCTCCTCGTTCGACC-3'(SEQ ID NO: 1) and pMSCV 3'-GAGACGTGCTACTTCCATTTGTC-5' (SEQ ID NO2). Virus carrying the desired fusion gene was produced by transfecting 293-T cells with target vector pMSCV-puro- AKT1-mCherry and packaging vector pCL-Ampho using the Mirus TranIT-293 transfection reagent and established protocols. Virus was collected 24 h following transfection. Before infection, cells were plated in a six-well plate in DMEM, 10% FCS. Infection was performed 24 h later by adding 0.5 mL DMEM, 10% FCS, 0.5mL pooled virus, and 1 μ L 1,000 $\times$ polybrene per well. A media change was performed the following day and cells were allowed to grow to confluency before splitting into a 10-cm dish and selection with 2 μ M puromycin. Following selection, cells were allowed to grow to confluency before clones were selected using single-cell sorting (Becton Dickinson FACS Aria II). Single cells were filtered by

gating on the brightest 5% of cells in the PETexas red channel and sorted into individual wells of a 96-well plate. Clones were harvested between 14 and 21 d.

[00167] *Immunofluorescence staining.* For bulk populations and for colonies, cells were grown directly on collagen IV-coated coverslips (Sigma). Cells were fixed in 3.7% formalin, permeabilized using 0.1% Triton X-100, and treated with 0.1% SDS. They were blocked in 1% BSA and then incubated with primary antibody (α -H3K9me2 (Abcam); α -MCM2 and α -Tubulin (Cell Signaling), α -Hes1 and α -TTC3 (Abnova)) diluted in blocking solution, washed, and incubated with the respective secondary antibody. Cells were mounted using hard-set mounting media containing DAPI (Vector Laboratories). All secondary antibodies were Alexa Fluor conjugates (488, 555, 568, 633, and 647) (Invitrogen). Immunofluorescence imaging (on a Nikon Eclipse Ti A1R-A1 confocal microscope) and live-cell imaging (on the Nikon Biostation CT platform) were performed as previously described (1).

[00168] *Generation of a HCT116-mCerulean-tagged cell line.* Virus carrying the pMSCV-CMV-NLSmCerulean construct was produced by transfecting 293-T cells plated at 500,000 cells per well in a six-well plate. Twenty four hours later, these cells were transfected with 1 μ g target vector pMSCV-CMV-NLS- mCerulean, 1 μ g packaging vector pCL-Ampho, and 3 μ L FuGENE HD mixed with 100 μ L reduced serum solution (Opti- MEM; Invitrogen). Virus was collected 24 h following transfection. Before infection with virus, HCT116 cells were plated at 50,000 cells per well in a six-well plate in DMEM, 10% FCS. Infection was performed 24 h later by adding 0.5 mL DMEM, 10% FCS, 0.5 mL pooled virus, and 1 μ L 1,000 $\times$ polybrene per well. A media change was performed the following day, and cells were allowed to grow to confluency before splitting into a 10-cm plate. MCF7/ NLS-mCerulean cells were selected using fluorescence-activated cell sorting (Becton Dickinson FACSARIA II) and gating on the brightest 5% cells in the Pacific blue channel.

[00169] *Live-cell imaging & time-lapse analysis.* In order to follow the fate of HCT-116 cells in vitro, we plated HCT-116 cells tagged with NLS-mCerulean and also a doxycycline-inducible non-silencing or Rictor knockdown shRNA (hp4) construct in glass-bottom 12-well plates (MatTek Product # P12G-1.0-10-F) treated with type IV collagen. Tagged HCT116 cells were plated in 2 μ g/ml of doxycycline at a density of 1000 cells per well along with unlabeled HCT-116 cells at a density of 4000 cells per well. All cells were initially grown in McCoy's 5-alpha + 10% FCS at t=0. Media changes were performed every day with 2 μ g/ml doxycycline. Multi-point serial imaging was performed using an inverted microscope fitted with a tissue culture incubator (Nikon Ti-Eclipse) every 20 minutes at 20 $\times$ magnification (CFI Plan Apo 20 $\times$) for 164 hours. Both phase and fluorescent images were captured. Cells were excited with an LED (Nikon C-HGFI Intensilight HG Illum) and passed through a filter series (Nikon, C-FL CFP and RFP HC HISN Zero Shift Filter Set). All cell division events were tracked manually using the CFP images by recording the following characteristics for each cell: ID based on initial frame of appearance and x/y coordinate, first frame, last frame, origin ID, progenitor IDs, and x/y coordinates for first and last frame, and end method

(division, lost in tracking, lost to wash out, or lost to cell death). Analysis was performed using R v2.8.0 (The R Foundation for Statistical Computing, 2008) by analyzing all division events.

[00170] *Xenografting & tumor propagation in vivo.* For *in vivo* RICTOR knockdown experiments, 5×10^5 cells (MCF7, HCT116, A375, PC9, MDA-MB-231 cell lines) carrying either doxycycline-inducible non-silencing or RICTOR-targeting shRNAs (120392, 225915) were injected subcutaneously into the flanks of 5-6 week old, female nude mice. The mice were given doxycycline in water at 20 mg/ml for hairpin induction. For induction of asymmetrically dividing cells and slow proliferators, cells were treated with AKT1/2 inhibitor and DMSO (vehicle) for 72 h and were harvested at 60–70% confluence, and then counted and washed twice in PBS and resuspended in 1:1 Media: Matrigel (BD Biosciences). 5×10^6 , 5×10^5 , 5×10^4 , 5×10^3 and 5×10^2 cells respectively were injected subcutaneously into the flanks of 5-6 week old, female nude mice (Nude/Nude) (Charles River Labs). For all experiments, growing tumors were measured weekly by caliper, and mice were killed after the tumor size reached 1 cm^3 . Mouse experiments were carried out under a Massachusetts General Hospital Institutional Review Board–approved protocol.

[00171] REFERENCES

1. I. Dey-Guha *et al.*, Asymmetric cancer cell division regulated by AKT. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 12845 (Aug 2, 2011).

[00172] Example 3: A Mechanism For Slowly Proliferating Cancer Cells that Promote Tumor Growth

[00173] Tumor growth is driven by rapidly dividing cancer cells that arise through mutation and natural selection. Clonal selection does not fully explain, however, why established tumors also contain slowly proliferating cancer cells. We now find that if a dividing cancer cell experiences an asymmetric decrease in $\beta 1$ -integrin signaling, it activates mTORC2 kinase signaling which induces degradation of AKT1 kinase through a TTC3 / proteasome mechanism, to produce a slowly proliferating AKT1low daughter cell. Remarkably, disrupting this mechanism for slowly proliferating cancer cells impedes tumor growth, while inducing slow proliferators enhances tumor formation, across a spectrum of cancer xenograft models. These results suggest that rapidly proliferating cancer cells retain a mechanism to spawn slow proliferators for selective advantage during tumorigenesis.

[00174] A dividing cancer cell generally produces two daughter cells that divide again in relative synchrony within hours of each other in cell culture. Occasionally, however, a cancer cell divides to produce progeny that are asynchronous, with one daughter cell having a markedly slower cell division time, on the order of days, compared to the other. As described above, this asynchronicity relates to cancer cells asymmetrically suppressing AKT protein kinase levels by about ninety percent during mitosis just before cytokinesis. This asymmetry produces one AKThigh daughter cell that rapidly

enters the next cell cycle and another AKTlow cell that remains dormant for a more prolonged time before dividing again. Slowly cycling AKTlow cells reduce their production of reactive oxygen species (i.e., ROSlow), down-regulate proliferation proteins (e.g., MKI67low, MCM2low), suppress multiple nuclear histone marks similar to quiescent cell populations (e.g., H3K9me2low), and up-regulate the HES1 transcription factor that may mark exit from the cell cycle into G0 (i.e., HES1high) (1). Since AKTlow cells do eventually divide, converting to an AKThigh proliferative phenotype over time, the term “G0-like” is used to describe this temporary and reversible cell state. It is described herein that AKTlow cancer cells are found within actual human breast tumors where they preferentially survive therapy with combination chemotherapy, suggesting that these cells may constitute an important but unappreciated reservoir of treatment resistance in patients with breast cancer (1). Since AKTlow cells share a number of conceptual features with putative cancer stem cell populations (e.g., asymmetric division, slow cycling, ROSlow, treatment resistance), it was reasoned that understanding in molecular detail how AKTlow slow proliferators arise might provide fundamental insight into the dynamics of tumor growth (1,2).

[00175] Given that AKTlow cancer cells only partially suppress total AKT protein levels, it first asked whether asymmetric division occurs in the complete absence of all three AKT isoforms (i.e., AKT1, AKT2, and AKT3). To do so, HCT116 colorectal cancer cells with adeno-associated virus (AAV)-mediated disruption of the AKT1 and AKT2 gene loci (i.e., AKT1/2-/- cells) were obtained (3). Importantly, AKT1/2-/- cells do not express either AKT1 or AKT2, nor do they express AKT3, and thus are able to survive and proliferate in the complete absence of AKT signaling, presumably through compensatory changes that arose during their initial selection. Confocal microscopy was used to score AKT1/2-/- cell populations for rare, asymmetrically dividing and G0-like cancer cells that express the previously validated MCM2low / H3K9me2low / HES1high marker profile (1). Interestingly, it was found that the AKT1/2-/- line had virtually no asymmetrically dividing or G0-like cells compared to wild type HCT116 (the parental line from which AKT1/2-/- cells are derived) (Fig. 1B). In addition, lentiviral-mediated overexpression of an AKT1 cDNA in AKT1/2-/- cells completely restored formation of both asymmetrically dividing and G0-like cells, while overexpression of AKT2 did not (Fig. 1B). These results indicate that AKT1 is necessary and sufficient for asymmetric cancer cell division and the production of G0-like cells.

[00176] Based on this result, site-directed mutagenesis was used to identify AKT1 domains that might be required for its partial suppression during asymmetric division. A series of AKT1 cDNA constructs with mutations in critical amino acids known to be important for various aspects of AKT1 signaling were created (Fig. 7A). Each mutant AKT1 construct was overexpressed in AKT1/2-/- cells and these engineered cells scored for both asymmetrically dividing and G0-like cancer cells. First, it was found that AKT1-K179M (a mutation in the kinase pocket that renders AKT1 catalytically inactive) failed to restore production of asymmetrically dividing and G0-like cells in the AKT1/2-/-

line, while AKT1-D292A (another kinase dead mutant) did so only weakly compared to wild-type AKT1 (Fig. 7B) (4). These results indicated that AKT1 enzymatic activity is necessary for asymmetric cancer cell division.

[00177] It was next asked how AKT1 protein is suppressed to produce slow proliferators. As described above, treating cancer cells with allosteric AKT inhibitors at low doses dramatically increases the frequency of both asymmetrically dividing and G0-like cells (i.e., AKT1/2, MK2206) (Fig. 7C) (1). These allosteric inhibitors are known to bind to the AKT1 pleckstrin homology domain, inducing conformational change and displacement of the protein from the cell membrane, promoting its ubiquitination and proteasome-mediated degradation (5). Therefore, it was hypothesized that asymmetric division might actually depend on the targeted degradation of the AKT1 protein. TTC3 is a RING-type E3 protein-ligase known to ubiquitinate AKT1 at its lysine-8 and lysine-14 residues leading to its destruction by the proteasome (6). Interestingly, it was found that G0-like cells express high levels of TTC3 protein compared to proliferating cells, consistent with a potential role for this E3 ligase in producing AKT1low cells (data not shown). In addition, inducible shRNA knockdown of TTC3 with three different short hairpin RNAs suppressed the frequency of G0-like cells in both HCT116 and MCF7 without affecting overall cell proliferation (Figs. 7D and 2A). Furthermore, AKT1-K8R, AKT1-K14R, and AKT1-K8R / K14R double mutant proteins (which cannot be ubiquitinated by TTC3) failed to rescue the formation of G0-like cells in the AKT1/2-/- line (Fig. 7D). In addition, two different small molecules that inhibit proteasome function reduced the frequency of G0-like cells in both HCT116 and MCF7 when used at doses that do not affect overall cell proliferation (i.e., MG-132, Bortezomib) (Fig. 7D). Overall, these results indicated that AKT1low slow proliferators are produced by TTC3-mediated ubiquitination of AKT1 followed by proteasomal degradation.

[00178] Two different upstream signaling pathways are known to activate AKT1 kinase: PDPK1 kinase phosphorylates AKT1 at the T308 residue, while the mTORC2 kinase complex phosphorylates the AKT1-S473 and AKT1-T450 sites (7,8). It was therefore asked whether any of these canonical AKT1 residues were necessary for asymmetric cancer cell division. Similar to wild-type AKT1, overexpression of the AKT1-T308A mutant (which cannot be phosphorylated by PDPK1) in AKT1/2-/- cells completely restored the production of asymmetrically dividing and G0-like cells (Fig. 7E). In contrast, AKT1-S473A, AKT1-T450A, and an AKT1-T308A / AKT1-S473A double mutant (all of which cannot be phosphorylated by mTORC2) did not produce phenotypic rescue (Fig. 7E). These results indicated that mTORC2 signaling might induce asymmetric division by partially phosphorylating and activating AKT1.

[00179] To further test this hypothesis, two complementary approaches were used to disrupt mTORC2 signaling and then cancer cell lines scored for changes in the frequency of asymmetrically dividing and G0-like cells. First, it was found that four structurally-different small molecules that

inhibit both mTORC1 and mTORC2 signaling reduced the frequency of asymmetrically dividing and G0-like cells in both HCT116 and MCF7 breast cancer cells when used at low doses that did not appreciably inhibit cell proliferation (i.e., TORIN1, AZD8055, INK-128, Palomid-529) (Fig. 7E). In contrast, production of these cells was not suppressed either by two different inhibitors that preferentially target the TORC1 signaling complex alone (i.e., Rapamycin, RAD-001), or by a pan-class I PI3 kinase inhibitor (i.e., BKM-120), at target-suppressing doses (Fig. 7E). In addition, inducible shRNA knockdown of RICTOR (an obligate member of the mTORC2 signaling complex) with two different short hairpin RNAs suppressed the production of both asymmetrically dividing and slowly proliferating G0-like cells in a panel of five different human cancer cell lines, including those with a functional dependency on mutant PI3K (i.e., HCT116 (PIK3CAmutant), MCF7 (PIK3CAmutant), MDA-MB-231 breast, PC9 lung, and A375 melanoma) (Figs. 7E, 1F, and 11C). However, RICTOR (-) cells did not differ from RICTOR (+) cells with respect to overall proliferation, response to stress (i.e., low serum, low glucose, or hypoxic conditions), or invasion in vitro. These results indicated that mTORC2 specifically induces asymmetric division and the production of slow proliferators, independent of PI3K or mTORC1 activity and without altering other important cancer cell functions (Figs. 12A-12O, 12A-13J and 14A-14D).

[00180] Live-cell imaging experiments were performed to confirm mTORC2 regulation of asymmetric cancer cell division. Serial images of HCT116 and MCF7 cells dividing over seven days in culture either with or without RICTOR knockdown were obtained. These images were analyzed to identify individual dividing cells, lineage traces of these cells and their progeny created in order to identify sibling pairs, and differences plotted in mitotic times between sister cells arising from the same precursor. In the control population, eighty to ninety percent of dividing cells produced two siblings that divided again within five hours of each other (Fig. 7I, 7J). However, approximately ten to twenty percent of cells divided more asymmetrically to produce daughters with larger differences in time to mitosis. Remarkably, inducible shRNA knockdown of RICTOR reduced this minority fraction of the most asymmetrically dividing cells, thus decreasing inter-sibling asynchronicity and proliferative heterogeneity in the population. These findings confirmed that mTORC2 signaling induces asymmetric division in a small fraction of cancer cells to produce slow proliferators.

[00181] To identify proteins that physically interact with and might activate mTORC2 signaling during asymmetric division an immunoprecipitation (IP) approach was used. Specifically, it was found that IP with a RICTOR antibody (under conditions that maintain integrity of the mTORC2 complex in whole cell lysates) pulled down focal adhesion kinase (FAK) protein in both HCT116 and MCF7 (Fig. 8). Reciprocally, IP with a FAK antibody pulled down both mTOR kinase and RICTOR, confirming that FAK directly interacts with mTORC2 complex in these cells (Fig. 8). This observation indicated that FAK activity might somehow modulate mTORC2 signaling during asymmetric cancer cell division. Consistent with this hypothesis, it was found that inducible shRNA

knockdown of FAK with two different short hairpins increased both asymmetrically dividing and G0-like cells in HCT116 and MCF7 (Figs. 7F and 11A). Similarly, inhibiting FAK activity with two different small molecules (at doses that do not inhibit proliferation) increased the frequency of both asymmetrically dividing and G0-like cells (i.e., PF-562271, NVP-TAE226) (Fig. 7F). After RICTOR knockdown, however, FAK inhibitors failed to increase asymmetries or slow proliferators (Fig. 7H). These findings indicated that a loss of FAK activity enables mTORC2-mediated asymmetric cancer cell division.

[00182] Integrins are a family of heterodimeric transmembrane receptors that transduce signals from the extracellular matrix by activating a number of well-described signaling intermediaries within the cell, including FAK, to regulate cell cycle, shape, and motility in cancer and normal cells (9). It was reasoned that decreased integrin signaling might cause a loss of FAK activity resulting in mTORC2 activation during asymmetric division. In fact, inducible shRNA knockdown of β 1-integrin (i.e., ITGB1, CD29) with two different short hairpins increased the fraction of asymmetrically dividing and G0-like cells in both HCT116 and MCF7 (Figs. 7G and 11B). In addition, inhibiting β 1-integrin function with two different monoclonal antibodies also increased both asymmetrically dividing and G0-like cells (i.e., A2B2, P4C10) (Fig. 7G) 910). In contrast, activating β 1-integrin signaling with two other monoclonal antibodies, which force β 1-integrin into an “on” state by imposing a conformational change, eliminated both asymmetries and slow proliferators in these cell lines (i.e., TS2/16, 12G10) (Fig. 7G) (10). These results indicated that an asymmetric loss in β 1-integrin signaling during mitosis is both necessary and sufficient for asymmetric division and slow proliferators.

[00183] It was noted, however, that asymmetrically dividing cancer cells express β 1-integrin protein uniformly on their cell membrane, suggesting that these cells might arise through the asymmetric β 1-integrin activity. Since Type-I collagen is the major extracellular matrix protein that binds to and activates β 1-integrin, it was hypothesized that polarities created by random variation in Type-I collagen might result in the asymmetric engagement of β 1-integrin on dividing cancer cells in cell culture (11). To explore this hypothesis, MCF7 cells were grown on engineered matrices that display Type-I collagen fibrils aligned in a stereotypical pattern (data not shown). This maximized the probability that these cancer cells would divide in a relatively uniform collagen microenvironment, thus assuring the symmetrical activation of β 1-integrin in all dividing cells within the population. Interestingly, MCF7 cells grown in this structured matrix did not produce asymmetries or G0-like cells unlike typical cell culture, indicating that a functional asymmetry in β 1-integrin signaling related to irregularities in Type I collagen produces slow proliferators in vitro (Fig. 7H).

[00184] These results indicate that cancer cells in long-term culture retain a signaling pathway to produce slow proliferators despite the selective pressure for rapid and uniform proliferation, suggesting that this pathway might provide cancer cells with some fundamental advantage. To test

this hypothesis, it was asked whether activating β 1-integrin signaling in cancer cells (to prevent the production of slow proliferators) affects tumor growth in vivo. five cancer cell lines were transplanted subcutaneously into nude mice. When palpable tumors formed (i.e., A375, MDA-MB-231, PC9, HCT116, and MCF7), tumor-bearing animals were treated with the TS2/16 antibody while the growth of established tumors was followed (100 μ l at 4mg/ml/wk x 5 weeks). Interestingly, TS2/16 treatment resulted in markedly slower tumor growth compared to control across this spectrum of solid tumor models, which included melanoma, lung, colorectal, and breast cancers (Fig. 9A). This finding was notable given that integrin signaling is generally thought to promote cancer cell proliferation, survival, and invasion (12). Since TS2/16 specifically activates human β 1-integrin, moreover, these anti-tumor effects most likely resulted from the direct targeting human cancer cells rather than mouse stroma in these xenografts. In addition, it was asked whether disrupting mTORC2 signaling with RNA interference (which also reduces asymmetric division and slow proliferators without altering general cancer cell viability) would retard tumor growth (Fig. 7E, 1F and 11C). The same cancer cell lines carrying doxycycline-inducible shRNAs for RICTOR were again injected subcutaneously in nude mice. These mice were fed doxycycline to induce continuous RICTOR knockdown in these cancer cells while tumor formation was monitored over time. Similar to β 1-integrin activation, it was found that inhibiting mTORC2 signaling to reduce the production of slow proliferators also decreased growth of these different solid tumor types, (Fig. 9B). Both of these striking results supported the idea that tumors derive a considerable growth advantage from continuously producing slow proliferating cancer cells.

[00185] It was also asked whether cancer cell populations with an increased number of slow proliferators form tumors more efficiently in vivo. A low dose of the allosteric inhibitor AKT1/2 was used to partially inhibit AKT signaling in HCT116 and MCF7 cells, thus increasing their proportion of slowly proliferating AKT1low cells ex vivo (see Fig. 7C) (1). Then, these pre-treated cells were implanted subcutaneously in immune-compromised nude mice to assess tumor formation without further manipulation in vivo. Interestingly, this transient increase in G0-like cells significantly promoted the engraftment of these moderately tumorigenic cell lines across a 100-fold range, resulting in notably larger tumors over time (Fig. 9C). These findings demonstrate that AKT1low slow proliferators increase the tumorigenicity of cancer cell populations as xenotransplants.

[00186] These findings demonstrate that a dividing cancer cell encountering an asymmetric decrease in β 1-integrin / FAK signaling during mitosis activates mTORC2 signaling, which induces AKT1 degradation by TTC3 and the proteasome, to asymmetrically produce an AKT1low daughter cell (Fig. 10). This AKT1low cancer cell temporarily arrests its cell cycle, and superficially expresses a quiescent marker profile (i.e., MCM2low, H3K9me2low, HES1high), but can begin cycling again if its β 1-integrin sensor is ligated optimally (1). Asymmetrically dividing cancer cells are not a fixed subpopulation, but rather appear to arise randomly depending on interaction with extracellular matrix

proteins like collagen. Furthermore, slowly proliferating AKT1low cancer cells do not differentiate as far as we know. Nevertheless, AKT1low slow proliferators mimic cancer stem cell properties in being ROSlow, slow cycling, differentially tumorigenic in nude mice, and resistant to cytotoxic drugs (13,14). Importantly, it was found that selectively inhibiting production of these slow proliferators (either by activating β 1-integrin with a monoclonal antibody or interfering with mTORC2 signaling) impedes the growth of biologically diverse solid tumor types in vivo. It was also noted that integrin receptors mark epithelial stem cells and dictate their self-renewal in normal tissues (11,15,16). Without wishing to be limited by theory, these observations suggest that a fundamental signaling pathway required for malignant stem cell homeostasis has been identified herein. It is speculated that rapidly dividing cancer cells facultatively trigger this stem-like pathway to spawn slow proliferators when they encounter disorganized extracellular matrix associated with a suboptimal tumor microenvironment (17). In turn, slow proliferators may buffer proliferating cancer cell populations against this cytotoxic stress within growing tumors, adding to tumor bulk as they accumulate over time, to proliferate as local conditions permit. This model of tumor dynamics complements clonal selection theory and may provide deeper insight into tumor progression, dormancy, and treatment resistance (18).

[00187] REFERENCES

1. I. Dey-Guha et al., Asymmetric cancer cell division regulated by AKT. *Proceedings of the National Academy of Sciences of the United States of America* 108, 12845 (Aug 2, 2011).
2. J. A. Magee, E. Piskounova, S. J. Morrison, Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer cell* 21, 283 (Mar 20, 2012).
3. K. Ericson et al., Genetic inactivation of AKT1, AKT2, and PDPK1 in human colorectal cancer cells clarifies their roles in tumor growth regulation. *Proceedings of the National Academy of Sciences of the United States of America* 107, 2598 (Feb 9, 2010).
4. T. Okuzumi et al., Inhibitor hijacking of Akt activation. *Nature chemical biology* 5, 484 (Jul, 2009).
5. H. Jo et al., Small molecule-induced cytosolic activation of protein kinase Akt rescues ischemia-elicited neuronal death. *Proceedings of the National Academy of Sciences of the United States of America* 109, 10581 (Jun 26, 2012).
6. F. Suizu et al., The E3 ligase TTC3 facilitates ubiquitination and degradation of phosphorylated Akt. *Developmental cell* 17, 800 (Dec, 2009).
7. D. D. Sarbassov, D. A. Guertin, S. M. Ali, D. M. Sabatini, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307, 1098 (Feb 18, 2005).
8. B. D. Manning, L. C. Cantley, AKT/PKB signaling: navigating downstream. *Cell* 129, 1261 (Jun 29, 2007).

9. R. O. Hynes, Integrins: bidirectional, allosteric signaling machines. *Cell* 110, 673 (Sep 20, 2002).
10. A. Byron et al., Anti-integrin monoclonal antibodies. *Journal of cell science* 122, 4009 (Nov 15, 2009).
11. R. O. Hynes, The extracellular matrix: not just pretty fibrils. *Science* 326, 1216 (Nov 27, 2009).
12. J. S. Desgrosellier, D. A. Cheresh, Integrins in cancer: biological implications and therapeutic opportunities. *Nature reviews. Cancer* 10, 9 (Jan, 2010).
13. M. Al-Hajj, M. S. Wicha, A. Benito-Hernandez, S. J. Morrison, M. F. Clarke, Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America* 100, 3983 (Apr 1, 2003).
14. M. Diehn et al., Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* 458, 780 (Apr 9, 2009).
15. F. M. Watt, B. L. Hogan, Out of Eden: stem cells and their niches. *Science* 287, 1427 (Feb 25, 2000).
16. S. Goulas, R. Conder, J. A. Knoblich, The Par complex and integrins direct asymmetric cell division in adult intestinal stem cells. *Cell stem cell* 11, 529 (Oct 5, 2012).
17. E. Brown et al., Dynamic imaging of collagen and its modulation in tumors in vivo using second-harmonic generation. *Nature medicine* 9, 796 (Jun, 2003).
18. P. C. Nowell, The clonal evolution of tumor cell populations. *Science* 194, 23 (Oct 1, 1976).

MATERIALS & METHODS

[00188] Cell culture. HCT116 colon, MCF7 breast, MDA-MB-231 breast, A375 melanoma, and PC9 lung cancer cells were purchased from the American Type Culture Collection (ATCC). HCT116 AKT1/2-/- cells we purchased from Horizon Discovery (Cambridge, UK). MCF7 and MDA-MB-231 cells were maintained in DMEM, 10% FCS, 40mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. HCT116 and HCT116 AKT1/2-/- cells were maintained in McCoy's 5α medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. PC9 cells were maintained in RPMI, 25% glucose, 1% sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin. A375 cells were maintained in DMEM supplemented with high glucose HEPES buffer, 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. All the cells were grown in a humidified atmosphere at 37°C and 5% CO₂.

[00189] Drug treatment in vitro. Cells were seeded onto collagen IV-coated coverslips, allowed to attach overnight, and treated with vehicle (DMSO) or AKT1/2 inhibitor (HCT116: 20µM; MCF7: 2µM) (Sigma), MK2206 (HCT116: 10µM; MCF7: 3µM) (Selleck Chemicals), TORIN1 (HCT116: 0.5µM; MCF7: 0.25µM) (Tocris Bioscience), AZD8055 (HCT116: 0.7µM; MCF7: 0.1µM) (Selleck

Chemicals), INK128 (HCT116: 0.05 μ M; MCF7: 0.01 μ M) (Active Biochem), Palomid 529 (HCT116: 10 μ M; MCF7: 20 μ M) (Selleck Chemicals), (Rapamycin (HCT116: 20 μ M; MCF7: 20 μ M)(Sigma), RAD-001 (HCT116: 10 μ M; MCF7: 5 μ M) (Selleck Chemicals), BKM-120 (HCT116: 1.5 μ M; MCF7: 0.5 μ M) (Active Biochem), FAK inhibitors (PF-562271: 1 μ M (Pfizer) and NVP-TAE226 : 1 μ M (Novartis), for both cell lines) for 72 h and Bortezomib (HCT116: 1 μ M; MCF7: 4 μ M) (Selleck Chemicals) MG-132 (vehicle: ethanol) (HCT116: 1 μ M; MCF7: 10 μ M), for 24 h.

[00190] shRNA constructs. Human TRIPZ lentiviral inducible shRNAmirs for RICTOR (Clone ID: V2THS_120392, V2THS_120389, V2THS_38014, V2THS_225915), FAK (Clone ID: V2THS_57326, V2THS_325805), β 1-integrin (Clone ID: V2THS_133469, V2THS_390997), non-silencing, and empty vector were purchased from Open Biosystems and virus was generated using a standard protocol. Infection was performed 24 h later in MCF7, HCT116, A375, PC9 and MDA-MB-231 cell lines with the lentiviral particles followed by selection with 2 μ M puromycin. Following selection, cells were allowed to grow to confluency. The shRNAs were induced using 2 μ g/ml doxycycline for 72 h. The TTC3 virus was purchased from Sigma-Aldrich and infected in HCT116 and MCF7 cells and the standard protocol for selection was followed.

[00191] Generation of AKT1 mutant cell lines. AKT1(WT) cDNA was purified using PCR after cutting PDD AKT1(WT) with restriction enzymes BamHI and XhoI. Following purification, the product was ligated into pMSCVpuro-C-tag-mCherry cut with BglII and SalI. All the AKT1mutants were generated using the QuikChange site directed mutagenesis kit (Agilent technologies) and the product was ligated into pMSCVpuro- C-tag-mCherry. The resulting vector pMSCV-puro-AKT1-mCherry was sub-cloned into DH5 α competent cells (Invitrogen). Sequencing verification of the fusion product was performed by the MGH DNA Core Facility with primers pMSCV 5'-CCCTTGAACCTCCTCGTTTCGACC-3' (SEQ ID NO: 1) and pMSCV 3'-GAGACGTGCTACTTCCATTTGTC-5' (SEQ ID NO: 2). Virus carrying the desired fusion gene was produced by transfecting 293-T cells with target vector pMSCV-puro- AKT1-mCherry and packaging vector pCL-Ampho using the Mirus TranIT-293 transfection reagent and established protocols. Virus was collected 24 h following transfection. Before infection, cells were plated in a six-well plate in DMEM, 10% FCS. Infection was performed 24 h later by adding 0.5 mL DMEM, 10% FCS, 0.5mL pooled virus, and 1 μ L 1,000 $\times$ polybrene per well. A media change was performed the following day and cells were allowed to grow to confluency before splitting into a 10-cm dish and selection with 2 μ M puromycin. Following selection, cells were allowed to grow to confluency before clones were selected using single-cell sorting (Becton Dickinson FACSaria II™). Single cells were filtered by gating on the brightest 5% of cells in the PETexas red channel and sorted into individual wells of a 96-well plate. Clones were harvested between 14 and 21 days.

[00192] Immunofluorescence staining. For bulk populations and for colonies, cells were grown directly on collagen IV-coated coverslips (Sigma). Cells were fixed in 3.7% formalin, permeabilized

using 0.1% Triton X-100, and treated with 0.1% SDS. They were blocked in 1% BSA and then incubated with primary antibody (α -H3K9me2 (Abcam); α -MCM2 and α -Tubulin (Cell Signaling), α -Hes1 and α -TTC3 (Abnova)) diluted in blocking solution, washed, and incubated with the respective secondary antibody. Cells were mounted using hard-set mounting media containing DAPI (Vector Laboratories). All secondary antibodies were Alexa Fluor conjugates (488, 555, 568, 633, and 647) (Invitrogen). Immunofluorescence imaging (on a Nikon Eclipse Ti A1R-A1™ confocal microscope) and live-cell imaging (on the Nikon Biostation CT platform) were performed as previously described⁷. Slides coated with Type-I collagen (control) and AlignCol™ woven with large collagen fibers (100-200nm) (Advanced Biomatrix) were incubated for 1 hour in 70% ethanol and then washed with PBS. Cells were then plated on the slides and processed for immunofluorescence.

[00193] Immunoprecipitation. Cells were rinsed with PBS, fixed with 0.37% formaldehyde and quenched with 0.25M glycine. Cell lysates were prepared in lysis buffer (1% Triton X-100, 150mM NaCl, 3mM MgCl, 40mM HEPES [pH 7.5], 50mM NaF, EDTA-Free protease inhibitor and phosphatase inhibitor [Roche]) and then incubated with rabbit serum as control or primary antibody (α -FAK (AbCam), α - RICTOR (Santa Cruz), for 4 hours followed by 50% slurry of protein G-sepharose (Roche) for 1 hour. The immunoprecipitates were washed and resolved by SDS-PAGE. For western blots: primary antibody: RICTOR, mTOR (Cell Signaling), Raptor, FAK, β 1-integrin, TTC3 (AbCam).

[00194] Antibody activation and inhibition. Cells were incubated in media containing 10%FBS and the antibody : inhibiting (A2B2:20 μ g/ml (Developmental Studies Hybridoma Bank), (P4C10: 10 μ g/ml (Millipore) and activating (TS2/16 and 12G10: 10 μ g/ml) (Santacruz), for 1 hour at 4oC. Cells were then plated on collagen IV-coated coverslips (Sigma) and incubated in the antibody at 37oC, for 24 hours.

[00195] Generation of mCerulean-tagged cell lines. Virus carrying the pMSCV-CMV-NLSmCerulean construct was produced by transfecting 293-T cells plated at 500,000 cells per well in a six-well plate. Twenty four hours later, these cells were transfected with 1 μ g target vector pMSCV-CMVNLS- mCerulean, 1 μ g packaging vector pCL-Ampho, and 3 μ L FuGENE HD mixed with 100 μ L reduced serum solution (Opti- MEM; Invitrogen). Virus was collected 24 h following transfection. Before infection with virus, HCT116 or MCF7 cells were plated at 50,000 cells per well in a six-well plate in DMEM, 10% FCS. Infection was performed 24 h later by adding 0.5 mL DMEM, 10% FCS, 0.5 mL pooled virus, and 1 μ L 1,000 $\times$ polybrene per well. A media change was performed the following day, and cells were allowed to grow to confluency before splitting into a 10-cm plate. HCT116 or MCF7 / NLS-mCerulean cells were selected using fluorescence-activated cell sorting (Becton Dickinson FACSaria II™) and gating on the brightest 5% cells in the Pacific blue channel.

[00196] Live-cell imaging & time-lapse analysis. In order to follow the fate of HCT116 cells in vitro, we plated HCT116 cells tagged with NLS-mCerulean and also a doxycycline-inducible non-

silencing or Rictor knockdown shRNA (hp4) construct in glass-bottom 12-well plates (MatTek Product # P12G-1.0-10-F) treated with type IV collagen. Tagged HCT116 cells were plated in 2µg/ml of doxycycline at a density of 1000 cells per well along with unlabeled HCT116 cells at a density of 4000 cells per well. All cells were initially grown in McCoy's 5-alpha + 10% FCS at t=0. We performed media changes every day with 2µg/ml doxycycline. Multi-point serial imaging was performed using an inverted microscope fitted with a tissue culture incubator (Nikon Ti-Eclipse) every 20 minutes at 20x magnification (CFI Plan Apo 20x) for 164 hours. Both phase and fluorescent images were captured. Cells were excited with an LED (Nikon C-HGFI Intensilight HG Illum™) and passed through a filter series (Nikon, C-FL CFP and RFP HC HISN Zero Shift Filter Set). All cell division events were tracked manually using the CFP images by recording the following characteristics for each cell: ID based on initial frame of appearance and x/y coordinate, first frame, last frame, origin ID, progenitor IDs, and x/y coordinates for first and last frame, and end method (division, lost in tracking, lost to wash out, or lost to cell death). Each point is calculated at 20-minute intervals and only shown if there was at least one event occurring. Analysis was performed using R v2.8.0 (The R Foundation for Statistical Computing, 2008) by analyzing all division events.

[00197] Proliferation assays. HCT116 colon, MCF7 breast, MDA-MB-231 breast, A375 melanoma, and PC9 lung cancer cells carrying either doxycycline-inducible non-silencing or RICTOR-targeting shRNAs (120392, 225915) were plated in a 12- well plate at a density of 50,000 cells/per well in triplicate with doxycycline containing medium on day 1 and the cells were counted every 24 hrs for 5 days. Doxycycline containing medium was replaced everyday. Cells were maintained at: 1) 21% oxygen, 10% fetal calf serum and 25mM D-glucose (normal condition), 2) 4% oxygen (hypoxia), 3) 1% serum (low serum), or 4) 5.56mM D-glucose (low glucose).

[00198] Clonogenic assays. HCT116 colon, MCF7 breast, MDA-MB-231 breast, A375 melanoma, and PC9 lung cancer cells carrying either doxycycline-inducible non-silencing or RICTOR-targeting shRNAs (120392, 225915) were plated in a 6- well plate at a density of 1,000 cells/per well in triplicate with doxycycline containing medium on day 1. Cells were allowed to grow into small colonies for 5 days and then irradiated at a dose of 2Gy. Colonies were then allowed to grow for another 2 weeks and were stained using 0.125% Coomassie Blue. Doxycycline containing medium was replaced everyday.

[00199] Invasion assays. HCT116 colon, MCF7 breast, MDA-MB-231 breast, A375 melanoma, and PC9 lung cancer cells carrying either doxycycline-inducible non-silencing or RICTOR-targeting shRNAs (120392, 225915) were induced with Doxycycline (2µg/ml) for 72 hrs and then seeded onto Matrigel invasion chambers at a density of 50,000 cells per well in triplicate. Doxycycline containing medium was replaced everyday. The invasion chambers were incubated for 24hrs at 37°C and 5% CO₂. The chamber filters were then stained using 0.125% Coomassie Blue and mounted onto glass slides.

[00200] Tumor studies in vivo. For TS2/16 antibody treatment studies in vivo, we injected 5×10^5 cells (A375, MDA-MB-231, PC9, HCT116, MCF7) subcutaneously into the flanks of 5-6 week old, female nude mice (Nude/Nude) (Charles River Labs). Once the tumors were palpable, mice were injected i.p. with TS2/16 (100 μ l at 4mg/ml/wk x 5 weeks) and the tumors were measured. For RICTOR knockdown experiments in vivo, we injected 5×10^5 cells (A375, MDA-MB-231, PC9, HCT116, MCF7) carrying either doxycycline-inducible non-silencing or RICTOR-targeting shRNAs (120392, 225915) subcutaneously into the flanks of 5-6 week old, female nude mice (Nude/Nude). The mice were given doxycycline in water at 20 mg/ml for hairpin induction starting immediately after implantation. For induction of slow proliferators, cells were treated with AKT1/2 inhibitor and DMSO (vehicle) for 72 h and were harvested at 60–70% confluence, and then counted and washed twice in PBS and resuspended in 1:1 Media: Matrigel (BD Biosciences). We then injected 5×10^6 , 5×10^5 , and 5×10^4 cells respectively subcutaneously into the flanks of 5-6 week old, female nude mice. For all experiments, growing tumors were measured weekly by caliper, and mice were killed when tumors reached approximately 1cm³ in size. Animal experiments were carried out under a Massachusetts General Hospital Institutional Review Board–approved protocol.

[00201] Example 4

[00202] All cancers contain an admixture of rapidly and slowly proliferating cancer cells. This proliferative heterogeneity complicates the diagnosis and treatment of patients with cancer because slow proliferators are hard to eradicate, can be difficult to detect, and may cause disease relapse sometimes years after apparently curative treatment. While clonal selection theory explains the presence and evolution of rapid proliferators within cancer cell populations, the circumstances and molecular details of how slow proliferators are produced is not well understood.

[00203] Described herein is the discovery of a β 1-integrin/FAK/mTORC2/AKT1–associated signaling pathway that can be triggered for rapidly proliferating cancer cells to undergo asymmetric cell division and produce slowly proliferating AKT1^{low} daughter cells. In addition, evidence indicates that the proliferative output of this signaling cascade involves a proteasome-dependent degradation process mediated by the E3 ubiquitin ligase TTC3. These findings reveal that proliferative heterogeneity within cancer cell populations, in part, is produced through a targetable signaling mechanism, with implications for understanding cancer progression, dormancy, and therapeutic resistance.

[00204] These findings provide a deeper understanding of the proliferative heterogeneity that exists in the tumor environment and highlight the importance of therapies against multiple proliferative contexts.

[00205] Introduction

[00206] In cell culture, dividing cancer cells usually produce two daughter cells that divide again in relative synchrony within a few hours of each other. Occasionally, however, a cancer cell divides to

produce progeny that are asynchronous with respect to the next cell cycle, with one daughter cell having a markedly slower cell division time than the other, on the order of days. As described herein, this proliferative heterogeneity correlates with cancer cells asymmetrically suppressing AKT protein kinase levels by about ninety percent during mitosis just before cytokinesis (1). These rare asymmetries produce one AKT^{normal} daughter cell that rapidly enters the next cell cycle and another AKT^{low} cell that remains dormant for a more prolonged time before dividing again. Slowly cycling AKT^{low} cells reduce their production of reactive oxygen species (i.e., ROS^{low}), downregulate proliferation proteins (e.g., MKI67^{low}, MCM2^{low}), suppress multiple nuclear histone marks similar to quiescent cell populations (e.g., H3K9me2^{low}), and transcriptionally upregulate the HES1 transcription factor that may mark exit from the cell cycle into G0 (i.e., HES1^{high}; ref. 1). As AKT^{low} cells do eventually divide, converting to a AKT^{normal} proliferative phenotype overtime, these cells are referred to herein as "G0-like" to describe this temporary and reversible cell state. Significantly, as described herein, AKT^{low} cancer cells within actual human breast tumors are highly resistant to prolonged treatment with combination chemotherapy using adriamycin, cyclophosphamide, and paclitaxel, indicating these slow proliferators constitute an important but unappreciated reservoir of treatment resistance in patients with breast cancer. Understanding more precisely how AKT^{low} cancer cells arise at a molecular level will provide fundamental insight into cancer biology with clinical relevance.

[00207] **Materials and Methods**

[00208] *Cell culture.* HCT116 colon and MCF7 breast were purchased from the ATCC where they were authenticated. HCT116-AKT1/2^{-/-} cells were purchased from Horizon Discovery where they were authenticated. MCF7 cells were maintained in DMEM, 10% FCS, 40 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. HCT116 and HCT116-AKT1/2^{-/-} cells were maintained in McCoy 5α medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were grown in a humidified atmosphere at 37°C and 5% CO₂.

[00209] *Generation of AKT1-mutant cell lines.* pDD AKT1(WT) and pMSCV-puro-Ctag-mCherry were utilized. AKT1(WT) cDNA was purified using PCR after cutting PDD AKT1(WT) with restriction enzymes BamHI and XhoI. After purification, the product was ligated into pMSCVpuro-C-tag-mCherry cut with BglII and SalI. All the AKT1 mutants were generated using the QuikChange Site Directed Mutagenesis Kit™ (Agilent Technologies) and the product was ligated into pMSCVpuro-C-tag-mCherry. The resulting vector pMSCV-puro-AKT1-mCherry was subcloned into DH5α-competent cells (Invitrogen). Sequencing verification of the fusion product was performed by the MGH DNA Core Facility with primers pMSCV 5'-CCCTTGAACCTCCTCGTTCGACC-3' (SEQ ID NO: 1) and pMSCV 3'-GAGACG-TGCTACTTCCATTTGTC-5' (SEQ ID NO: 2). Virus carrying the desired fusion gene was produced by transfecting HEK 293T cells with target vector pMSCV-puro-AKT1-mCherry and packaging vector pCL-Ampho using the Mirus TransIT-293™ transfection reagent and established protocols. Virus was collected 24 hours after transfection. Before infection,

cells were plated in a 6-well plate in DMEM, 10% FCS. Infection was performed 24 hours later by adding 0.5 mL DMEM, 10% FCS, 0.5 mL pooled virus, and 1 μ L 1,000x polybrene per well. A media change was performed the following day and cells were allowed to grow to confluency before splitting into a 10 cm dish and selection with 2 μ mol/L puromycin. After selection, cells were allowed to grow to confluency before clones were selected using single-cell sorting (Becton Dickinson FACSaria II™). Single cells were filtered by gating on the brightest 5% of cells in the PE Texas red channel and sorted into individual wells of a 96-well plate. Clones were harvested between 14 and 21 days.

[00210] *Drug treatment in vitro.* Cells were seeded onto collagen IV–coated coverslips, allowed to attach overnight, and treated with vehicle (DMSO) or AKT1/2 inhibitor (HCT116: 20 μ mol/L; MCF7: 2 μ mol/L; Sigma), MK2206 (HCT116: 10 μ mol/L; MCF7: 3 μ mol/L; Selleck Chemicals), TORIN1 (HCT116: 0.5 μ mol/L; MCF7: 0.25 μ mol/L; Tocris Bio-science), AZD8055 (HCT116: 0.7 μ mol/L; MCF7: 0.1 μ mol/L; Selleck Chemicals), INK128 (HCT116: 0.05 μ mol/L; MCF7: 0.01 μ mol/L; Active Biochem), Palomid 529 (HCT116: 10 μ mol/L; MCF7: 20 μ mol/L; Selleck Chemicals), Rapamycin (HCT116: 20 μ mol/L; MCF7: 20 μ mol/L; Sigma), RAD-001 (HCT116: 10 μ mol/L; MCF7: 5 μ mol/L; Selleck Chemicals), BKM-120 (HCT116: 1.5 μ mol/L; MCF7: 0.5 μ mol/L; Active Bio-chem), FAK inhibitors [PF-562271: 1 μ mol/L (Pfizer) and NVP-TAE226 : 1 μ mol/L (Novartis), for both cell lines] for 72 hours or 144 hours and bortezomib (HCT116: 1 μ mol/L; MCF7: 4 μ mol/L; Selleck Chemicals) MG-132 (vehicle: ethanol; HCT116: 1 μ mol/L; MCF7: 10 μ mol/L), for 24 hours.

[00211] *shRNA constructs.* Human TRIPZ lentiviral inducible shRNAmirs for RICTOR (clone ID: V2THS_120392, V2THS_120389, V2THS_38014, V2THS_225915), FAK (clone ID: V2THS_57326, V2THS_325805), β 1-integrin (clone ID: V2THS_133469, V2THS_390997), nonsilencing, and empty vector were purchased from Open Biosystems and virus was generated using a standard protocol. Infection was performed 24 hours later in MCF7 and HCT116 cells with the lentiviral particles followed by selection with 2 μ mol/L puromycin. After selection, cells were allowed to grow to confluency. The shRNAs were induced using 2 μ g/mL doxycycline for 72 hours. The TTC3 virus was purchased from Sigma-Aldrich and infected in HCT116 and MCF7 cells and the standard protocol for selection was followed.

[00212] *Antibody activation and inhibition.* Cells were incubated in media containing 10% FBS and the respective β 1-integrin antibody: inhibiting [AIIB2: 20 μ g/mL (Developmental Studies Hybridoma Bank), P4C10: 10 μ g/mL (Millipore)], and activating (TS2/16 and 12G10: 10 μ g/mL; Santa Cruz Biotechnology), for 1 hour at 4°C. Cells were then plated on collagen IV–coated coverslips (Sigma) and incubated in the antibody at 37°C for 24 hours.

[00213] *Immunofluorescence staining.* Cells were grown directly on collagen IV–coated coverslips (Sigma). Cells were fixed in 3.7% formalin, permeabilized using 0.1% Triton X-100, and

treated with 0.1% SDS. They were blocked in 1% BSA and then incubated with primary antibody (α -H3K9me2, α -Hes1 α -TTC3, and α -AKT(phospho-T308; Abcam); α -MCM2, α -Tubulin, α -pan-AKT, and α -AKT(phos-pho-S473; Cell Signaling Technology), diluted in blocking solution, washed, and incubated with the respective secondary antibody. Cells were mounted using hardset mounting media containing DAPI (Vector Laboratories). All secondary antibodies were Alexa Fluor conjugates (488, 555, 568, 633, and 647; Invitrogen).

[00214] *Collagen matrix studies.* Slides coated with type-I collagen (control) and AlignCol™ woven with large collagen fibers (100–200 nm; Advanced Bio-matrix) were incubated for 1 hour in 70% ethanol and then washed with PBS. Cells were then plated on the slides, incubated for 24 hours, and processed for immunofluorescence.

[00215] *Confocal imaging.* Immunofluorescence imaging was performed on a Nikon Eclipse Ti A1R-A1™ confocal microscope. G0-like slow proliferators were specifically identified as cells in the bottom 10% of coincident staining for MCM2, H3K9me2, and HES1. Cells were scored by counting G0-like versus other proliferating cancer cells among 10,000 cells from multiple fields of view at 20x magnification.

[00216] *Western blotting and immunoprecipitation.* Standard protocols were used for SDS-PAGE electrophoresis and the following primary antibodies: α -RICTOR, α -mTOR, Phospho-AKT-Ser473 (D9E; Cell Signaling Technology) and α -RAPTOR, α -FAK, α - β 1-integrin, α -TTC3, Pan-AKT, and GAPDH (Abcam). For immunoprecipitation studies, cells were synchronized with 200 ng/mL of nocodazole for 12 hours and then released for 2 hours. Cells were rinsed with PBS, fixed with 0.37% formalde-hyde, and quenched with 0.25 mol/L glycine. Cell lysates were prepared in lysis buffer [1% Triton X-100, 150 mmol/L NaCl, 3 mmol/L MgCl, 40 mmol/L HEPES (pH 7.5), 50 mmol/L NaF, EDTA-free protease inhibitor and phosphatase inhibitor (Roche)] and centrifuged at 14,000 x g for 10 minutes. Supernatant (250 μ g) was incubated with the indicated antibodies [α -FAK (AbCam), α -RICTOR (Santa Cruz Biotechnology)], for 4 hours at 4°C with rotation and then with 50 μ L of a 50% slurry of protein G-sepharose (Roche) for 1 hour. Immunoprecipitates were washed and resolved by SDS-PAGE electrophoresis.

[00217] Results

[00218] As AKTlow cancer cells only partially suppress total AKT protein levels, it was first asked whether asymmetric division occurs in the complete absence of all three AKT isoforms (i.e., AKT1, AKT2, and AKT3). To do so, HCT116 colorectal cancer cells with adeno-associated virus (AAV)-mediated disruption of the AKT1 and AKT2 gene loci (i.e., AKT1/2-/- cells; ref. 2) were obtained. Importantly, AKT1/2-/- cells do not express either AKT1 or AKT2, nor do they express AKT3, and thus survive and proliferate in the complete absence of AKT signaling, presumably through compensatory changes that arose during their initial selection. Confocal microscopy was used to score AKT1/2-/- cell populations for rare, asymmetrically dividing, and G0-like cancer cells that

express the previously validated H3K9me2^{low}/MCM2^{low}/HES1^{high} molecular profile, which specifically marks AKT^{low} slow proliferators previously shown (data not shown; ref. 1).

Interestingly, this AKT1/2^{-/-} line had virtually no asymmetrically dividing or G0-like cells compared with wild-type HCT116 (the parental line from which AKT1/2^{-/-} cells are derived; Fig. 15A).

However, lentiviral-mediated overexpression of an AKT1 cDNA in AKT1/2^{-/-} cells completely restored formation of both asymmetrically dividing and G0-like cells, while overexpression of AKT2 did not, indicating that AKT1 is both necessary and sufficient for the production of G0-like cells (Fig. 15A).

[00219] On the basis of this result, site-directed mutagenesis was used to identify AKT1 domains that might be required for its partial suppression during asymmetric division. A series of AKT1 cDNA constructs with mutations in critical amino acids known to be important for various aspects of AKT1 signaling were created (Fig. 15B). Each mutant AKT1 construct was then overexpressed in AKT1/2^{-/-} cells and these engineered cells scored for both asymmetrically dividing and G0-like cancer cells. It was first asked whether AKT1 kinase activity was necessary for production of these slow proliferators. It was found that AKT1-K179M (a commonly studied mutation in the kinase pocket that renders AKT1 catalytically dead) failed to restore production of asymmetrically dividing and G0-like cells in the AKT1/2^{-/-} line (Fig. 15A). In addition, AKT1-D292A (a mutant hypomorph with diminished kinase catalytic activity) did so only weakly compared with wild-type AKT1 (Fig. 15A; refs. 3, 4). These results were consistent with AKT1 kinase activity being necessary for asymmetric division.

[00220] Treating wild-type cancer cells with allosteric AKT inhibitors at low doses dramatically increases the frequency of both asymmetrically dividing and G0-like cells in HCT116 and MCF7 breast cancer cells (i.e., AKT1/2, MK2206; Fig. 15C; ref. 1). These allosteric inhibitors are known to bind to the AKT1 pleckstrin homology domain, inducing conformational change and protein displacement from the cell membrane, thus promoting its ubiquitination and proteasome-mediated degradation (5). It was therefore hypothesized that asymmetric division might depend on targeted degradation of AKT1 protein.

[00221] TTC3 is a RING-type E3 protein-ligase known to ubiquitinate AKT1 at the lysine-8 and lysine-14 residues leading to its destruction by the proteasome (6). It was found that G0-like cells from wild-type MCF7 express high levels of TTC3 protein compared with proliferating cells, consistent with a potential role for this E3 ligase in producing AKT1^{low} cells (data not shown). In addition, inducible shRNA knock-down of TTC3 suppressed the frequency of G0-like cells in both wild-type HCT116 and MCF7 (Fig. 15C). Furthermore, AKT1-K8R, AKT1-K14R, and AKT1-K8R/K14R double mutant proteins (which cannot be ubiquitinated by TTC3) failed to rescue the formation of G0-like cells in the AKT1/2^{-/-} line (Fig. 15C-left). Moreover, two different small molecules that inhibit proteasome function reduced the frequency of G0-like cells in both wild-type

HCT116 and MCF7 when used at doses that do not affect overall cell proliferation (i.e., MG-132, bortezomib; Fig. 15C). Overall, these results were consistent with enzymatically active AKT1 being ubiquitinated by TTC3 and degraded by the proteasome during cell division to produce slow proliferators.

[00222] AKT1 is usually activated by two different upstream kinases: PDK1 phosphorylates AKT1 at the T308 residue, whereas the mTORC2 kinase complex phosphorylates the AKT1-S473 and AKT1-T450 sites (7, 8). Similar to AKT1 cDNA, overexpression of the AKT1-T308A cDNA mutant (which cannot be phosphorylated by PDK1) completely restored the production of asymmetrically dividing and G0-like cells in AKT1/2^{-/-} cells (Fig. 16A, left). In contrast, AKT1-S473A, AKT1-T450A, and an AKT1-T308A/AKT1-S473A double mutant (all of which cannot be phosphorylated by mTORC2) did not produce phenotypic rescue in these cells (Fig. 6A, left). It was also found that four structurally different small molecules that inhibit both mTORC2 and mTORC1 signaling reduced the frequency of asymmetrically dividing and G0-like cells in both wild-type HCT116 and MCF7 cancer cells at low doses that did not appreciably inhibit cell proliferation (i.e., TORIN1, AZD8055, INK-128, Palomid-529; Fig. 16A). In contrast, the production of G0-like cells was not suppressed either by inhibitors that preferentially target the TORC1 signaling complex alone (i.e., rapamycin, RAD-001) or by a pan-class I PI3K inhibitor (i.e., BKM-120), when used at target-suppressing doses in these wild-type cells (Fig. 16A). In addition, inducible shRNA knockdown of RICTOR (an obligate member of the mTORC2 signaling complex) suppressed the production of both asymmetrically dividing and slowly proliferating G0-like cells in both wild-type HCT116 and MCF7 (Fig. 16A and 16B). It was also found in asymmetrically dividing cells, that the slow proliferator daughter cells (i.e., H3K9me2^{low}) were phospho-AKT1-S473^{high} but phospho-AKT1-T308^{normal} (data not shown). In contrast, after cytokinesis these slow proliferators (i.e., H3K9me2^{low}) were AKT^{low} and commensurately phospho-AKT1-S473^{low} and phospho-AKT1-T308^{low} (data not shown; ref. 1). In aggregate, these results support a dynamic model whereby differential phosphorylation of AKT1 by mTORC2 precedes the production of slow proliferators with low levels of AKT1 protein.

[00223] To identify an upstream regulator that might activate mTORC2 signaling during asymmetric division, an immunoprecipitation approach was used to identify proteins that physically interact with the mTORC2 complex during mitosis. HCT116 and MCF7 cells were treated with nocodazole, to synchronize cells in metaphase, and then whole-cell protein lysates prepared 2 hours after release of this synchronization with the cells still in mitosis. It was found that immunoprecipitation with a RICTOR antibody (under conditions that maintain integrity of the mTORC2 complex in whole-cell lysates) pulled down focal adhesion kinase (FAK) protein in both HCT116 and MCF7. Reciprocally, immunoprecipitation with a FAK antibody pulled down both mTOR kinase and RICTOR, but not RAPTOR (an obligate member of the related mTORC1

complex), confirming the specific interaction of FAK with mTORC2 complex in these cells (Fig. 16C). This observation suggested that FAK activity might regulate mTORC2-associated AKT1 degradation and asymmetric cancer cell division. Furthermore, inducible shRNA knockdown of FAK increased both asymmetrically dividing and G0-like cells in HCT116 and MCF7 (Fig. 17A and 17D). Similarly, inhibiting FAK enzymatic activity with two different small molecules increased the frequency of both asymmetrically dividing and G0-like cells (i.e., PF-562271, NVP-TAE226; Fig. 17A). However, FAK inhibitors failed to increase asymmetries or slow proliferators after RICTOR knockdown (Fig. 17A). These findings were consistent with a model whereby a loss of FAK activity induces mTORC2-mediated asymmetric cancer cell division.

[00224] Integrins are a family of heterodimeric transmembrane receptors that transduce signals from the extracellular matrix, by activating signaling intermediaries, including FAK, to increase the cell cycle, survival, and motility of cancer and normal cells (9). It was therefore reasoned that decreased integrin signaling might be the proximate cause for a loss in FAK activity resulting in asymmetric mitosis. In fact, shRNA knockdown of β 1-integrin (i.e., ITGB1) increased the fraction of asymmetrically dividing and G0-like cells in both HCT116 and MCF7 (Fig. 17B and 17E). In addition, blocking β 1-integrin function with two different monoclonal antibodies also increased both asymmetrically dividing and G0-like cells (i.e., A2B2, P4C10; Fig. 17B; ref. 10). However, activating β 1-integrin signaling with two other monoclonal antibodies, which force β 1-integrin into a constitutive "on" state by imposing a conformational change, eliminated both asymmetries and slow proliferators in these cell lines (i.e., TS2/16, 12G10; Fig. 17B; ref. 10).

[00225] These observations indicated that the asymmetric cancer cell divisions can result from random variation in β 1-integrin signaling related to extracellular irregularities within cell culture. Cancer cells were grown on engineered matrices displaying type-I collagen (a major extracellular matrix protein that activates β 1-integrin) closely aligned in a regular fibrillar pattern, to assure uniform β 1-integrin activation in any cancer cell undergoing mitosis (11). Notably, cancer cells proliferating in this structured collagen matrix did not produce asymmetries or G0-like cells, in contrast with typical cell culture (Fig. 17C). In the aggregate, these results were consistent with loss in β 1-integrin signaling during mitosis (likely resulting from random irregularity in extracellular type-I collagen) triggering a conserved pathway to produce slow proliferators in vitro.

[00226] Discussion

[00227] The proliferative heterogeneity among cancer cells within tumors generally correlates with differences in growth, response to treatment, and disease relapse in patients with cancer (12). As described herein, cancer cells occasionally divide asymmetrically to spawn AKT_{low}, MCM2_{low}, H3K9me2_{low}, HES1_{high} progeny that proliferate slowly and are resistant to cytotoxic chemotherapy in cell culture (1% of cell divisions; ref. 1). The existence of these AKT_{low} cancer cells within actual human breast tumors where they appear to survive intensive, combination chemotherapy is also

demonstrated herein, indicating that these cells can mediate clinically important chemoresistance (1). Described herein is a signaling pathway that is triggered in dividing cancer cells to spawn these slow proliferators in vitro. This pathway involves a decrease in β 1-integrin/FAK activity, activation of the mTORC2 complex, and suppression of AKT1 protein levels through TTC3/proteasome-mediated degradation. Interestingly, any dividing cancer cell appears capable of triggering the β 1-integrin pathway that is described herein to produce AKT1low slow proliferators. This facultative behavior presumably occurs if dividing cancer cells encounter irregularities in extracellular type I collagen, although additional cooperative factors yet to be discovered may also be required. Moreover, it is described herein that activation of β 1-integrin signaling with monoclonal antibodies or inhibition of mTORC2 signaling with small molecules reduces asymmetric cancer cell division and the production of these slow proliferators. These findings permit avenues for experimentally or therapeutically manipulating and studying the production of AKT1low slow proliferators both in vitro and in vivo.

[00228] These results also offer useful molecular insight into different signaling molecules that are under intensive investigation as therapeutic targets for various cancer types, which may carry implications for the development and use of clinical inhibitors that target these important molecules. For example, the MCF7 and HCT116 cancer cells have activating mutations in PIK3CA, and are thus dependent on constitutive PI3K/AKT signaling for their proliferation and survival. Despite this dependency, however, it is described herein that these ER β breast and colorectal cancers retain the β 1-integrin pathway that produces AKT1low slow proliferators. This indicates that cancer cells can actually derive some indispensable advantage from suppressing AKT1 to produce slow proliferators in this way. In addition, it is described herein that a quantitative reduction in β 1-integrin, FAK, or AKT1 (rather than AKT2/3) signaling in cancer cells produces this reversible cell-cycle arrest through a conserved pathway, compared with complete suppression of these targets that generally results in cell death or senescence.

[00229] Our results also indicate that FAK can physically interact with and functionally suppress the mTORC2 signaling complex during cell division. Moreover, while mTORC2 activity is normally required for AKT1 activation, this multifunctional signaling complex is also necessary for triggering AKT1 degradation during asymmetric cancer cell division. Finally, TTC3-mediated proteasome degradation of AKT1 is necessary for the production of AKT1low slow proliferators.

[00230] References

1. Dey-Guha I, Wolfer A, Yeh AC, Albeck JG, Darp R, Leon E, et al. Asymmetric cancer cell division regulated by AKT. *Proc Natl Acad Sci U S A* 2011;108:12845–50.
2. Ericson K, Gan C, Cheong I, Rago C, Samuels Y, Velculescu VE, et al. Genetic inactivation of AKT1, AKT2, and PDPK1 in human colorectal cancer cells clarifies their roles in tumor growth regulation. *Proc Natl Acad Sci U S A* 2010;107:2598–603.

3. Okuzumi T, Fiedler D, Zhang C, Gray DC, Aizenstein B, Hoffman R, et al. Inhibitor hijacking of Akt activation. *Nat Chem Biol* 2009;5: 484-93.
4. Aoki M, Batista O, Bellacosa A, Tsichlis P, Vogt PK. The akt kinase: molecular determinants of oncogenicity. *Proc Natl Acad Sci U S A* 1998; 95:14950-5.
5. Jo H, Mondal S, Tan D, Nagata E, Takizawa S, Sharma AK, et al. Small molecule-induced cytosolic activation of protein kinase Akt rescues ischemia-elicited neuronal death. *Proc Natl Acad Sci U S A* 2012;109: 10581-6.
6. Suizu F, Hiramuki Y, Okumura F, Matsuda M, Okumura AJ, Hirata N, et al. The E3 ligase TTC3 facilitates ubiquitination and degradation of phosphorylated Akt. *Dev Cell* 2009;17:800-10.
7. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 2005;307: 1098-101.
8. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007;129:1261-74.
9. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002;110:673-87.
10. Byron A, Humphries JD, Askari JA, Craig SE, Mould AP, Humphries MJ. Anti-integrin monoclonal antibodies. *J Cell Sci* 2009;122(Pt 22): 4009-11.
11. Bessea L, Coulomb B, Lebreton-Decoster C, Giraud-Guille MM. Production of ordered collagen matrices for three-dimensional cell culture. *Biomaterials* 2002;23:27-36.
12. Hong WK, Bast RC, Hait WN, Kufe DW, Pollock RE, Weichselbaum RR, et al. *Cancer Medicine*, 8th Edition. Shelton, CT: People's Medical Publishing House, 2010. xxv, 2021 pp.

[00231] Example 5: AKT Inhibition Promotes Non-autonomous Cancer Cell Survival

[00232] Small-molecule inhibitors of AKT signaling are being evaluated in patients with various cancer types, but have so far proven therapeutically disappointing for reasons that remain unclear. Described herein is the treatment of cancer cells with sub-therapeutic doses of Akti-1/2, an allosteric small molecule AKT inhibitor, in order to experimentally model pharmacologic inhibition of AKT signaling in vitro. A combined RNA, protein, and metabolite profiling approach was applied to develop an integrated, multi-scale, molecular snapshot of this "AKTlow" cancer cell state. AKT-inhibited cancer cells suppress thousands of mRNA transcripts, and proteins related to the cell cycle, ribosome, and protein translation. Surprisingly, however, these AKT-inhibited cells simultaneously up-regulate a host of other proteins and metabolites post-transcriptionally, reflecting activation of their endo-vesiculo-membrane system, secretion of inflammatory proteins, and elaboration of extracellular microvesicles. Importantly, these microvesicles enable rapidly proliferating cancer cells of various types to better withstand different stress conditions, including serum deprivation, hypoxia, or cytotoxic chemotherapy in vitro and xenografting in vivo. These findings indicate a model whereby

cancer cells experiencing a partial inhibition of AKT signaling may actually promote the survival of neighbors through non-cell autonomous communication.

[00233] INTRODUCTION

[00234] Most human cancers activate the AKT kinase signaling pathway either directly through somatic mutation of PTEN, PI3 kinase, or AKT itself, or indirectly through the activation of intersecting oncogenic pathways (1-3). In turn, the AKT kinase activates myriad downstream targets that promote tumor growth, survival, and progression (1). Therefore, most human tumors are thought to depend on AKT signaling to a varying degree for their viability. Based on these observations, AKT-selective small molecule inhibitors have been developed and are currently being evaluated as cancer therapeutics for patients with many different types of malignancy. In pre-clinical xenograft models, however, many AKT inhibitors produce tumor stasis instead of regression (4-6). Moreover, rare patients treated with these inhibitors will occasionally show a significant clinical response to small-molecule AKT inhibition, but most either have partial or minimal responses regardless of PTEN / PI3K / AKT tumor mutational status for reasons that remain unclear (7, 8).

[00235] We recently discovered that epithelial cancer cells growing in culture occasionally divide asymmetrically by suppressing AKT signaling in one emerging “AKTlow” daughter cell (9, 10). This unusual type of cell division is triggered by an asymmetric decrease in Type I collagen- β 1-integrin-FAK signaling, resulting in activation of the mTORC2 signaling complex, partial phosphorylation of AKT1 kinase, and its activation-induced degradation mediated by the E3-ubiquitin ligase TTC3 and the proteasome (9). Asymmetric signaling thus produces one normally proliferating daughter cell and another AKT1low daughter expressing a MCM2low, H3K9me2low, HES1high marker profile (9, 10). Importantly, suppression of AKT signaling is both necessary and sufficient to produce these slow proliferators (9). AKT1low cancer cells are not apoptotic, autophagic, or senescent, nor do they express cancer stem cell markers or differentiate (10). Rather, they are quiescent but able to eventually resume their cell cycle after a prolonged period of dormancy in vitro (i.e., ~ 7-10 days) (10).

[00236] Interestingly, we have also found that human cancer cell lines treated with allosteric small-molecule AKT inhibitors (e.g., Akti-1/2, MK-2206), at a sub-therapeutic dose (i.e., which only partially suppresses AKT signaling by about 80-90%), dramatically increase their fraction of AKTlow, MCM2low, H3K9me2low, HES1high cancer cells (6, 9-11). These quiescent cancer cells rapidly resume their cell cycle with inhibitor washout, consistent with a temporary rather than permanent cell cycle arrest, which is identical to spontaneously arising AKTlow slow proliferators (10). In fact, malignant cells of various types can be made quiescent this way regardless of their PTEN / PI3K / AKT mutation status or general dependency on PI3K / AKT signaling pathway for their growth (9). Based on these observations, we sought to understand this AKT-induced quiescent

cancer cell state in further molecular detail using a combined RNA, protein, and metabolite profiling approach to develop an integrated, multi-scale, molecular snapshot of small molecule AKT inhibition.

[00237] MATERIALS AND METHODS

[00238] Cell lines. HCT116 colon, MCF7 breast, MDA-MB-231 breast, A375 melanoma, and PC9 lung were purchased from ATCC, where they were validated. HCT116 AKT1/2^{-/-} was purchased from Horizon Discovery (Cambridge, UK), where it was validated. AG11726 skin fibroblasts were purchased from Coriell Repositories, where they were validated. MCF7, MDA-MB-231 and AG11726 were maintained in DMEM, 10% FCS, 40mM glutamine, 100 U/mL penicillin, and 100µg/mL streptomycin; HCT116 and HCT116 AKT1/2^{-/-} in McCoy's 5α medium supplemented with 10% FCS, 100U/mL penicillin, and 100µg/mL streptomycin; PC9 in RPMI, 25% glucose, 1% sodium pyruvate, 100U/mL penicillin, and 100µg/mL streptomycin; A375 in DMEM supplemented with high glucose HEPES buffer, 10% FCS, 100U/mL penicillin, and 100µg/mL streptomycin. All the cells were grown at 37°C and 5% CO₂.

[00239] Induction of AKTlow cancer cells in vitro. Cells were treated for 72h with vehicle (DMSO), Akti-1/2 inhibitor (HCT116: 20µM; MCF7: 2µM; MDA-MB-231: 20µM; A375: 20µM; PC9: 20µM) (Sigma) or MK-2206 (HCT116: 10µM; MCF7: 3µM; MDA-MB-231: 5µM; A375: 10µM; PC9: 3µM) (Selleckchem).

[00240] Induction of AKTlow cancer cells followed by xenografting in vivo. HCT116 and MCF7 were treated for 72h with vehicle (DMSO) and Akti-1/2 inhibitor; 500,000 cells were injected subcutaneously into the flanks of 5-6 week old, female, immunocompromised Nu/Nu mice (Charles River Laboratories), and then growing tumors were measured weekly by caliper.

[00241] GRO-sequencing (global run-on). HCT116 or MCF7 were treated with DMSO and Akti-1/2 for 72h and cells were collected. Isolation of nuclei and nuclear run-on was carried out as described previously (8). Nascent RNAs were on average approximately 100nt long. The immunopurified RNA was resuspended in 8.5µl water and 5'- or 3'- adapters ligated using Tru-Seq™ Small RNA Kit, Illumina. RNAs were reverse transcribed and amplified. The NRO-cDNA libraries were then run on a non-denaturing 1XTBE, 8% acrylamide gel, and cDNAs greater than 90 nucleotides were excised from the gel and eluted, precipitated and sequenced on the Illumina HiSeq 2000™ Sequencing System.

[00242] RNA-Sequencing. We created a dUTP strand-specific cDNA library for RNA-Seq. Total RNA was purified for all the above experiments using RNeasy™ Mini Kit (Qiagen), and RNA integrity was checked using RNA 6000 Nano™ Kit on Agilent 2100™ Bioanalyzer. Akti-1/2 treated cells showed only a mild decrease (e.g., ~10%) in total RNA concentration compared to DMSO treated cells (i.e., MCF7 DMSO - 38.7µg; MCF7 Akti-1/2 - 35.69µg; HCT116 DMSO - 45.08µg; HCT116 Akti-1/2 - 40.3µg). We used 4µg of total RNA for library construction. The purification, fragmentation and first strand synthesis were performed as described in the Illumina TruSeq™ RNA

Library Prep Kit v2. The second strand cDNA synthesis was modified using the dUTP second strand method (12). End repair, 3' adenylation and adapter ligation steps were done using TruSeq protocol. The libraries were validated using a High Sensitivity DNA Kit on Agilent 2100™ Bioanalyzer, and sequenced using 1 lane of 101bp (for batch 1), or 51bp (for batch 2) paired end reads with the Illumina HiSeq 2000™ Sequencing System.

[00243] Quantitative Proteomics. Tandem mass tag reagents (TMT; Thermo Scientific) and a synchronous precursor selection-based MS3 method was used on an Orbitrap Fusion™ mass spectrometer (Thermo Scientific) as described previously (13).

[00244] Antibody array profiling. MCF7 and HCT116 were treated with DMSO and Akti-1/2 for 6 days and culture supernatant were screened for secreted proteins using the RayBiotech™ L-Series Human Antibody Array 493 and 507 biotin label-based kits (RayBiotech).

[00245] Immunofluorescence staining. Cells were grown directly on collagen IV-coated coverslips. Cells were fixed in 3.7% formalin, permeabilized using 0.1% Triton X-100, and treated with 0.1% SDS. They were blocked in 1% BSA and then incubated with primary antibody (MCM2, Cell Signaling, H3K9me2, Abcam, CD63 (H-193), Santa Cruz, FDFT1, Abcam), followed by the respective secondary antibody, Alexa Fluor™ conjugates (Invitrogen). Cells were mounted using hard-set mounting media containing DAPI (Vector Laboratories). Cells were stained with Filipin (Sigma) for cholesterol and Alexa 594-conjugated CTXB (Invitrogen) for lipid rafts.

Immunofluorescence imaging was performed on a Nikon Eclipse Ti A1R-A1™ confocal microscope.

[00246] Western blots. Standard protocols were used for SDS-PAGE electrophoresis and the following primary antibody: CD63 (H-193) and CD81 (H-121), Santa Cruz; Calnexin, GM130 and TNFSF10, Abcam. Microvesicle fractions used for the Western Blots were isolated from equivalent number of cells (1x10⁶).

[00247] Microvesicle extraction from cell media. Extracellular microvesicles (30-120nm) were isolated from the media of cells treated with DMSO, Akti-1/2 or MK-2206 for 72h as per instructions using the Total Exosome Isolation Reagent™ (Life Technologies). Cells were cultured in exosome-free media (complete media containing Exosome-depleted FCS). Microvesicle pellets were re-suspended in 100µl PBS.

[00248] RNA isolation from microvesicles. Exosomal RNA was isolated as per manufacturer's instructions using the Total Exosome RNA and Protein Isolation Kit™ (Life Technologies). Recovered RNA was characterized using Agilent's RNA 6000 Pico™ Kit on an Agilent 2100 Bioanalyzer™.

[00249] Small RNA sequencing. The libraries of cellular and microvesicular small RNA were made using Illumina's TruSeq™ Small RNA Kit. The 3' and 5' adaptors were ligated, and an RT reaction was used to create single stranded cDNA, which was subsequently PCR amplified using a common primer and one index sequence before size selection on 6% native polyacrylamide gel.

Fragment range of 105-150bp, corresponding to the small RNA population, were excised, eluted, precipitated, and resuspended in 20µl of nuclease-free water. The size, quality and quantity of the DNA in each final small RNA library were verified using the High Sensitivity DNA Kit (Agilent).

[00250] In vitro cell survival assays with microvesicles. Microvesicles were incubated with recipient cells for 1h at 37°C. Pre-conditioned cells were analyzed for growth for 120h, different stress conditions including growth in 1% fetal calf serum supplemented media, low oxygen (4%), and paclitaxel for 72h. The total number of cells was counted in triplicates. The standard MTS assays were also done for the growth curves. For colony formation assays, cells were treated with microvesicles for 1h and seeded at a density of 400 cells per well in six-well plates, allowed to attach overnight. Cells were then incubated under different stress conditions for an additional 6 days. Colonies were fixed and stained with Coomassie blue and counted in triplicates. In the long-term experiment, these cells were passaged for two additional weeks and then challenged with stress conditions. In addition, microvesicles from the parent cell lines were also incubated with cell lines of different cancer models and skin fibroblasts before exposing them to different stress conditions.

[00251] In vivo xenograft tumors with microvesicles. 1x10⁵ cells were incubated with microvesicles derived from equivalent number of cells (1x10⁶) for 1h at 37°C. Cells were then injected subcutaneously into the flanks of 5-6 week old, female immunocompromised Nu/Nu mice (Charles River Laboratories), and the growing tumors were measured weekly by caliper.

[00252] GRO-Seq analysis. Single-end reads were 50bp long. Clipping of contaminating adapter sequences was done with Cutadapt™ v1.2.1 (14). Reads with poor overall quality were further removed with fastq_quality_filter v0.0.13 (available on the world wide web at hannonlab.cshl.edu/fastx_toolkit/). Reads were aligned to the hg19 human genome with Bowtie™ v0.12.9 (15), allowing no mismatches and discarding multiple mapping reads. An average of ~30M of uniquely aligned reads per sample was obtained. Stranded read counts were obtained using coverageBed™ v2.17.0 (16). In order to specifically quantify the amount of actively elongated polymerase, the expression level of each gene was quantified based on the number of reads mapping to the region starting at +500 bp downstream the transcription start site (TSS) up to the transcription end site (TES). Genes less than 1 kb long were considered too short to reliably estimate their expression levels and thus were removed from further analyses. Reads per kilobase per million reads (RPKM) were estimated based on the number reads sequenced per sample, the number of reads mapping to each gene, and the gene mappable length. Each cell line and condition was done in duplicate. Log2-RPKM correlation levels between replicates were between 0.71 and 0.85.

[00253] RNA-Seq analysis. Paired-end reads were either 101bp long (1st batch of replicates) or 51bp long (2nd batch of replicates). Clipping of contaminating adapter sequences and trimming of low-quality read ends was done with Trimmomatic™ v0.25 (6). Average fragment length and its standard deviation were empirically determined for each sample with Bowtie™ v2.1.0.

Hg19/GRCh37 (Feb. 2009) transcriptome was obtained from table “knowGene” in the UCSC Table Browser website (available on the world wide web at genome.ucsc.edu/cgi-bin/hgTables). Tophat™ v2.0.8b (5) was used to align the reads to hg19 version of the human genome and transcriptome. Multiple mapping reads were excluded from subsequent analyses. Human UCSC hg19 genome annotation was downloaded from Illumina’s FTP repository on Feb 21, 2013, corresponding to the UCSC freeze of March 9, 2012. At least 20M uniquely aligned reads were obtained for each sample. Fragments per kilobase per million reads (FPKMs) estimations for each annotated genomic feature were obtained with Cufflinks v2.1.1 (17). Ribosomal, mitochondrial and transfer RNAs were masked in the analysis. Each cell line and condition was done in duplicate. Log2-FPKM correlation levels between replicates were above 0.9.

[00254] Quantitative proteomic analysis. Data analysis was done on an in-house developed software suite. MS2 spectra were assigned using the SEQUEST algorithm to search against the human UniProt protein sequence database using a target-decoy database search approach allowing to filter peptide and protein assignments to false-discovery rate of less than 1% (2, 3). MS3 spectra were used for peptide quantification only if the summed signal-to-noise ratios of all 8 TMT ions was greater than 310 and the proportion of non-target ions in the isolation m/z window applied for isolating the target ion was less than 25%. For protein quantification the TMT ion intensities for each TMT channel from each peptide assigned to a protein were summed up and the protein TMT intensities were normalized based on the median TMT intensities of the TMT channel intensities from the pooled standard peptide mixtures (TMT-126 and TMT-131). Each cell line and condition was done in triplicate. Correlation of protein abundance in log2 space between replicates was around 0.8.

[00255] Metabolomics analysis. Metabolites were identified by automated comparison in the experimental samples to a reference library of chemical standard entries developed at Metabolon, Inc. Each sample was profiled six times. Most correlation coefficients between replicate pairs fell within the 0.8-0.9 range.

[00256] Secreted protein analysis. RayBiotech provided background-subtracted, positive-control normalized intensities for both L-507 and L-493 antibody arrays. For each one of the sub-arrays, all samples were mean-centered to a log2 value of 4.5. Data from both sub-arrays were combined after normalization.

[00257] Cellular and microvesicle small RNA-Seq analysis. Single-end reads were 50bp long. Reads with poor overall quality were further removed from downstream analyses with fastq_quality_filter tool. Clipping of contaminating adapter sequences was done with Cutadapt™ v1.2.1 (14). Reads were aligned to the hg19 human genome (GRCh37.p13) with Bowtie™ v1.0 (15), allowing no mismatches and discarding multiple mapping reads. More than 20M uniquely aligned reads were obtained for the cellular samples and between 1-3M for the microvesicle libraries. For every annotated feature in the GENCODE v19 database, read counts were obtained using the HTSeq

Python package (7). Reads normalized per million sequenced reads (RPMs) were subsequently estimated based on the number of million reads sequenced per sample and the number of reads mapping to each gene in that sample. Each cell line and condition was done in duplicate for the cellular samples (biological replicates), while library preparations were sequenced in duplicate for the microvesicle samples (technical replicates). Log2-RPM correlation range between replicates was 0.85-0.99 for the cells and 0.96 for the microvesicles.

[00258] Enrichment analyses. All enrichment analyses were computed with GSEA v2.0.14 (18). Paired T-scores comparing AKTi vs DMSO treated samples were used to pre-rank genes. When there were multiple possible pairing combinations, T-scores were computed for all of them and the median T-score was selected. Gene sets with a FWER < 5% were selected as significant. Only canonical gene sets (i.e., KEGG, REACTOME, BIOCARTA, PID, GO) were included in the analyses.

[00259] The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (19) and are accessible through GEO Series accession number GSE71901.

[00260] RESULTS

[00261] HCT116 (colon) or MCF7 (breast) cells were treated with a low, non-lethal, cytostatic dose of the allosteric inhibitor Akti-1/2 for three days in vitro, which partially suppresses AKT kinase activity, to induce the AKTlow quiescent cell state rather than killing these cells (i.e., HCT116 = 20 μ M; MCF7 = 2 μ M) (10). 5x10⁵ pre-treated cancer cells were then injected subcutaneously in nude mice and their ability to form tumors compared to vehicle-treated control cells (i.e., DMSO, n=5 mice per condition). Akti-1/2 inhibitor treatment increases the fraction of AKTlow slow proliferators within these cancer cell populations from a baseline of 1% up to 60% within 3 days of treatment (10). Surprisingly, increasing the fraction of AKTlow cells, by suppressing AKT signaling in this way, resulted in a substantially improved engraftment of these poorly tumorigenic cell lines compared to DMSO-treated control cells (Fig. 25A-25B). This result was counterintuitive since both HCT116 and MCF7 have activating mutations in PIK3CA, rendering them constitutively dependent on AKT signaling for their proliferation, growth, and survival (4).

[00262] To determine the basis of this paradox, a battery of RNA, protein, and metabolite profiling technologies were applied to Akti-1/2-treated cells in order to define the AKTlow cell state in further molecular detail. These included: 1) genome-wide GRO-sequencing (global run-on) to examine active transcription across the genome; 2) RNA-sequencing to measure genome-wide steady-state mRNA levels; 3) multiplexed, quantitative mass spectrometry-based proteomics to assess levels of approximately 10,000 proteins at steady state; and 4) mass spectrometry-based metabolite profiling to assess levels of approximately 375 metabolites at steady state. Integration of these datasets allowed the definition of a multi-scale molecular snapshot of AKT-inhibited cell quiescence (Fig. 25C-25H). Details on experimental procedures, data quality, bioinformatics, and computational analyses for these different data types can be found in the methods section.

[00263] Transcriptional profiles from AKTi-treated and DMSO-treated control cells were compared. Transcripts or proteins with greater than an average 2-fold change after AKTi treatment in both HCT116 and MCF7 cells were focused on. AKTi-treated cells displayed only a subtle increase in the expression of a few transcripts compared to control cells at the GRO-Seq level ($n = 128$) (Fig. 25C). Furthermore, gene-set enrichment analysis (i.e., GSEA) applied to this GRO-Seq profile did not reveal significant enrichment in any gene sets out of the 2000+ that were tested ($\text{FWER} < 5\%$) (18). In addition, computational analysis of GRO-Seq profiles comparing pausing indexes for all genes across conditions also failed to reveal global changes transcriptional activity in AKTi-treated versus control cells (Fig. 29A-29B). These results indicated that AKTi-induced quiescence was likely not associated with programmatic changes in RNA PolII-associated transcriptional activity.

[00264] Consistent with these findings, RNA-Seq profiling further confirmed that AKTlow cancer cells did not up-regulate many transcripts at steady-state (Fig. 25D). In contrast, however, slow proliferators suppressed thousands of mRNAs at steady state compared to rapidly proliferating cells (i.e., 2913 genes < -2 -fold across both HCT116 and MCF7 cells, $\sim 16\%$ of total number of profiled transcripts) (Fig. 25D). However, most of the transcripts beyond this threshold (i.e., $85\% - 2483/2913$) showed mild expression levels (i.e., average $\log_2$ expression < 4), while many of the higher expressed genes that account for most of the sequenced reads remain stable after Akti-1/2 treatment. GSEA applied to this down-regulated RNA-Seq signature, however, only revealed statistically significant enrichment in a single gene set related to XBP1-mediated protein folding ($\text{FWER} < 5\%$). Moreover, this mRNA suppression did not relate to decreases in the active transcription of these genes as determined by GRO-seq analysis (Fig. 25F). Overall, these results were consistent with AKT inhibition producing a global, post-transcriptional, and largely random degradation of many mRNA transcripts.

[00265] Against this transcriptional backdrop, 192 proteins were identified that were also down-regulated after AKTi treatment in both HCT116 and MCF7 cells (i.e., < -2 -fold) (Fig. 25E). For some of these proteins, corresponding mRNA transcripts were also suppressed, while for others we found that protein and mRNA levels correlated poorly, indicating a mixed transcriptional and post-transcriptional effect (Fig. 25H). Moreover, GSEA applied to this down-regulated protein signature was associated with statistically significant enrichments in 13 gene sets related to cell cycle transit, ribosomal activity, and translational regulation ($\text{FWER} < 5\%$) (Fig. 25I). These findings were consistent with known effects that inhibition of AKT signaling might be predicted to have on cell cycle, ribosome function, and RNA stability (1).

[00266] 294 proteins were identified that were up-regulated after AKTi treatment in both HCT116 and MCF7 cells (> 2 -fold) (Fig. 25E). The expression of these proteins correlated poorly with changes in either active transcription or steady state mRNA levels, however, suggesting that these changes more likely related to post-translational protein stabilization (Fig. 25G-25H). Furthermore, GSEA of

this up-regulated protein signature revealed a significant enrichment in 23 gene sets related to cholesterol biosynthesis, lipid metabolism, the endoplasmic reticulum, vesiculo-membrane transport, trafficking, secretion, along with membrane and extracellular matrix proteins (FWER < 5%) (Fig. 25J). Metabolite profiling further suggested the up-regulation of a select set of 13 out of 379 metabolites analyzed, but did not reveal major changes in metabolites related to cellular energetics in AKTi-treated versus control cells (i.e., > an average 2-fold change for both HCT116 and MCF7 cells) (Fig. 26A). Eleven of these thirteen metabolites were lysolipid derivatives that are major components of cell membranes. Furthermore, cholesterol and cholesterol-like molecules (e.g., lathosterol) also showed milder increases in quiescent cells. Consistent with this finding, it was also noted the corresponding up-regulation of FDFT1 protein (which is a rate-limiting enzyme in the cholesterol biosynthetic pathway) with proteomic profiling.

[00267] In addition, a highly validated antibody array platform was used to measure the expression of approximately one thousand different cytokines, chemokines, growth factors, and receptors in conditioned media from Akti-1/2-treated HCT116 and MCF7 cells. These experiments suggested the up-regulation of a small set of 13 secreted proteins (Fig. 26B). This set included multiple TNF, VEGF, and WNT family members known to powerfully modulate a spectrum of cell types, including epithelial, mesenchymal, vascular, and immune cells (e.g., TNFSF10) (20). Overall, these results were consistent with the notion that AKTi-induced slow proliferators might broadly activate their endo-vesiculo-membrane system, membrane formation, membrane remodeling, and secretion of bioactive factors. Initial validation experiments using immunofluorescence confocal microscopy confirmed increase in the expression of CD63 (i.e., a strongly up-regulated membrane protein), FDFT1, cell membrane cholesterol, and membrane lipid rafts after Akti-1/2 treatment in HCT116 and MCF7 cells (data not shown).

[00268] CD63 is not only expressed on cell membranes but also marks exosomes, which are extracellular microvesicles that are secreted by both cancer and normal cells (21). These microvesicles are known to mediate cell-cell communication within cancer microenvironments through complex mechanisms that have yet to be fully elucidated (22). It was therefore asked whether AKTlow cancer cells also increase their secretion of extracellular microvesicles. Differential solubility was used to biochemically isolate secreted microvesicles ranging in size from 30-120nm (which includes the CD63/CD81+ exosome fraction) in conditioned media from Akti-1/2 treated HCT116 and MCF7 cells compared to control. These experiments, which compared whole cell lysates to microvesicles isolated from equivalent numbers of either treated or untreated cells, confirmed the increased secretion of CD63/CD81-expressing microvesicles by Akti-1/2-treated cells in HCT116 and MCF7 cell lines. Further immunoblotting for Calnexin (i.e., an ER-vesicle marker) and GM130 (i.e., a Golgi vesicle marker) excluded other potential vesicle contaminants in enriched microvesicle fractions (Fig. 26C). Also isolated were microvesicles from the HCT116-AKT1/2-/- cell line, which has adeno-associated

virus (AAV)-mediated disruption of the AKT1 and AKT2 gene loci (23). AKT1/2^{-/-} cells do not express either AKT1 or AKT2, nor do they express AKT3, and thus survive and proliferate albeit poorly in the complete absence of AKT signaling, presumably through compensatory changes that arose during their initial selection. Akti-1/2 treatment of these HCT116-AKT1/2^{-/-} cells did not produce an increase in CD63/CD81+ microvesicle secretion as observed with wild-type HCT116 cells (Fig. 26D). Overall, these results supported the idea that AKT signaling negatively regulates microvesicle secretion by cancer cells. In addition, these microvesicles displayed a time-dependent increase in the expression of TNFSF10 (Fig. 26E), which was identified as up-regulated in conditioned media of Akti-1/2-treated cells using antibody arrays. RNA-sequencing also revealed an increase in the expression of various small RNAs in microvesicles from Akti-1/2-treated compared to control cells (> an average 2-fold change for both HCT116 and MCF7) (Fig. 26F). These same microRNAs were suppressed within AKTi-treated cells themselves, however, indicating their active export in microvesicles. (R=-0.49) (Fig. 26G-26H).

[00269] Next, it was asked whether microvesicles isolated from AKT-inhibited cancer cells have functional effects either in vitro or in vivo. Akti-1/2 inhibition was used to produce AKTi-induced microvesicles from five different human cancer cell lines of different molecular types (i.e., HCT116, MCF7, A375 (melanoma) (20 μ M), MDA-MB-231 (breast) (20 μ M), and PC9 (lung) (20 μ M)). Either Akti-1/2-induced or control microvesicles were admixed with untreated, isogenic cancer cells for one hour, and the behavior of these pre-treated cells examined in a variety of functional assays in vitro. It was found that pre-conditioning with AKTi-induced microvesicles did not increase the growth of target cells in culture over time, as assessed by either direct cell count (Fig. 27A-27E) or vitality (i.e. MTS) assay (Figs. 30A-30E). Based on both cell and colony count assays, pre-treatment with microvesicles derived from AKTi-treated cells mostly increased the resistance of proliferating cancer cells to various stress conditions, however, including in vitro serum deprivation (1%), hypoxia (4%), and paclitaxel chemotherapy (0.05 μ M, HCT116; 0.5 μ M, MCF7; 0.001 μ M, MDA-MB-231; 10 μ M, PC9 and 0.05 μ M, A375; and 2.5 μ M, AG11726) (Fig. 27F-27J, Figs. 30F-30J). Importantly, similar results were obtained when pre-treating cancer cells with MK-2206, a second allosteric AKT1/2 small molecule inhibitor, further confirming that these effect likely related to small molecule inhibition of AKT signaling (Figs. 31A-31J). In contrast, cancer cells pre-treated with Akti-1/2 microvesicles, but passaged for two weeks before challenge, did not display an increased resistance to stress, suggesting a transient rather than prolonged effects in vitro (Fig. 27F-27J). Additionally, microvesicles from one cell type (e.g., HCT116) could not pre-condition virgin cancer cells of other types (i.e., MCF7, MDA-MB-231, PC9, A375) or normal human fibroblasts (i.e., AG11726) to withstand these stress conditions in vitro (Fig. 32). Finally, cancer cells were exposed for 1 hour to microvesicles derived from 1x10⁶ Akti-1/2 or DMSO treated cells. In order to assess xenograft efficiency, 100,000 pre-treated cells were subcutaneously injected in nude mice (n=6, initially per condition). Remarkably,

pre-treatment with AKTi-induced microvesicles resulted in the increased engraftment of isogenic cancer cells in most cell lines tested relative to control (Fig. 28A-28E). These functional experiments further indicated that microvesicles secreted by AKT-inhibited slow proliferators promote context-specific, non-cell autonomous survival of rapidly proliferating cancer cells exposed to a variety of experimental stresses including xenotransplantation both in vitro and in vivo.

[00270] DISCUSSION

[00271] Small molecule drugs that are designed to target specific aspects of cell behavior may produce unanticipated biological effects that are interesting but might ultimately compromise their therapeutic utility. Strategies to systematically understand these effects can therefore prove valuable for both biological research and pre-clinical drug development. Described herein is a multi-scale profiling approach to functionally assess inhibition of AKT signaling in human cancer cell lines. In these experiments, a single, sub-therapeutic drug dose of Akti-1/2, which is a well-studied, prototypic, small molecule, allosteric AKT inhibitor is used to partially inhibit AKT signaling, and a time of exposure carefully chosen in each individual cell line to induce a reversibly quiescent cell state rather than cell death (i.e., 3 days) (10). Allosteric AKT inhibitors were used herein, since we previously found that catalytic AKT inhibitors do not induce the same quiescent cell phenotype, suggesting a class-specific inhibitor effect, likely related to the ability of allosteric but not catalytic inhibitors to induce degradation of AKT protein (24).

[00272] Combined RNA, protein, and metabolite profiling was used in this highly validated experimental system in order to develop an integrative molecular view of the AKT-low cell state. This multi-scale profiling strategy reveals a rich and complex landscape of molecular activity in AKT-inhibited cancer cells. Surprisingly, AKT-inhibited cancer cells continue to actively transcribe most genes similar to rapidly proliferating cells, but post-transcriptionally suppress several thousand mRNAs and proteins, consistent with prior observations regarding AKT signaling and its regulation of cell cycle transit, transcript stability, ribosomal activity, and protein translation (1). In addition, AKT-inhibited slow proliferators appear to post-translationally increase their expression of endo-vesiculo-membrane proteins, membrane remodeling, secretion of inflammatory proteins, and elaboration of extracellular microvesicles. It was further found that microvesicles from both Akti-1/2 and MK-2206-inhibited cells functionally increase the resistance of a molecularly diverse panel of target cancer cell types to various stress conditions including serum deprivation, hypoxia, and chemotherapy exposure in vitro. Several miRNAs that were identified within Akti-1/2-induced microvesicles have been previously reported to play roles in response to hypoxia (i.e., miR-210 (25, 26)); stress response (i.e., miR-320a (27), miR-574 (28)); and chemotherapy resistance (i.e., miR-92b (29), miR-375 (30, 31), miR-345 (32), miR-197 (33, 34), and miR-140 (35)). It is therefore possible that these or other individual miRNAs promote stress-resistance in trans, and that mild differences in biological effect size that we observe across different cell types might relate to multiple factors

including specific microvesicle content or the molecular profiles of target cells. Nevertheless, microvesicles from one cancer cell line do not induce stress resistance when applied to cancer or normal cells of different types in vitro, suggesting additional, complex, and context-specific effects yet to be fully elucidated. Intriguingly, cancer cells of various types, either pre-treated with a sub-therapeutic dose of Akti-1/2, or with microvesicles isolated from Akti-1/2-treated cells, also display increased engraftment upon xenografting into nude mice in vivo. While a transient ability of microvesicle-treated cells to withstand xenotransplantation-associated stress might account for increased experimental tumorigenesis, additional experiments are required to determine the possibility of more sustained effects on tumor growth in vivo.

[00273] Cancer cells growing in culture or within tumors continuously encounter both internal (e.g., oncogenic, proteotoxic) and external (e.g., hypoxic) stresses, and use a range of cellular programs to survive this constant pressure (36, 37). It was recently discovered that dividing epithelial cancer cells encountering a loss of integrin signaling trigger a conserved mechanism to partially suppress AKT signaling and produce a newborn “AKTlow” daughter cell (9, 10). These “G0-like” daughters remain quiescent for a period of time within the population before eventually resuming their cell cycle (10). Interestingly, we have also identified these AKTlow slow cyclers within actual human breast tumors, where they appear to survive exposure to intensive, prolonged, combination chemotherapy (10). The current findings indicate that epithelial cancer cells in this AKT-inhibited state can in fact instruct rapidly proliferating neighbors to increase their resistance to stressful challenge. This view might explain why rapid proliferators, which have evolved through years of mutation and clonal selection, continuously produce small fractions of naturally-arising AKTlow slow proliferators via a conserved signaling mechanism (9, 10). Slow proliferators, while only marginally reducing overall population expansion, may not only resist cytotoxic challenge themselves because they are slowly cycling, but might also provide a survival advantage to more rapidly proliferating neighbors. Consistent with this model, recent findings suggest that targeted inhibition of growth factor signaling in cancer cells (i.e., EGFR, HER2, ALK, MET, KRAS) might contribute to the drug resistance of neighboring cells through secreted factors such as IFN- γ (38). Similarly, targeted inhibition BRAF, ALK, or EGFR in cancer cells may induce a complex, reactive secretome that both enhances cancer cell drug resistance and also supports the expansion and dissemination of drug resistant clones in vivo (39). It is also noted that cells undergoing programmed senescence secrete various inflammatory proteins, most notably IL6 and IL8 (i.e., the senescence-associated secretory phenotype) (40). Unlike senescent or drug tolerant phenotypes, however, AKT-inhibited quiescent cells apparently increase their secretion of different factors including exosomes and WNT-, TNF, and VEGF-related proteins (41). Nevertheless, this emerging body of work suggests that various cell stresses, either naturally arising or iatrogenic, may trigger cell-cell interactions of various types within tumors with potentially important consequences.

[00274] Without wishing to be bound by theory, given that allosteric AKT inhibitors have proven disappointing when used clinically despite strong pre-clinical rationale for their development, it is contemplated herein that incomplete pharmacologic inhibition of AKT signaling with small molecule AKT inhibitors within tumors may paradoxically increase cancer cell survival through the type of non-cell autonomous communication described herein, rather than inducing cell death as intended (4). Finally, no single type of molecular profiling technology was sufficient to reveal the biological insight provided by the present multi-scale approach. For example, RNA-profiling alone would suggest that Akti-1/2 treatment generally results in transcriptional repression, which is not consistent with the fuller picture revealed through a multi-scale profiling approach. Additionally, while proteomic profiling appears to be a richer source of information in this context, suggesting the broad-scale activation of biological pathways related to endo-membrane trafficking, metabolite and secreted protein profiling adds layers of useful information that further sharpen the focus of down-stream biological validation experiments. Additional experiments with related or different drugs, across full dose and time ranges, in many additional cell lines, and using additional profiling technologies (e.g., translational profiling) may therefore prove useful as a general approach that complements ongoing efforts aimed at understanding the molecular action of cancer therapeutics (12, 15).

[00275] REFERENCES

1. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell*. 2007;129:1261-74.
2. Elias JE, Gygi SP. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nature methods*. 2007;4:207-14.
3. Huttlin EL, Jedrychowski MP, Elias JE, Goswami T, Rad R, Beausoleil SA, et al. A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell*. 2010;143:1174-89.
4. She QB, Chandralapaty S, Ye Q, Lobo J, Haskell KM, Leander KR, et al. Breast tumor cells with PI3K mutation or HER2 amplification are selectively addicted to Akt signaling. *PloS one*. 2008;3:e3065.
5. Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome biology*. 2013;14:R36.
6. Lohse M, Bolger AM, Nagel A, Fernie AR, Lunn JE, Stitt M, et al. RobiNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. *Nucleic acids research*. 2012;40:W622-7.
7. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics*. 2015;31:166-9.
8. Core LJ, Waterfall JJ, Lis JT. Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters. *Science*. 2008;322:1845-8.

9. Dey-Guha I, Alves CP, Yeh AC, Salony, Sole X, Darp R, et al. A mechanism for asymmetric cell division resulting in proliferative asynchronicity. *Molecular cancer research : MCR*. 2015;13:223-30.
10. Dey-Guha I, Wolfer A, Yeh AC, J GA, Darp R, Leon E, et al. Asymmetric cancer cell division regulated by AKT. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108:12845-50.
11. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25:2078-9.
12. Levin JZ, Yassour M, Adiconis X, Nusbaum C, Thompson DA, Friedman N, et al. Comprehensive comparative analysis of strand-specific RNA sequencing methods. *Nature methods*. 2010;7:709-15.
13. Van Rechem C, Black JC, Boukhali M, Aryee MJ, Graslund S, Haas W, et al. Lysine demethylase KDM4A associates with translation machinery and regulates protein synthesis. *Cancer discovery*. 2015;5:255-63.
14. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal*. 2011;17:10-2.
15. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome biology*. 2009;10:R25.
16. Quinlan AR, Hall IM. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*. 2010;26:841-2.
17. Roberts A, Pimentel H, Trapnell C, Pachter L. Identification of novel transcripts in annotated genomes using RNA-Seq. *Bioinformatics*. 2011;27:2325-9.
18. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102:15545-50.
19. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic acids research*. 2002;30:207-10.
20. Chen G, Goeddel DV. TNF-R1 signaling: a beautiful pathway. *Science*. 2002;296:1634-5.
21. Kim DK, Lee J, Simpson RJ, Lotvall J, Gho YS. EVpedia: A community web resource for prokaryotic and eukaryotic extracellular vesicles research. *Seminars in cell & developmental biology*. 2015.
22. Webber J, Yeung V, Clayton A. Extracellular vesicles as modulators of the cancer microenvironment. *Seminars in cell & developmental biology*. 2015.
23. Ericson K, Gan C, Cheong I, Rago C, Samuels Y, Velculescu VE, et al. Genetic inactivation of AKT1, AKT2, and PDPK1 in human colorectal cancer cells clarifies their roles in tumor growth

regulation. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107:2598-603.

24. Jo H, Mondal S, Tan D, Nagata E, Takizawa S, Sharma AK, et al. Small molecule-induced cytosolic activation of protein kinase Akt rescues ischemia-elicited neuronal death. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109:10581-6.
25. Huang X, Le QT, Giaccia AJ. MiR-210--micromanager of the hypoxia pathway. *Trends in molecular medicine*. 2010;16:230-7.
26. Kim JH, Park SG, Song SY, Kim JK, Sung JH. Reactive oxygen species-responsive miR-210 regulates proliferation and migration of adipose-derived stem cells via PTPN2. *Cell death & disease*. 2013;4:e588.
27. Ren XP, Wu J, Wang X, Sartor MA, Qian J, Jones K, et al. MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20. *Circulation*. 2009;119:2357-66.
28. Ishikawa K, Ishikawa A, Shoji Y, Imai T. A genotoxic stress-responsive miRNA, miR-574-3p, delays cell growth by suppressing the enhancer of rudimentary homolog gene in vitro. *International journal of molecular sciences*. 2014;15:2971-90.
29. Li Y, Li L, Guan Y, Liu X, Meng Q, Guo Q. MiR-92b regulates the cell growth, cisplatin chemosensitivity of A549 non small cell lung cancer cell line and target PTEN. *Biochemical and biophysical research communications*. 2013;440:604-10.
30. Shen Y, Wang P, Li Y, Ye F, Wang F, Wan X, et al. miR-375 is upregulated in acquired paclitaxel resistance in cervical cancer. *British journal of cancer*. 2013;109:92-9.
31. Shen Y, Zhou J, Li Y, Ye F, Wan X, Lu W, et al. miR-375 mediated acquired chemo-resistance in cervical cancer by facilitating EMT. *PloS one*. 2014;9:e109299.
32. Schou JV, Rossi S, Jensen BV, Nielsen DL, Pfeiffer P, Hogdall E, et al. miR-345 in metastatic colorectal cancer: a non-invasive biomarker for clinical outcome in non-KRAS mutant patients treated with 3rd line cetuximab and irinotecan. *PloS one*. 2014;9:e99886.
33. Fujita Y, Yagishita S, Hagiwara K, Yoshioka Y, Kosaka N, Takeshita F, et al. The clinical relevance of the miR-197/CKS1B/STAT3-mediated PD-L1 network in chemoresistant non-small-cell lung cancer. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2015;23:717-27.
34. Zou D, Wang D, Li R, Tang Y, Yuan L, Long X, et al. MiR-197 induces Taxol resistance in human ovarian cancer cells by regulating NLK. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2015.
35. Song B, Wang Y, Xi Y, Kudo K, Bruheim S, Botchkina GI, et al. Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells. *Oncogene*. 2009;28:4065-74.

36. Green DR, Galluzzi L, Kroemer G. Cell biology. Metabolic control of cell death. *Science*. 2014;345:1250256.
37. Whitesell L, Lindquist SL. HSP90 and the chaperoning of cancer. *Nature reviews Cancer*. 2005;5:761-72.
38. Lee HJ, Zhuang G, Cao Y, Du P, Kim HJ, Settleman J. Drug resistance via feedback activation of Stat3 in oncogene-addicted cancer cells. *Cancer cell*. 2014;26:207-21.
39. Obenauf AC, Zou Y, Ji AL, Vanharanta S, Shu W, Shi H, et al. Therapy-induced tumour secretomes promote resistance and tumour progression. *Nature*. 2015;520:368-72.
40. Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annual review of pathology*. 2010;5:99-118.
41. McAlister GC, Huttlin EL, Haas W, Ting L, Jedrychowski MP, Rogers JC, et al. Increasing the multiplexing capacity of TMTs using reporter ion isotopologues with isobaric masses. *Analytical chemistry*. 2012;84:7469-78.

[00276] Example 6: JARID1B mediates transition between distinct cell states within the oral cancer stem cell pool

[00277] The degree of heterogeneity among cancer stem cells (CSCs) remains ill-defined and may hinder effective anti-CSC therapy. Evaluation of oral cancers for such heterogeneity identified two compartments within the CSC pool. One compartment was detected using a reporter for expression of the H3K4me3 demethylase JARID1B to isolate a JARID1B^{high} fraction of cells with stem cell-like function. JARID1B^{high} cells expressed oral CSC markers including CD44 and ALDH1 and showed increased PI3-kinase (PI3K) pathway activation. They were distinguished from a distinct fraction in a G0-like cell cycle state characterized by low reactive oxygen species and diminished PI3K signaling. G0-like cells lacked conventional CSC markers but were primed to acquire stem cell-like function by upregulating JARID1B, which directly mediated transition to a state expressing known oral CSC markers. The transition was regulated by PI3K signals acting upstream of JARID1B expression, resulting in PI3K inhibition depleting JARID1B^{high} cells but expanding the G0-like subset.

[00278] These findings define a novel developmental relationship between two distinct cell phenotypes contributing jointly to CSC maintenance. The G0-like phenotype's expansion during targeted depletion of JARID1B^{high} cells further implicates it as a distinct therapeutic target within the oral CSC pool.

[00279] Stem cell-like subpopulations of tumor cells have been functionally defined by the capacity for unlimited self-renewal together with differentiation to states lacking growth potential. In oral squamous cell carcinomas (OSCCs), such cancer stem cells (CSCs) have been isolated based on expression of CD44 and ALDH1 (1, 2) as well as multiple other markers (3). They have also been shown to upregulate factors related to embryonic stem cell self-renewal or differentiation, including

Oct4, Sox2, Nanog, and Bmi-1 (4-6). However, application of functional criteria for CSCs has not produced a clear consensus marker profile for them in OSCC and other carcinoma types.

[00280] Contributing to this uncertainty may be the presence of epigenetic heterogeneity within a functionally-defined oral CSC pool. Such heterogeneity exists in the normal epithelial stem cell pool, which can contain both quiescent and proliferative populations that dynamically interconvert to support tissue homeostasis and injury repair (7, 8). Thus, similar phenotypic heterogeneity and plasticity among oral CSCs seems likely to drive cancer progression and shape the therapy response.

[00281] Although quiescence is not an essential stem cell trait, it is commonly attributed to both normal adult stem cells and CSCs, where it may underlie generalized resistance to radiation and drugs that target proliferating cells. As such, quiescence may be defined as exit from the cell cycle while retaining potential to divide and give rise to progeny. An approach to defining cancer cells with quiescent traits is use of Pyronin-Y staining to gate cells with the lowest total RNA within the G0/G1 cell cycle fraction. Such cells exhibit the high p27kip1 levels and low reactive oxygen species (ROS) of the G0 cell cycle status (9, 10). A related population in breast cancer isolated based on low levels of ROS also exhibits molecular features of quiescence including loss of proliferation markers and increased Hes1 (11), an inhibitor of senescence and differentiation (12). Not meeting strict criteria for a G0 state, these "G0-like" cells were also characterized by reversible downregulation of the phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR axis through Akt degradation (11, 13). The central role of PI3K-Akt activation in allowing certain adult stem cells to exit quiescence (14) suggests that the pathway might similarly permit G0-like cancer cells to exert stem cell-like function.

[00282] However, the molecular traits of G0-like cells seem to diverge from those described for CSCs of multiple tumor types, making their relationship to the CSC pool unclear. Specifically, high PI3K activation has been noted in putative CSCs of various cancers and can render them sensitive to PI3K inhibitors (15-18). Furthermore, the ROS_{low} feature of G0-like cells is incongruous with the elevated oxidative metabolism observed in stem cell-like melanoma cells defined by high levels of the H3K4me3 demethylase JARID1B (19). This small subset of JARID1B_{high} melanoma cells resides in a slow cycling state (20) but thus far has not been shown to express molecular markers of quiescence. Recently JARID1B silencing in OSCC cell lines was shown to attenuate sphere forming capacity and multiple stem cell molecular markers, whereas high JARID1B expression in human OSCCs correlated with decreased survival (21). Additional findings have led to appreciation of JARID1B's role in either maintenance or differentiation of multiple normal and malignant stem cell phenotypes (22-25), suggesting its broader utility as a CSC marker.

[00283] Despite some similarities, the unknown degree of identity between G0-like and JARID1B_{high} tumor cells led us to evaluate their function, overlap, and developmental relationship. Here we demonstrate that the two subsets are represented in human OSCCs as distinct populations. Despite showing similar stem cell-like functional capacities, they had disparate molecular features,

with only JARID1B^{high} cells displaying conventional CSC-associated molecular markers, PI3K pathway activation, and PI3K inhibitor sensitivity. However, the G0-like compartment was primed to enter a JARID1B^{high} state and remained dependent on JARID1B to exert its stem cell-like function. These results have novel implications for maintenance of a dynamic CSC pool and provide new insight into the adaptive plasticity of a heterogeneous oral CSC population to targeted therapy.

[00284] RESULTS

[00285] G0-like cells within OSCCs exhibit stem cell-like function. G0-like cells were identified by flow cytometry (FC) within a panel of OSCC cell lines by using Hoechst-33342 and Pyronin-Y to detect an RNA^{low} fraction within the G0/G1 peak of the cell cycle profile (Fig. 18A left.).

H2DCFDA staining confirmed this fraction to be ROS^{low} (Fig. 18A right.), consistent with the H2DCFDA-based definition of G0-like cells as a ROS^{low} subset in breast cancer (11). As in this definition, G0-like cells in OSCC cell lines showed the traits of high p27Kip1 and Hes1, along with a decrease in total Akt protein (Fig. 18B). Their continued cyclin D1 expression in culture implied a lack of complete G0 exit from the cell cycle, supporting the "G0-like" designation. Applying the same Pyronin-Y-based isolation strategy to tumor cells purified from clinical specimens and patient-derived xenografts (PDXs) also detected G0-like cells with low levels of ROS and total Akt (Fig. 18C-8D).

G0-like cells were further characterized in 9 human tumors and 2 derivative PDXs by confocal immunofluorescence (IF) using existing methodology to detect G0-like cells in fixed tumor tissue. Tumor cells that showed the high Hes1, low Akt, and low H3K9me2 staining of the ROS^{low} G0-like marker profile in breast cancer (11) were detectable as a minority fraction of OSCC cells in most samples (Fig. 18E). The G0-like fraction from OSCC cell lines was subsequently tested for stem cell-like functional properties. In clonal sphere formation assays, G0-like cells had enhanced sphere-forming capacity as primary spheres and upon disaggregation and propagation as secondary spheres (Fig. 18F). G0-like cells from OSCC cell lines also formed tumors at higher incidence and shorter latency than the non-G0-like OSCC fraction upon transplantation at limiting dose in NSG mice (Fig. 18G). These results established that G0-like cells are detectable in OSCCs and display functional properties attributed to CSCs.

[00286] High JARID1B is a distinct basis for detecting stem cell-like function in OSCC. Based on their stem cell-like features, G0-like OSCC cells seemed comparable to a fraction of low turnover cells described in malignant melanoma that highly express the H3K4me3 demethylase JARID1B (20). To assess the role of JARID1B in the G0-like fraction and the overall oral CSC pool, a promoter-based fluorescent reporter for JARID1B transcription, J1BpromEGFP (20), was stably expressed in OSCC cell lines. As described in melanoma (20), OSCC cells with 5% highest EGFP fluorescence in monolayer culture showed elevated JARID1B mRNA and protein levels (Fig. 19A), establishing this gate to define JARID1B^{high} OSCC cells in subsequent studies. Retention of the fluorescent membrane dyes PKH26 or CellTraceTM Violet was used to evaluate the growth properties of

JARID1B^{high} OSCC cells. These cells were distinguished from the majority cell fraction by retention of a higher and more uniform fluorescence level over 10 days post-labeling, supporting their low-turnover state (Fig. 19B). Use of multicolor FC to assess overlap between the JARID1B^{high} and G0-like fractions detected lower JARID1B reporter function in the G0-like gate (data not shown). The JARID1B^{high} cells instead were largely distributed outside the G0-like gate and instead showed an expanded G2M fraction (Fig. 19C). Based on the possibility that the two subsets are distinct cell states, they were isolated for subsequent experiments using mutually exclusive gates and compared against a reference bulk tumor fraction defined as being both JARID1B^{low} and non-G0-like (data not shown). JARID1B^{high} OSCC cells isolated by this methodology displayed CSC-associated functional traits comparable to those of the G0-like fraction. Specifically, the JARID1B^{high} fraction was enriched in tumor spheres made from J1BpromEGFP-expressing OSCC cell lines relative to conventional monolayer culture (Fig. 19D). Similar to G0-like cells, purified JARID1B^{high} cells showed enhanced primary and secondary sphere formation (Fig. 19E). JARID1B^{high} and G0-like cells also formed xenograft tumors with comparable efficiency and with higher incidence and shorter latency than the bulk tumor cell pool (Fig. 19F). All three fractions generated tumors that retained the histology of the original tumor (data not shown). Together these data indicated that JARID1B^{high} and G0-like cells represent molecularly distinct, minority subpopulations within OSCCs that display similar stem cell-like functional properties.

[00287] JARID1B^{high} and G0-like cells show divergent molecular markers and PI3K pathway function. The shared stem cell-like functional traits between G0-like and JARID1B^{high} fractions led to their comparative molecular analysis, starting with the oral CSC markers CD44 and ALDH1. Analyses of OSCC cell lines expressing J1BpromEGFP showed that JARID1B^{high} cells reside at the high end of the cell surface CD44 distribution, whereas G0-like cells in cell lines or patient tumor specimens showed decreased surface CD44 (Fig. 20A). Similarly, only JARID1B^{high} cells expressed elevated mRNA for ALDH1A1 (Fig. 20B), the isoform associated with increased ALDH activity in OSCC (26). Subsequent assessment of pluripotency-related factors in the two populations revealed that only JARID1B^{high} cells have increased Oct4 and Bmi1 protein levels (Fig. 20C).

[00288] Broader comparative assessment of G0-like cells, JARID1B^{high} cells, and the bulk fraction was performed by transcriptome analysis with mRNA sequencing. Pairwise comparison of bulk cells to the G0-like and JARID1B^{high} subsets revealed a common core of genes upregulated by both subsets (Fig. 3D). This significant 61-gene overlap (data not shown) included the majority of gene transcripts increased in G0-like cells and suggested a close relationship between G0-like and JARID1B^{high} cells, despite clear molecular distinctions. Minimal overlap was seen between genes downregulated by G0-like versus JARID1B^{high} cells relative to the bulk fraction (data not shown). Consistent with their shared stem cell-like function, G0-like and JARID1B^{high} cells both upregulated gene signatures defined in CD31⁺ stromal stem cells (27) and CD29^{high}CD24^{low}CD61⁺ mammary

stem cells (28) (not shown). Importantly, these gene set enrichment analysis (GSEA) profiles were more strongly represented in JARID1B^{high} cells over G0-like cells (data not shown). In addition, JARID1B^{high} cells showed increased mesenchymal gene expression relative to G0-like cells, based on an epithelial-to-mesenchymal transition-related profile strongly associated with breast CSCs (29) (data not shown). These findings support a clear molecular distinction between G0-like and JARID1B^{high} cells despite their functional similarity and shared expression of a core set of genes, with JARID1B^{high} cells exhibiting a more conventional CSC-associated molecular profile.

[00289] Transcriptional profiling was combined with cell signaling analysis to confirm that distinct proliferative states underlie any quiescent attributes shared by the G0-like and JARID1B^{high} fractions. Despite JARID1B^{high} cells being label-retaining in culture (Fig. 19B), only the G0-like cells downregulated cell cycle-related gene transcription relative to the bulk population based on a gene set curated from the Reactome database (data not shown). To further characterize this difference, oncogenic signals known to drive cell cycle progression in OSCC were compared across cell fractions. No differences were evident in MAPK or JAK/STAT signaling based on ERK activation and STAT3 phosphorylation, respectively (data not shown). The reduced PI3K activity anticipated in G0-like cells based on low total AKT levels (Fig. 18B) was confirmed at the level of total and phospho-AKT (data not shown). However, high phosphorylation of Akt and its downstream target GSK3 β indicated that JARID1B^{high} cells hyper-activate the PI3K pathway relative to bulk cells (Fig. 20E). Because similar PI3K activation is noted in numerous CSC phenotypes (15-18) these findings added to the observed similarities between existing CSC definitions and the JARID1B^{high} subset. By contrast, suppressed PI3K signaling and other molecular distinctions in G0-like cells suggested a distinct role for this fraction within the oral CSC pool.

[00290] G0-like cells are primed to enter the JARID1B^{high} state. Shared upregulation of a large gene set between G0-like and JARID1B^{high} subsets relative to the broader tumor cell pool (Fig. 20D) led to the hypothesis that the two subsets are closely related developmentally. Specifically, an enhanced capacity to acquire the molecular features seen in the JARID1B^{high} state might underlie the stem cell-like function of G0-like cells. To test the relative capacity of G0-like versus bulk cells to become JARID1B^{high}, the two fractions were purified by fluorescence-activated cell sorting (FACS) from LNT14_J1BpromEGFP cells and re-cultured. Tracking JARID1B reporter function showed that G0-like cells reconstituted the JARID1B^{high} fraction by day 4 and restored the original EGFP distribution by day 7 (Fig. 21A). By contrast, even after 14 days, bulk cells failed to fully regenerate the JARID1B^{high} fraction (Fig. 21A), suggesting that the G0-like subset is uniquely poised to become JARID1B^{high}. Despite this enhanced capacity for entry into the slow cycling JARID1B^{high} pool, G0-like cells display high proliferative potential comparable to that of bulk cells in culture (data not shown). Thus it was possible that a rapidly proliferative intermediate precedes entry into the JARID1B^{high} state. To test whether G0-like cells can become JARID1B^{high} without extensive

proliferation, FACS-purified G0-like or bulk cells were labeled with CellTrace™ membrane dye and analyzed for label retention versus JARID1B reporter function upon re-culture. A schematic is shown to represent possible outcomes of this assay (Figure 21B left).

[00291] Here, cells that become EGFP^{high} while remaining CellTrace^{high} most likely enter the JARID1B^{high} subset without significant loss of label from division and remain in this state over the course of the assay. G0-like cells produced more CellTrace^{high}/JARID1B^{high} cells overall (Fig. 21B right, top) and two-fold more CellTrace^{high} cells in the JARID1B^{high} fraction (Fig. 21B right, bottom), demonstrating their enhanced capacity to become JARID1B^{high} without loss of label. The efficiency of the G0-like to JARID1B^{high} transition was most apparent in the sphere formation assay, where dilution of these states by rapid proliferation in standard culture is constrained. Here, G0-like cells generated spheres containing more JARID1B^{high} cells than the spheres arising less frequently from bulk cells (Fig. 21C). Rapid re-expression of JARID1B by G0-like cells during sphere formation was also indicated by appearance of a large, second EGFP peak in a bimodal distribution, which was absent or less prominent in the spheres forming from bulk cells (Fig. 21D). Together, these data support a proximate developmental relationship between the two stem cell-like subpopulations, with G0-like cells being primed to enter a JARID1B^{high} state.

[00292] The G0-like fraction exerts stem cell-like function by a JARID1B-dependent mechanism. Given JARID1B's role as a chromatin regulator, the enhanced efficiency with which G0-like cells become JARID1B^{high} suggested that JARID1B directly contributes to this cell state transition. To test this possibility, JARID1B expression in OSCC cell lines was reduced by approximately 50% using a lentiviral shRNA (Fig. 22A) known to produce specific silencing (20). Cells in the G0-like FC gate after JARID1B knockdown retained their characteristic low ROS and AKT levels (data not shown); however, sphere formation by these cells was attenuated to that of bulk cells, whose sphere-forming capacity was unaltered by JARID1B depletion (Fig. 22B). Similarly, *in vivo* tumor formation by G0-like cells after JARID1B silencing was diminished to match the lower incidence and longer latency shown by bulk cells at limiting dose (Fig. 22C). These results may be explained by JARID1B reduction preventing transition of cells from the G0-like pool into a state with conventional oral CSC markers. Alternatively, JARID1B loss might not prevent G0-like cells from acquiring CSC markers but only impair subsequent stem cell function. The former possibility was supported by the accumulation of G0-like cells upon JARID1B silencing (Fig. 22D). Under these conditions, we also analyzed EGFP^{high} cells, which exhibit high JARID1B transcription as detected by J1BpromEGFP reporter function, despite reduced JARID1B levels by shRNA. These cells were similar in frequency to control EGFP^{high} (i.e., JARID1B^{high}) cells but, as anticipated, did not express high levels of JARID1B mRNA (data not shown). This population not only lost enhanced sphere-forming capacity (Fig. 22E left) but also failed to upregulate Oct4 and Bmi1 (Fig. 22E right). This ability of JARID1B silencing to prevent acquisition of other stem cell-associated markers further supported a role in the

transition of G0-like cells to a JARID1B^{high} CSC state. The finding agreed with other shifts in CSC marker expression and function observed during JARID1B knockdown or over-expression.

Specifically, knockdown decreased expression of cell surface CD44 and ALDH1A1 mRNA (Fig. 22F), while transient over-expression increased CD44 and ALDH1A1 levels coordinately with sphere-forming capacity (Fig. 22G). In sum, these results indicate that JARID1B directly contributes to the stem cell-like function of G0-like cells by driving their transition toward the molecular profile of a JARID1B^{high} cell.

[00293] The PI3-kinase pathway regulates the dynamics between G0-like and JARID1B^{high} states.

Although JARID1B silencing blocked acquisition of CSC markers by EGFP^{high} cells (Fig. 22E), global pAKT levels were unaltered (Fig. 23A left) and AKT hyper-activation was maintained by the EGFP^{high} fraction (right). This result suggested that PI3K signals might act proximally to JARID1B upregulation to drive transition to the JARID1B^{high} state. To test this possibility, AKT was inducibly activated in LNT14_J1BpromEGFP in cells carrying a myristoylated form of Akt fused to an altered estrogen receptor domain (myrAktER) that blocks AKT activation in absence of 4-hydroxytamoxifen (4-OHT) (data not shown) (30). Addition of 4-OHT expanded the JARID1B^{high} subset (Fig. 23B), supporting that PI3K signaling drives entry into this state and predicting that PI3K inhibitors may alter the balance between the G0-like and JARID1B^{high} fractions. PI3K inhibition in vitro with LY294002 at a ~25% growth-inhibitory dose (data not shown) decreased both JARID1B protein levels in LNT14 cells (Fig. 23C right) and the size of the JARID1B^{high} fraction detected by EGFP reporter function (left). By contrast, the fraction of cells in the G0-like gate was expanded over two-fold by LY294002 treatment (Fig. 23D left). Importantly, the G0-like fraction isolated from OSCC cells treated with the pan-PI3K inhibitor GDC-0941 retained both sphere-forming capacity (Fig. 23D middle) and enhanced tumorigenic potential (Fig. 23D right), providing further evidence of their resistance to PI3K inhibition. To define whether PI3K targeting in vivo produces similar dynamics between the subpopulations, xenograft tumors were established from LNT14_J1BpromEGFP cells. GDC-0941 treatment caused significant growth inhibition (Fig. 23E left). Subsequent disaggregation and FC analysis of the treated tumors revealed no enrichment of JARID1B^{high} cells and a trend toward their decreased percentage (Fig. 23E middle). Concurrently, there was greater than two-fold expansion of the G0-like fraction (right). The expanded G0-like subset in treated tumors was verified histologically based on confocal IF quantitation of increased Hes1^{high}/Akt^{low}/H3K9me2^{low} tumor cells in the GDC-treated group (Fig. 23F), as detected previously for treatment-naïve tumors (Fig. 18E).

[00294] In sum, these findings support a model in which PI3K signals regulate the plasticity within a heterogeneous oral CSC pool by functioning proximal to JARID1B expression in driving cells out of the G0-like fraction and toward the JARID1B^{high} state (Fig. 24). By this model, G0-like

cells exert stem cell-like function by efficiently re-expressing JARID1B, which promotes transition to a CSC-associated gene expression profile and upregulation of the CD44 and ALDH1 markers. The model further indicates that PI3K signals regulate the composition of the oral CSC pool in a manner that has implications for therapeutic inhibition of this pathway, which is prone to expand the G0-like compartment while depleting the JARID1B^{high} fraction.

[00295] DISCUSSION

[00296] A potential barrier to CSC-directed therapy arises from evidence that some tumor cells lacking CSC markers retain enough phenotypic plasticity to exert stem cell-like function (20, 31, 32). It is demonstrated herein that a G0-like subset lacking the conventional oral CSC markers is primed to exert stem cell-like function by transitioning into a slow-cycling JARID1B^{high} state that expresses such markers. The shift from G0-like to JARID1B^{high} was mediated by upregulation of the H3K4me3 demethylase JARID1B in a PI3K-dependent manner. Regulation of a heterogeneous CSC pool by this mechanism offers a novel conceptual framework for understanding homeostasis of oral CSCs during cancer progression and therapy responses.

[00297] Detection of stem cell-like properties in JARID1B^{high} OSCC cells here adds to growing evidence that links this chromatin regulator to stem cell biology. By establishing JARID1B's importance in mediating the stem cell-like potential of G0-like cells, the present results expand understanding of its role in malignant and normal stem cell homeostasis, which appears varied and context specific. In normal development, JARID1B can either promote maintenance or drive differentiation of various stem cell populations (25, 33). These functions derive at least in part from its H3K4me3 demethylase activity directly silencing promoters of genes involved in lineage specification (22, 34). In malignancy, JARID1B overexpression or amplification has been described in multiple tumor types (35-37). JARID1B oncogenic function is perhaps best characterized in luminal type breast cancer, where its overexpression drives a luminal cell-specific gene expression program (37). By contrast, JARID1B is not highly expressed in melanomas but still underlies the function of a small subset of JARID1B^{high} cells with a role in tumor maintenance and drug resistance (19, 20). Similar to findings in OSCC, neuroblastoma tumor spheres express increased JARID1B, which was associated with ALDH activity (38). Because JARID1B protein is widely detectable in both normal squamous epithelia and OSCCs (21), CSC function associated with the small JARID1B^{high} subset studied here is likely to be dose-dependent. Thus fine-tuning of JARID1B levels is needed to pursue its target genes specifically associated with oral CSC function. Such genes are also impacted by JARID1B's known interactions with other transcriptional and epigenetic regulators, which can vary based on cellular context (36, 39-41). Defining these dose- and context-dependent effects may have implications for therapeutic application of JARID1B inhibitors (42) in preclinical development.

[00298] Inclusion of G0-like cells within the oral CSC pool in the model here (Fig. 24) emphasizes their stem cell-like properties arising from rapid entry into the JARID1B^{high} state bearing standard oral CSC markers. While defining the G0-like to JARID1B^{high} transition, the model retains questions regarding what mechanisms regulate entry into or exit from the G0-like state. As illustrated in Fig. 24, G0-like cells could in principle arise from either the JARID1B^{high} subset or the rapid cycling subset. The latter possibility is well supported by prior studies, which directly visualized them arising by asymmetric division of rapidly proliferative cells through a low frequency stochastic event (11, 13). Divisions producing G0-like cells were driven by a novel integrin-regulated signaling cascade that leads to proteasome degradation of Akt1 via the E3 ubiquitin ligase TTC3 (13). These mechanisms might allow G0-like cells to serve as an intermediate for other cell phenotypes transitioning to the slow-cycling JARID1B^{high} state. Although PI3K activation was shown to underlie entry into the JARID1B^{high} pool, it is unclear what signals initiate this transition or if additional mechanisms permit G0-like cells to return to rapid proliferation without a JARID1B^{high} intermediate. Detailing such molecular mechanisms may provide multiple approaches to addressing the G0-like subset as a potential basis for innate therapy resistance. In addition, tracing single cell fates to precisely delineate the network of cell state transitions into and out of the G0-like subset may further help define strategies to deplete this population.

[00299] The dichotomous levels of PI3K activation detected here in G0-like versus JARID1B^{high} cells offer insight into the pathway's role in homeostasis of the oral CSC pool and its adaptation to PI3K inhibition. Putative CSCs in multiple tumor types are known to exhibit PI3K hyper-activation and small molecule inhibitor sensitivity (15-18). Our similar finding in JARID1B^{high} OSCC cells is particularly relevant given the recent appreciation of frequent driving PI3K pathway alterations in OSCC (43), which include the presence of isolated PIK3CA mutations for some tumors of the HPV-positive subtype (44). The present results further indicate that G0-like cells can serve as a reservoir that replenishes the JARID1B^{high} CSC state if it is depleted by PI3K inhibition or other therapies. Such effects may explain why PI3K pathway inhibitors generally produce modest initial clinical responses despite apparent sensitivity of the CSC pool. In this context, expansion of the G0-like subset may play a role in therapy resistance parallel to that of reserve stem cells that are recruited during the injury responses of normal epithelia. For example, depletion of the proliferative Lgr5-positive intestinal stem cell pool through injury leads to its regeneration by a quiescent Bmi1-positive cell type that has limited role in normal homeostasis (45). Similar plasticity among quiescent and proliferative adult stem cells has been defined in multiple tissue types (7, 8) and thus is likely to be maintained and exploited by solid tumors.

[00300] Although absence of rapid proliferation in G0-like and JARID1B^{high} cells may contribute to therapy resistance in both subsets, their divergent PI3K pathway function and cell cycle-related gene expression suggest a need for differing targeting strategies. Without wishing to be bound

by theory, one general approach can be to block entry into the G0-like state pharmacologically in combination with inhibition of PI3K signals.

[00301] MATERIALS AND METHODS

[00302] Cell lines, PDXs, clinical specimens, and vectors. Cells were maintained in 1:1 Dulbecco's modified Eagle/Ham's F12 media with 400 ng/ml hydrocortisone, 10% fetal bovine serum (FBS), and 50 µg/ml gentimycin. SCC9 cells were obtained from ATCC (CRL-1629). LNT14 and OCTT2 lines and PDXs are previously described (46). Tumor specimens were obtained with informed consent from advanced stage head and neck SCC patients under University of Pennsylvania IRB protocol #417200 or Philadelphia VA Medical Center protocol #01090. Lentiviral vectors pLU-JARID1Bprom-EGFP-Blast, pLKO-shJARID1B (20) and transient expression vector pBIND-RBP2H1(JARID1B) (47) are previously described. Retroviral vectors pWZLneo-myrAKT 4-129-ER and A2myrAKT 4-129-ER (30) were used as described (48).

[00303] Detection of G0-like cells and ROS. Tumor cells were suspended at 10⁶ cells/ml and incubated with 4 µM Hoechst-33342 (Life Technologies, Grand Island, NY) at 37°C for 30 min followed by 1 µg/ml Pyronin-Y (Sigma-Aldrich, St. Louis, MO) at 37°C for 30 min. G0-like cells were FACS-purified by setting a PyroninYlow gate within the G0/G1 (2n DNA) peak of the Hoechst-33342 fluorescence profile. To detect ROS, cell suspensions were subsequently incubated with 2.5 µM H2DCFDA (Life Technologies) for 20 min at 37°C.

[00304] Flow cytometry. FC was performed using a MoFlo AstriosEQ™ (Beckman-Coulter, Inc., Miami, FL) or LSR II™ (BD Biosciences, San Jose, CA) and FlowJo™ Analysis Software (FlowJo, LLC, Ashland, OR). JARID1B promoter-driven EGFP signals were measured as described (20). Unfractionated J1BpromEGFP-expressing cells grown as a monolayer were used to set the reference JARID1Bhigh gate in all experiments. Cell surface CD44 was defined using anti-CD44-APC (BD) or anti-CD44-FITC (eBioscience, San Diego, CA). The reference CD44high gate was defined as the 10% of unfractionated cells with the highest signal. Dead cells were excluded by 7-AAD.

[00305] Tumor cell purification from human tumors or PDXs. Tumors were minced, transferred to gentleMACS™ C-Tubes (Miltenyi Biotech Inc., San Diego, CA) containing 1mg/ml collagenase-IV solution, and mechanically disrupted using a gentleMACS™ Dissociator (Miltenyi). Suspensions were incubated at 37°C for 1 hour with rocking. Mechanical disruption was repeated, and suspensions were passed through 40 µm filters. To remove mouse stromal cells from PDXs, suspensions were pretreated with anti-mouse CD16/32 (Fc-Block, Miltenyi) and human cells were positively selected by FACS using APC-conjugated mouse anti-human major histocompatibility complex (MHC) class I, HLA-ABC (eBioscience). Periodic co-staining with anti-mouse MHC-I (H-2Kd) (eBioscience) was conducted to confirm purity of the tumor cell fraction. For human tissues, tumor cells were purified as described previously by negative selection with anti-human CD45-FITC and anti-human CD31-FITC (Miltenyi) (49).

[00306] Western blotting. Protein was extracted by directly lysing equal cell numbers in Laemmli buffer (125mM Tris-HCl pH 6.8, 4% (w/v) SDS, 200 mM β -mercaptoethanol, 10% (v/v) glycerol and 0.2% (w/v) bromophenol blue). Lysates were separated on 10% ECL gels (GE Healthcare Life Sciences, Pittsburgh, PA) and transferred to nitrocellulose using the Trans-Blot® Turbo™ System (Bio-Rad, Hercules, CA). Primary antibodies (Supplementary Table S3) were incubated at 4°C overnight. After washing, blots were incubated with anti-Rabbit IgG-DyLight™800 or anti-Mouse IgG-DyLight680 and imaged/quantified using an Odyssey Infrared Imaging System and Image Studio software (LI-COR, Lincoln, NE).

[00307] Immunofluorescent staining and quantification of G0-like cells in tissue. Formalin-fixed, paraffin-embedded specimens were cut as 5 μ m sections, stained, and imaged as previously described (11). Briefly, sections were de-paraffinized and rehydrated, followed by target antigen retrieval via a single microwave step, blocking with 5% FBS, and overnight incubation of primary antibodies. Secondary fluorophores used were AlexaFluor 488, 555, and 633 (Life Technologies). A Nikon Eclipse Ti A1R-A™1 confocal microscope was used to identify G0-like cells that were positive for DAPI and cytokeratin (tumor cells) and pan-AKTlow/MCM2low/H3K9me2low/HES1high based on co-staining the same or sequential sections. A species-specific isotype control served as a negative control. Images of 10 randomly selected fields per section were taken at 60x magnification, and cells counted using Image J™ software. Degree of fluorescence (high vs. low) was semi-quantitatively assessed in comparison to background in a negative control image. Signals were confirmed as ‘high’ if the ratio of corrected total fluorescence was >2x compared to three ‘low’ cells in the same image. A single observer was blind to the clinical and treatment allocation of each specimen to avoid bias in counting.

[00308] Sphere formation assay. For sphere culture, 10 cells/well were seeded on ultralow attachment 96-well plates (Corning, Corning, NY) in serum-free MEGM™ with hEGF, hydrocortisone, bovine pituitary extract, and insulin (Lonza, Basel, Switzerland) and cultured for 14 days. Spheres were counted by phase-contrast microscopy at 10x magnification. Phase contrast and EGFP-fluorescent sphere images were generated using a Leica DM IRB inverted microscope and iVision™ software (Biovision Technologies, Chester Springs, PA). For propagation, spheres were pooled, washed, and digested in 1 ml StemPro® Accutase solution (Life Technologies) for 20 min at 37°C. Dissociated cells were washed, counted with Trypan Blue for dead cell exclusion, and re-plated under sphere-forming conditions.

[00309] In vivo experiments. Non-obese diabetic/severe combined immunodeficient/interleukin-2 receptor γ -chain-deficient (NSG) mice were bred and used at the Wistar Institute animal facility under protocols approved by the Institutional Animal Care and Use Committee (#112652, 112653, 112655). For tumorigenicity studies, a limiting dose of purified cells was suspended in 100 μ l Matrigel (Corning) and injected subcutaneously into the mouse flank. For drug studies, xenograft tumors were

established in NSG mice by subcutaneous flank injection of 106 cells in 100 μ l Matrigel. Treatment was started when average tumor volume reached $\sim$ 100mm³. GDC-0941 (SelleckChem, Houston, TX) was dissolved in 0.5% methylcellulose/0.2% Tween 80 (MCT). The treatment group received 100 mg/kg GDC-0941 by daily oral gavage. Control mice received MCT. Tumor volumes were measured every three days, and mice were euthanized after 2 weeks of treatment prior to harvesting tumors.

[00310] Label retention (LR). LR was determined using the PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) or CellTrace™ Violet Cell Proliferation Kit (Life Technologies) per manufacturer instructions. 100% labeling efficiency was confirmed immediately post-labeling. Labeled cells were cultured for 10 days and LR measured by FC as previously described (20).

[00311] Real-time reverse transcription PCR and RNA-Seq. RNA was isolated and treated with DNase using the Qiagen RNeasy™ kit (Qiagen, Valencia, PA). cDNA was synthesized from 1 μ g RNA using an RNA-to-DNA kit (Applied Biosystems by Life Technologies) and purified with a PCR purification kit (Qiagen). Gene expression was quantified using Power SYBR™ green Master Mix and a Step-One Real-Time PCR System (Applied Biosystems).

[00312] Multiplexed Illumina libraries were prepared from total RNA using the Illumina stranded mRNA kit. Libraries were pooled and sequenced to 100bp from one end of the insert. The resulting reads were aligned against the human genome (hg19) using RUM (version 2.0.4) for a total of 236,908,957 aligning reads. The 215,252,518 uniquely-aligning reads were used to quantify expression of transcripts. Differentially-expressed genes were identified using EdgeR with a generalized linear model that took the donor linkage into account and then looked for inter-treatment differences. A multidimensional scaling plot confirmed a significant donor effect supporting the choice of this analysis strategy. Differentially-expressed genes had an FDR better than 10%.

[00313] To identify biological functions enriched in the subpopulations, a GSEA approach using the MSigDB C2 v5.0 collection of curated gene sets (>3000) was applied. JARID1B^{high} and G0-like cells were compared to bulk cells, as well as each other. The log₂-fold change for all genes between each pair of conditions was used as a ranking variable.

[00314] Statistical Methods. Data are expressed as mean $\pm$ standard error. At least three replicates were performed per experiment. Analysis of variance (ANOVA) or a t-test was used to evaluate differences among group means. If ANOVA was significant, Tukey's procedure tested for significant pairwise differences. An F-statistic was used to test for equal variances; when variances were unequal, Welch's ANOVA or t-statistic was used. The Mann-Whitney U test was used when variables were not normally distributed. Cumulative distributions for latency times were estimated using the Kaplan-Meier procedure. Exact log-rank tests were evaluated differences in latency times.

[00315] REFERENCES

1. Prince ME, Sivanandan R, Kaczorowski A, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci U S A* 2007;104(3):973-8.
2. Clay MR, Tabor M, Owen JH, et al. Single-marker identification of head and neck squamous cell carcinoma cancer stem cells with aldehyde dehydrogenase. *Head Neck* 2010;32(9):1195-201.
3. Facompre N, Nakagawa H, Herlyn M, Basu D. Stem-like cells and therapy resistance in squamous cell carcinomas. *Adv Pharmacol* 2012;65:235-65.
4. Chiou SH, Yu CC, Huang CY, et al. Positive correlations of oct-4 and nanog in oral cancer stem-like cells and high-grade oral squamous cell carcinoma. *Clin Cancer Res* 2008;14(13):4085-95.
5. Chien CS, Wang ML, Chu PY, et al. Lin28B/let-7 regulates expression of Oct4 and Sox2 and reprograms oral squamous cell carcinoma cells to a stem-like state. *Cancer Res* 2015;75(12):2553-65.
6. He Q, Liu Z, Zhao T, Zhao L, Zhou X, Wang A. Bmi1 drives stem-like properties and is associated with migration, invasion, and poor prognosis in tongue squamous cell carcinoma. *Int J Biol Sci* 2015;11(1):1-10.
7. Li L, Clevers H. Coexistence of quiescent and active adult stem cells in mammals. *Science* 2010;327(5965):542-5.
8. Blanpain C, Fuchs E. Epidermal homeostasis: A balancing act of stem cells in the skin. *Nat Rev Mol Cell Biol* 2009;10(3):207-17.
9. Deng X, Ewton DZ, Friedman E. Mirk/Dyrk1B maintains the viability of quiescent pancreatic cancer cells by reducing levels of reactive oxygen species. *Cancer Res* 2009;69(8):3317-24.
10. Jin K, Ewton DZ, Park S, Hu J, Friedman E. Mirk regulates the exit of colon cancer cells from quiescence. *J Biol Chem* 2009;284(34):22916-25.
11. Dey-Guha I, Wolfer A, Yeh AC, et al. Asymmetric cancer cell division regulated by AKT. *Proc Natl Acad Sci U S A* 2011;108(31):12845-50.
12. Sang L, Roberts JM, Collier HA. Hijacking HES1: How tumors co-opt the anti-differentiation strategies of quiescent cells. *Trends Mol Med* 2010;16(1):17-26.
13. Dey-Guha I, Alves CP, Yeh AC, et al. A mechanism for asymmetric cell division resulting in proliferative asynchronicity. *Mol Cancer Res* 2015;13(2):223-30.
14. Lechman ER, Gentner B, van Galen P, et al. Attenuation of miR-126 activity expands HSC in vivo without exhaustion. *Cell Stem Cell* 2012;11(6):799-811.
15. Ma S, Lee TK, Zheng BJ, Chan KW, Guan XY. CD133+ HCC cancer stem cells confer chemoresistance by preferential expression of the akt/PKB survival pathway. *Oncogene* 2008;27(12):1749-58.
16. Kolev VN, Wright QG, Vidal CM, et al. PI3K/mTOR dual inhibitor VS-5584 preferentially targets cancer stem cells. *Cancer Res* 2015;75(2):446-55.

17. Dubrowska A, Kim S, Salamone RJ, et al. The role of PTEN/akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. *Proc Natl Acad Sci U S A* 2009;106(1):268-73.
18. Hardt O, Wild S, Oerlecke I, et al. Highly sensitive profiling of CD44+/CD24- breast cancer stem cells by combining global mRNA amplification and next generation sequencing: Evidence for a hyperactive PI3K pathway. *Cancer Lett* 2012;325(2):165-74.
19. Roesch A, Vultur A, Bogeski I, et al. Overcoming intrinsic multidrug resistance in melanoma by blocking the mitochondrial respiratory chain of slow-cycling JARID1B(high) cells. *Cancer Cell* 2013;23(6):811-25.
20. Roesch A, Fukunaga-Kalabis M, Schmidt EC, et al. A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. *Cell* 2010;141(4):583-94.
21. Lin CS, Lin YC, Adebayo BO, et al. Silencing JARID1B suppresses oncogenicity, stemness and increases radiation sensitivity in human oral carcinoma. *Cancer Lett* 2015.
22. Schmitz SU, Albert M, Malatesta M, et al. Jarid1b targets genes regulating development and is involved in neural differentiation. *EMBO J* 2011;30(22):4586-600.
23. Cellot S, Hope KJ, Chagraoui J, et al. RNAi screen identifies Jarid1b as a major regulator of mouse HSC activity. *Blood* 2013;122(9):1545-55.
24. Kuo YT, Liu YL, Adebayo BO, et al. JARID1B expression plays a critical role in chemoresistance and stem cell-like phenotype of neuroblastoma cells. *PLoS One* 2015;10(5):e0125343.
25. Stewart MH, Albert M, Sroczynska P, et al. The histone demethylase Jarid1b is required for hematopoietic stem cell self-renewal in mice. *Blood* 2015;125(13):2075-8.
26. Visus C, Ito D, Amoscato A, et al. Identification of human aldehyde dehydrogenase 1 family member A1 as a novel CD8+ T-cell-defined tumor antigen in squamous cell carcinoma of the head and neck. *Cancer Res* 2007;67(21):10538-45.
27. Boquest AC, Shahdadfar A, Fronsdal K, et al. Isolation and transcription profiling of purified uncultured human stromal stem cells: Alteration of gene expression after in vitro cell culture. *Mol Biol Cell* 2005;16(3):1131-41.
28. Lim E, Wu D, Pal B, et al. Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. *Breast Cancer Res* 2010;12(2):R21.
29. Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G, Palacios J. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. *Cancer Res* 2008;68(4):989-97.
30. Kohn AD, Takeuchi F, Roth RA. Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J Biol Chem* 1996;271(36):21920-6.
31. Gupta PB, Fillmore CM, Jiang G, et al. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell* 2011;146(4):633-44.

32. Chaffer CL, Marjanovic ND, Lee T, et al. Poised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. *Cell* 2013;154(1):61-74.
33. Kidder BL, Hu G, Yu ZX, Liu C, Zhao K. Extended self-renewal and accelerated reprogramming in the absence of kdm5b. *Mol Cell Biol* 2013;33(24):4793-810.
34. Dey BK, Stalker L, Schnerch A, Bhatia M, Taylor-Papadimitriou J, Wynder C. The histone demethylase KDM5b/JARID1b plays a role in cell fate decisions by blocking terminal differentiation. *Mol Cell Biol* 2008;28(17):5312-27.
35. Wang Z, Tang F, Qi G, et al. KDM5B is overexpressed in gastric cancer and is required for gastric cancer cell proliferation and metastasis. *Am J Cancer Res* 2014;5(1):87-100.
36. Xiang Y, Zhu Z, Han G, et al. JARID1B is a histone H3 lysine 4 demethylase up-regulated in prostate cancer. *Proc Natl Acad Sci U S A* 2007;104(49):19226-31.
37. Yamamoto S, Wu Z, Russnes HG, et al. JARID1B is a luminal lineage-driving oncogene in breast cancer. *Cancer Cell* 2014;25(6):762-77.
38. Kuo YT, Liu YL, Adebayo BO, et al. JARID1B expression plays a critical role in chemoresistance and stem cell-like phenotype of neuroblastoma cells. *PLoS One* 2015;10(5):e0125343.
39. Wong PP, Miranda F, Chan KV, Berlatto C, Hurst HC, Scibetta AG. Histone demethylase KDM5B collaborates with TFAP2C and myc to repress the cell cycle inhibitor p21(cip) (CDKN1A). *Mol Cell Biol* 2012;32(9):1633-44.
40. Zhang Y, Liang J, Li Q. Coordinated regulation of retinoic acid signaling pathway by KDM5B and polycomb repressive complex 2. *J Cell Biochem* 2014;115(9):1528-38.
41. Barrett A, Santangelo S, Tan K, et al. Breast cancer associated transcriptional repressor PLU-1/JARID1B interacts directly with histone deacetylases. *Int J Cancer* 2007;121(2):265-75.
42. Sayegh J, Cao J, Zou MR, et al. Identification of small molecule inhibitors of jumonji AT-rich interactive domain 1B (JARID1B) histone demethylase by a sensitive high throughput screen. *J Biol Chem* 2013;288(13):9408-17.
43. Lui VW, Hedberg ML, Li H, et al. Frequent mutation of the PI3K pathway in head and neck cancer defines predictive biomarkers. *Cancer Discov* 2013;3(7):761-9.
44. Seiwert TY, Zuo Z, Keck MK, et al. Integrative and comparative genomic analysis of HPV-positive and HPV-negative head and neck squamous cell carcinomas. *Clin Cancer Res* 2015;21(3):632-41.
45. Tian H, Biehs B, Warming S, et al. A reserve stem cell population in small intestine renders Lgr5-positive cells dispensable. *Nature* 2011;478(7368):255-9.
46. Basu D, Bewley AF, Sperry SM, et al. EGFR inhibition promotes an aggressive invasion pattern mediated by mesenchymal-like tumor cells within squamous cell carcinomas. *Mol Cancer Ther* 2013;12(10):2176-86.

47. Roesch A, Mueller AM, Stempf T, Moehle C, Landthaler M, Vogt T. RBP2-H1/JARID1B is a transcriptional regulator with a tumor suppressive potential in melanoma cells. *Int J Cancer* 2008;122(5):1047-57.
48. Oyama K, Okawa T, Nakagawa H, et al. AKT induces senescence in primary esophageal epithelial cells but is permissive for differentiation as revealed in organotypic culture. *Oncogene* 2007;26(16):2353-64.
49. Judd NP, Winkler AE, Murillo-Sauca O, et al. ERK1/2 regulation of CD44 modulates oral cancer aggressiveness. *Cancer Res* 2012;72(1):365-74.

What is claimed herein is:

1. A method of increasing the rate of asymmetric proliferation in a cancer cell, the method comprising:
 - contacting the cancer cell with an agonist of AKT1 degradation selected from the group consisting of:
 - an inhibitor of FAK; an inhibitor of β 1-integrin; PF-562271; and NVP-TAE226.
 - wherein an increase in AKT1 degradation increases the rate of asymmetric proliferation in the cancer cell.
2. The method of claim 1, whereby slow proliferator cancer cells are produced.
3. A method of decreasing the rate of asymmetric proliferation in a cancer cell, the method comprising:
 - contacting the cancer cell with an inhibitor of AKT1 degradation selected from the group consisting of:
 - an inhibitor of AKT1 expression; an agonist of β 1-integrin; and a cell medium comprising a fibrillar pattern of collagen.
 - wherein a decrease in AKT1 degradation decreases the rate of asymmetric proliferation in the cancer cell.
4. A method of treating cancer in a subject in need thereof, the method comprising:
 - administering an inhibitor of AKT1 degradation to the subject, wherein the inhibitor of AKT1 degradation is selected from the group consisting of:
 - an inhibitor of AKT1 expression and an agonist of β 1-integrin.
5. The method of claim 4, wherein the method further comprises administering a cancer therapy that targets fast proliferator cancer cells.
6. The method of claim 5, wherein the inhibitor of AKT1 degradation is administered before the administration of a cancer therapy that targets fast proliferator cancer cells.
7. The method of claim 6, wherein the inhibitor of AKT1 degradation is administered at least 1 day before the administration of a cancer therapy that targets fast proliferator cancer cells.
8. The method of claim 6, wherein the inhibitor of AKT1 degradation is administered at least 3 days before the administration of a cancer therapy that targets fast proliferator cancer cells.
9. The method of any of claims 4-8, wherein the subject has been determined to have a subpopulation of cancer cells expressing increased levels of one or more genes selected from the group consisting of:
 - Hes1 and TTC3;
 - or decreased levels of one or more genes selected from the group consisting of:

AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph; H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.

wherein an increased level is a level statistically significantly higher than the level of expression found in at least 70% of cancer cells obtained from the same tumor and a decreased level is a level statistically significantly lower than the level of expression found in at least 70% of cancer cells obtained from the same tumor.

10. The method of claim 9, wherein the subject has been determined to have cancer cells expressing increased levels of TTC3; and optionally,
increased levels of Hes1;
or decreased levels of one or more genes selected from the group consisting of:
AKT1; H3K9me2; MCM2; MK167; CDC6; GMNN; AURKA; PLK1; H3S10ph;
H3K4me2; H3K9me2; H3K27me3; H4K12ac; and H4K16ac.
11. The method of claim 9, wherein the subject has been determined to have cancer cells expressing increased levels of Hes1 and TTC3 and decreased levels AKT1; H3K9me2; and MCM2.
12. The method of claim 9, wherein the expression level of the one or more genes is the level of polypeptide expression product.
13. The method of claim 9, wherein the expression level is determined by immunochemistry.
14. The method of any of claims 1-13, wherein the agonist of β 1-integrin is a β 1-integrin activating antibody reagent.
15. The method of claim 14, wherein the β 1-integrin activating antibody reagent is TS2/16 monoclonal antibody or 12G10 monoclonal antibody.

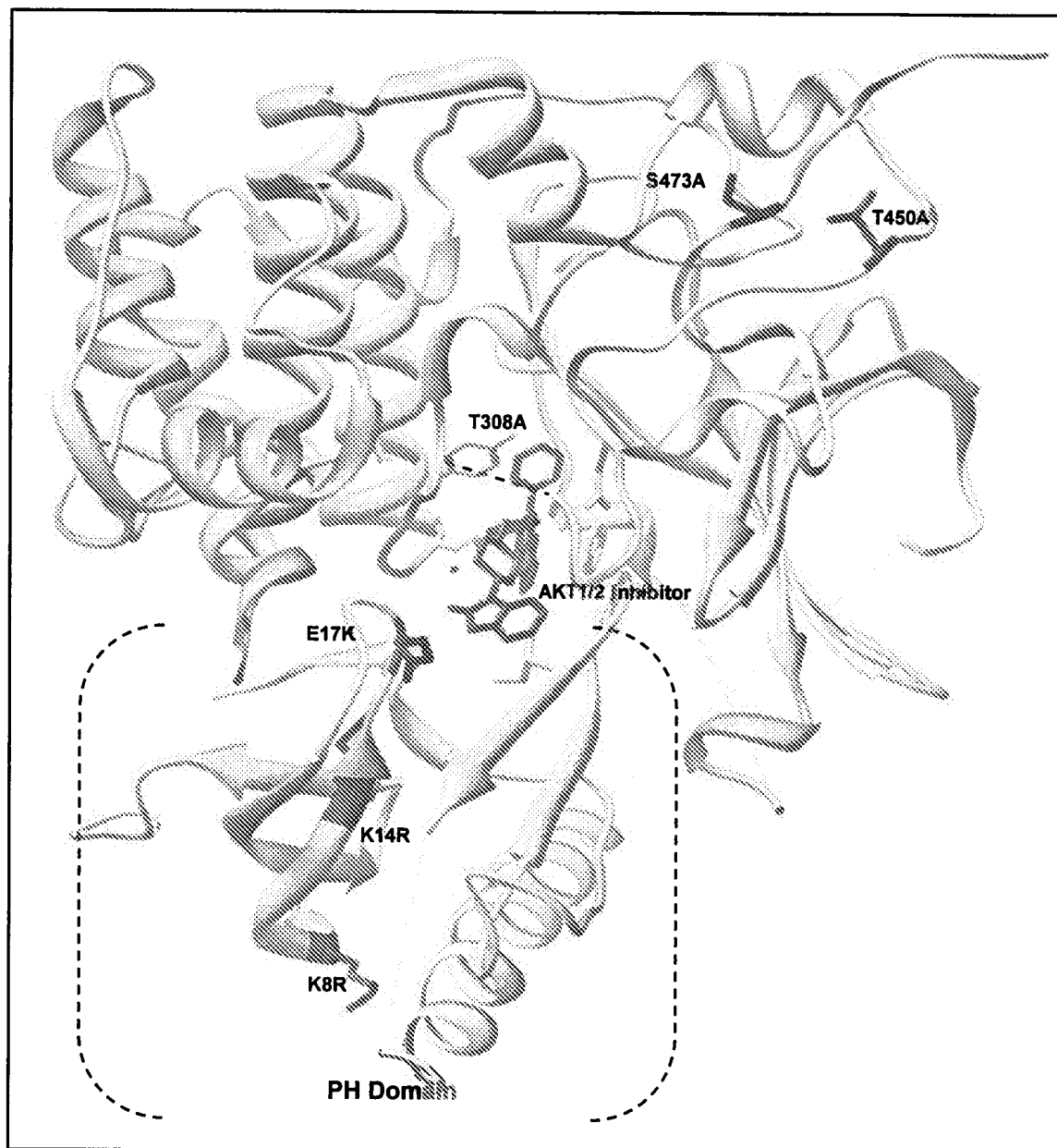


Figure 1A

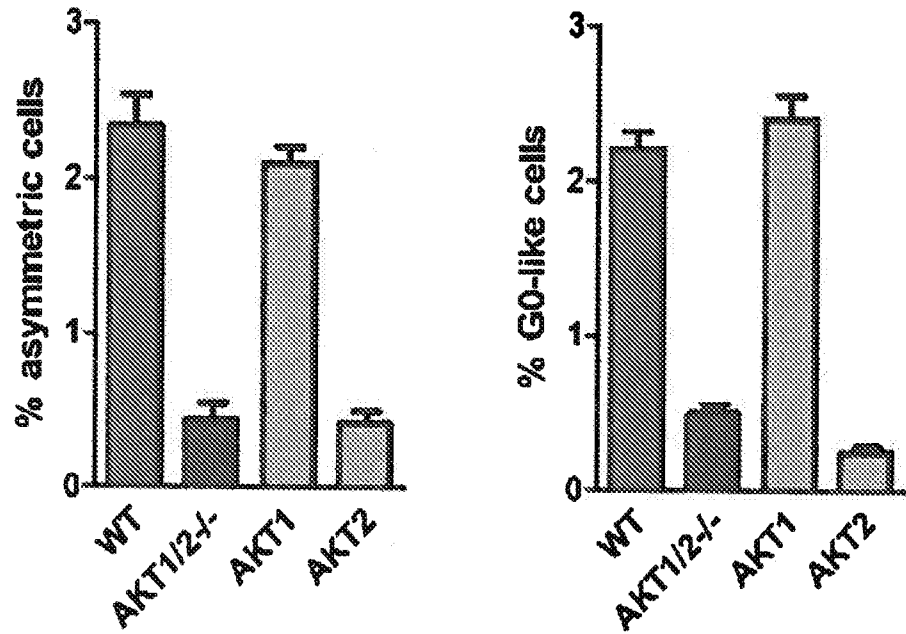


Figure 1B

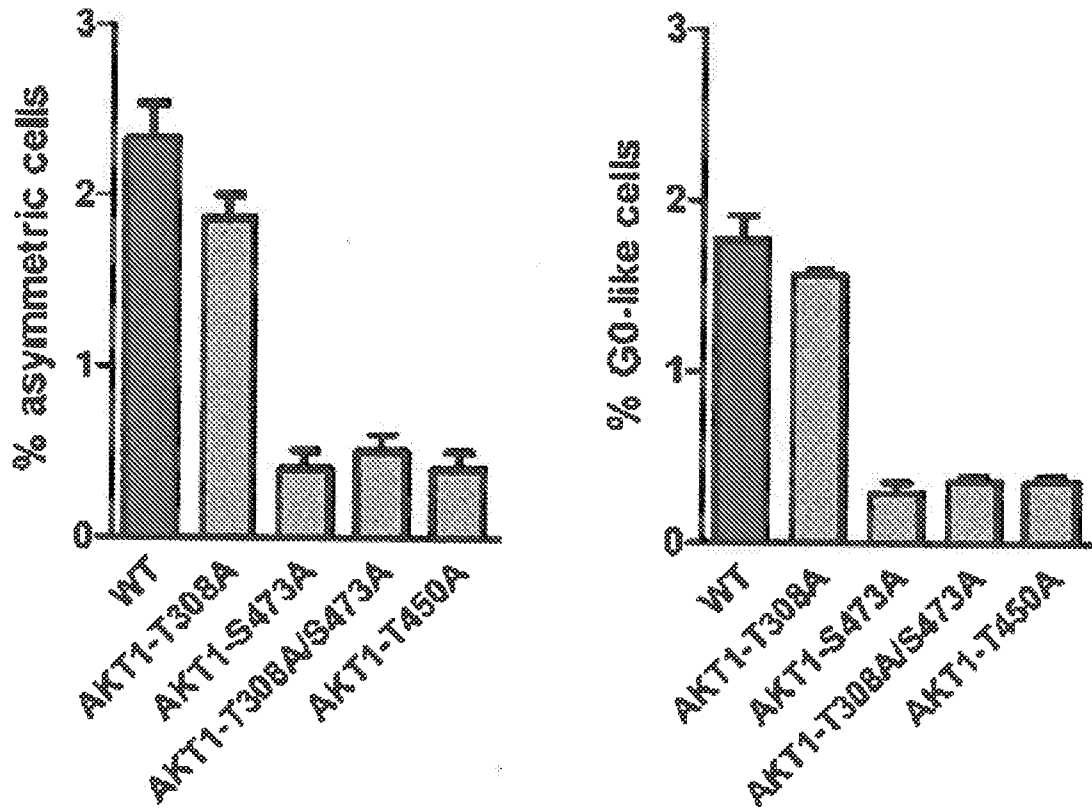


Figure 1C

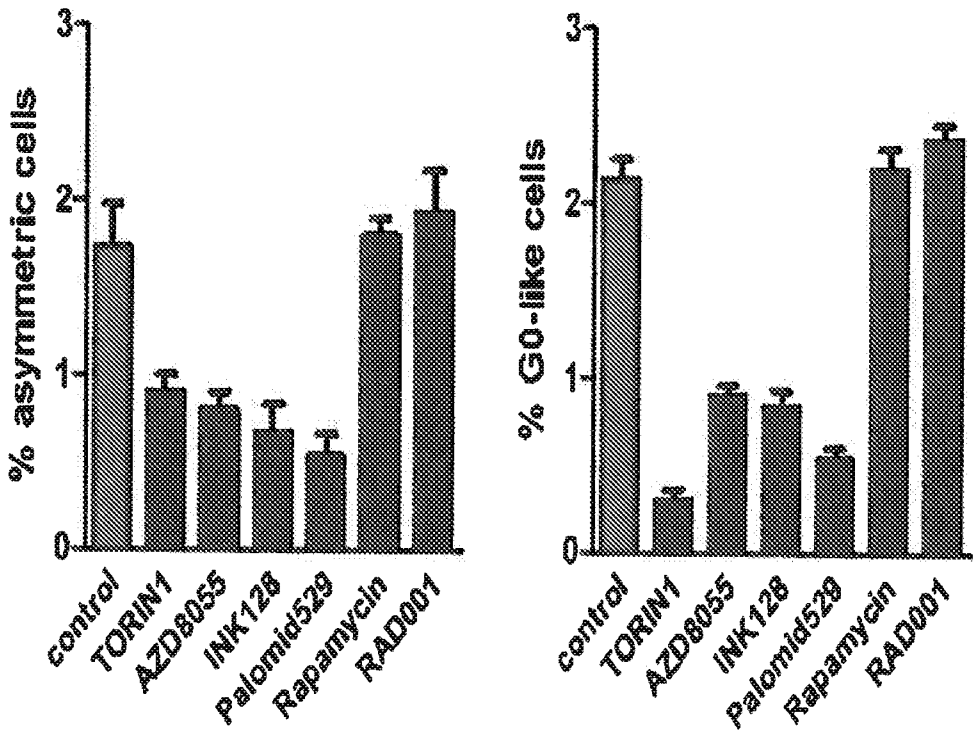


Figure 1D

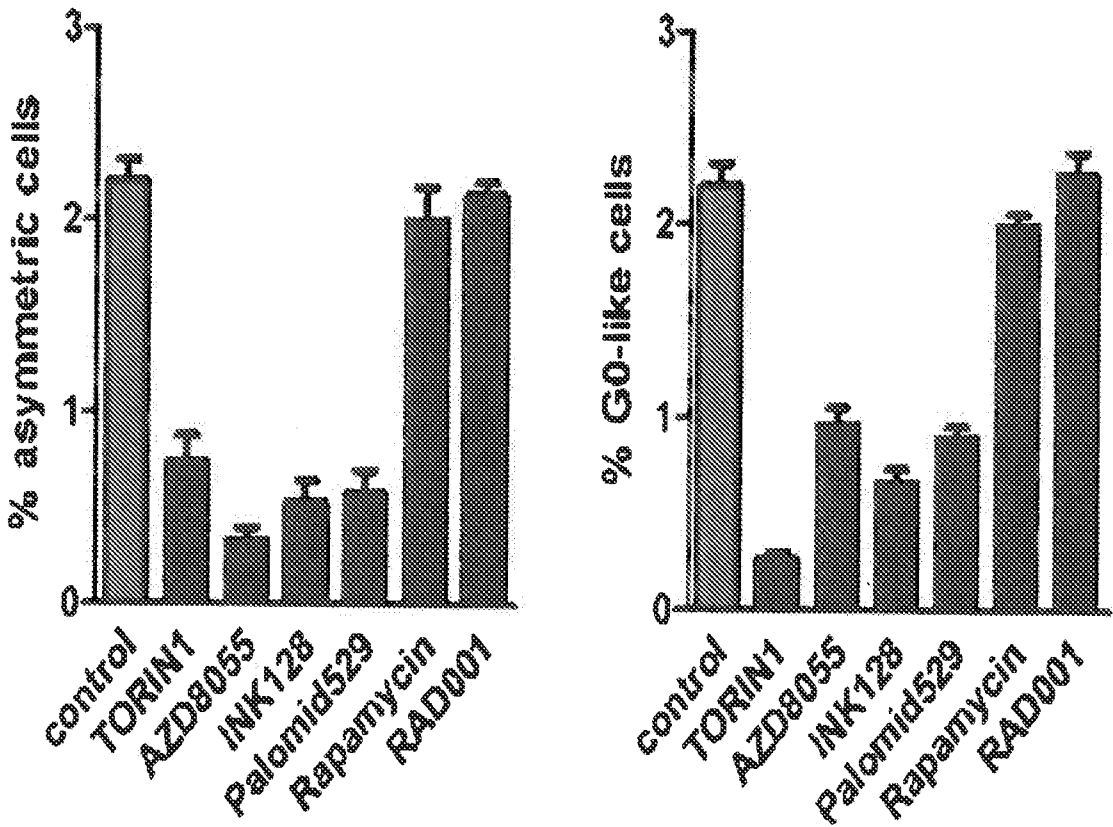


Figure 1E

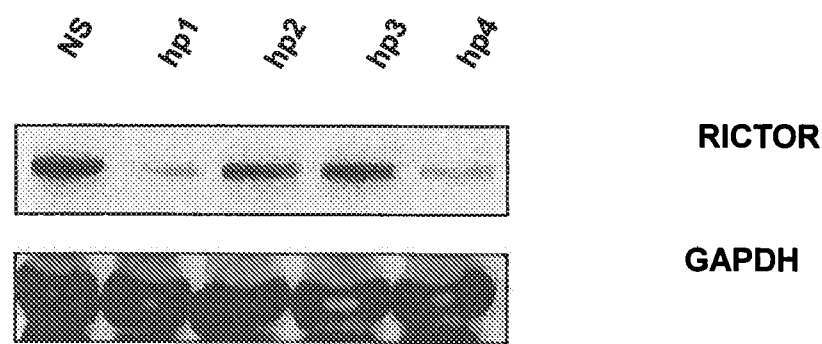


Figure 1F

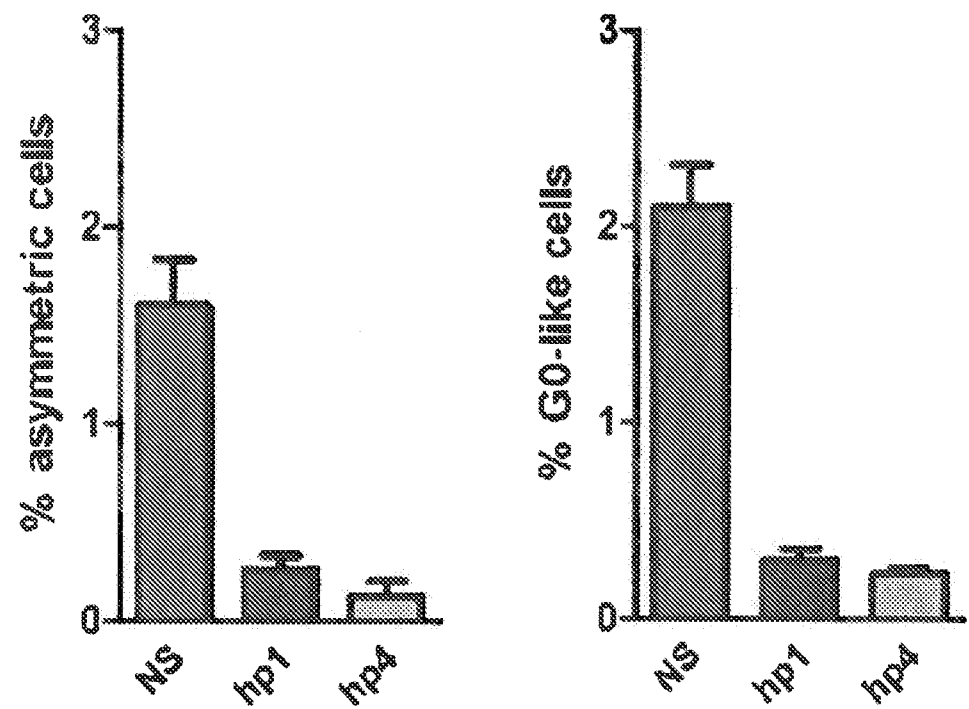


Figure 1G

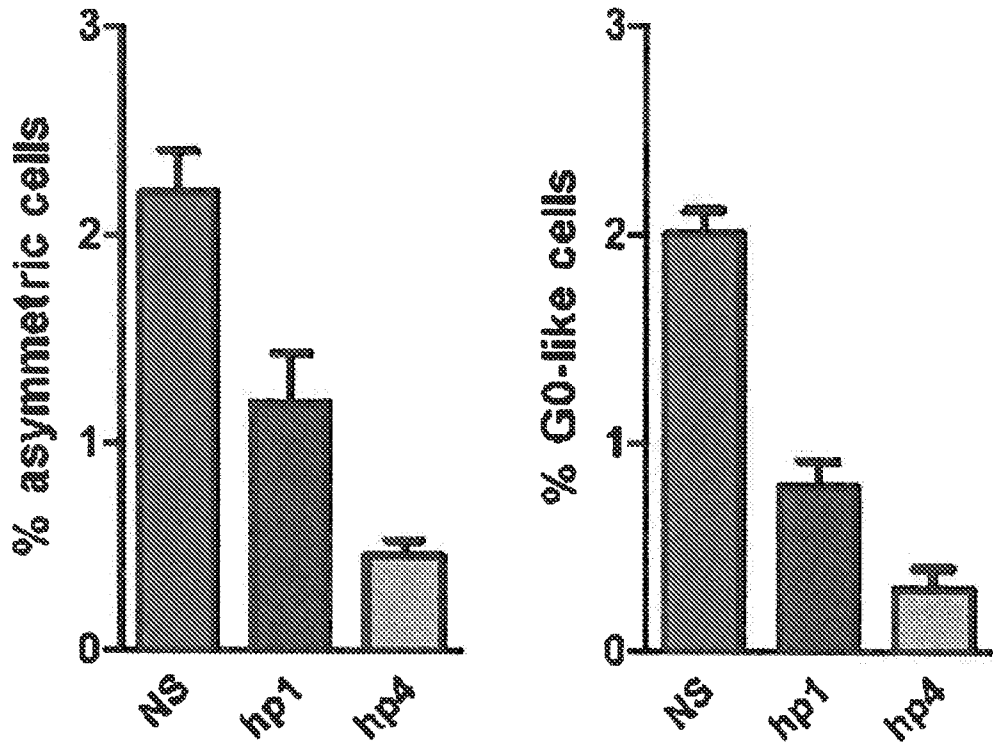


Figure 1H

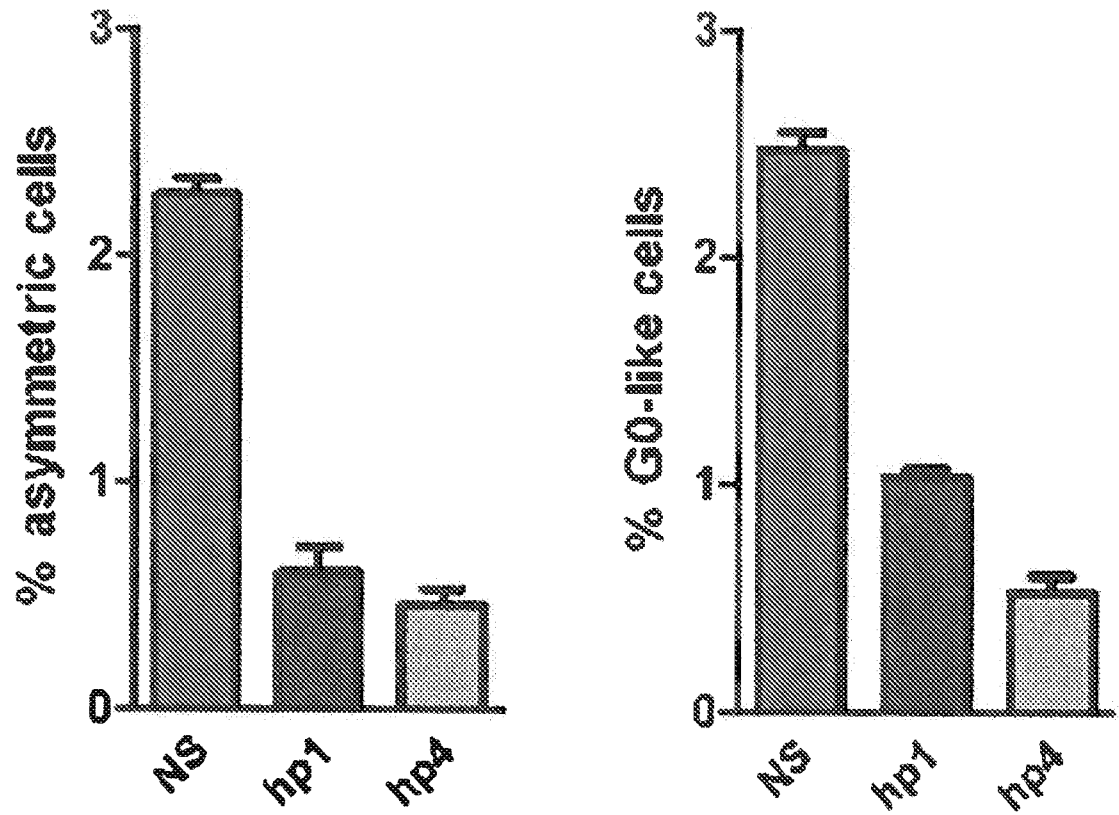


Figure 1I

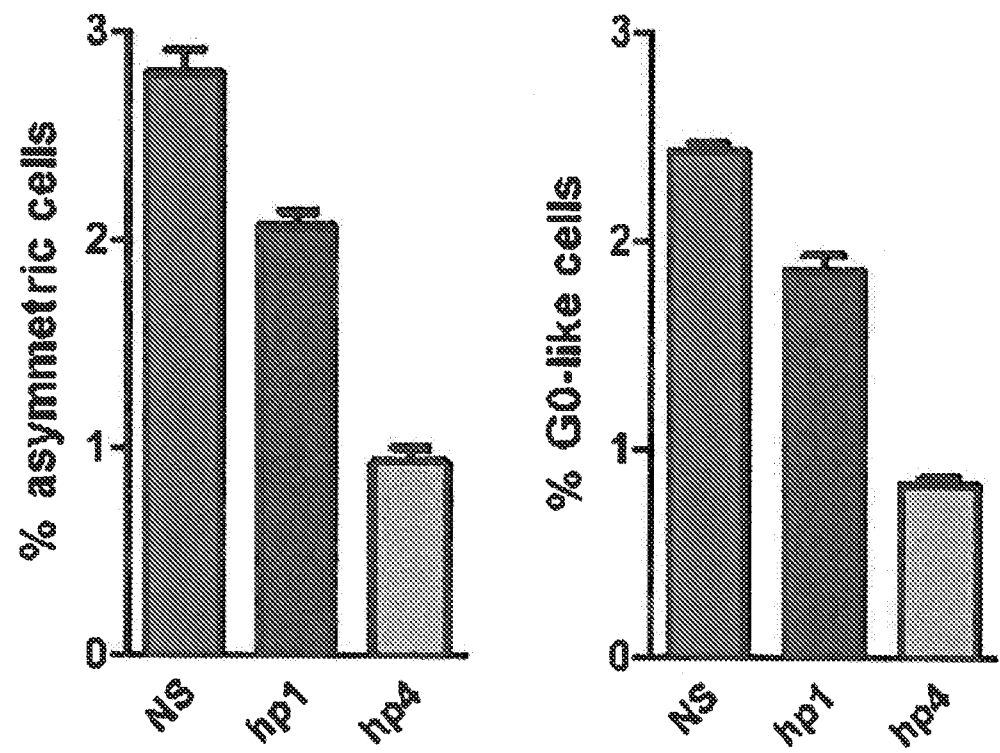


Figure 1J

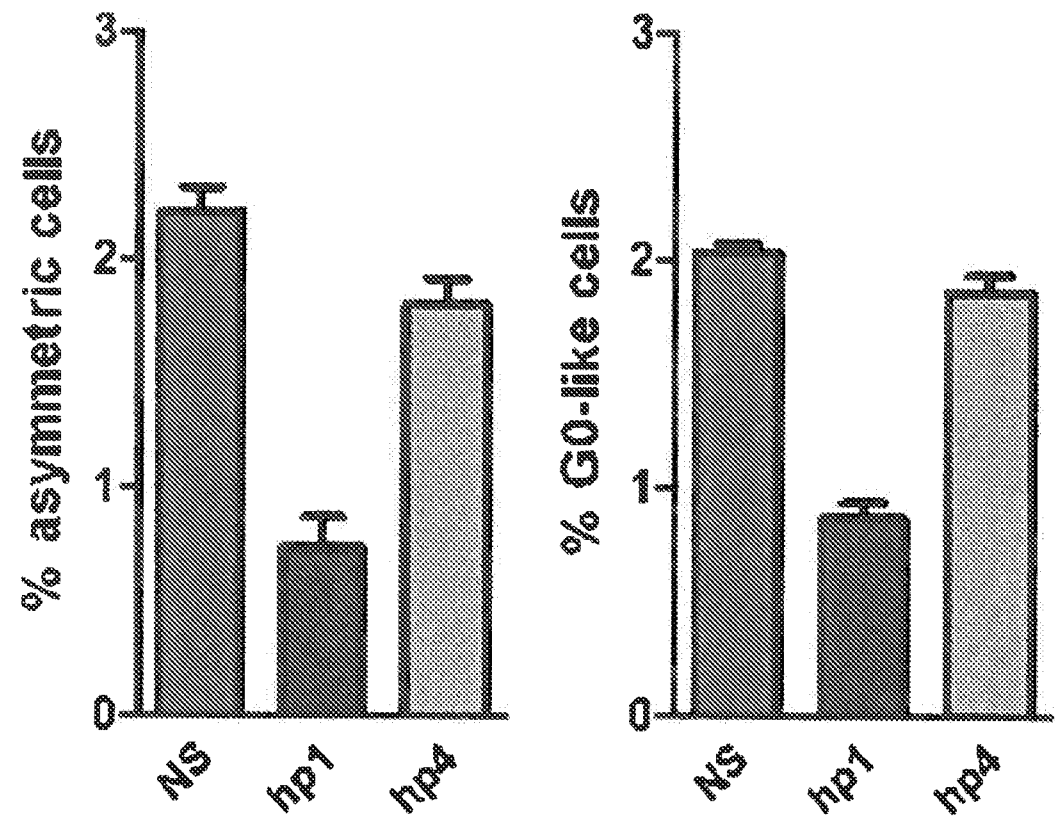


Figure 1K

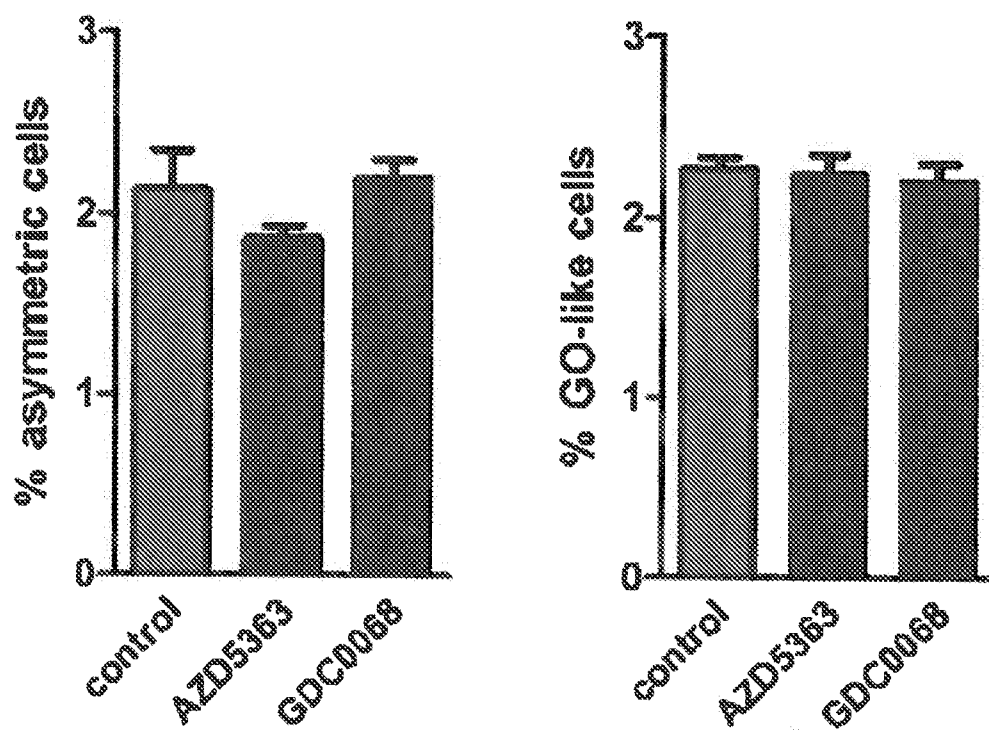


Figure 1L

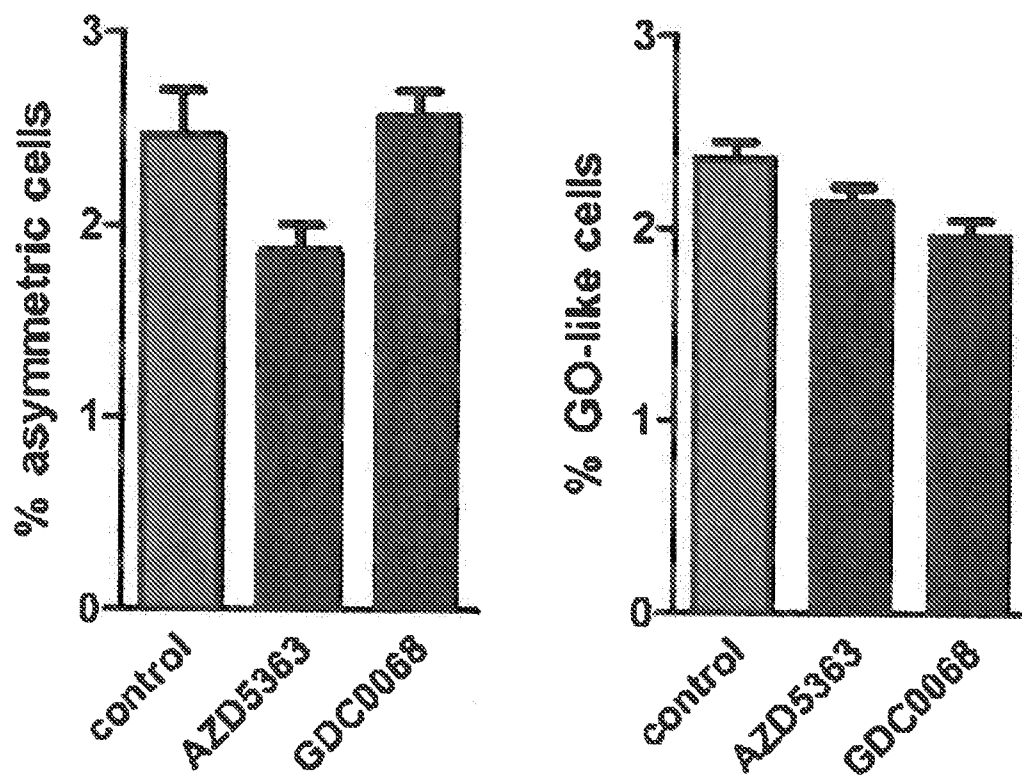


Figure 1M

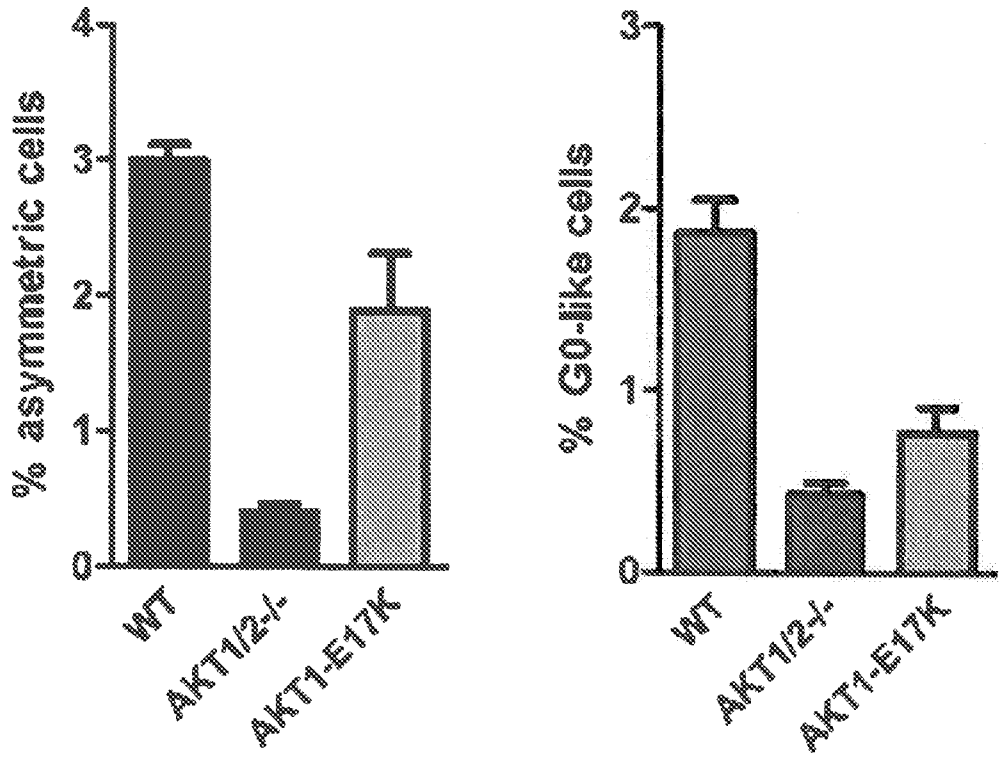


Figure 1N

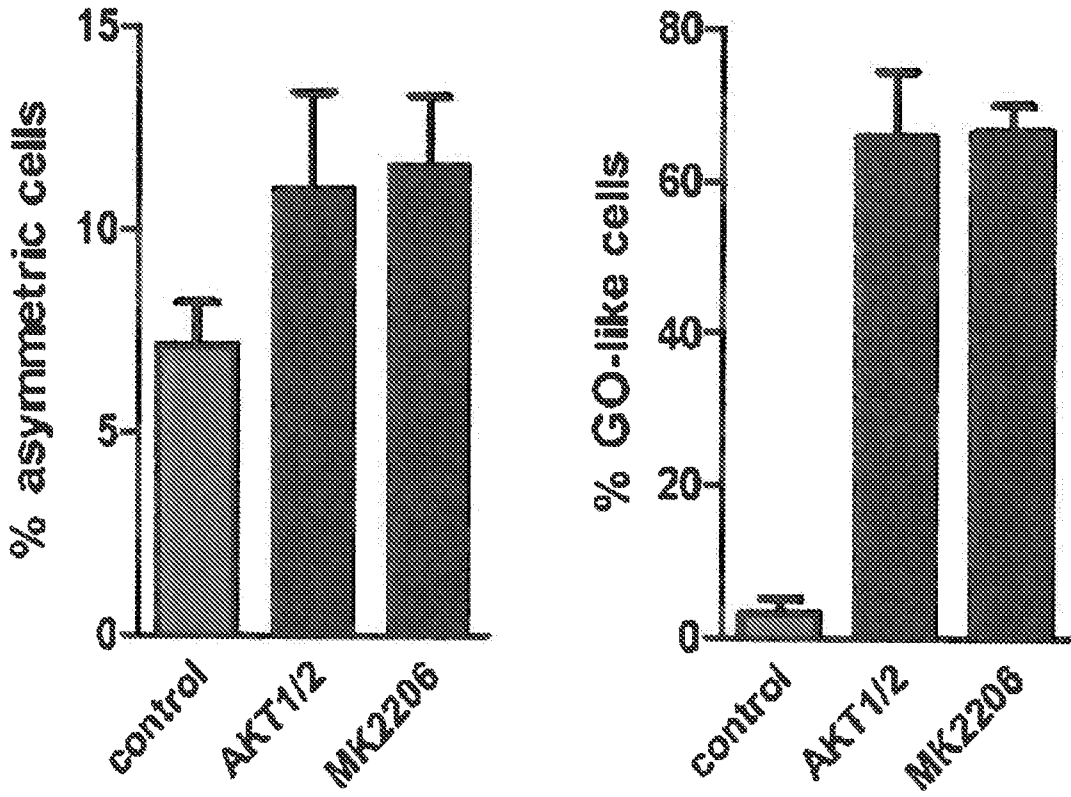


Figure 1O

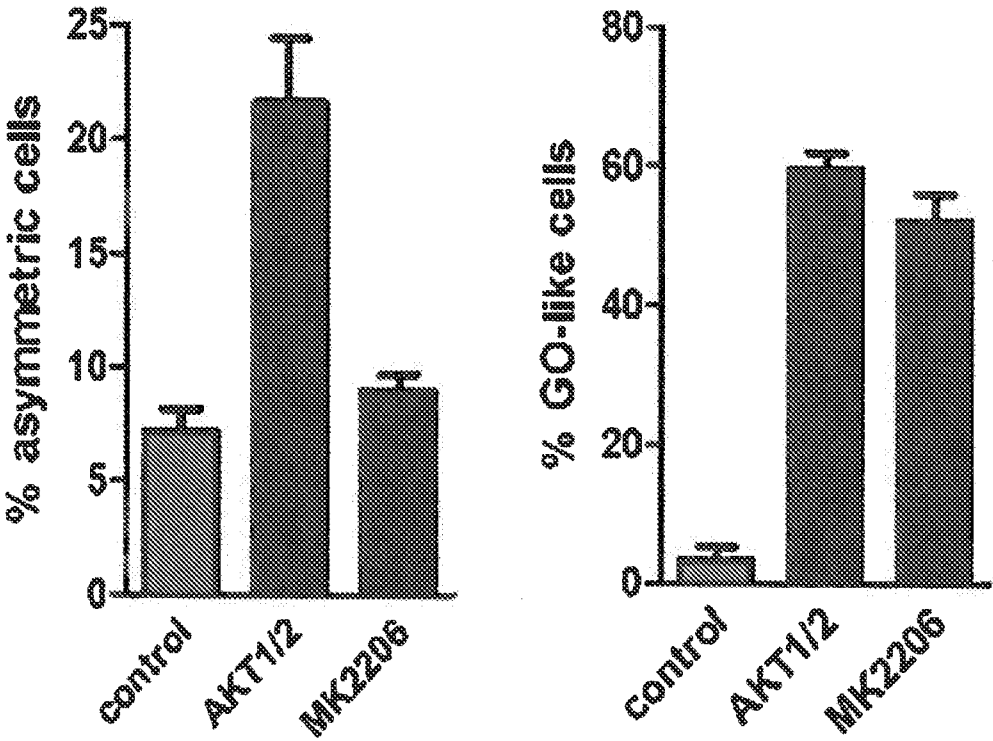


Figure 1P

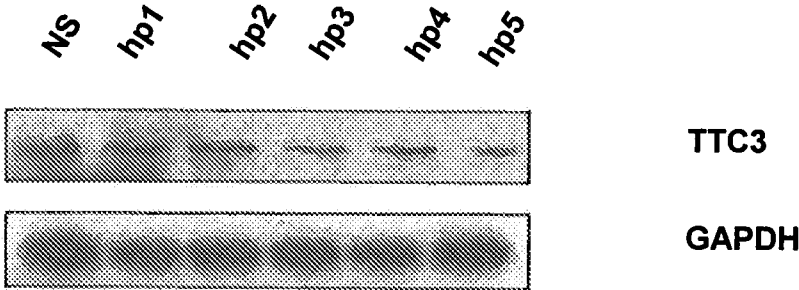


Figure 2A

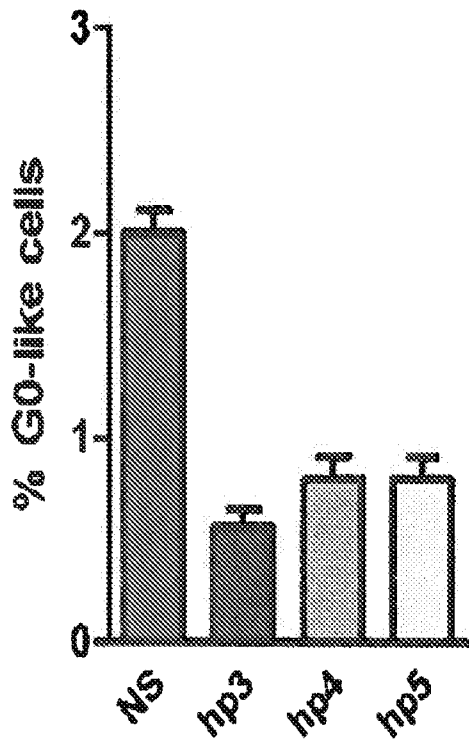


Figure 2B

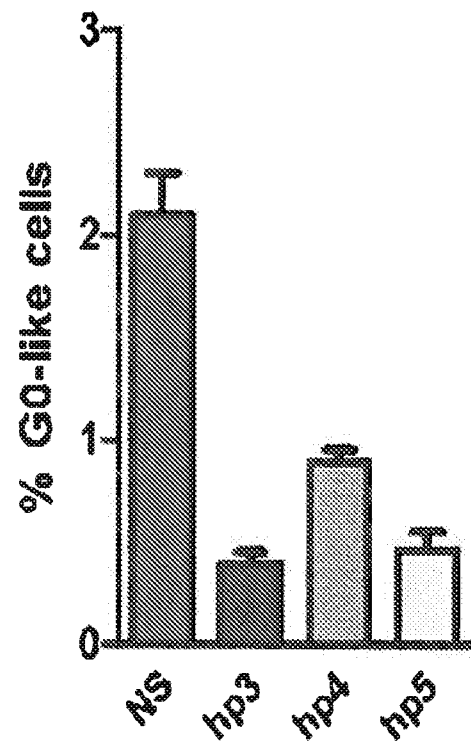


Figure 2C

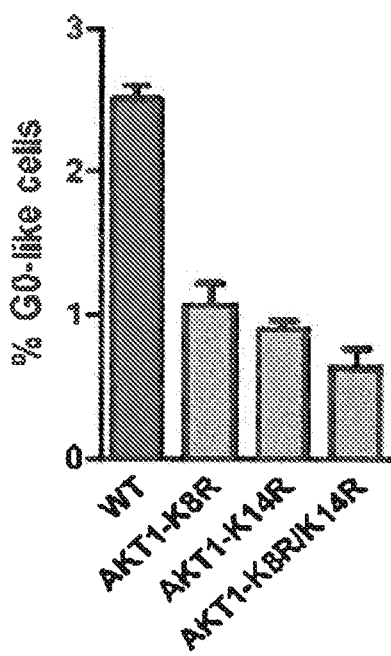


Figure 2D

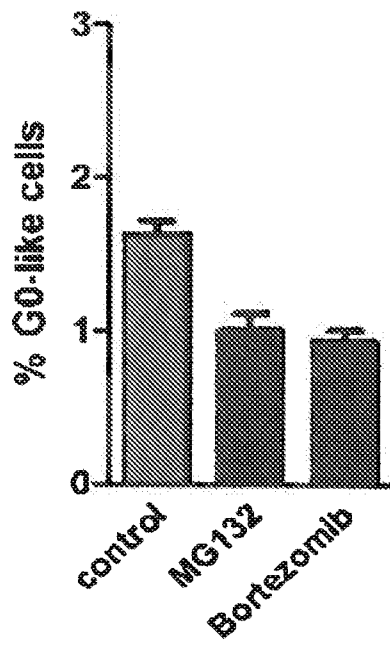


Figure 2E

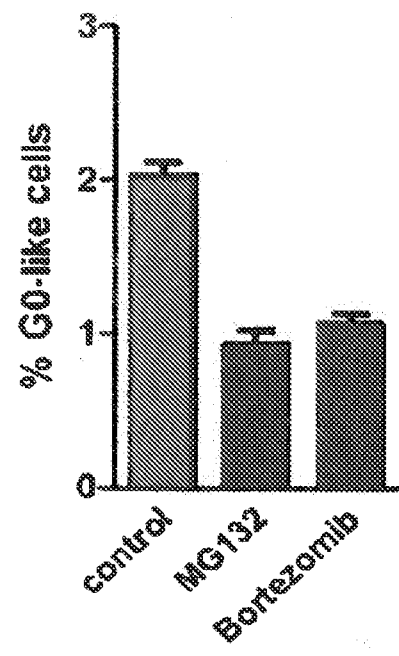


Figure 2F

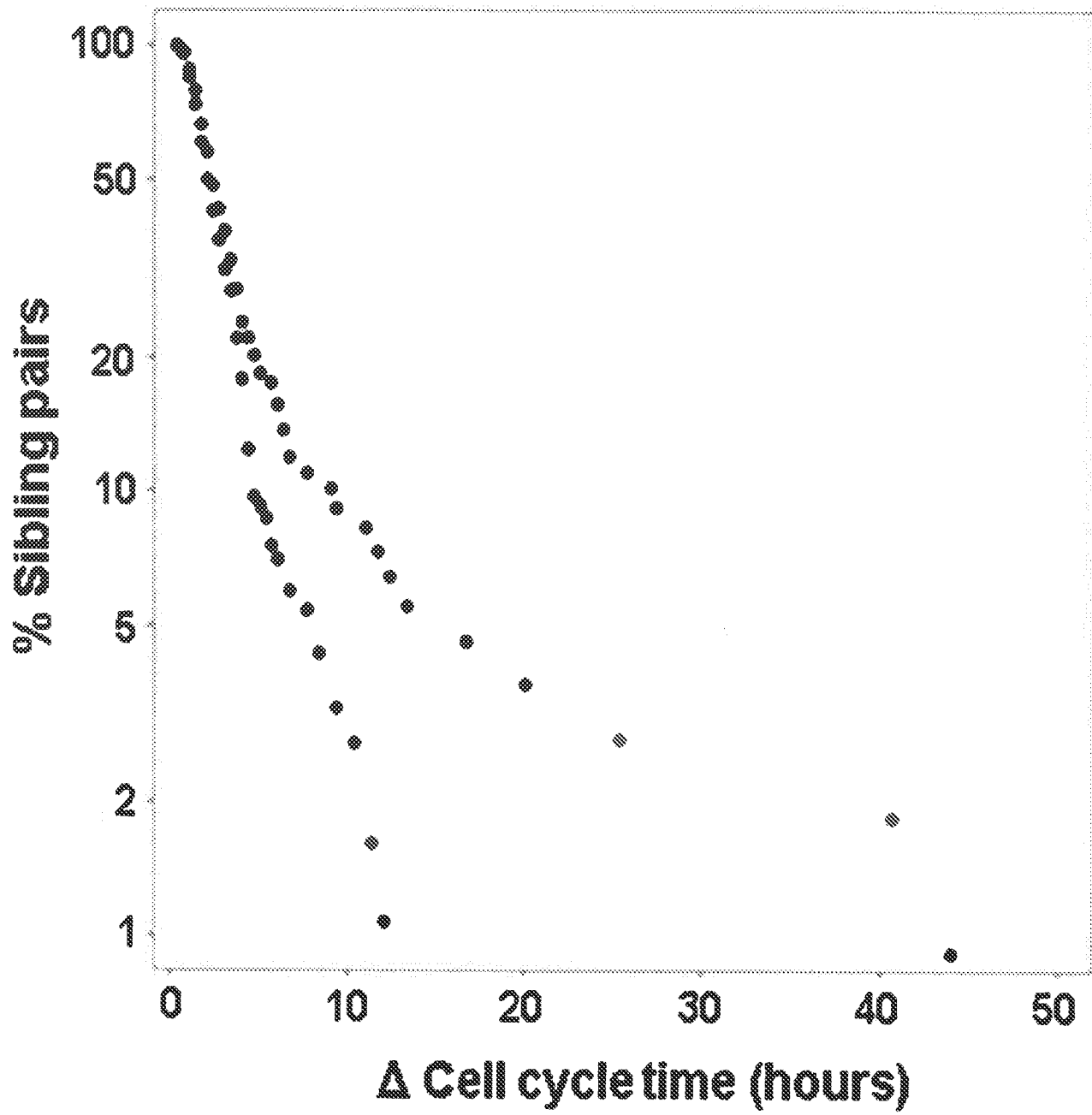


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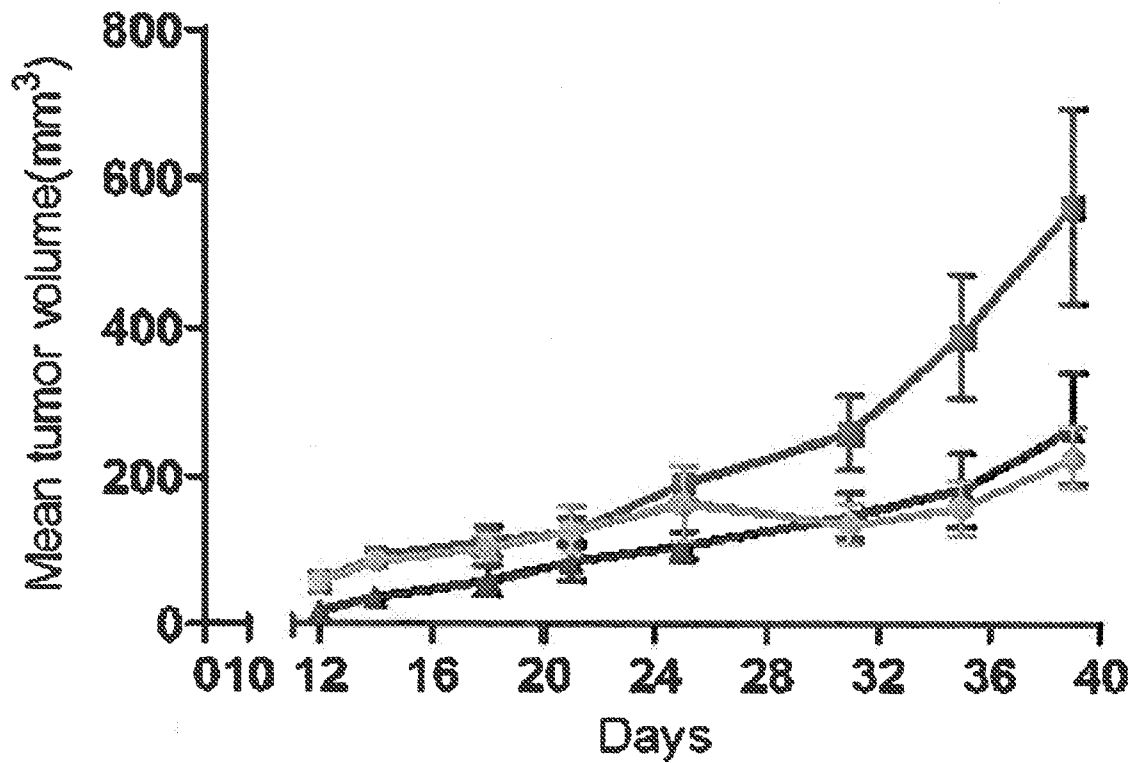


Figure 4A

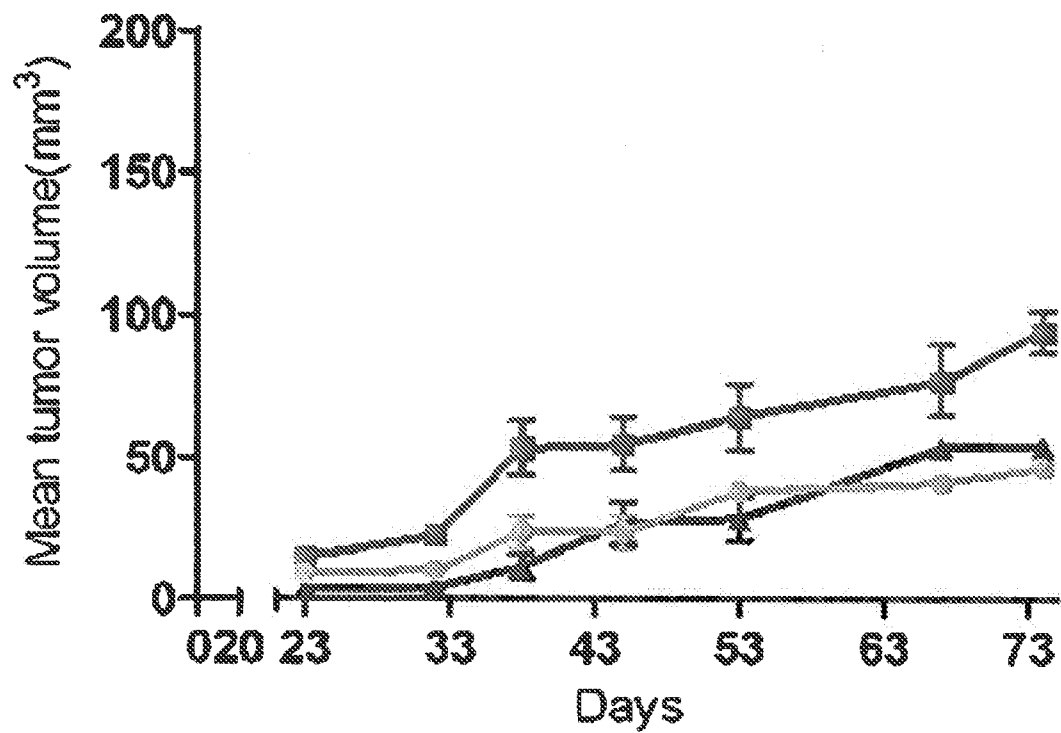


Figure 4B

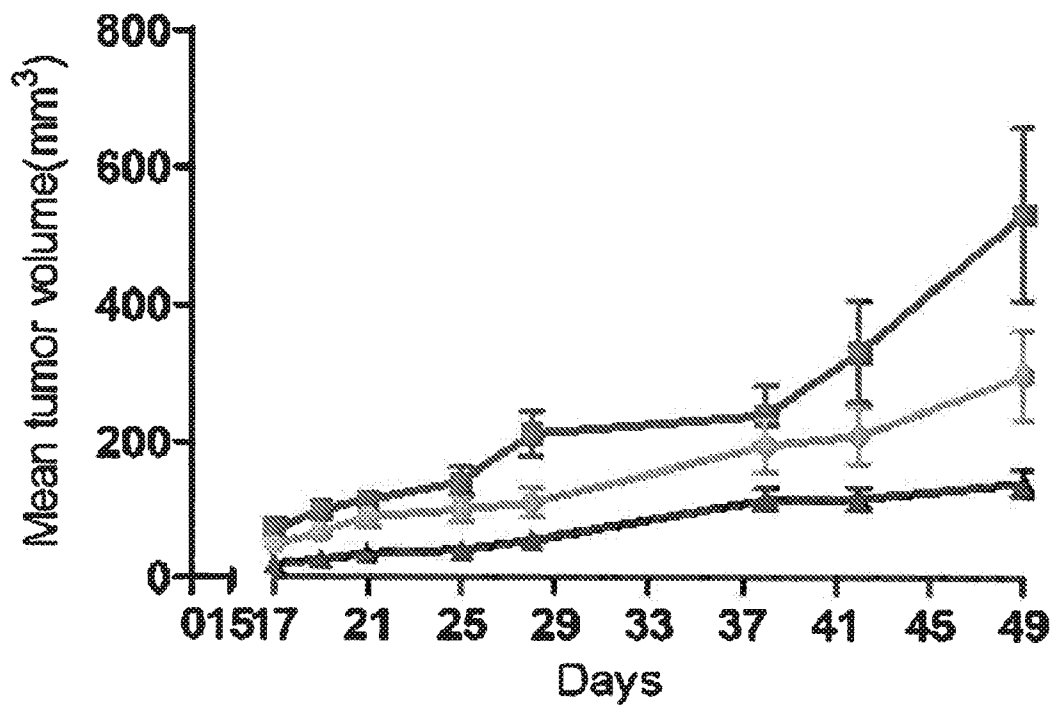


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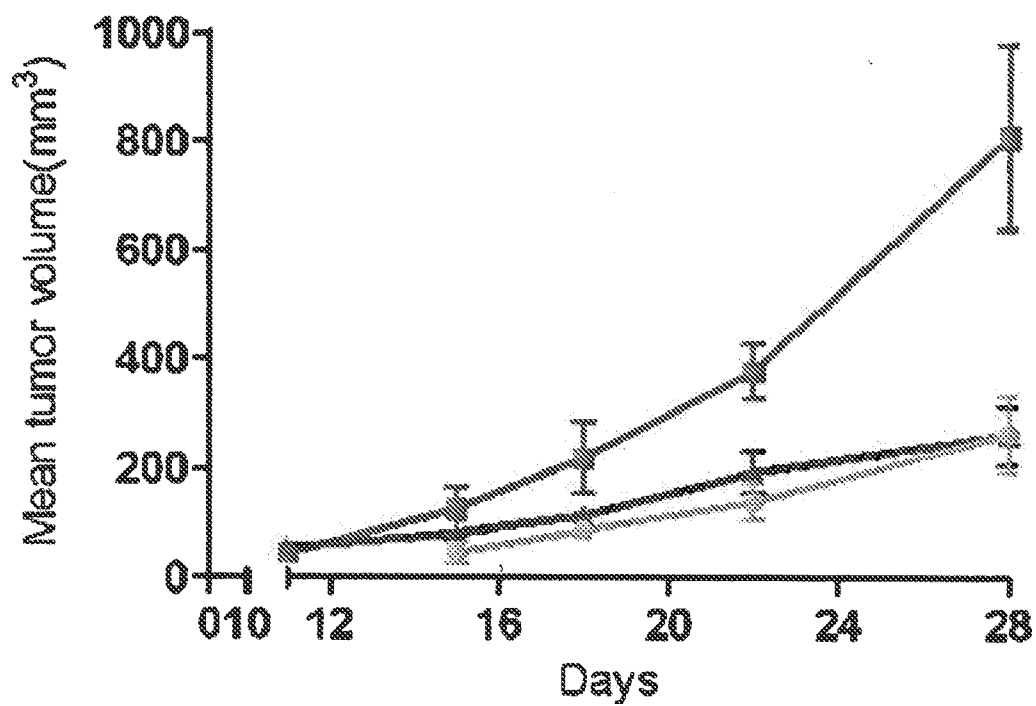


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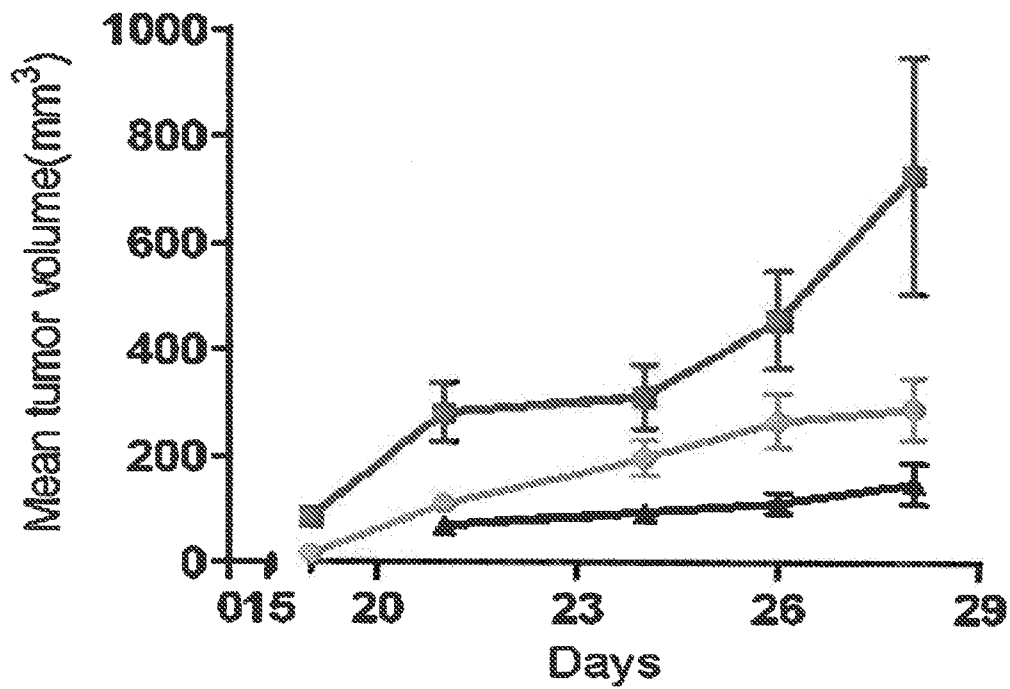


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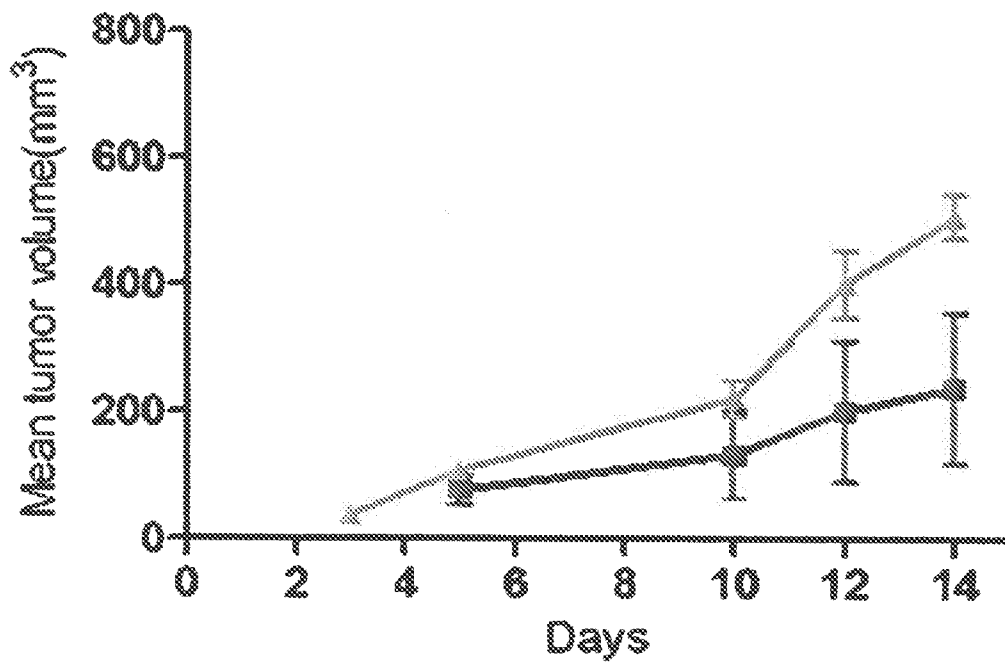


Figure 4F

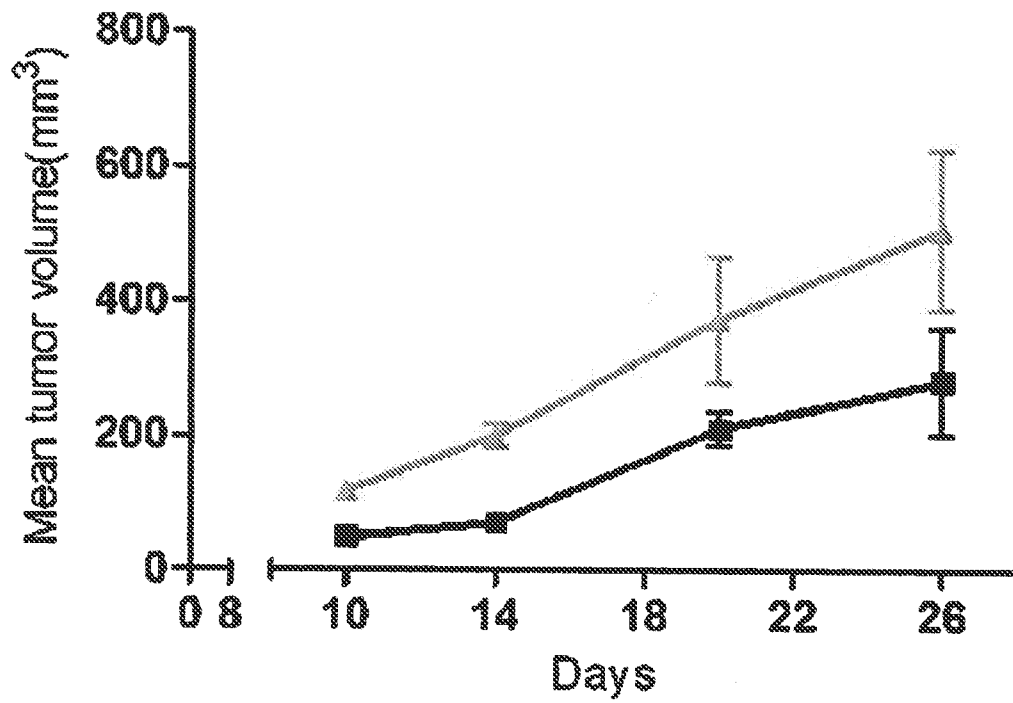


Figure 4G

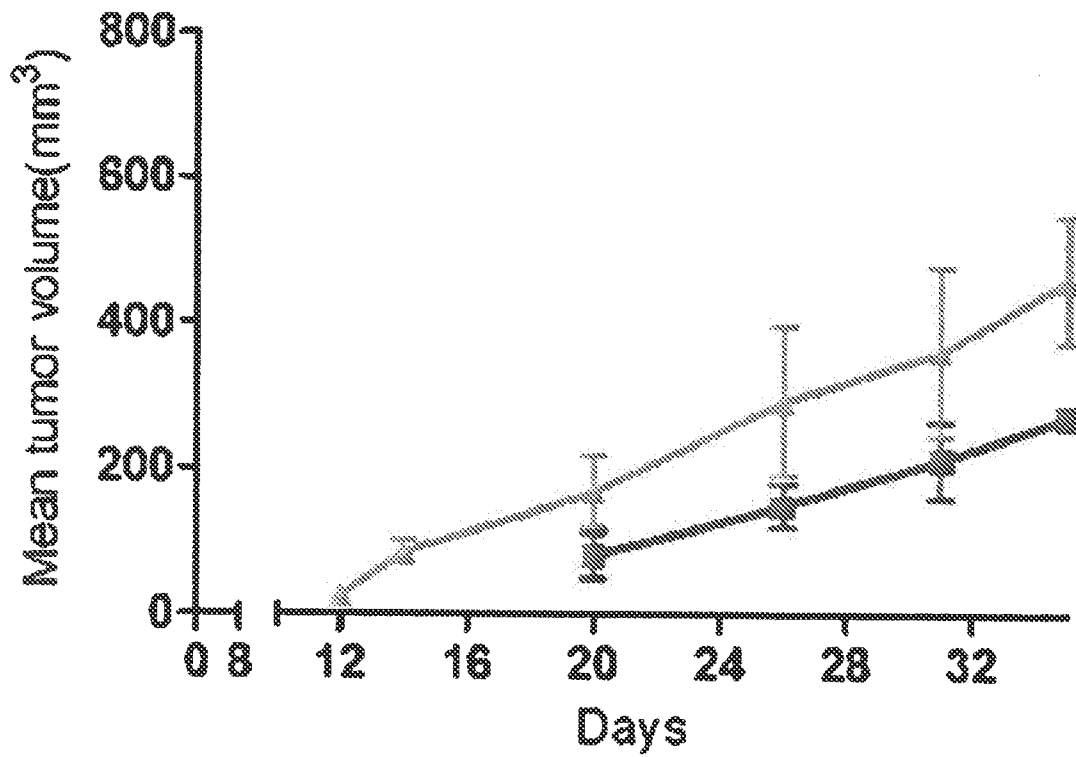


Figure 4H

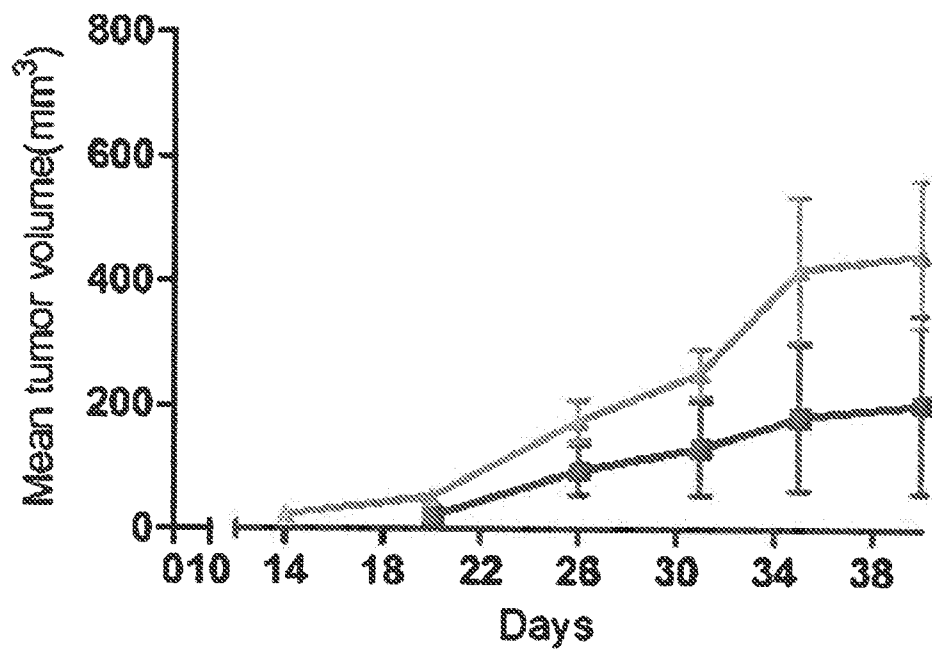


Figure 4I

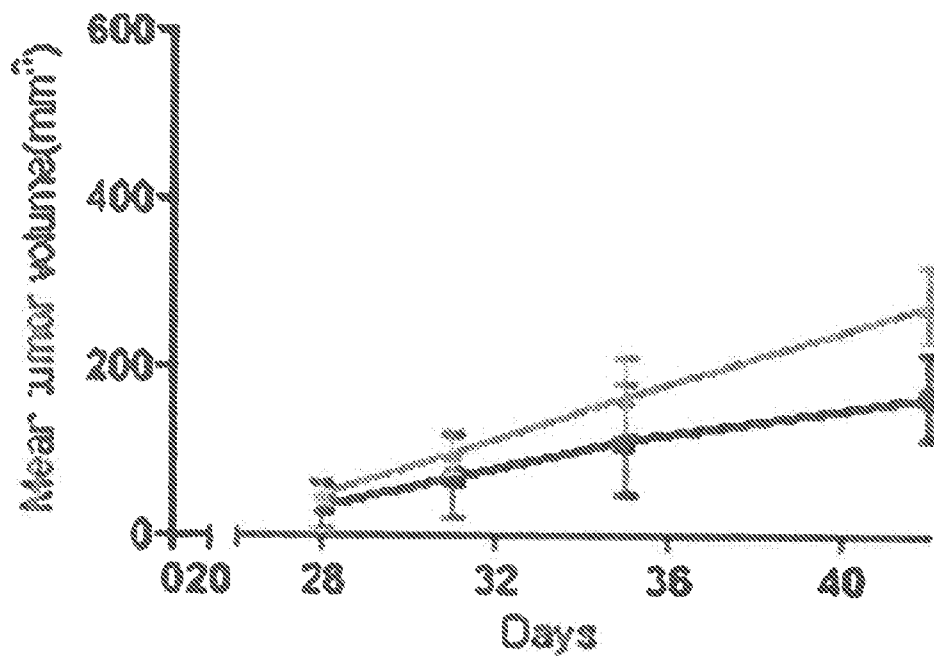


Figure 4J

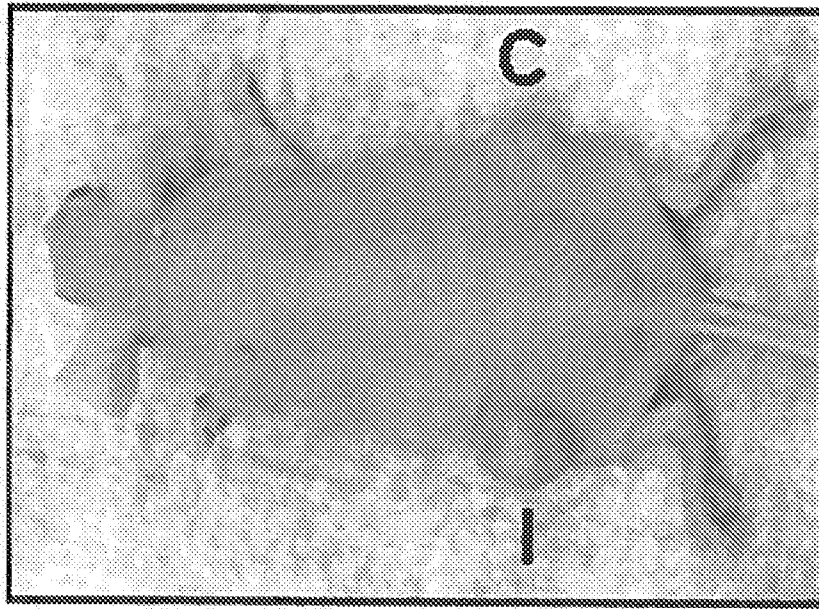


Figure 4K

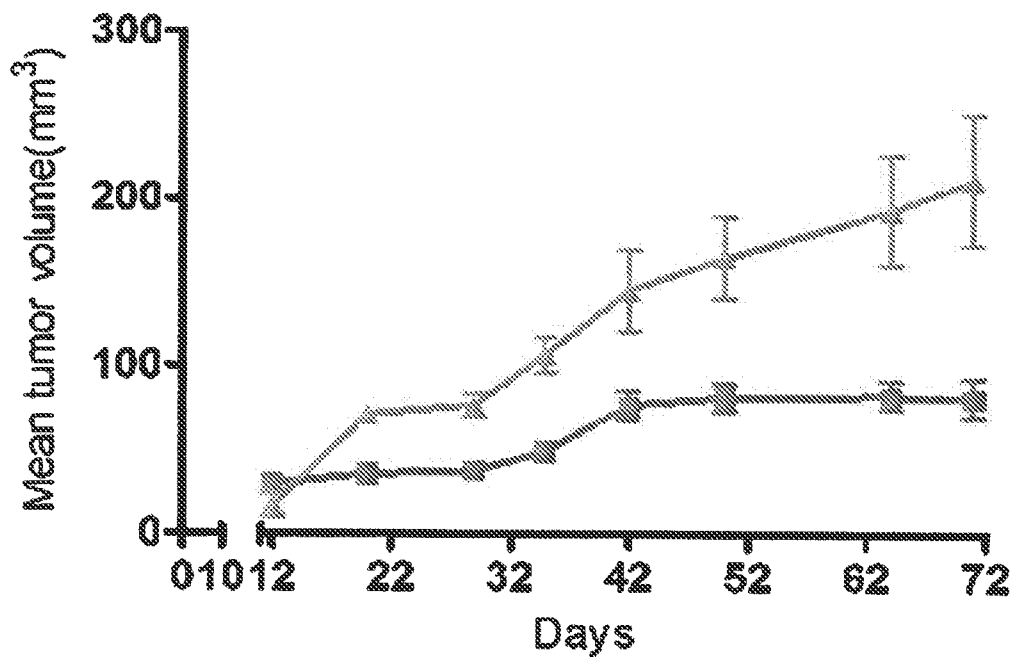


Figure 4L

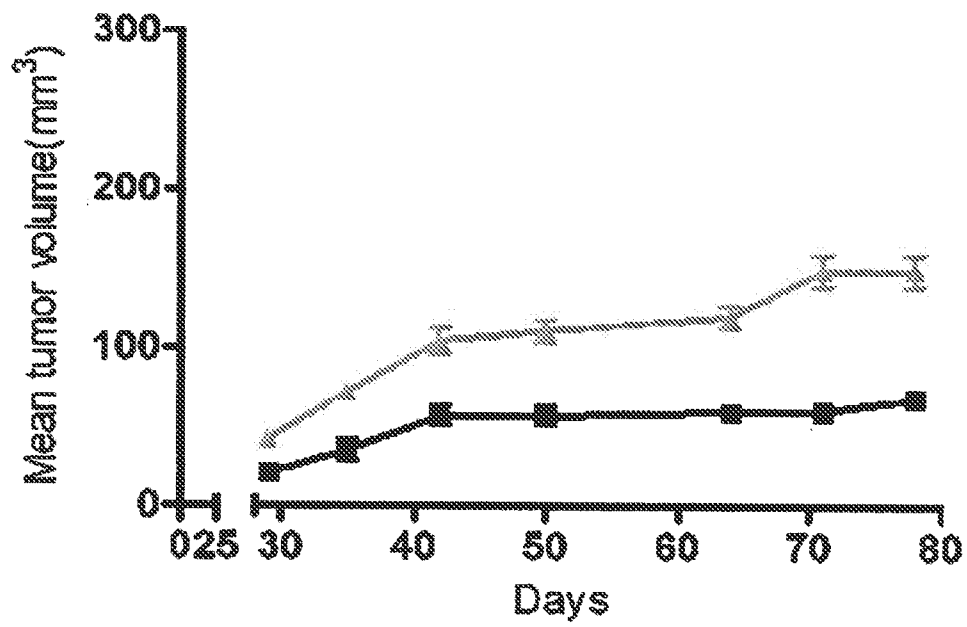


Figure 4M

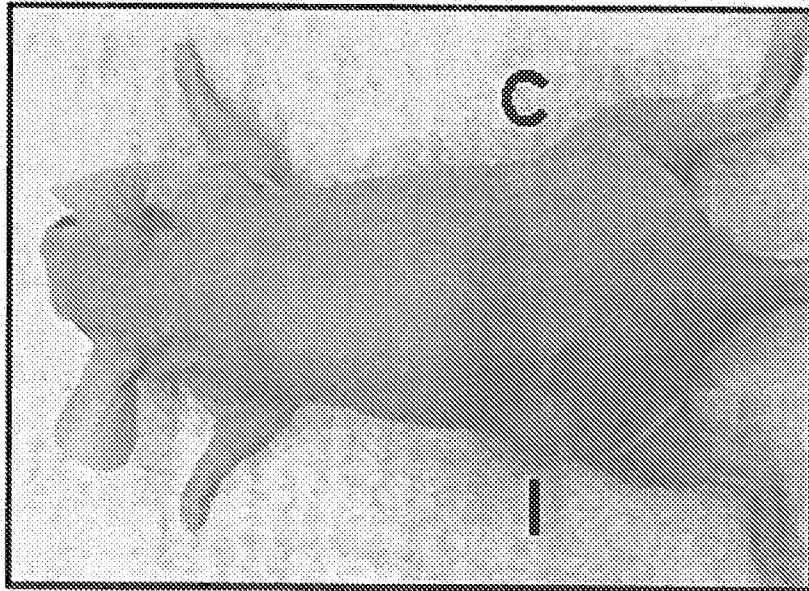


Figure 4N

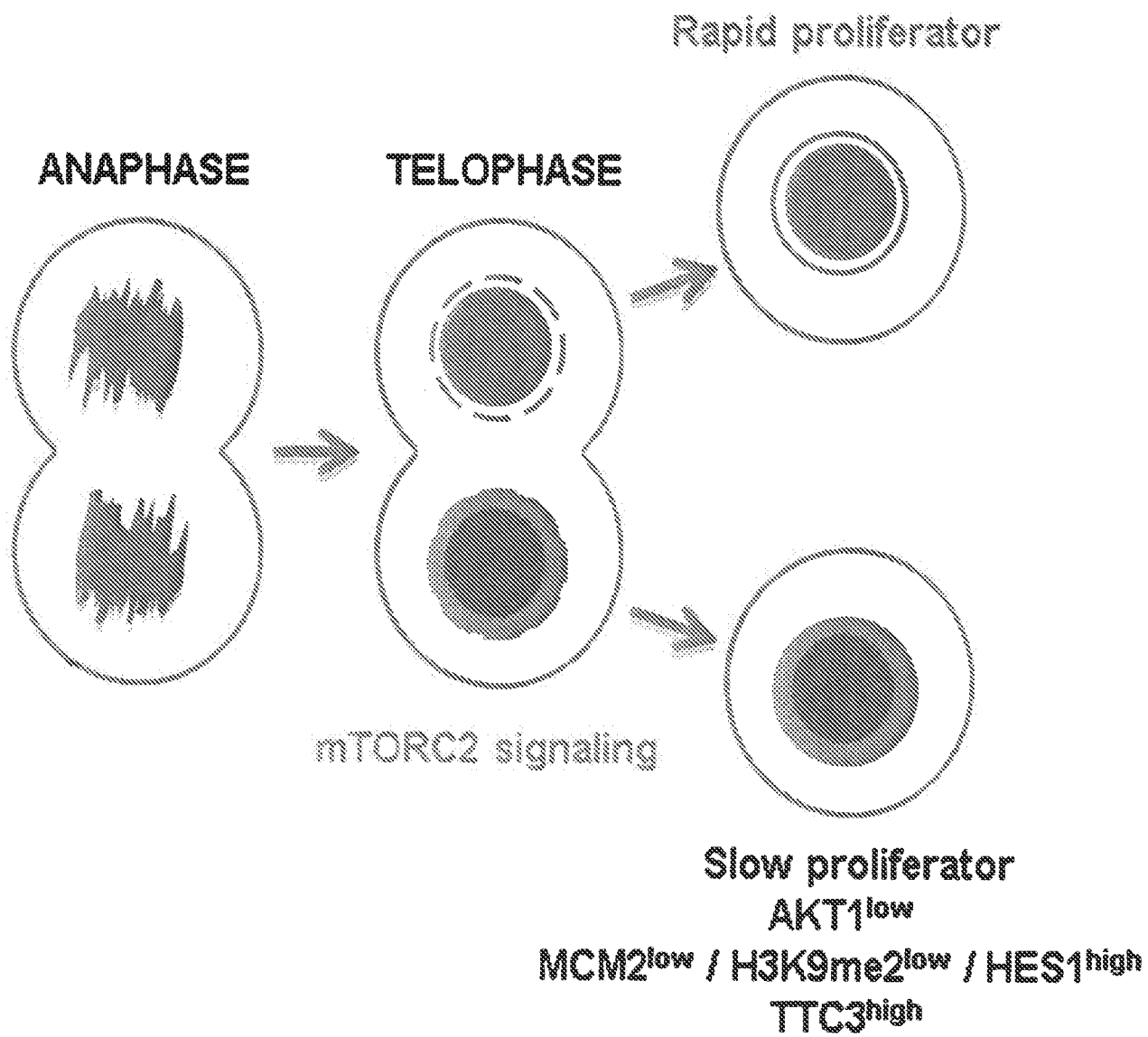


Figure 5

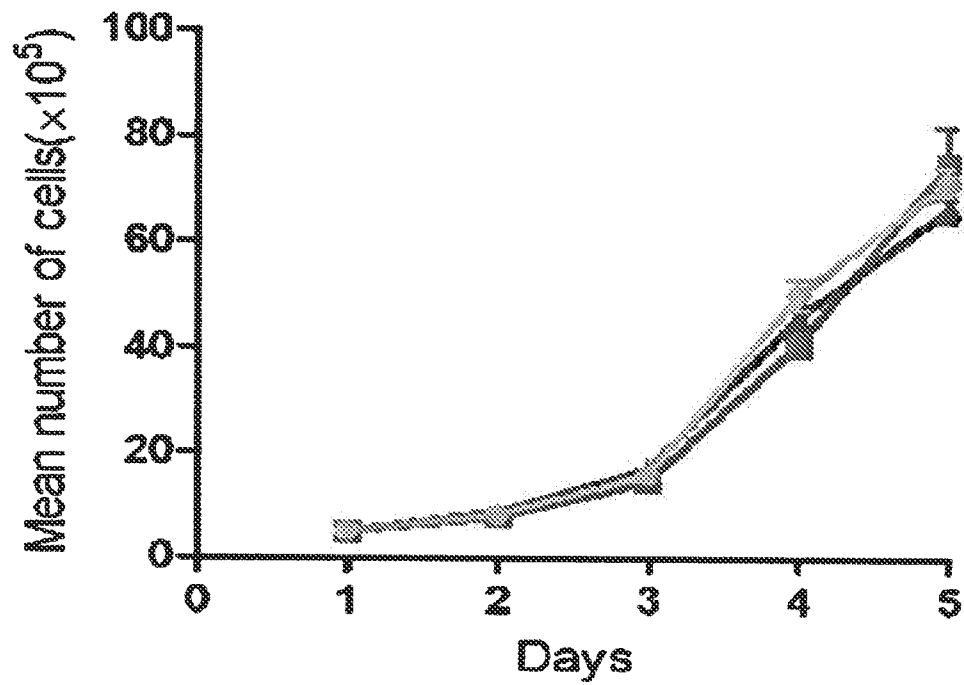


Figure 6A

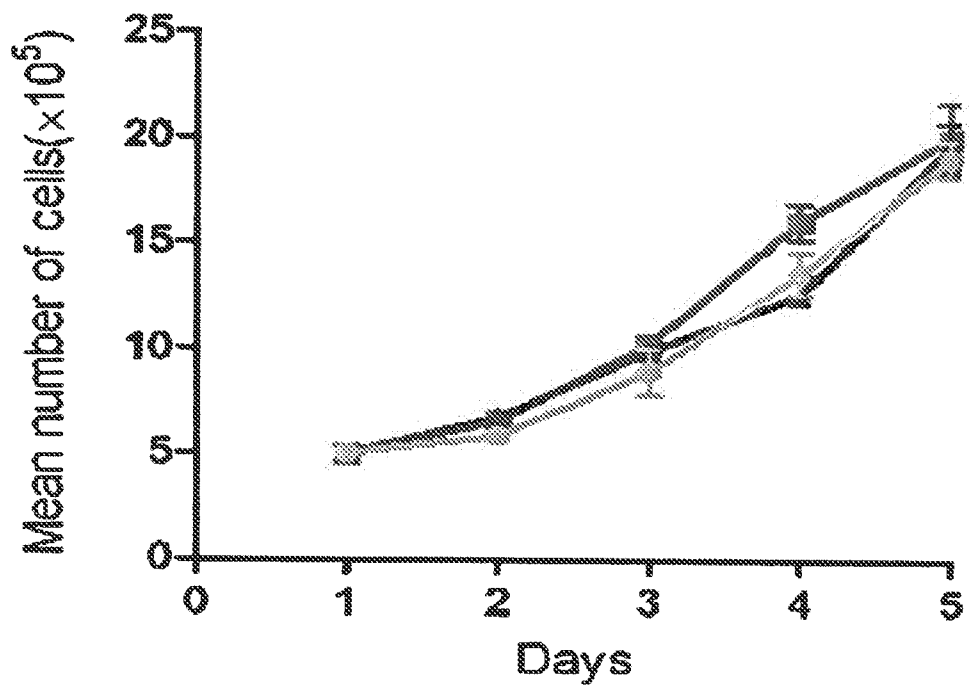


Figure 6B

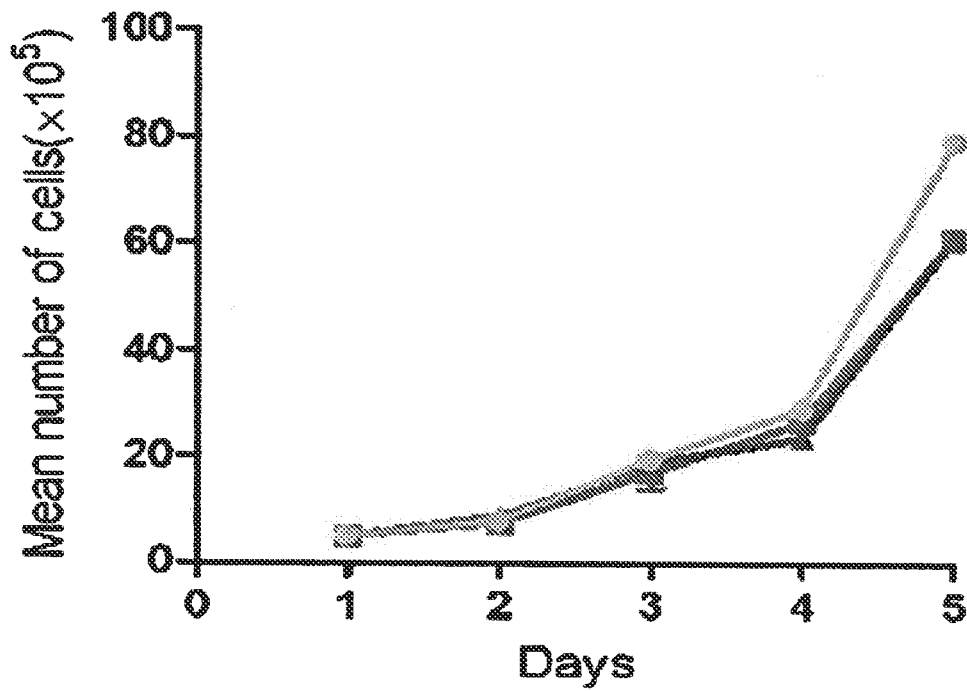


Figure 6C

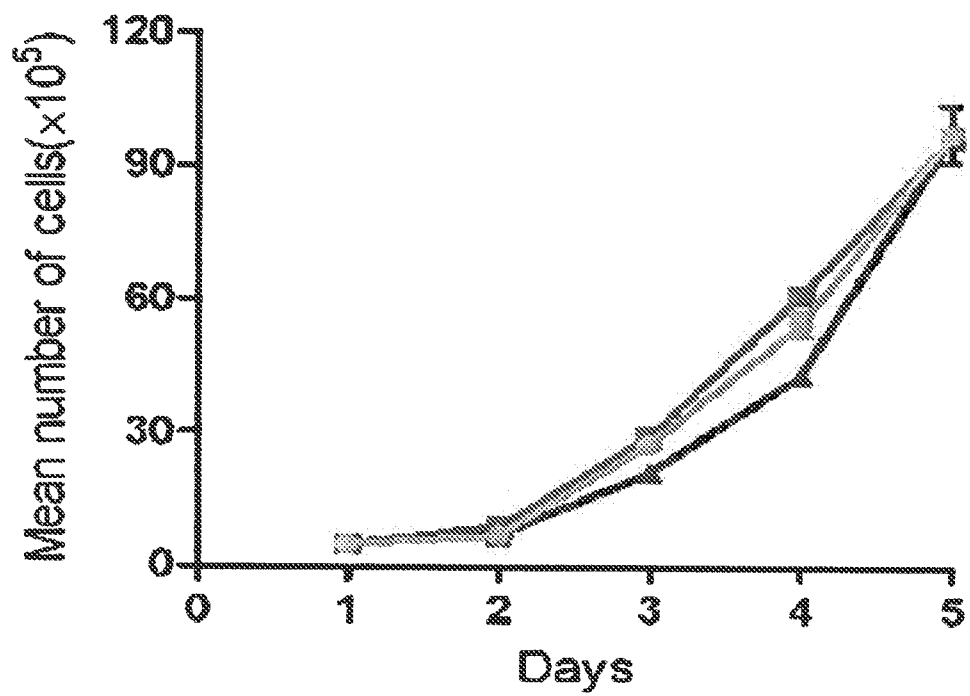


Figure 6D

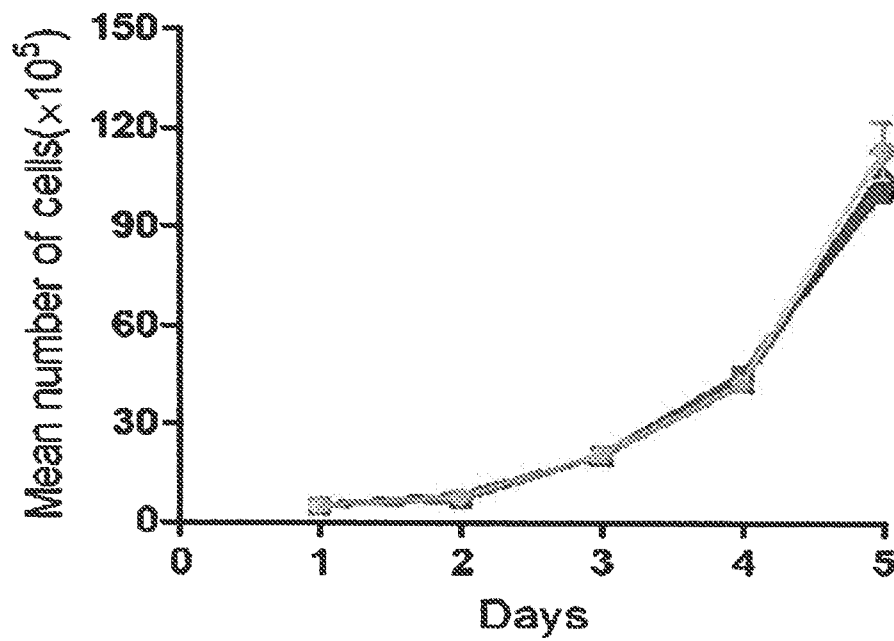


Figure 6E

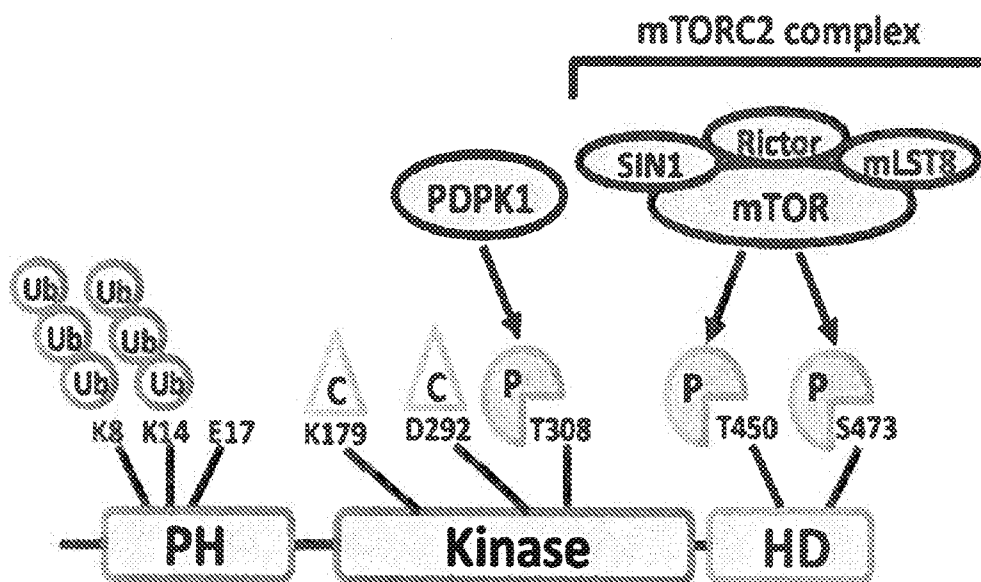
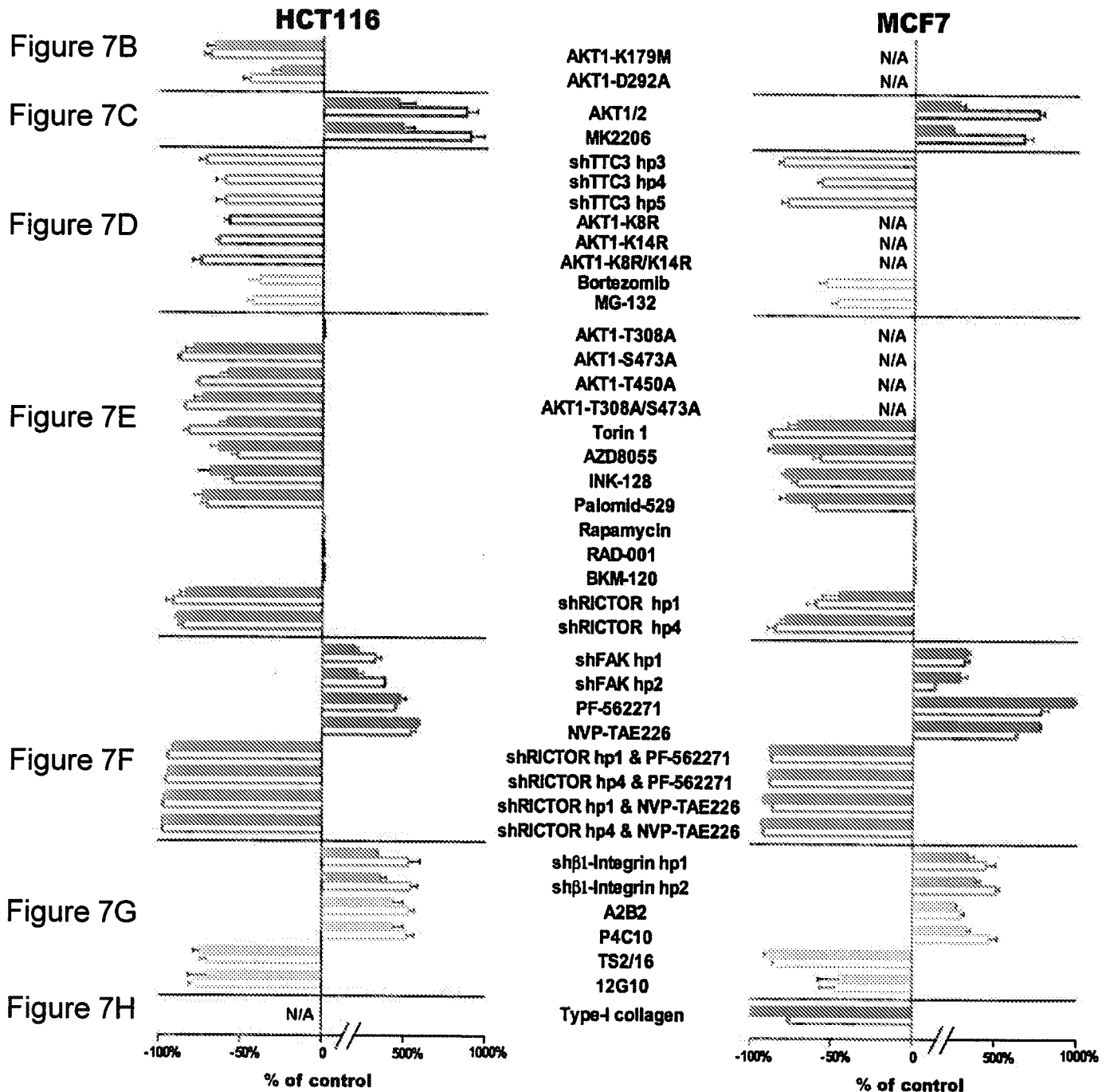


Figure 7A



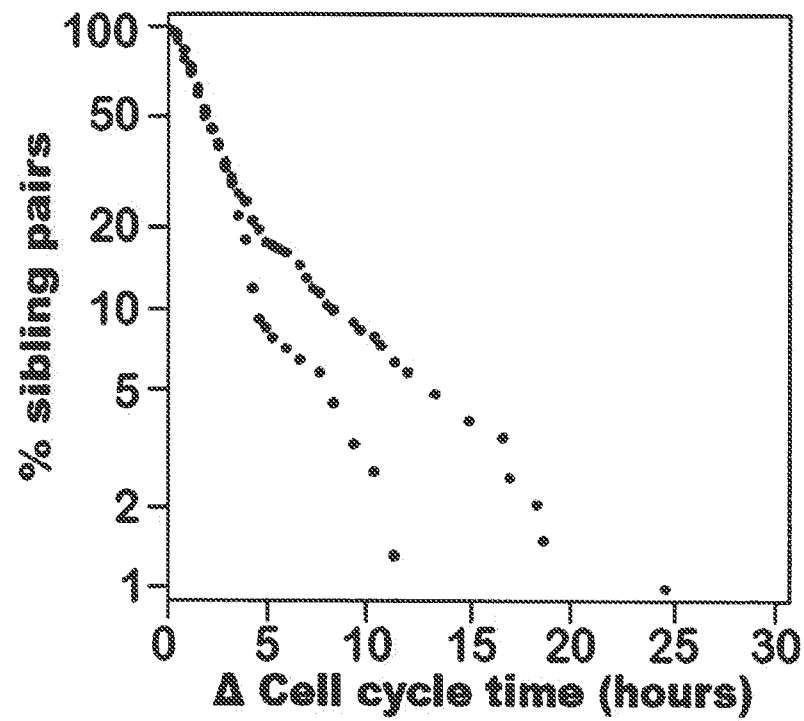


Figure 7I

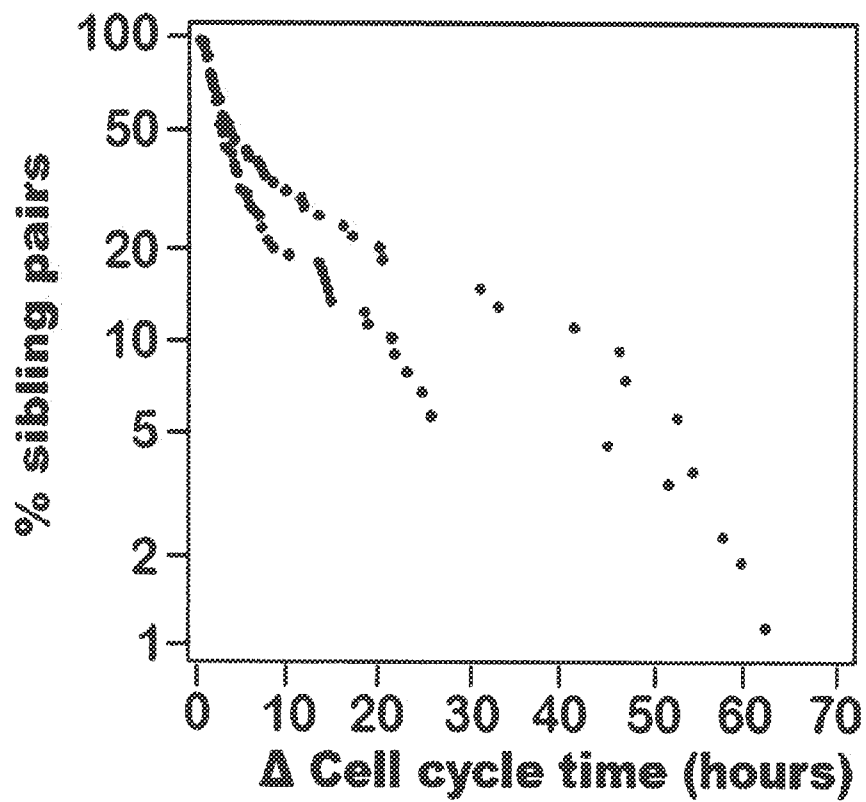


Figure 7J

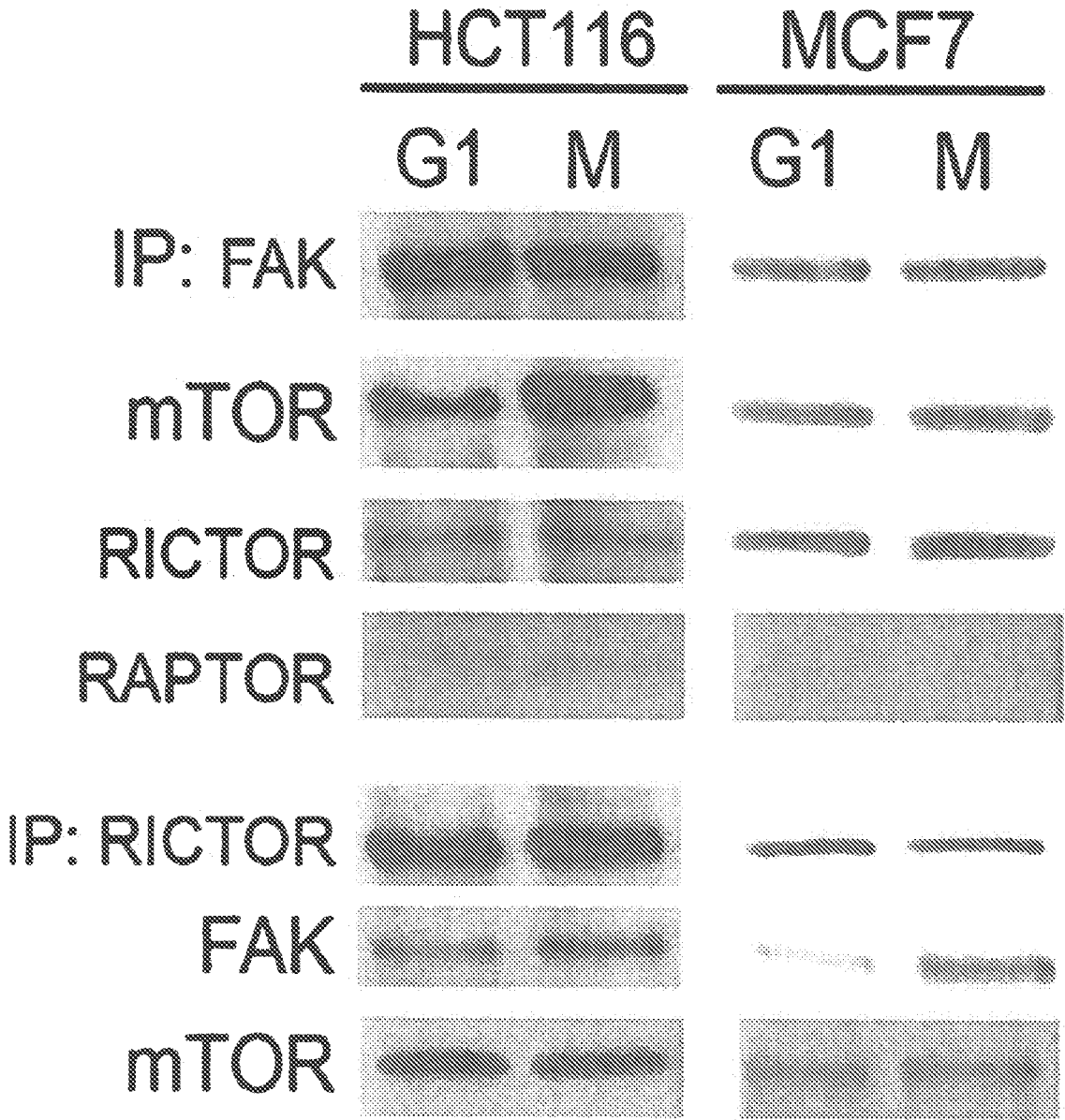


Figure 8

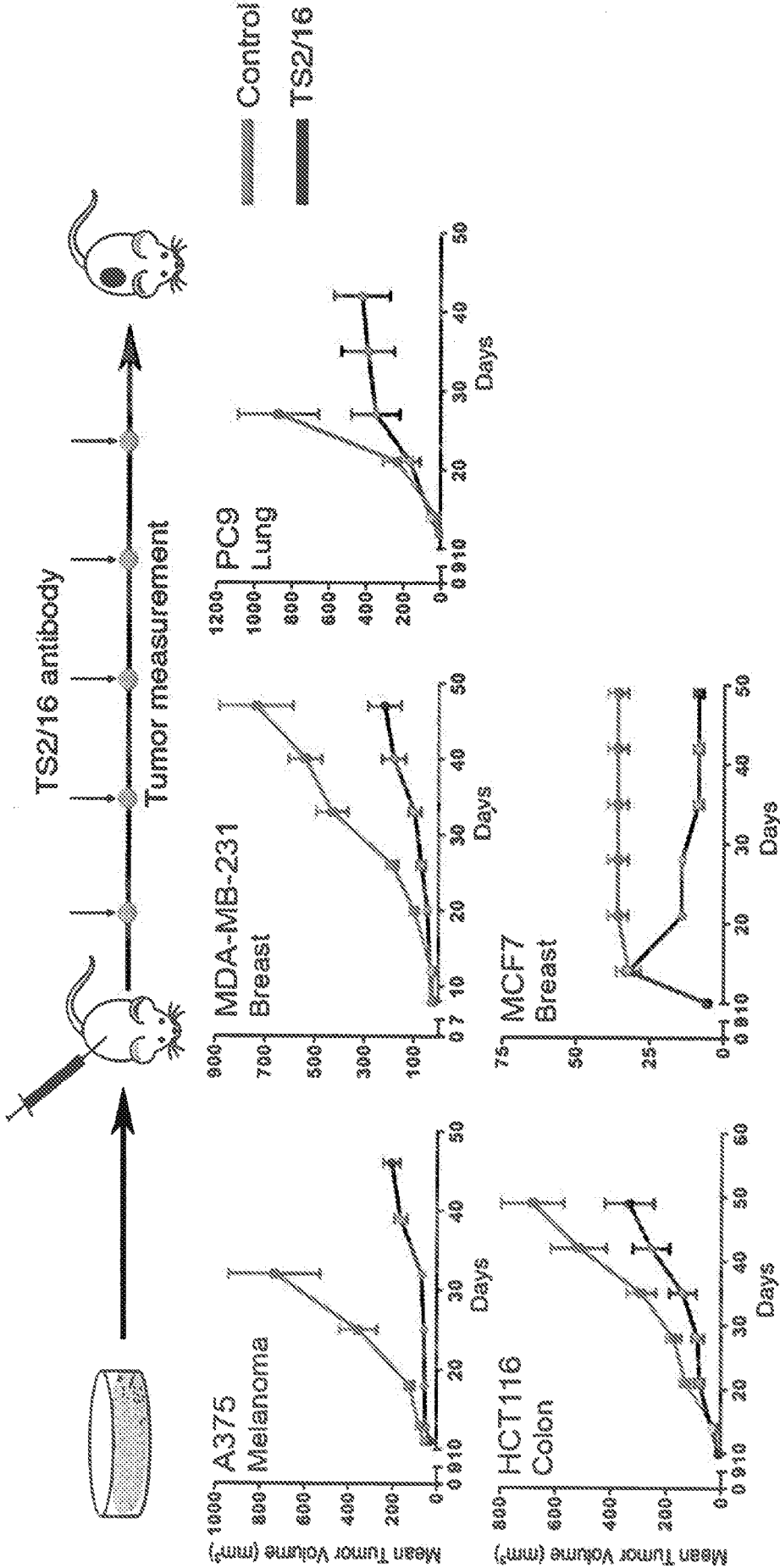


Figure 9A

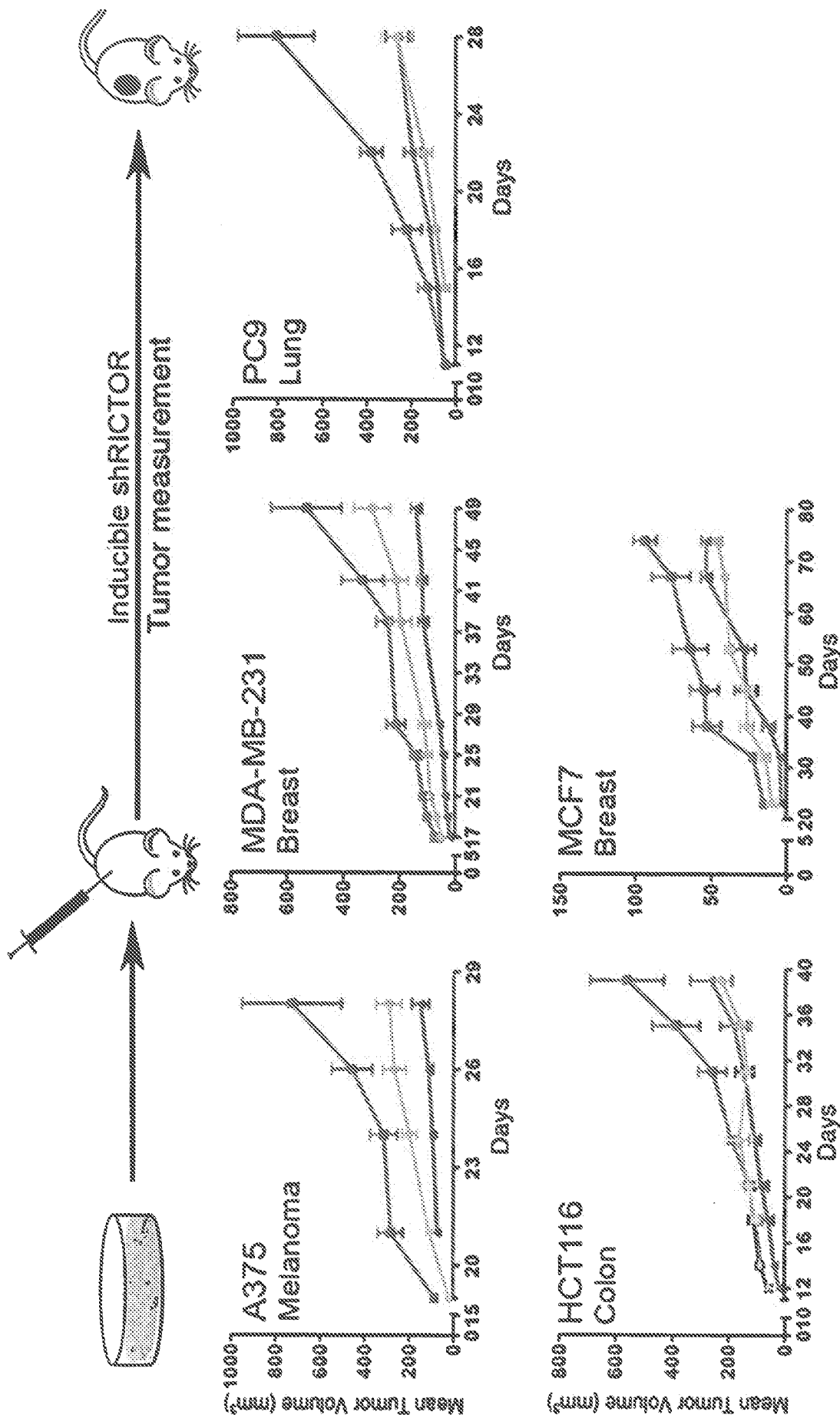


Figure 9B

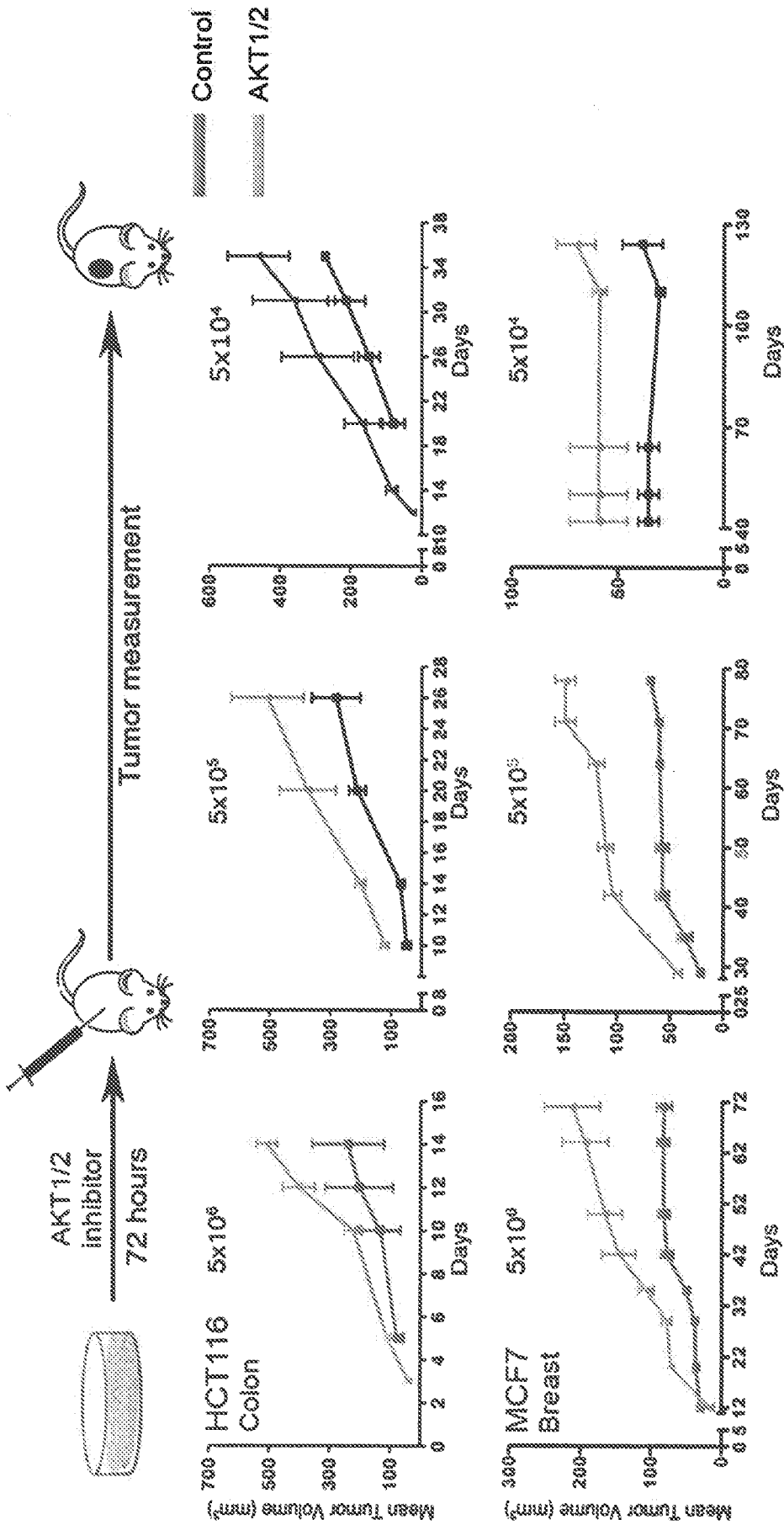


Figure 9C

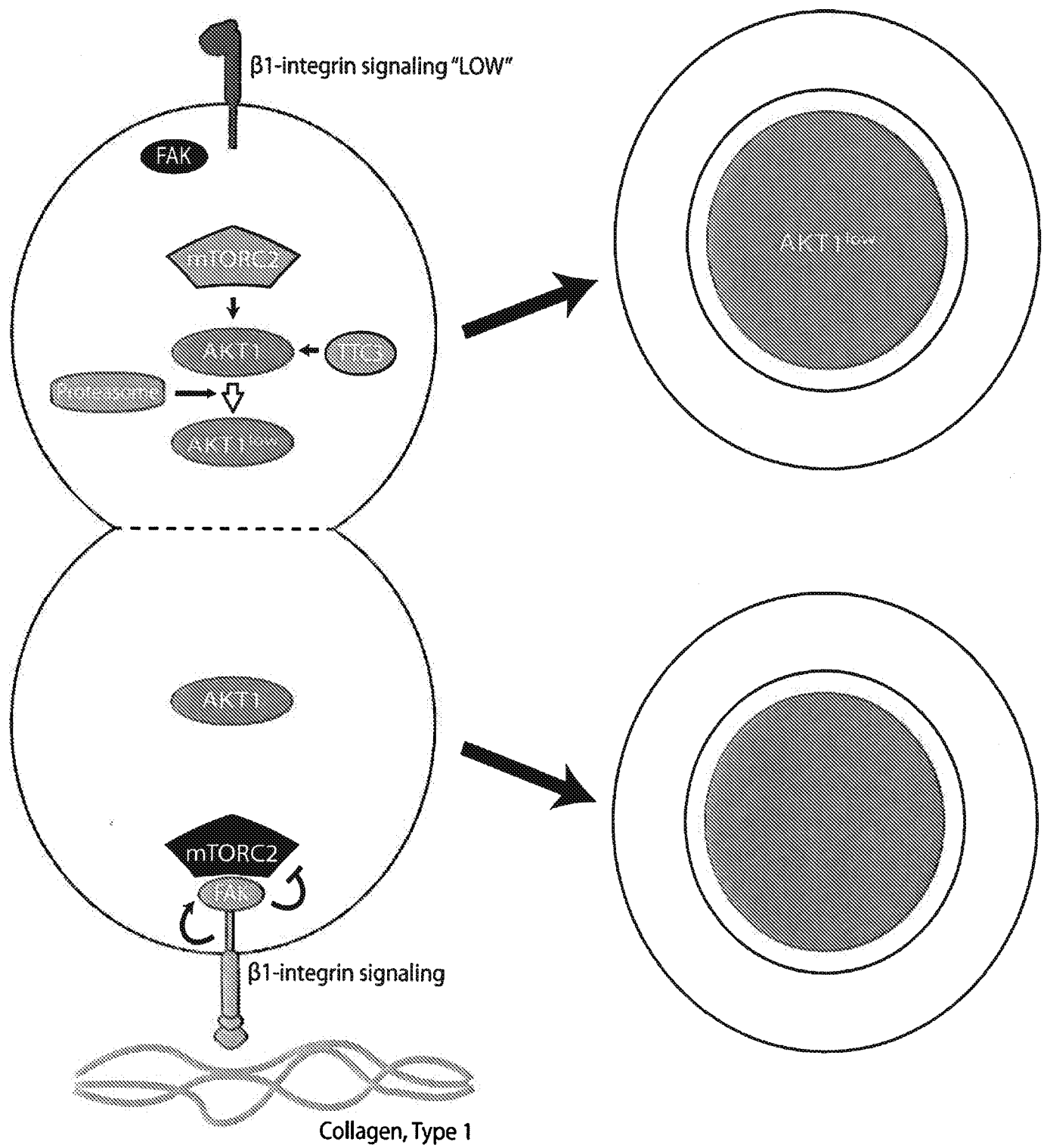


Figure 10

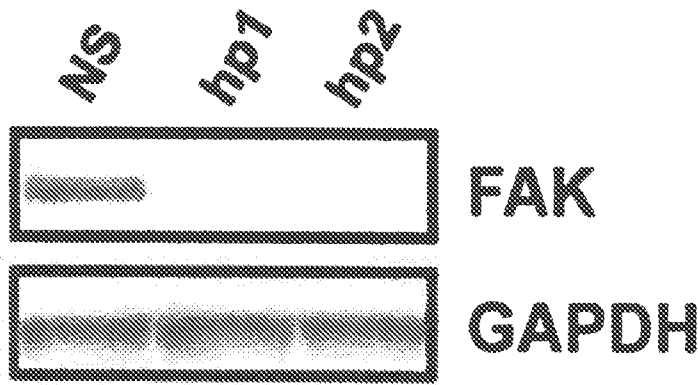


Figure 11A

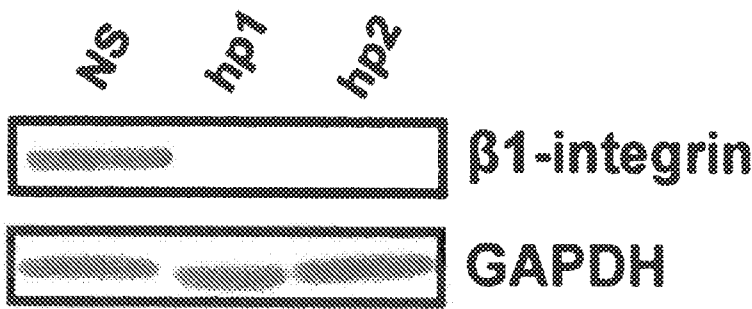


Figure 11B

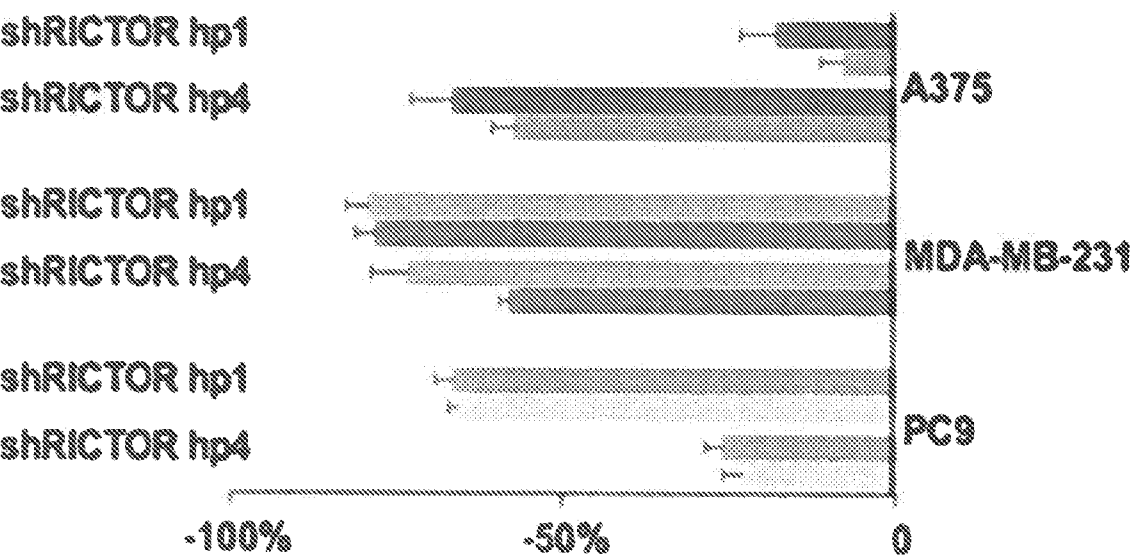


Figure 11C

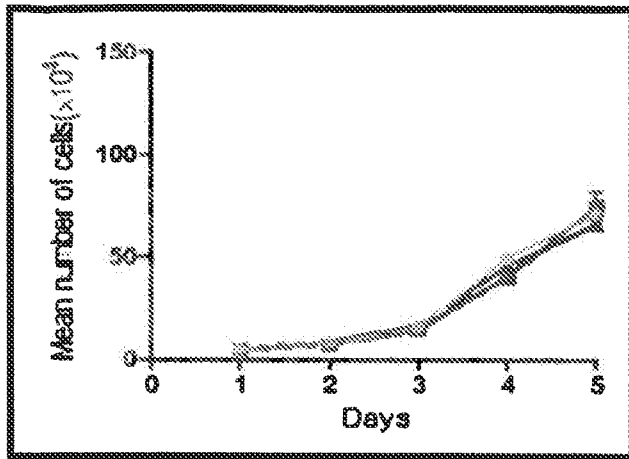


Figure 12A

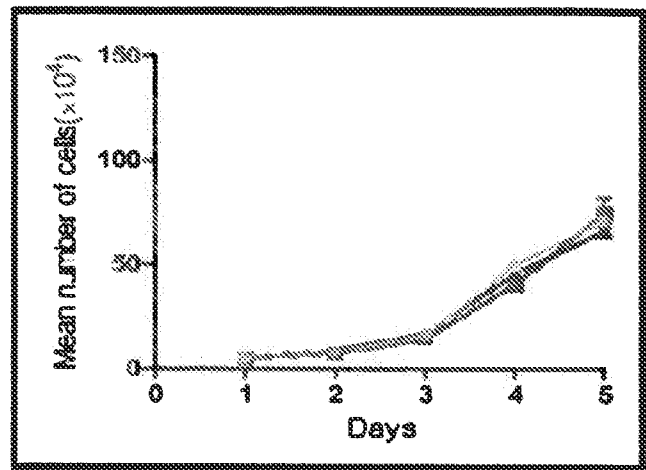


Figure 12B

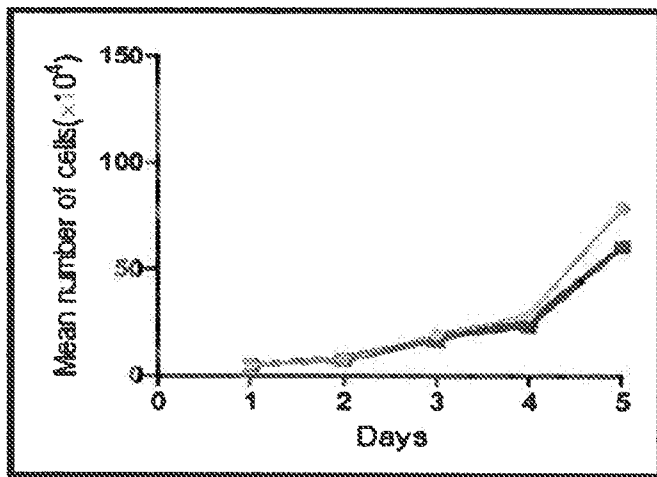


Figure 12C

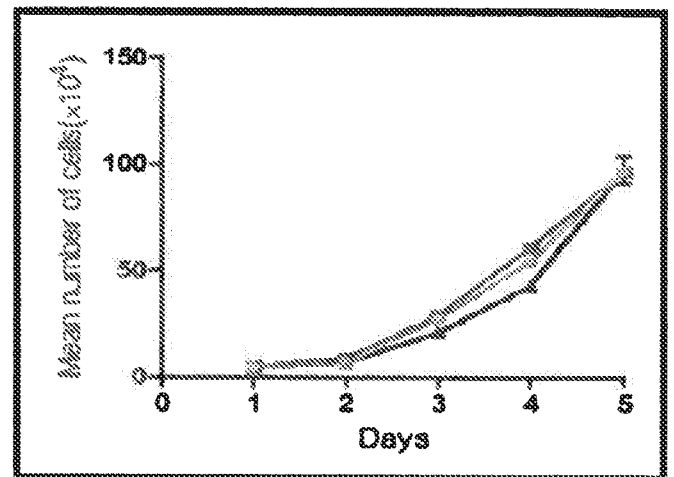


Figure 12D

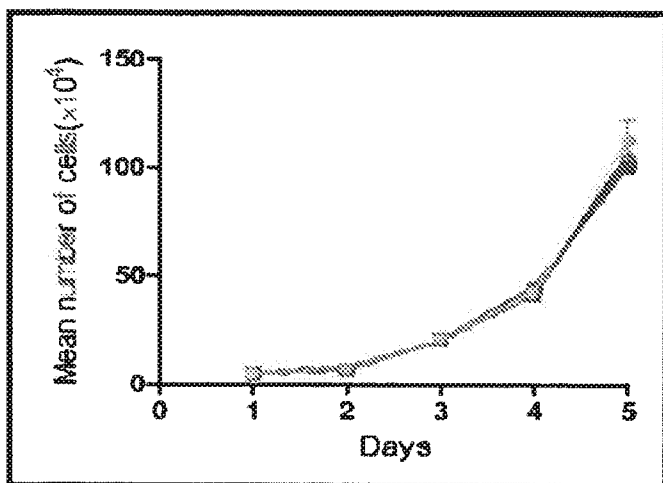


Figure 12E

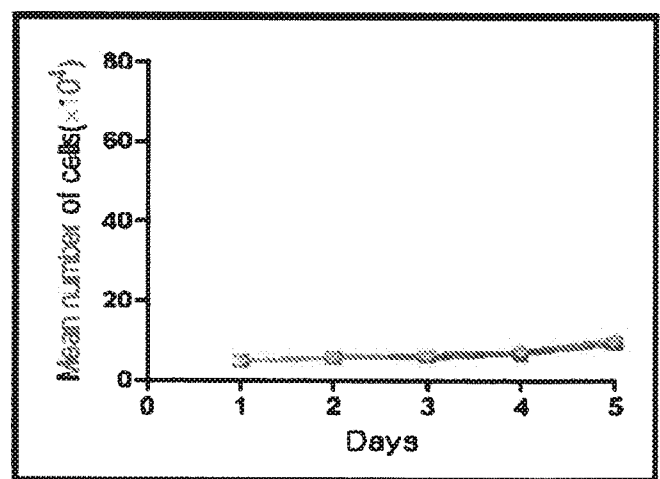


Figure 12F

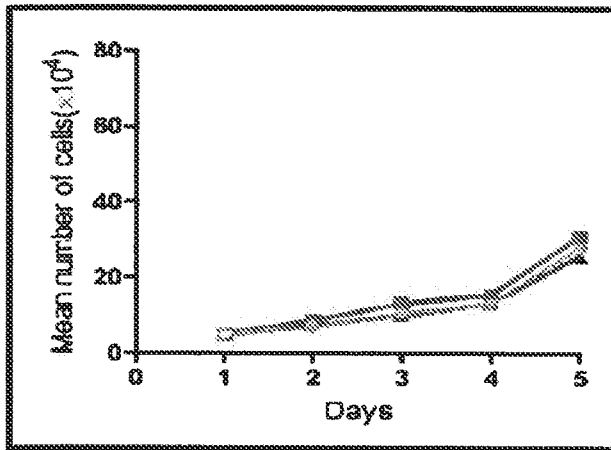


Figure 12G

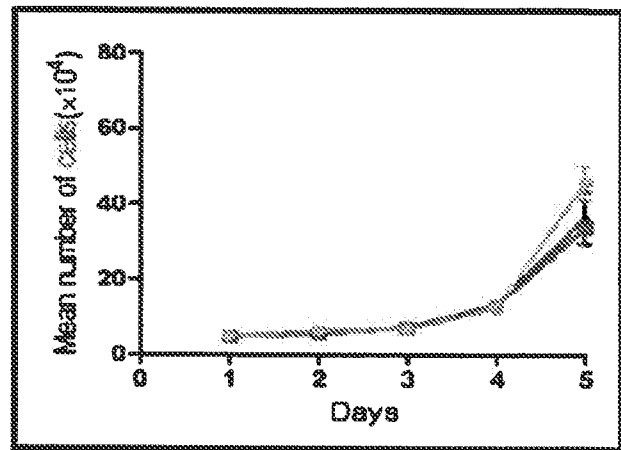


Figure 12H

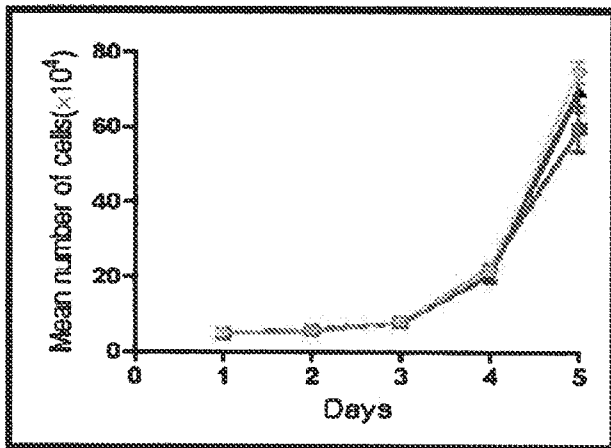


Figure 12I

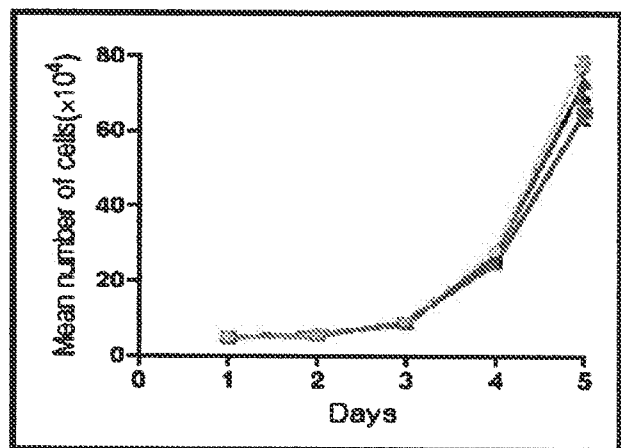


Figure 12J

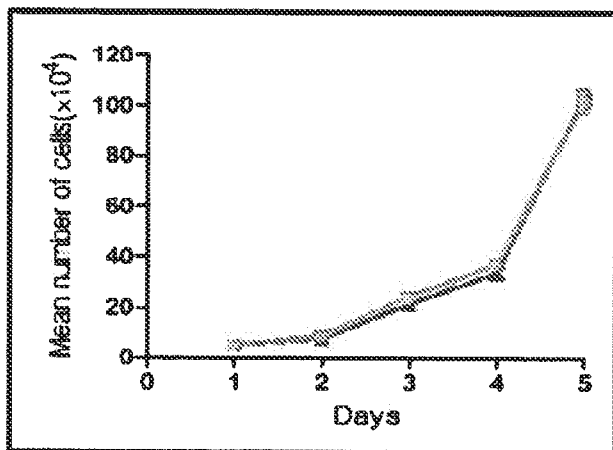


Figure 12K

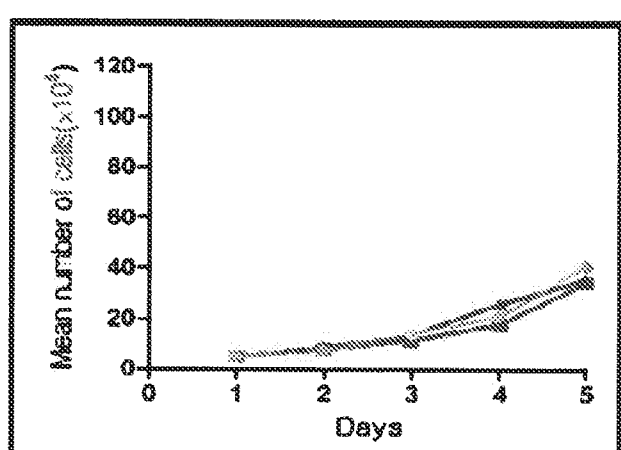


Figure 12L

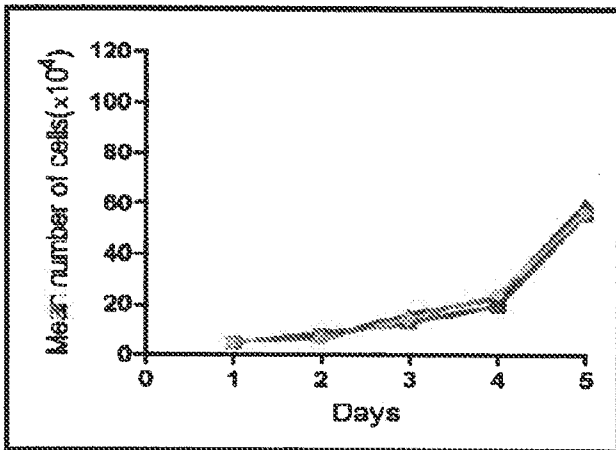


Figure 12M

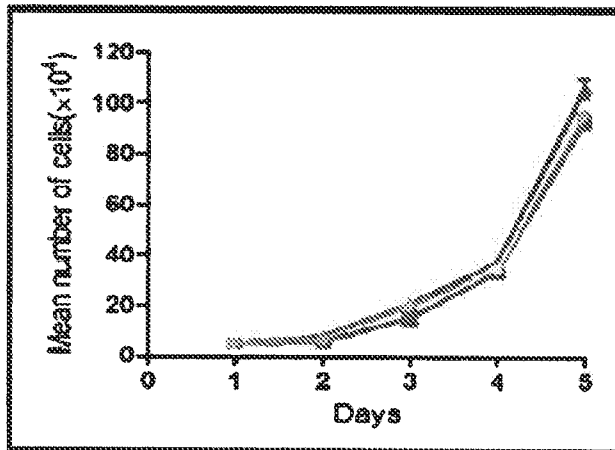


Figure 12N

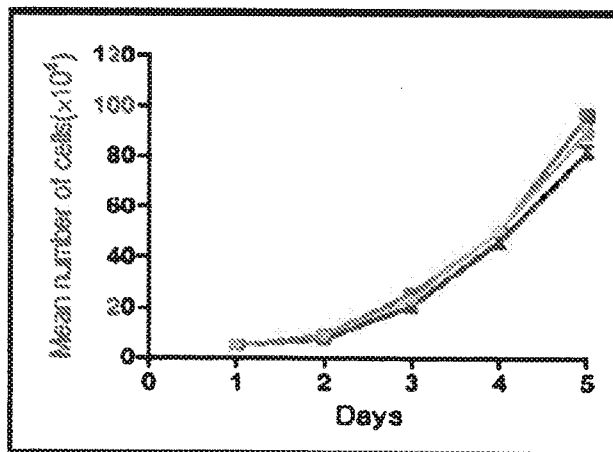
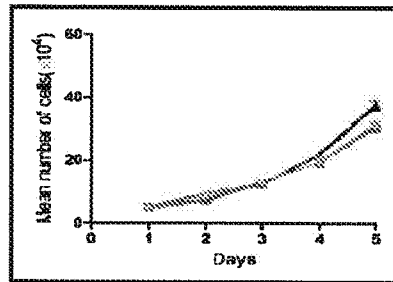
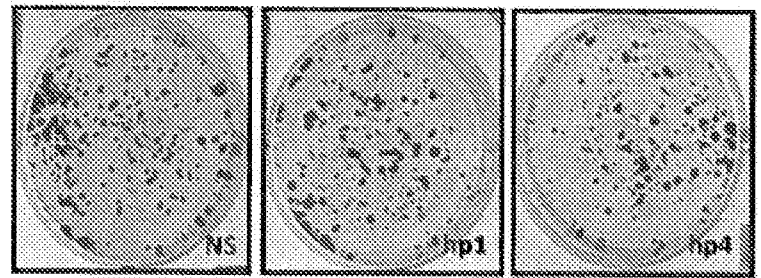
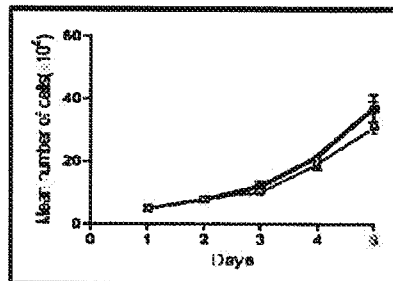
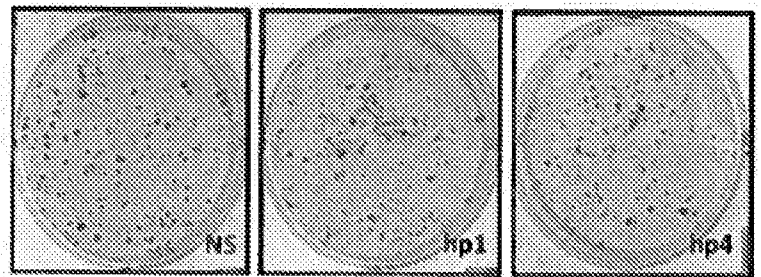
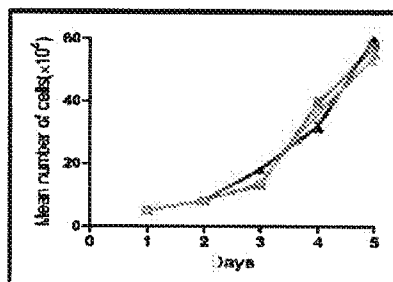
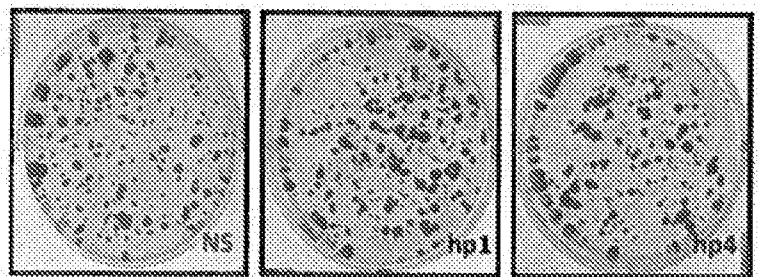
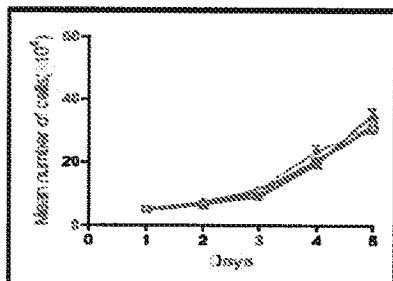
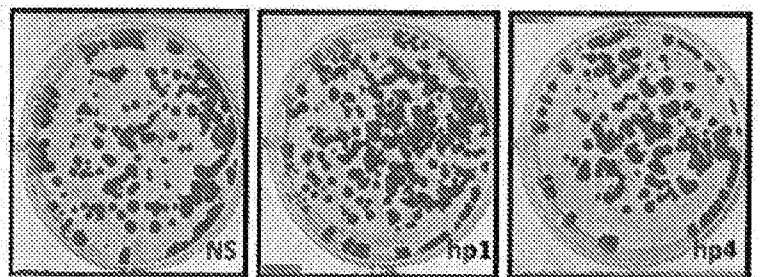
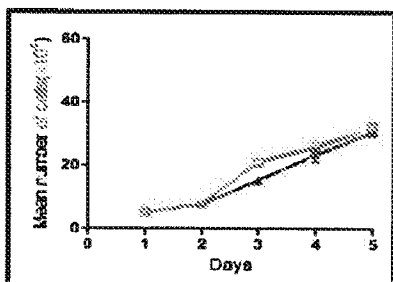
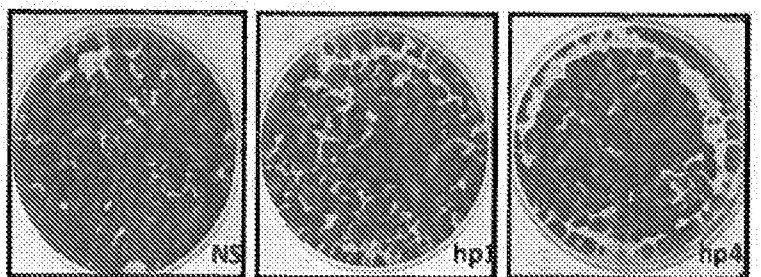


Figure 12O

Figure
13AFigure
13FFigure
13BFigure
13GFigure
13CFigure
13HFigure
13DFigure
13IFigure
13EFigure
13J

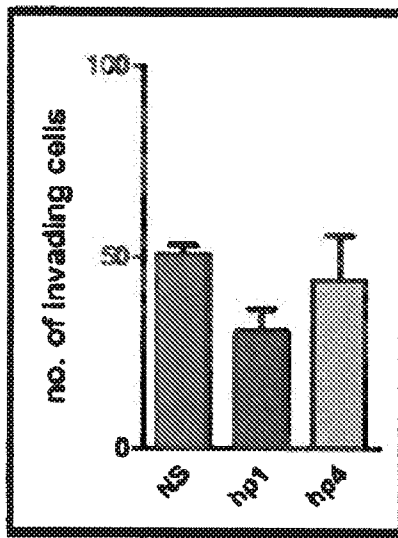


Figure 14A

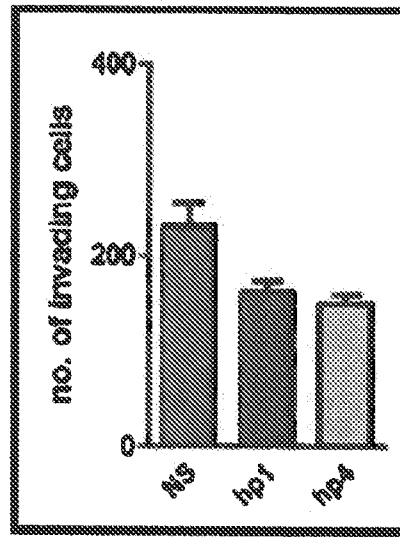


Figure 14B

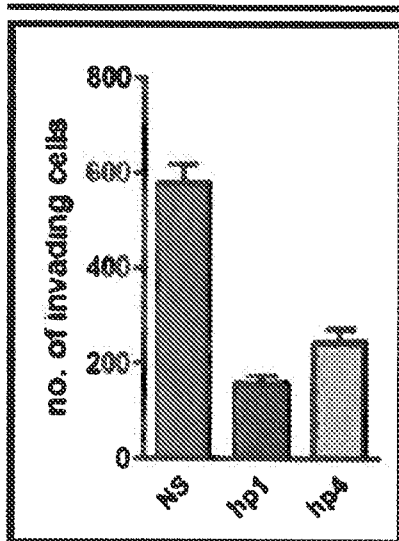


Figure 14C

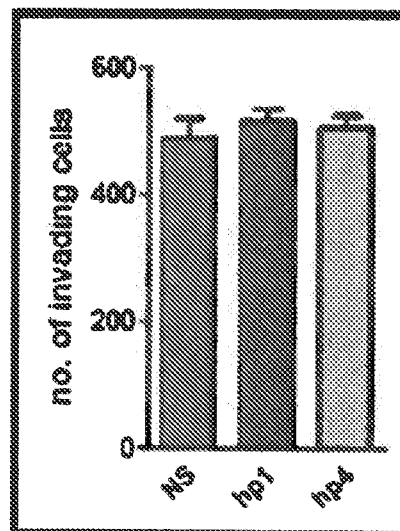


Figure 14D

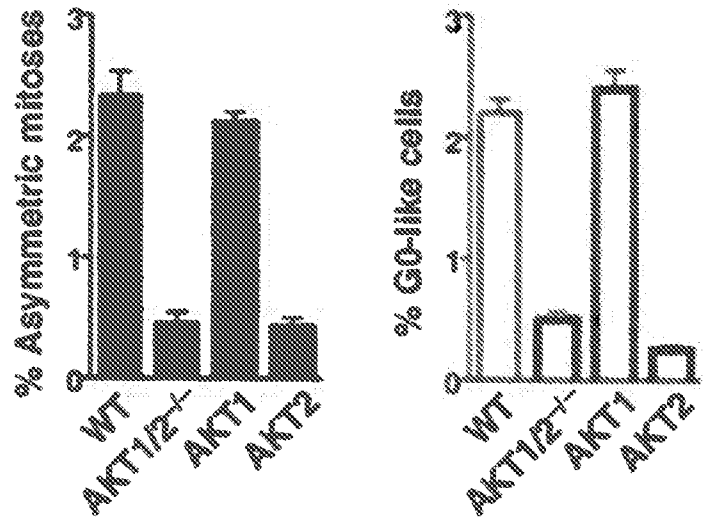


Figure 15A

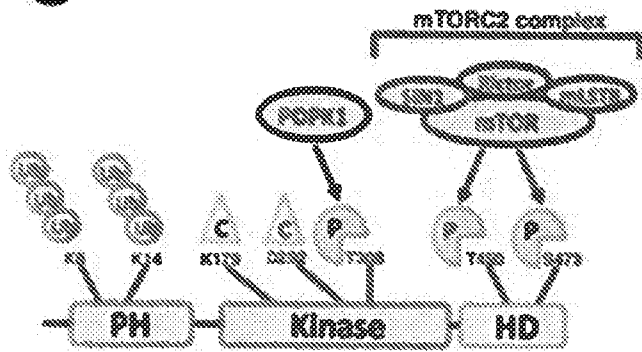


Figure 15B

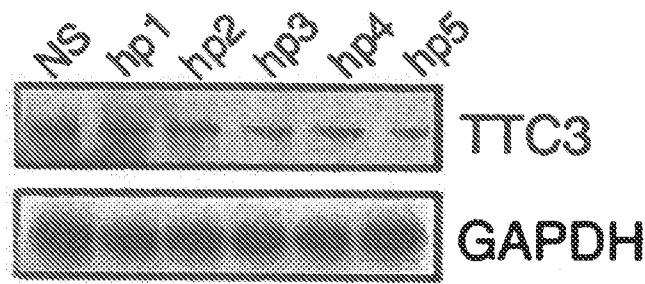


Figure 15D

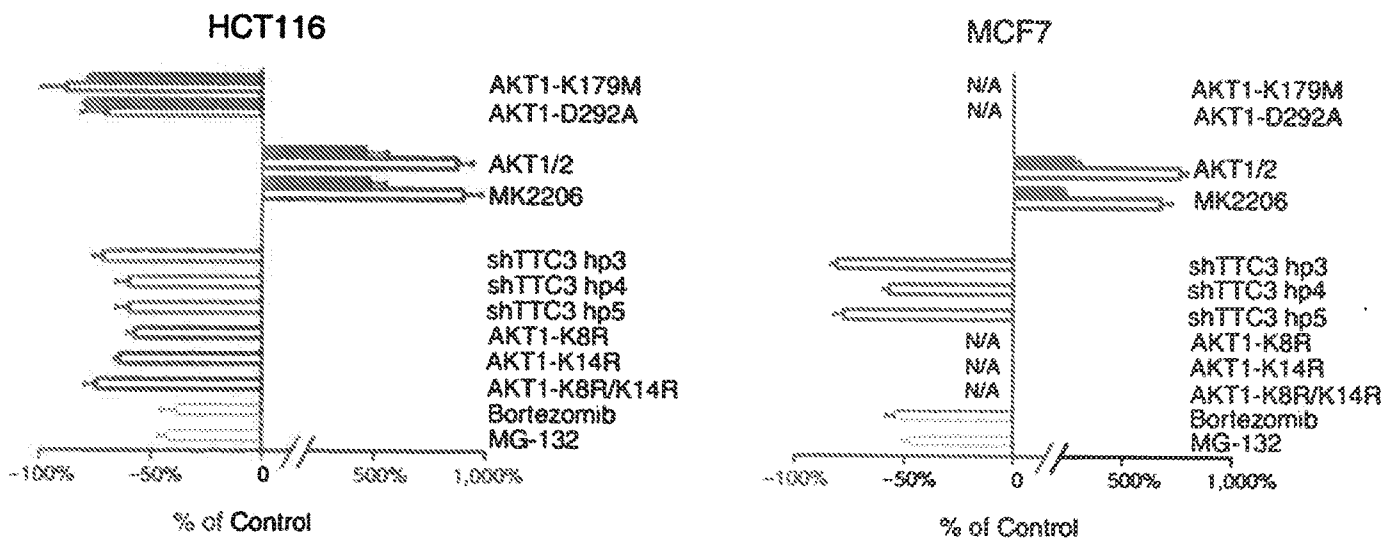


Figure 15C

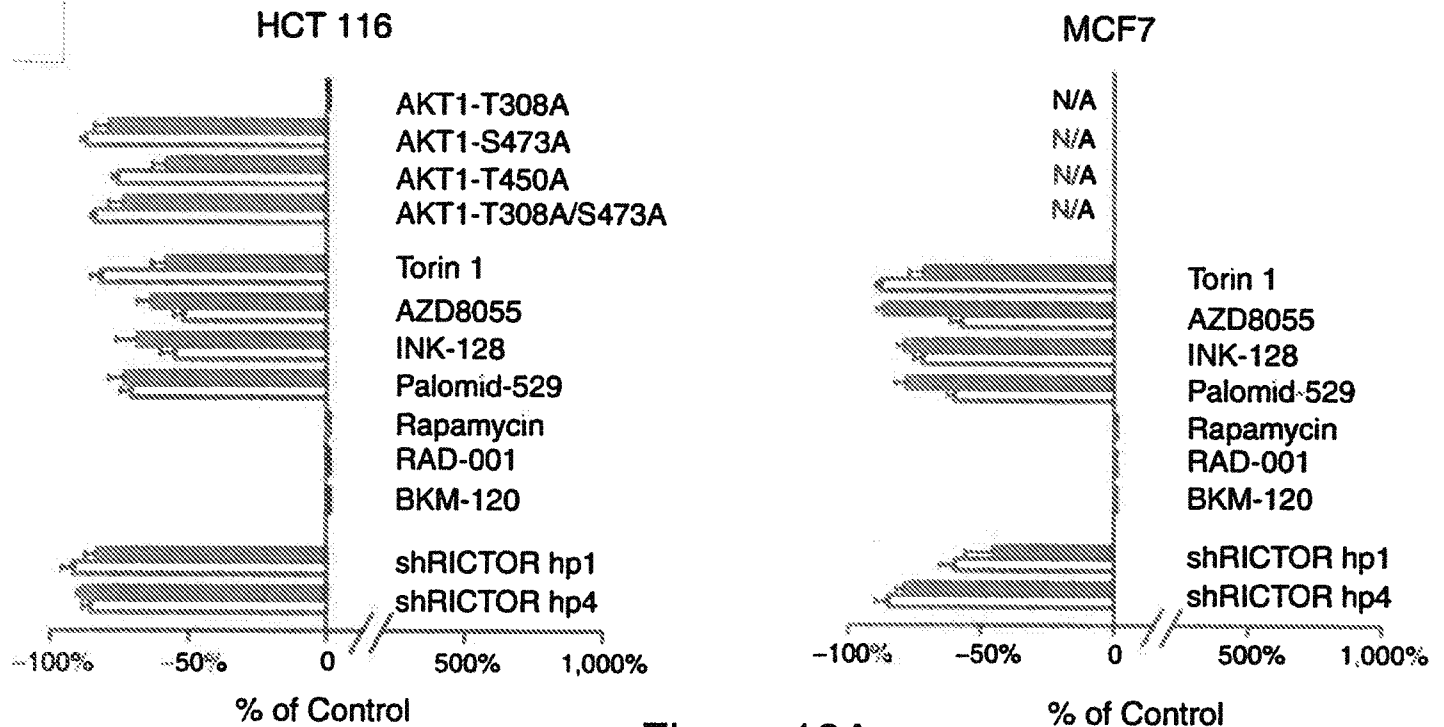


Figure 16A

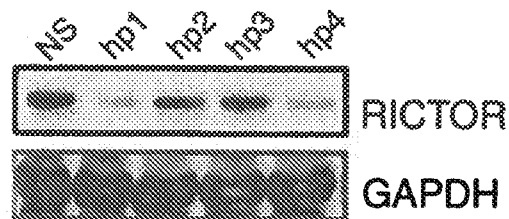


Figure 16B

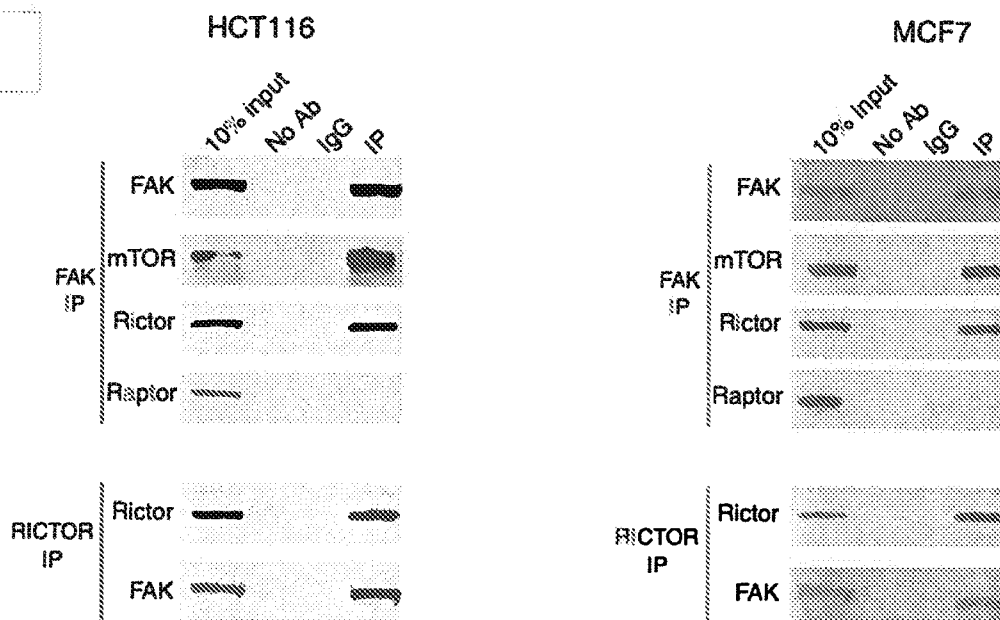


Figure 16C

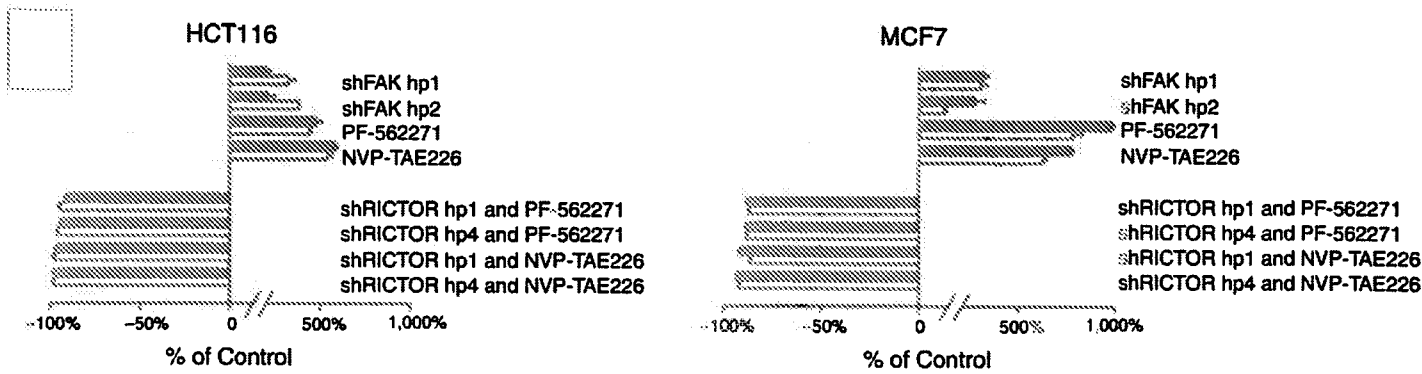


Figure 17A

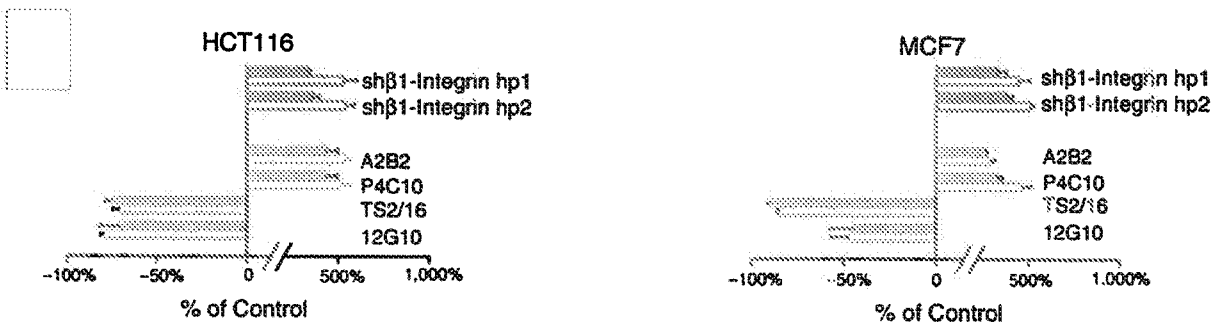


Figure 17B

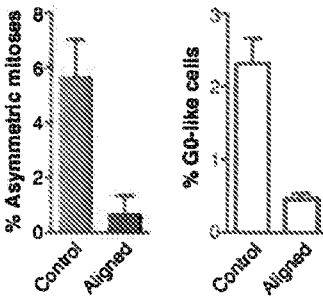


Figure 17C

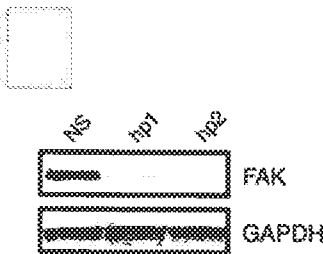


Figure 17D

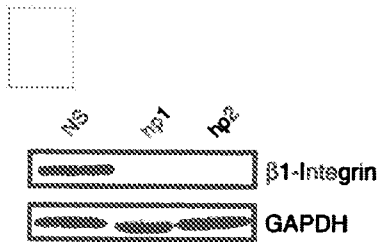
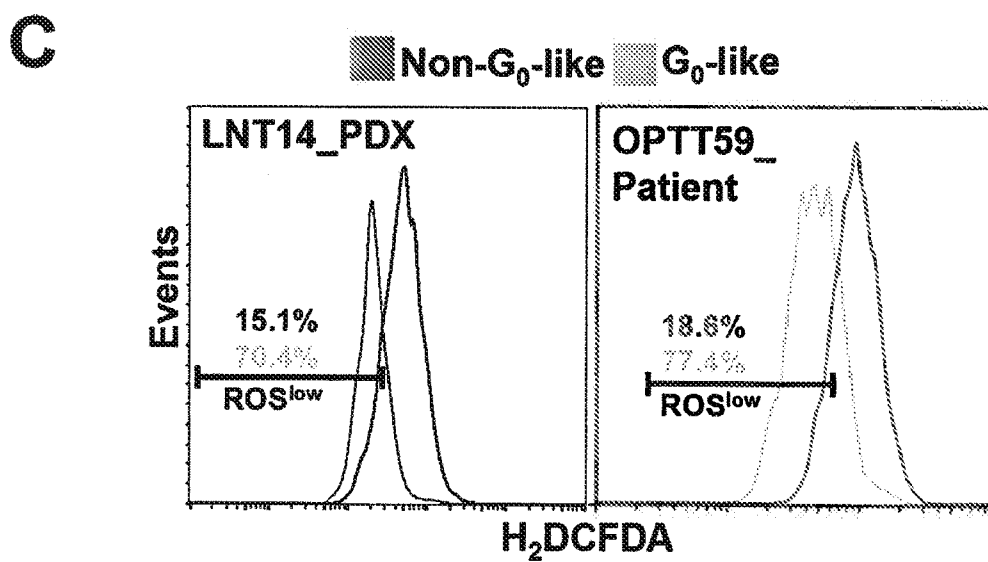
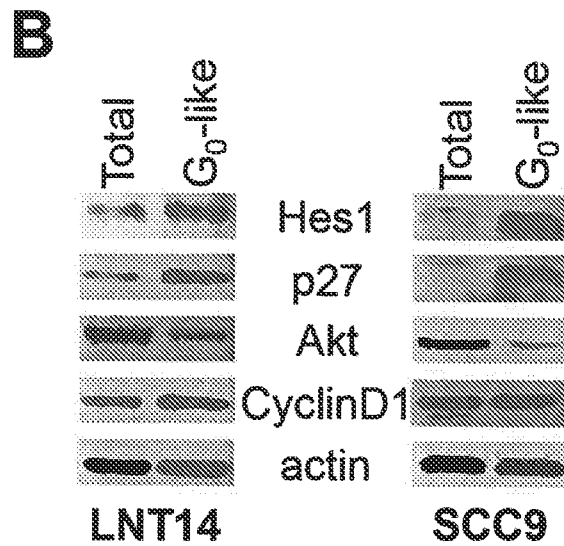
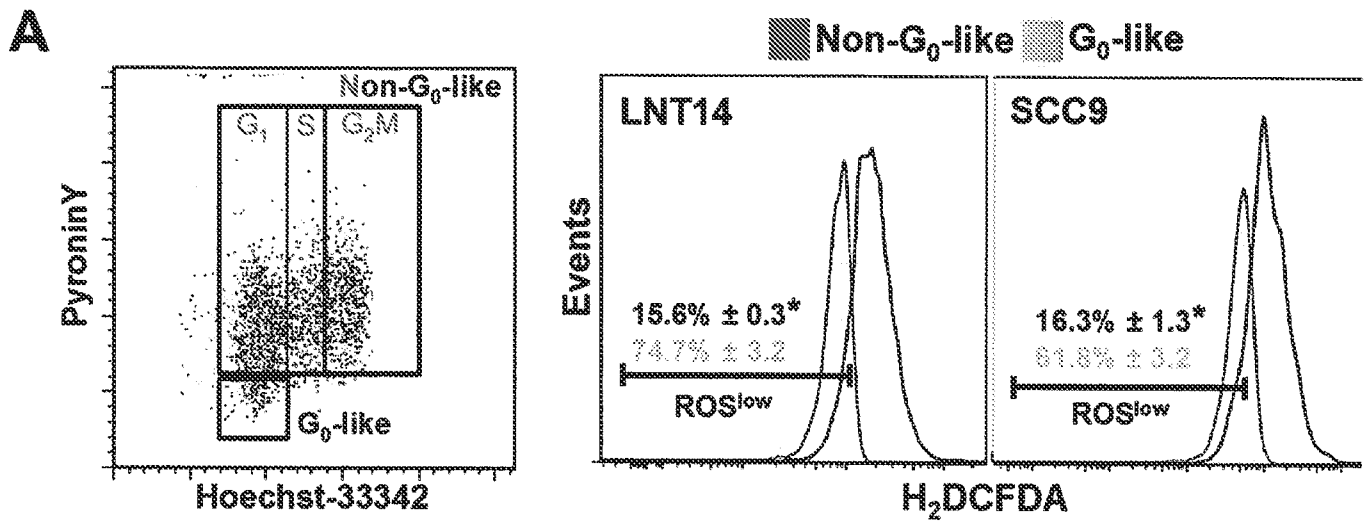
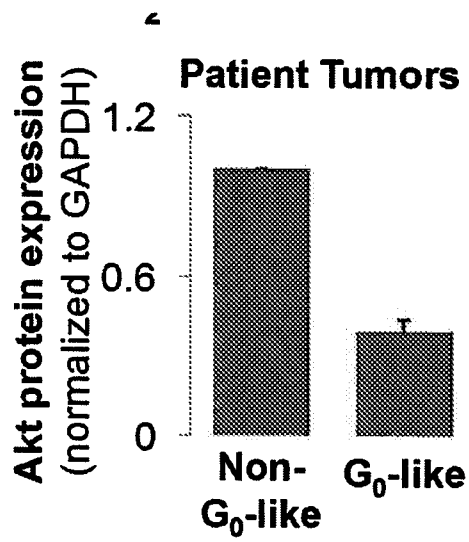
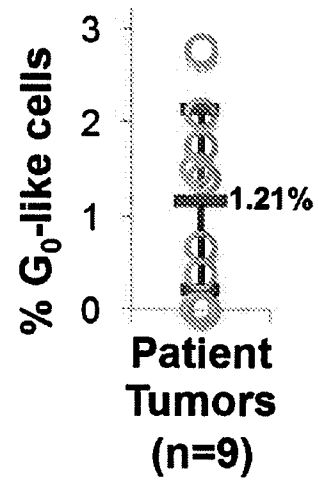
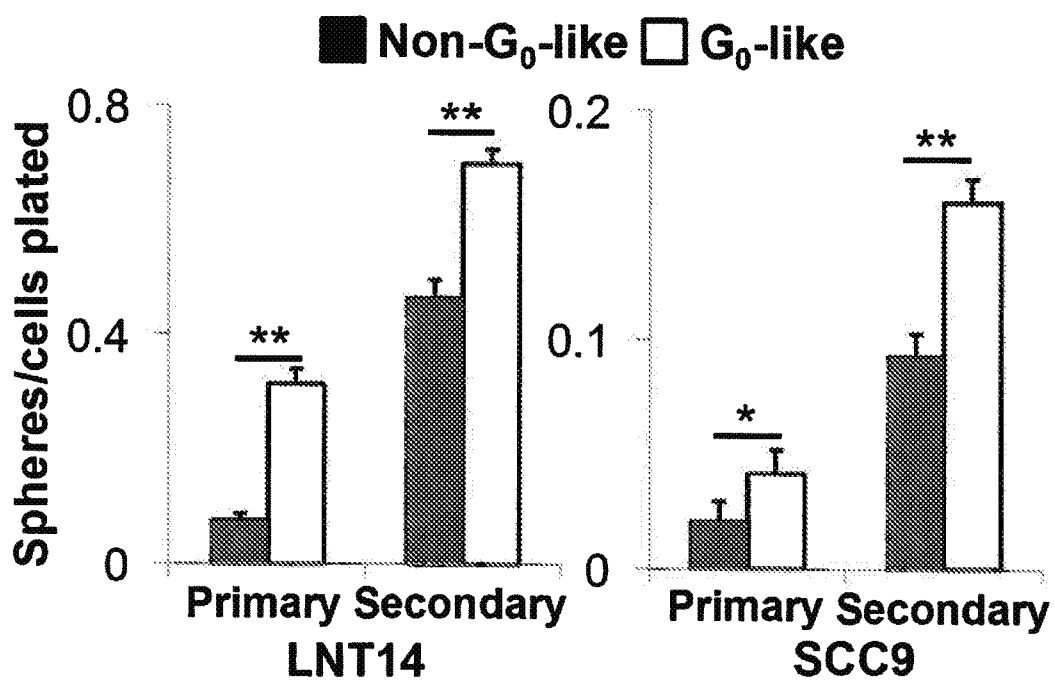


Figure 17E



Figures 18A-18C

D**E****F**

Figures 18D-18F

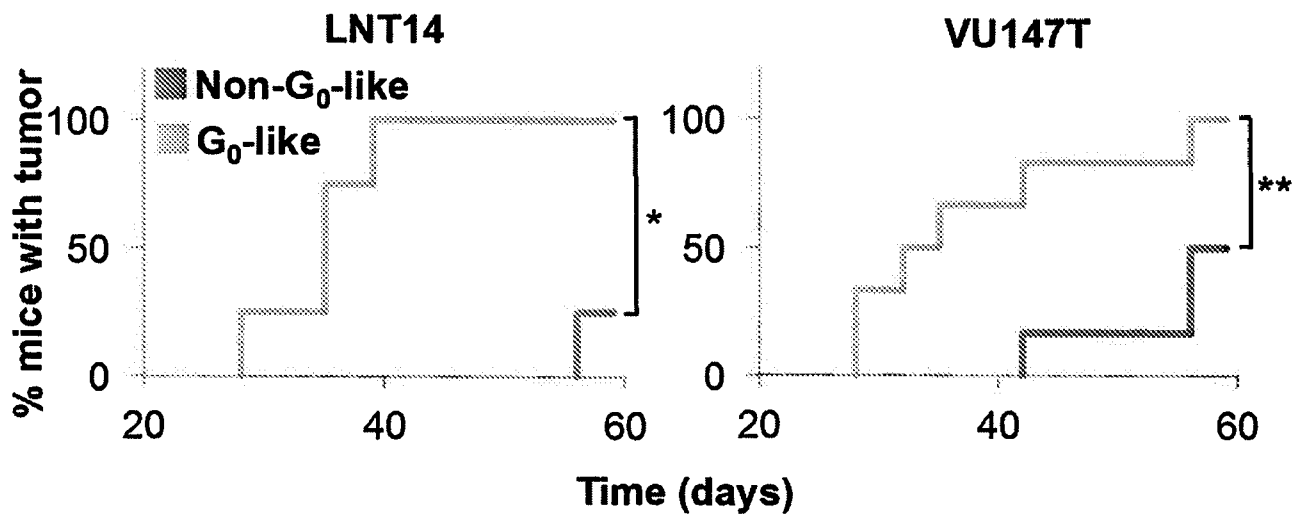


Figure 18G

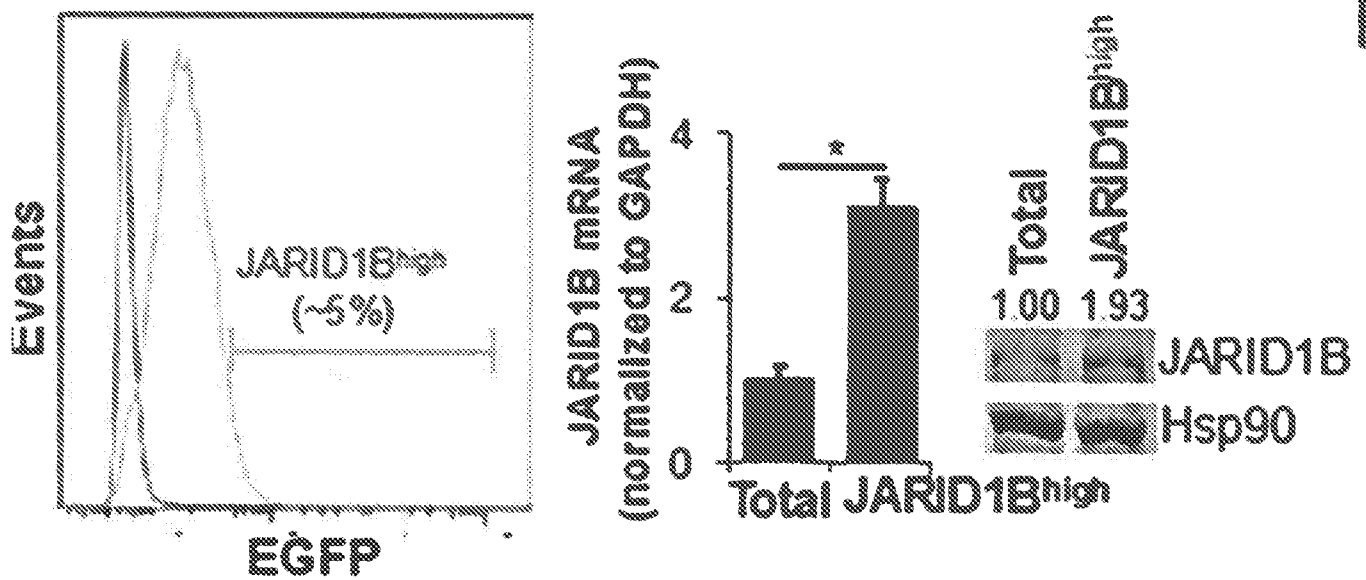


Figure 19A

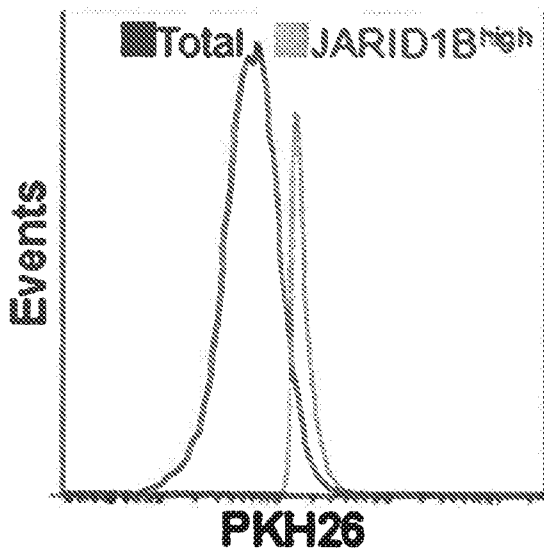


Figure 19B

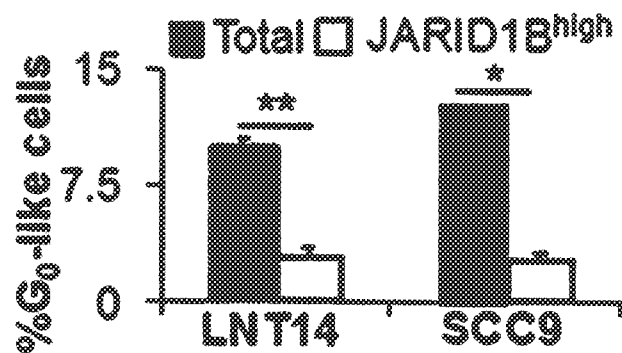


Figure 19C

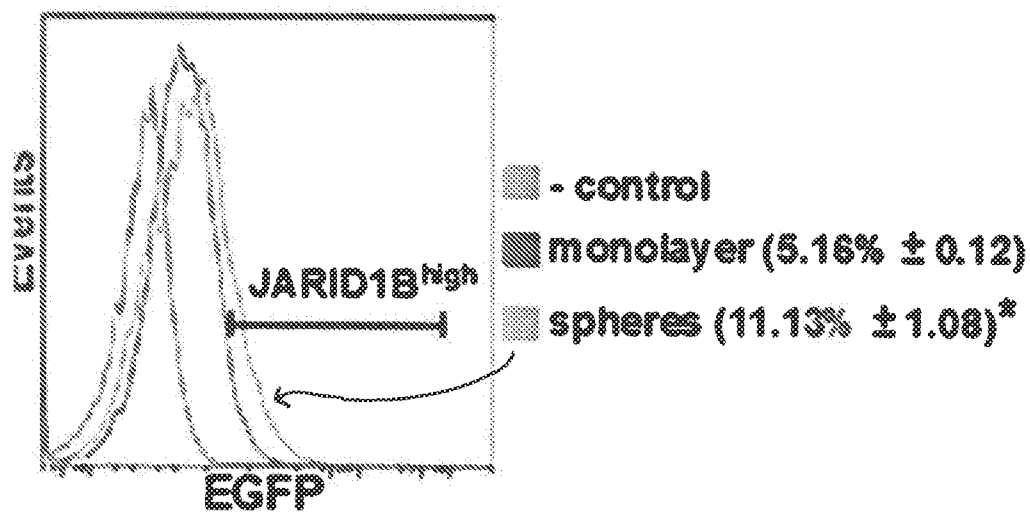


Figure 19D

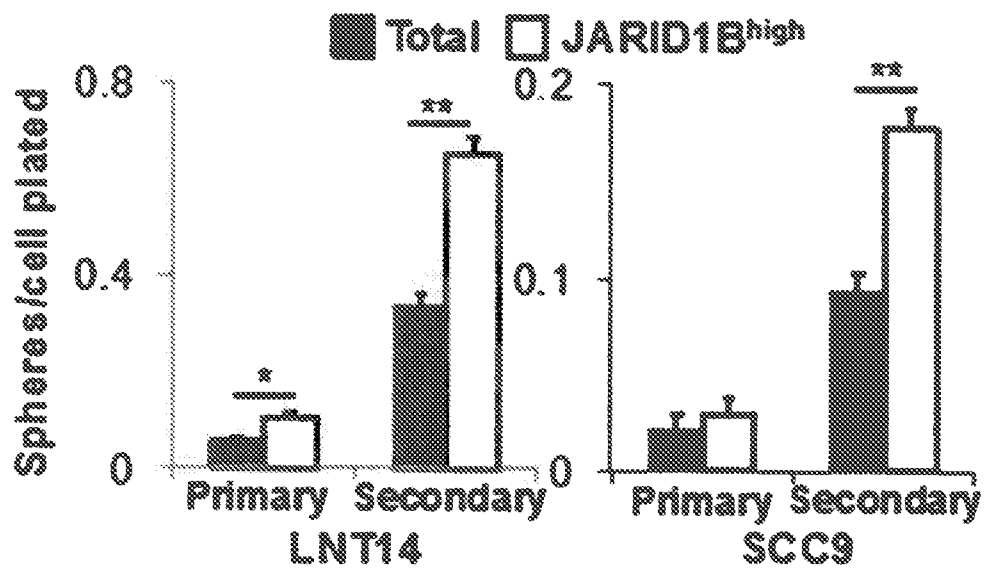


Figure 19E

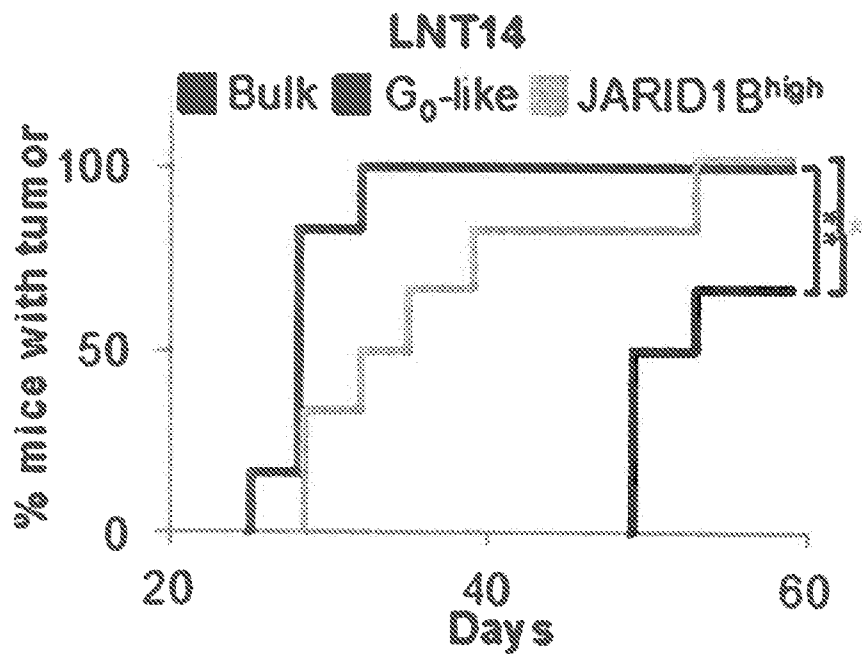


Figure 19F

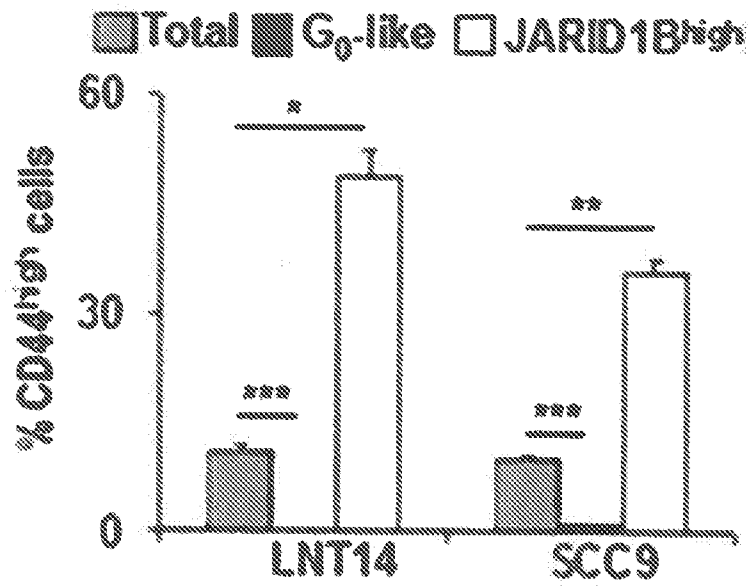


Figure 20A

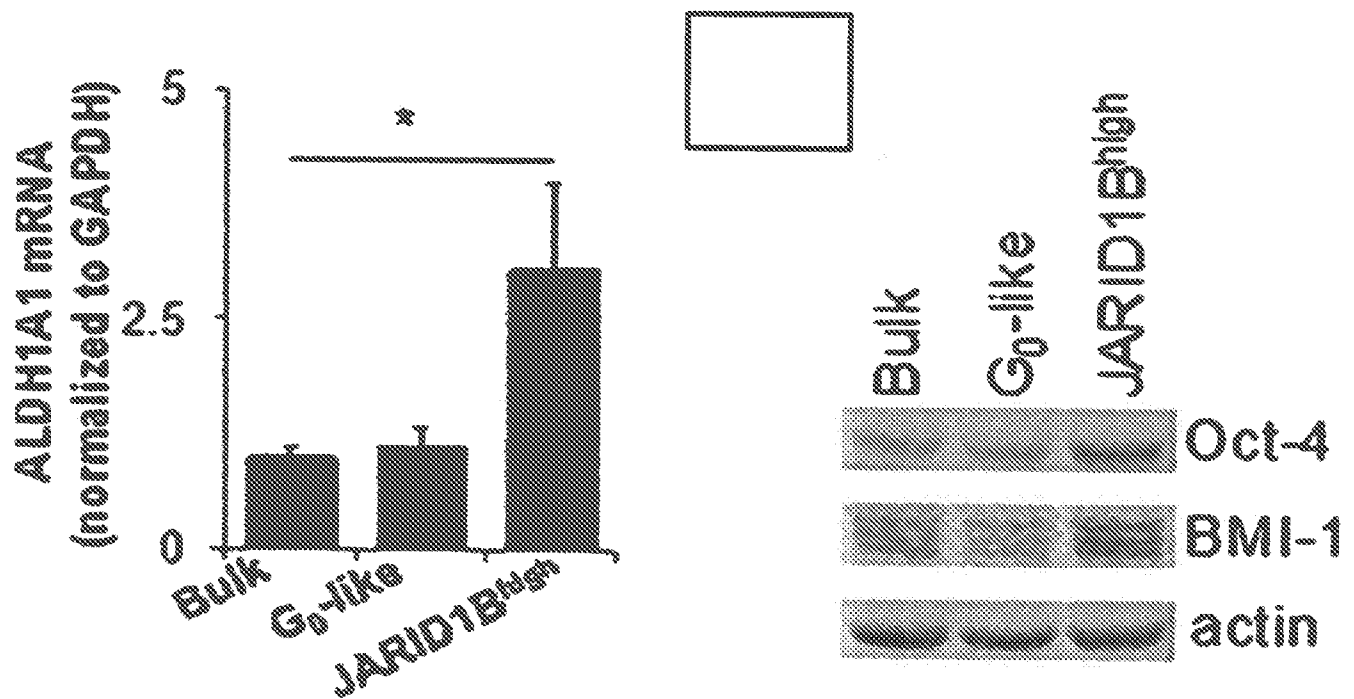


Figure 20B

Figure 20C

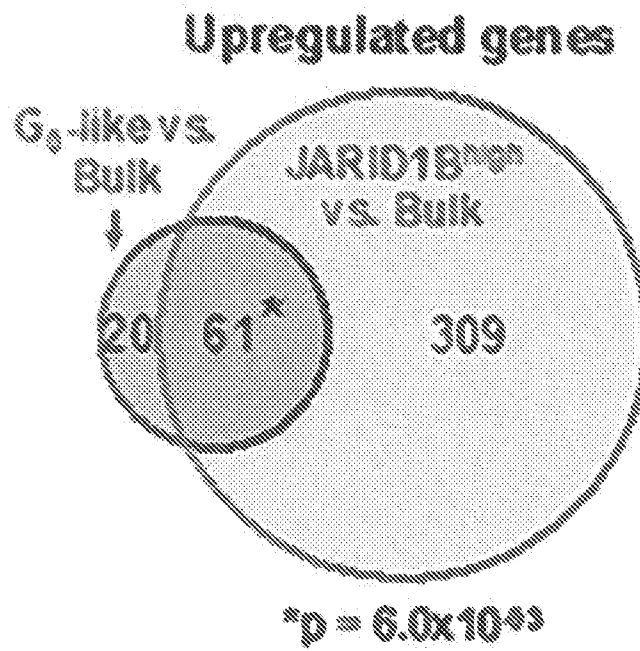


Figure 20D

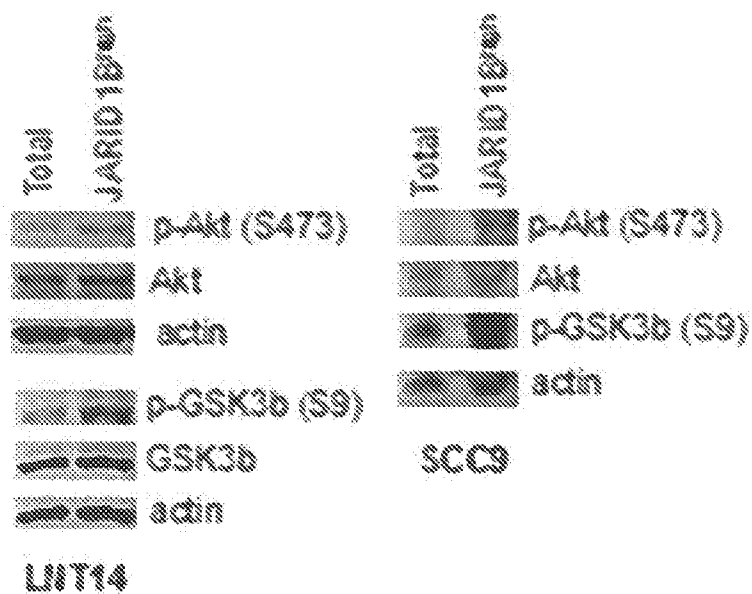


Figure 20E

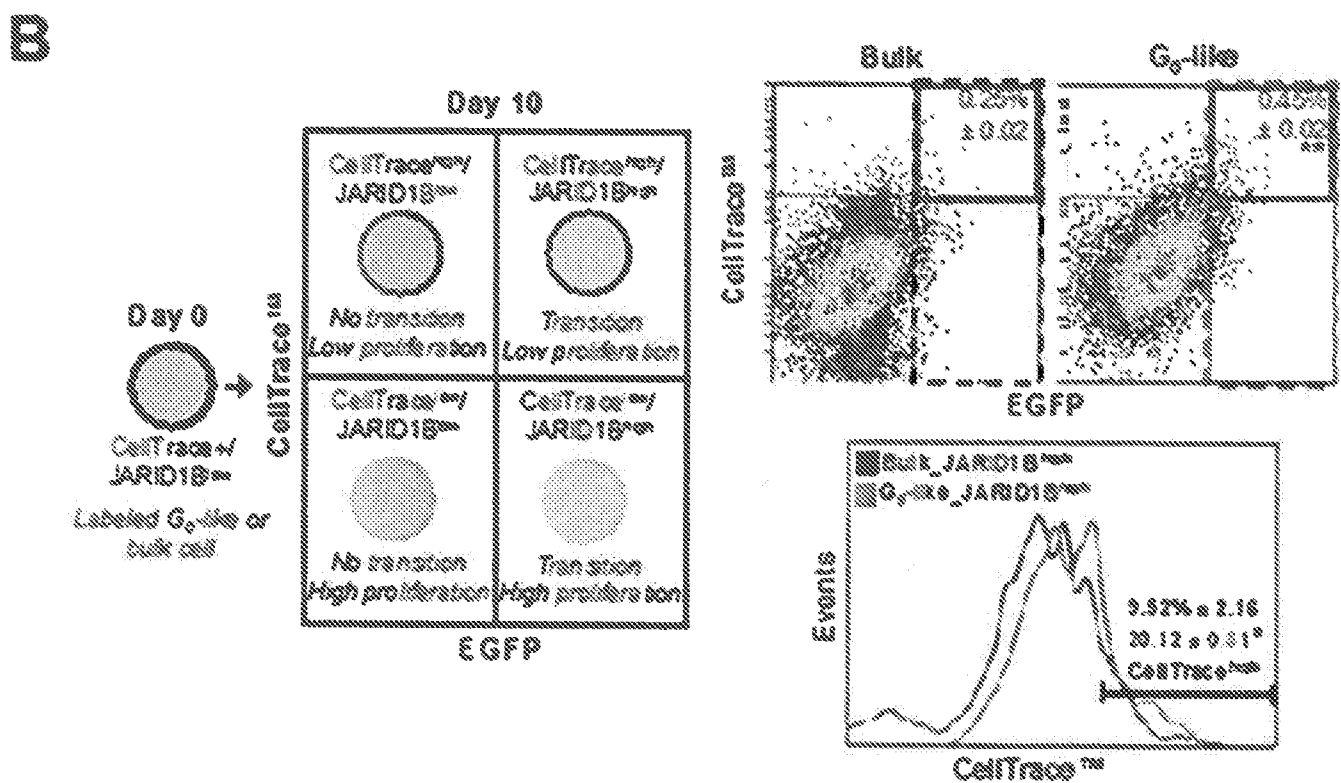
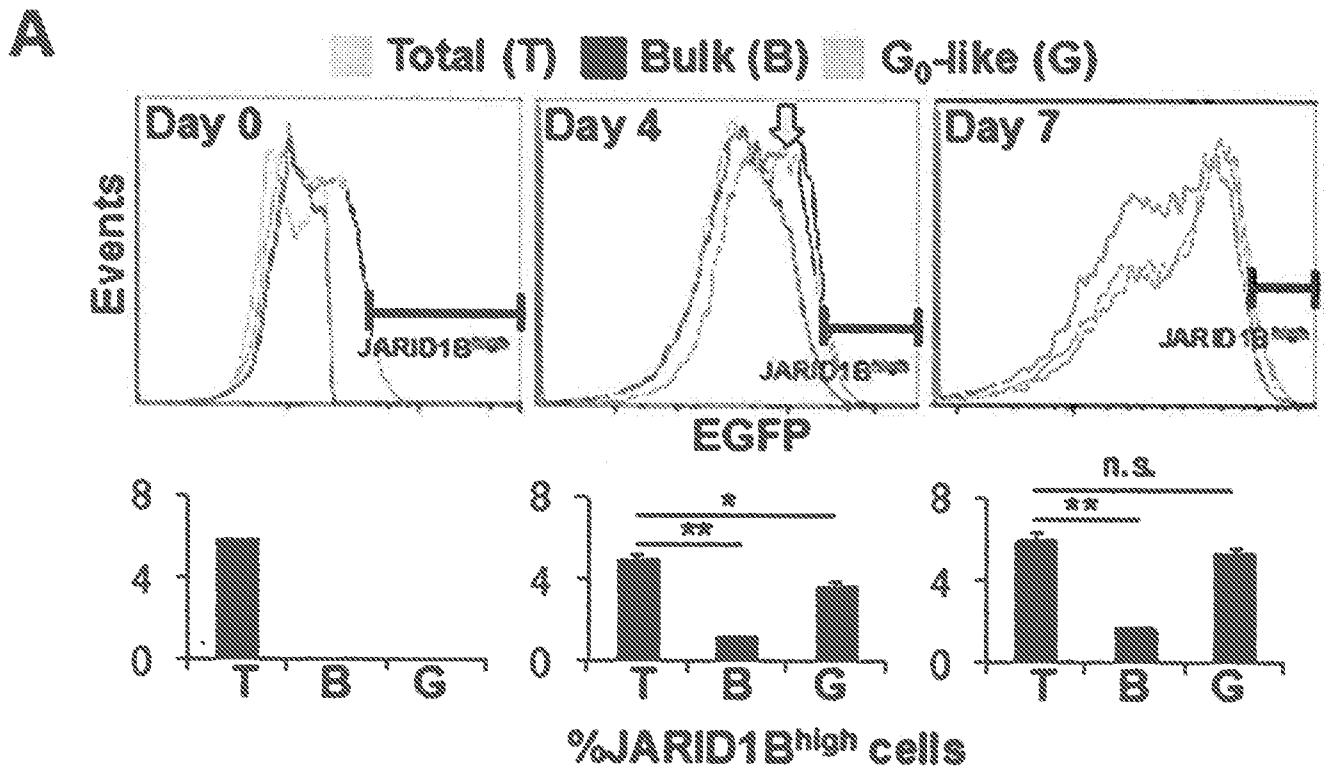


Figure 21A-21B

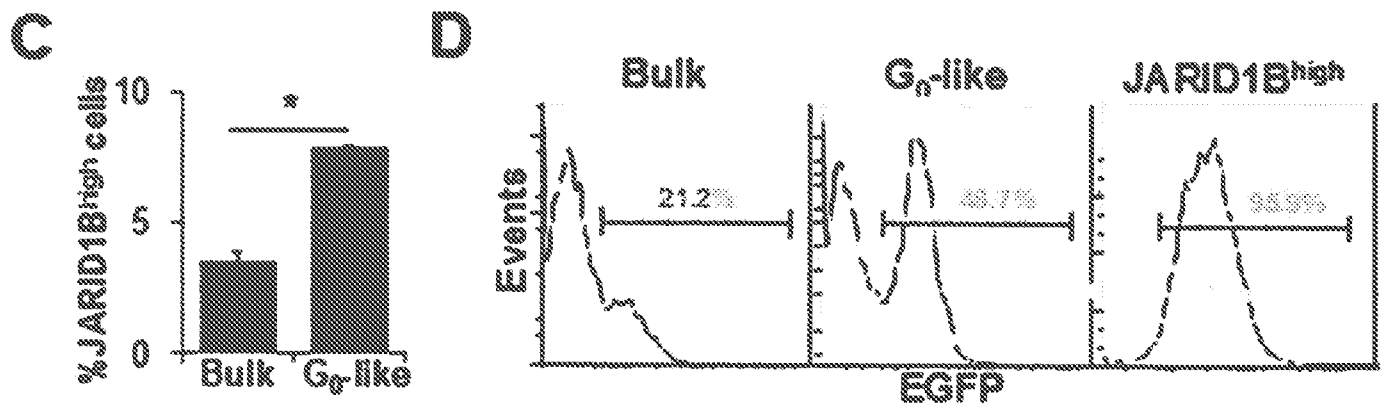


Figure 21C-21D

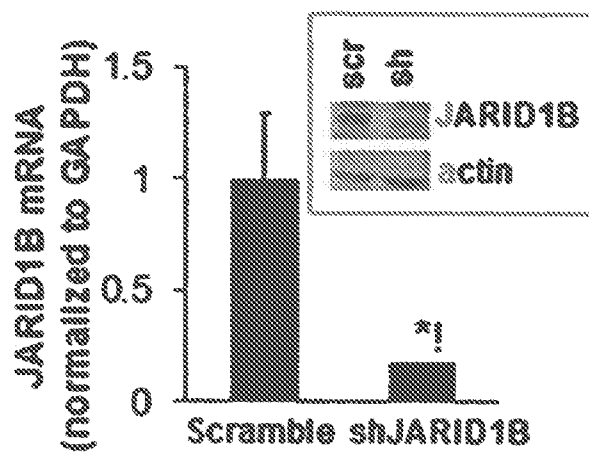


Figure 22A

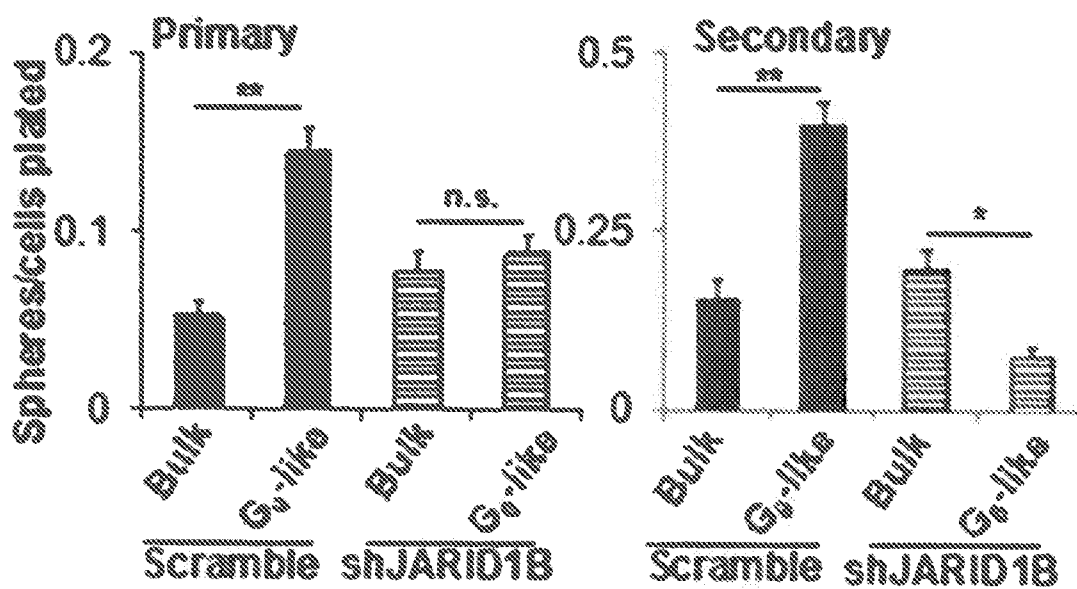


Figure 22B

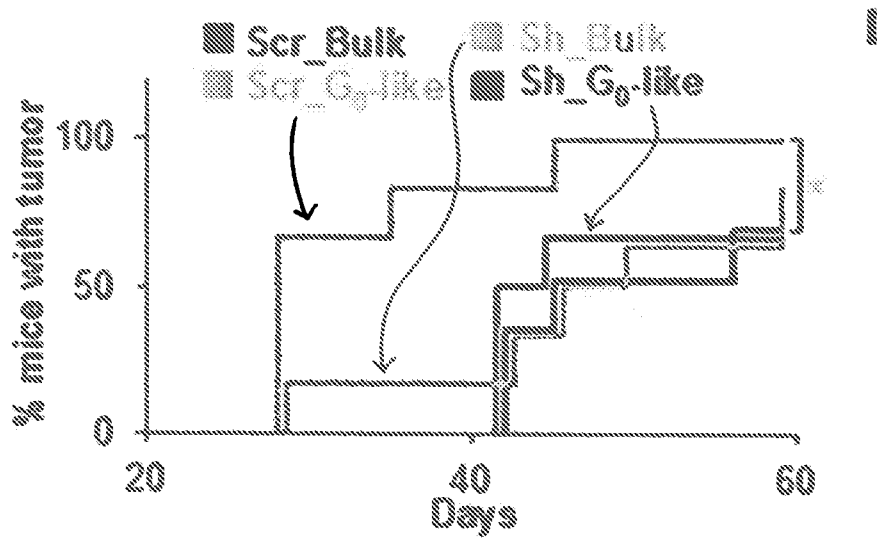


Figure 22C

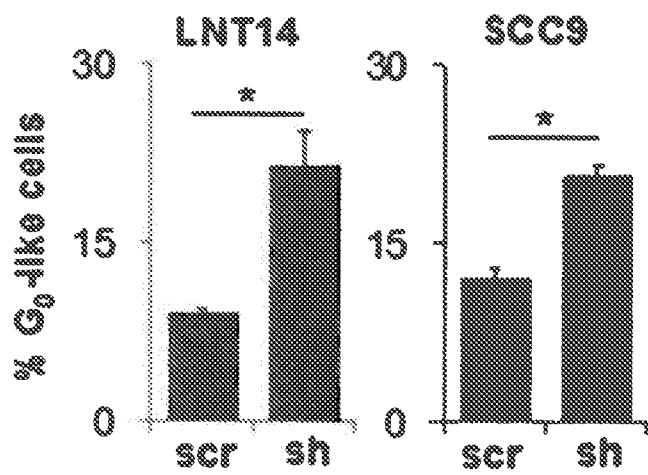


Figure 22D

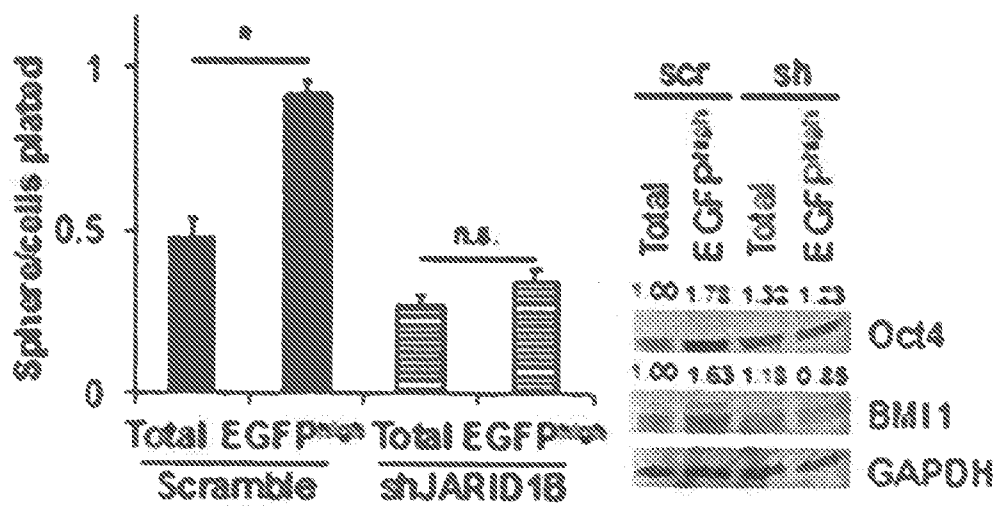


Figure 22E

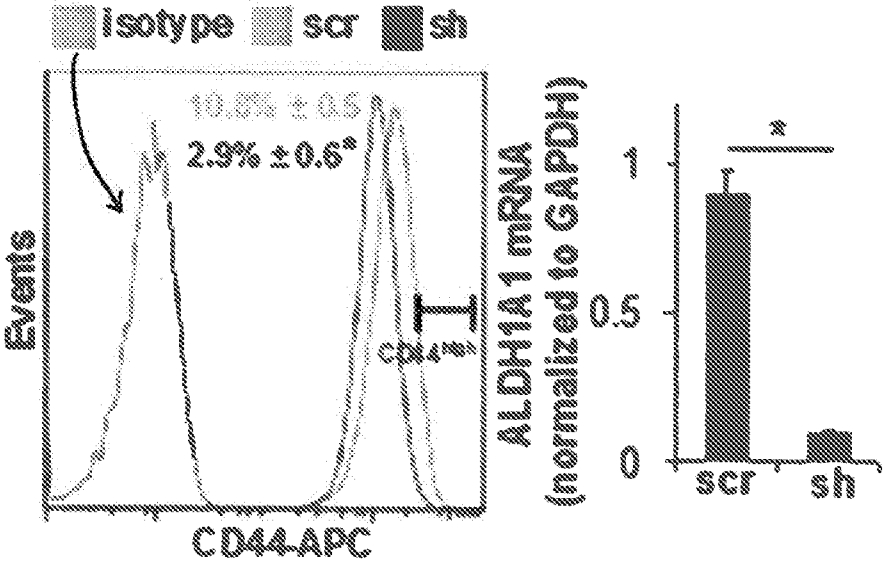


Figure 22F

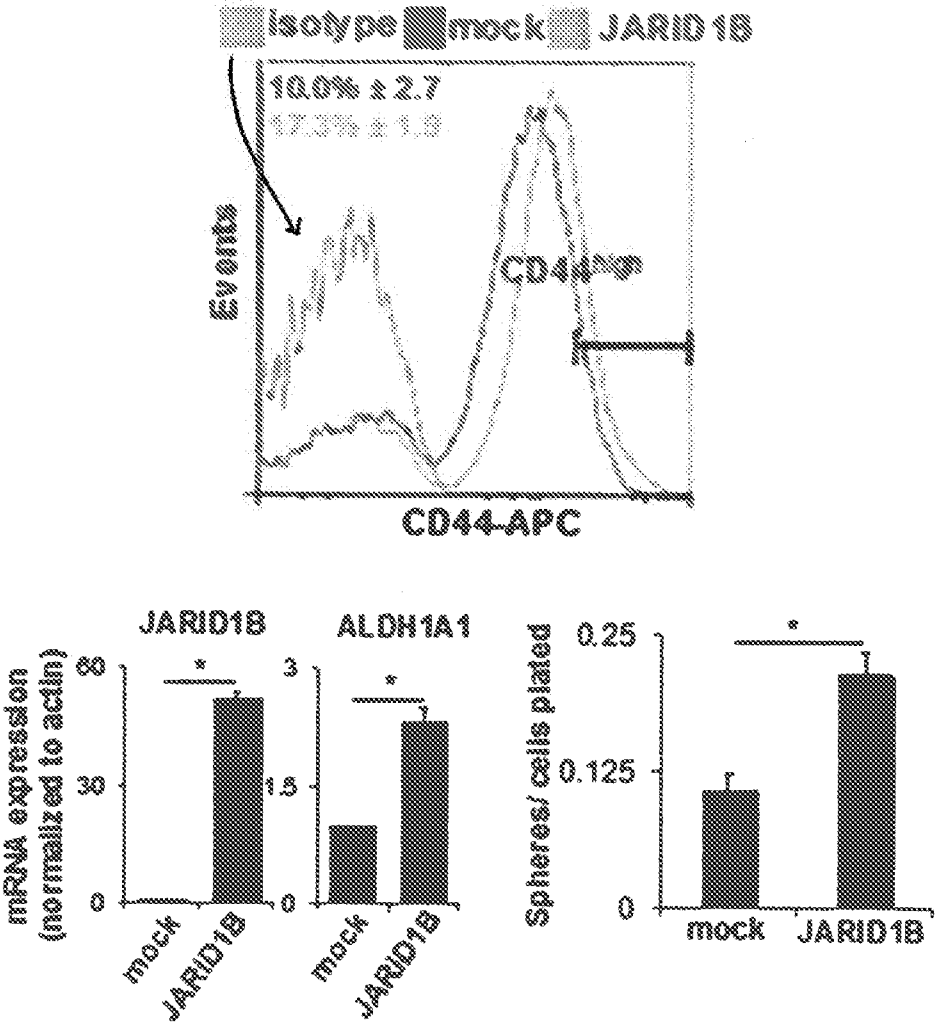


Figure 22G



Figure 23A

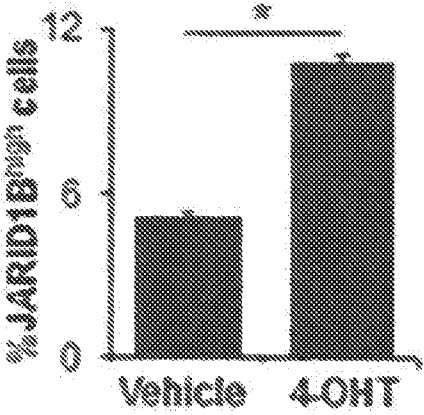


Figure 23B

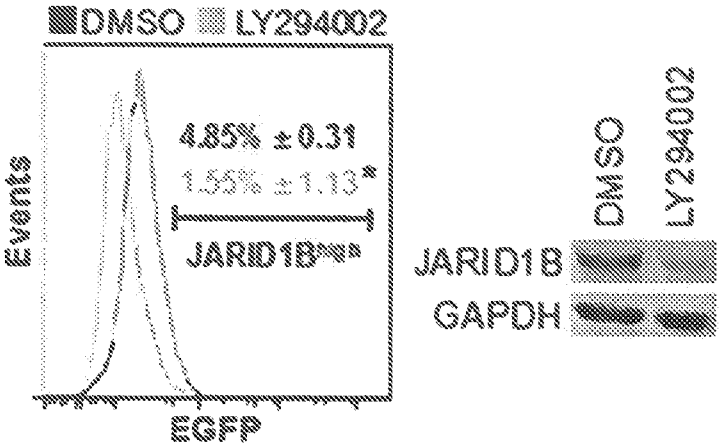


Figure 23C

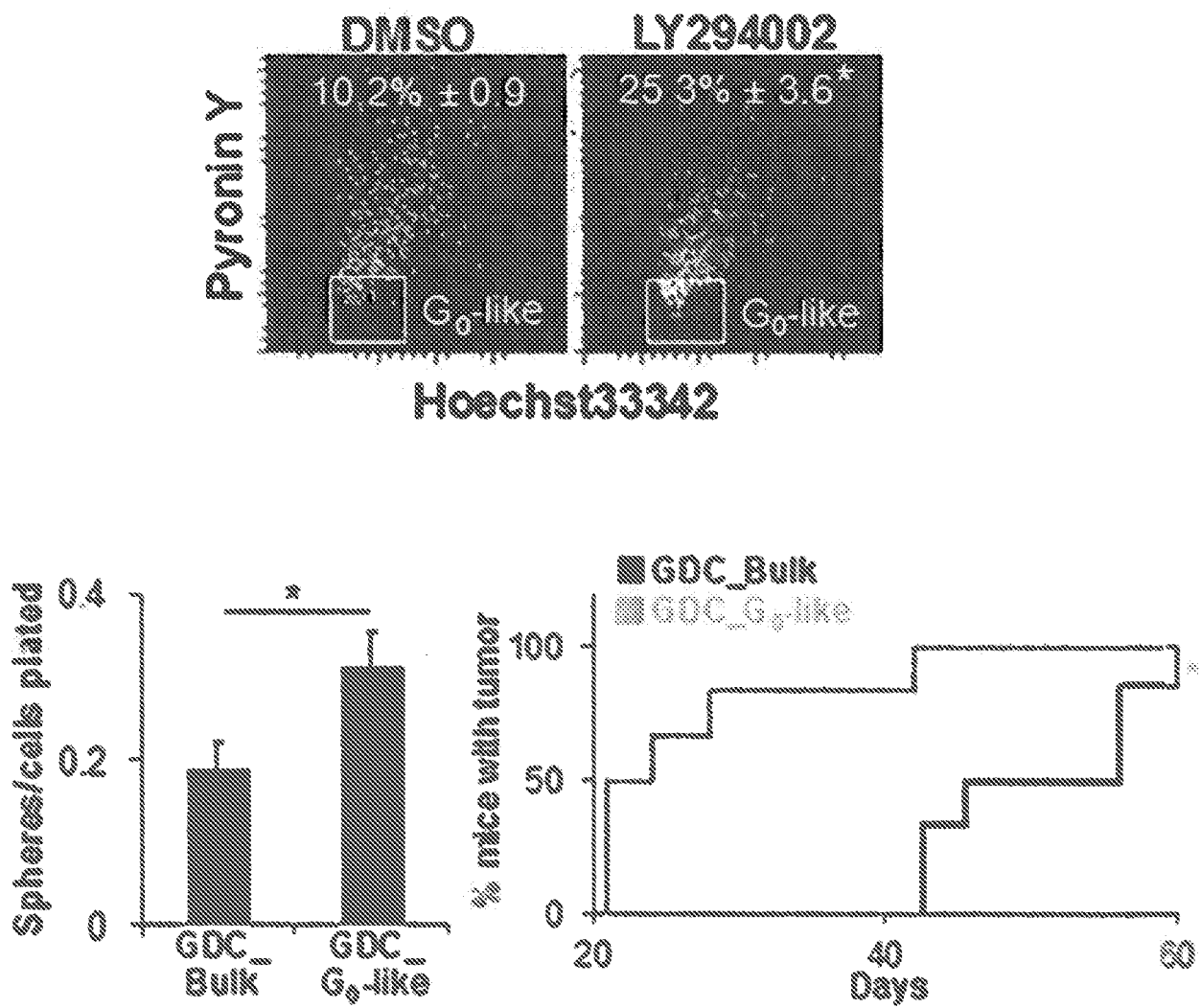


Figure 23D

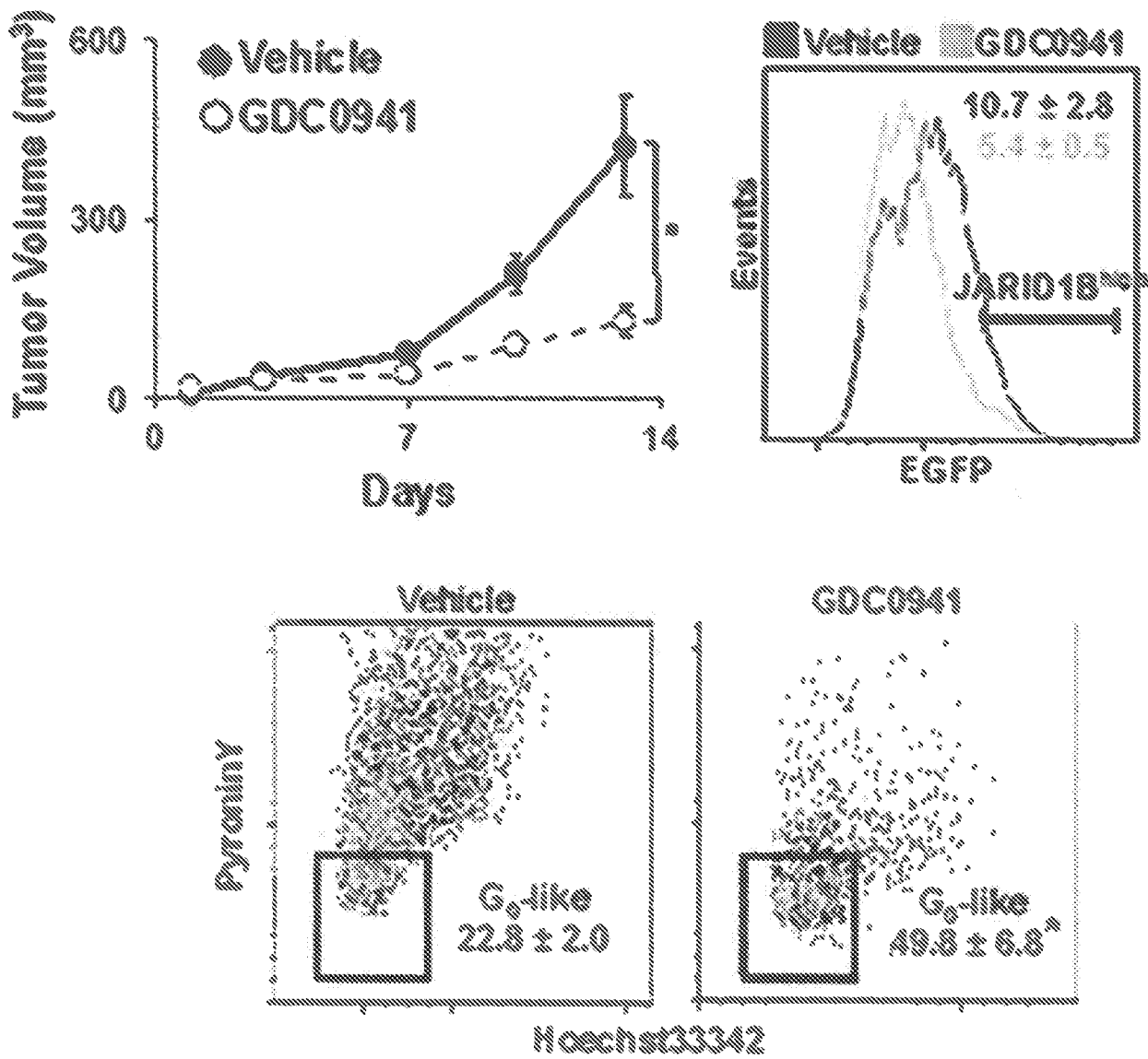


Figure 23E

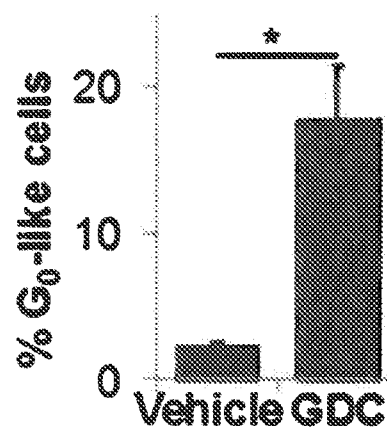


Figure 23F

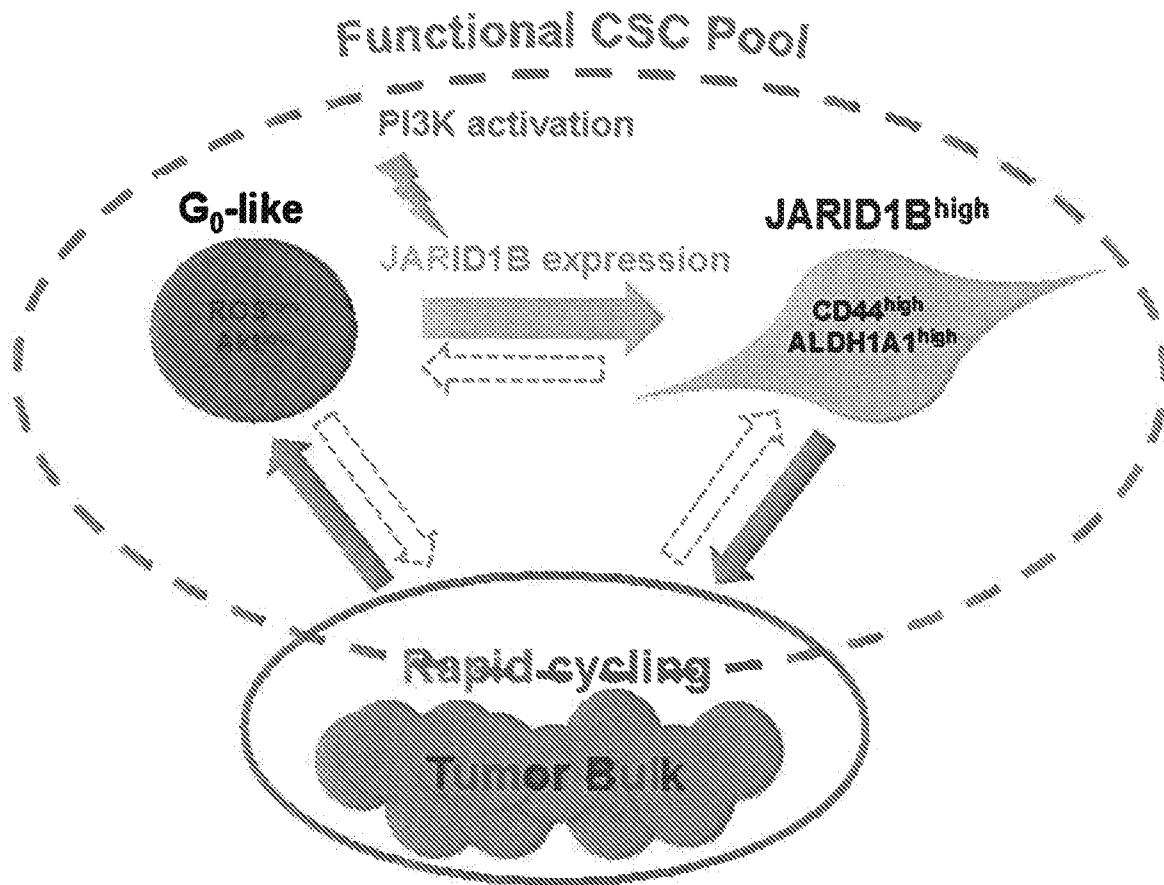
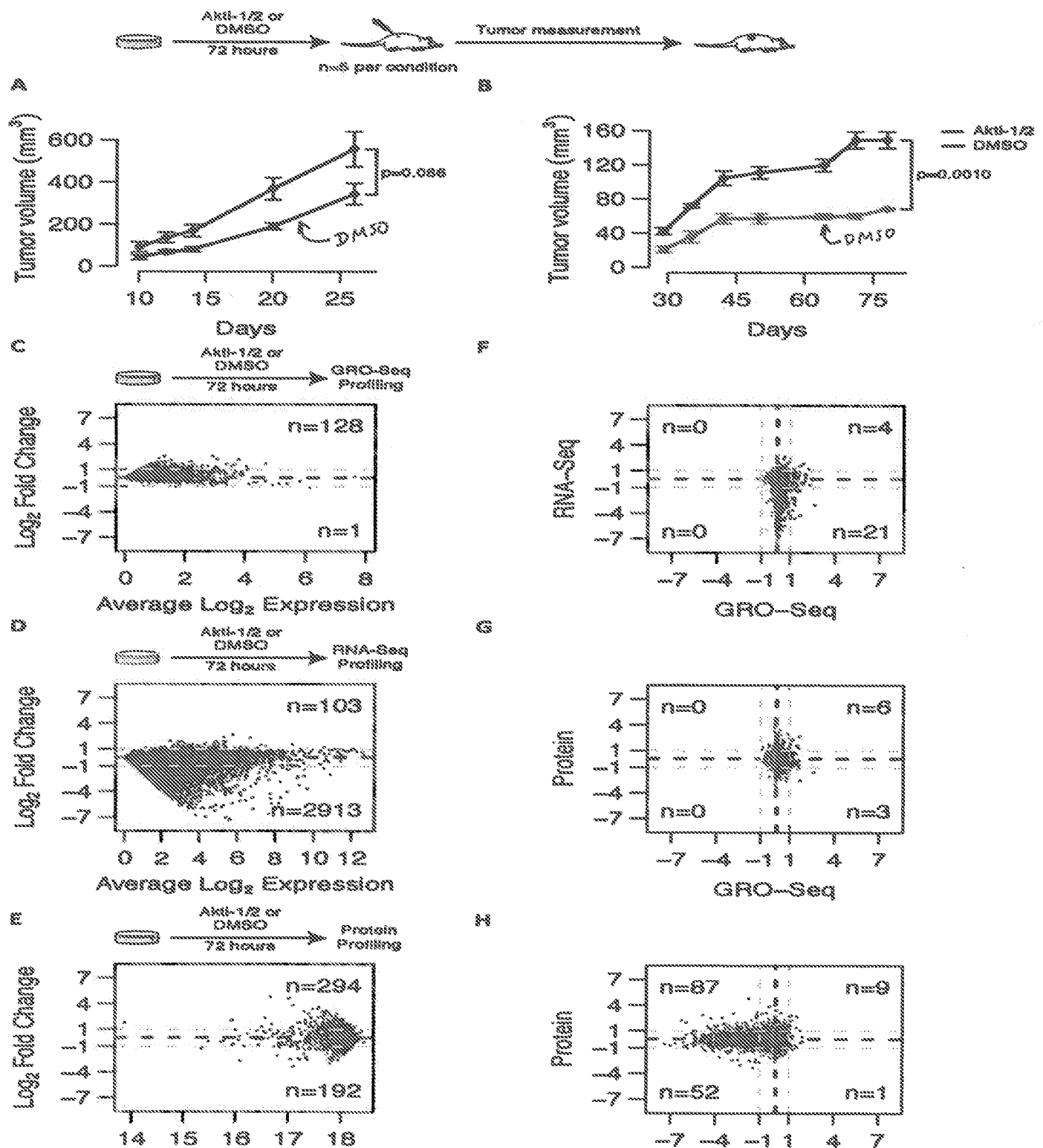


Figure 24



Figures 25A-25H

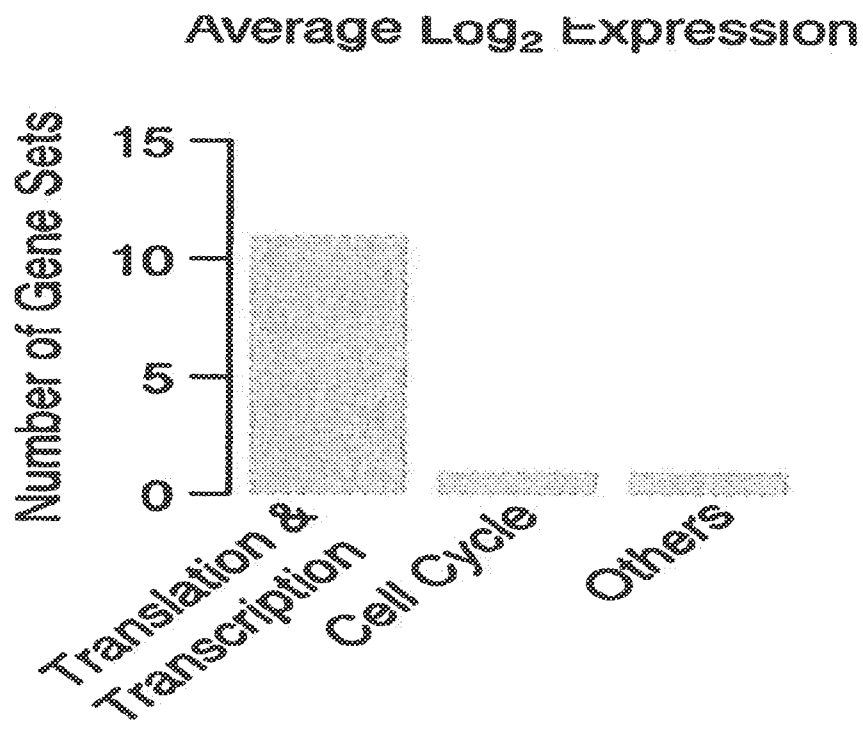


Figure 25I

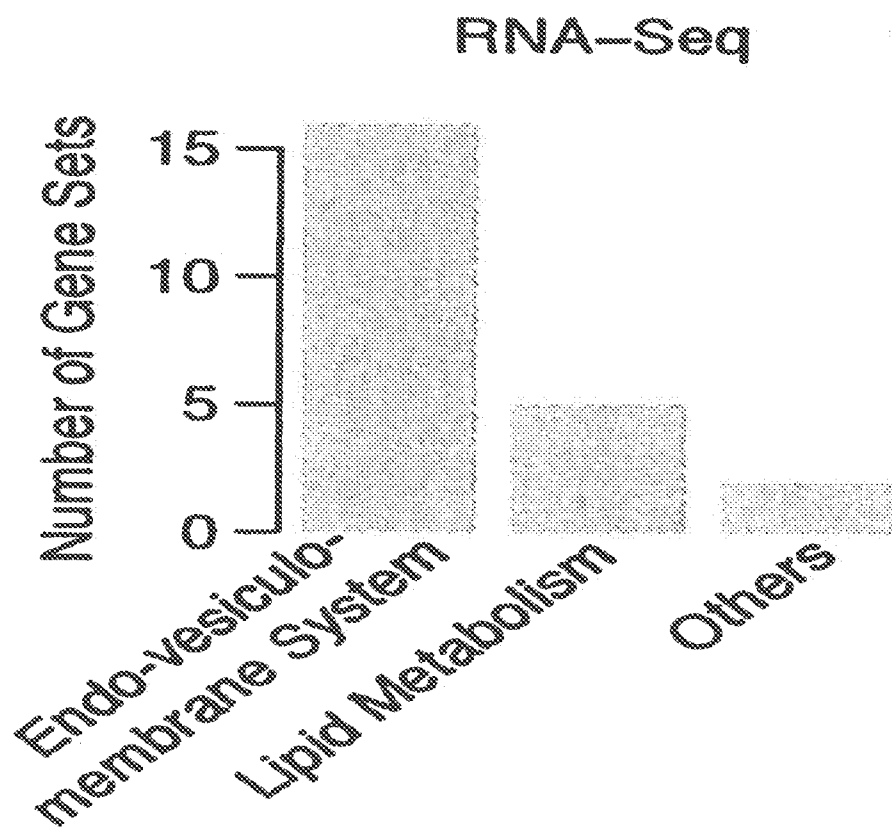


Figure 25J

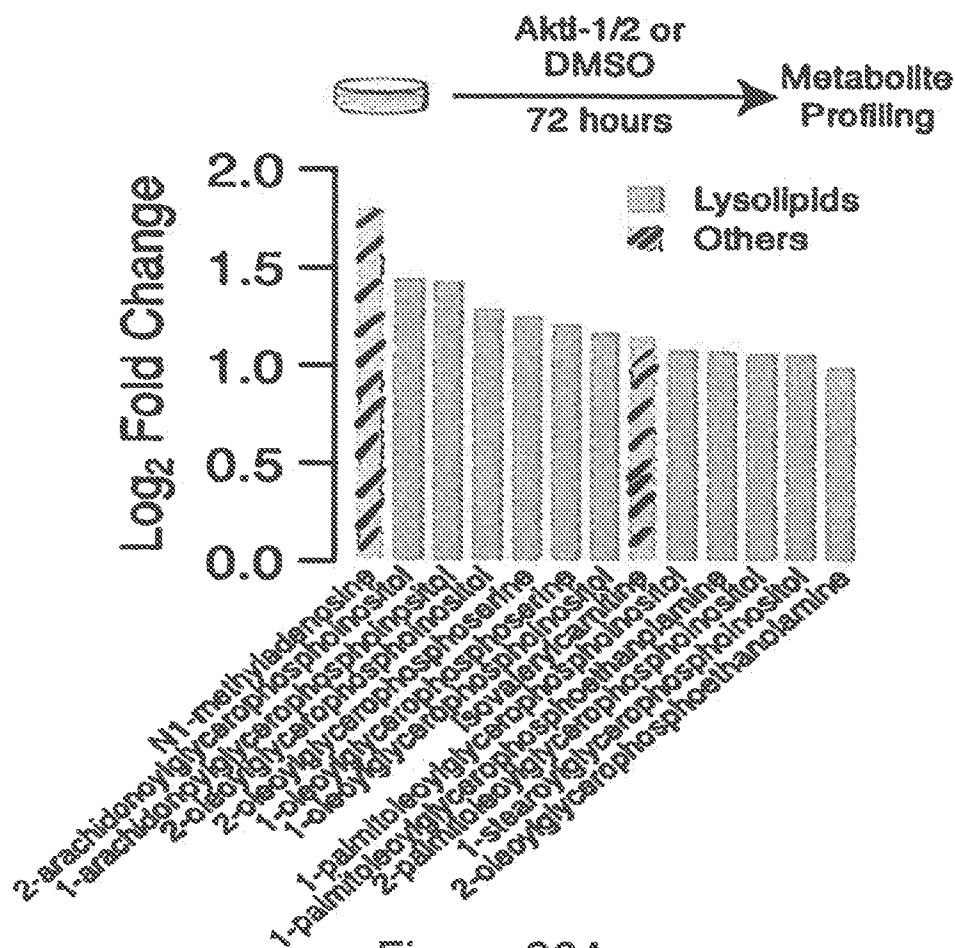


Figure 26A

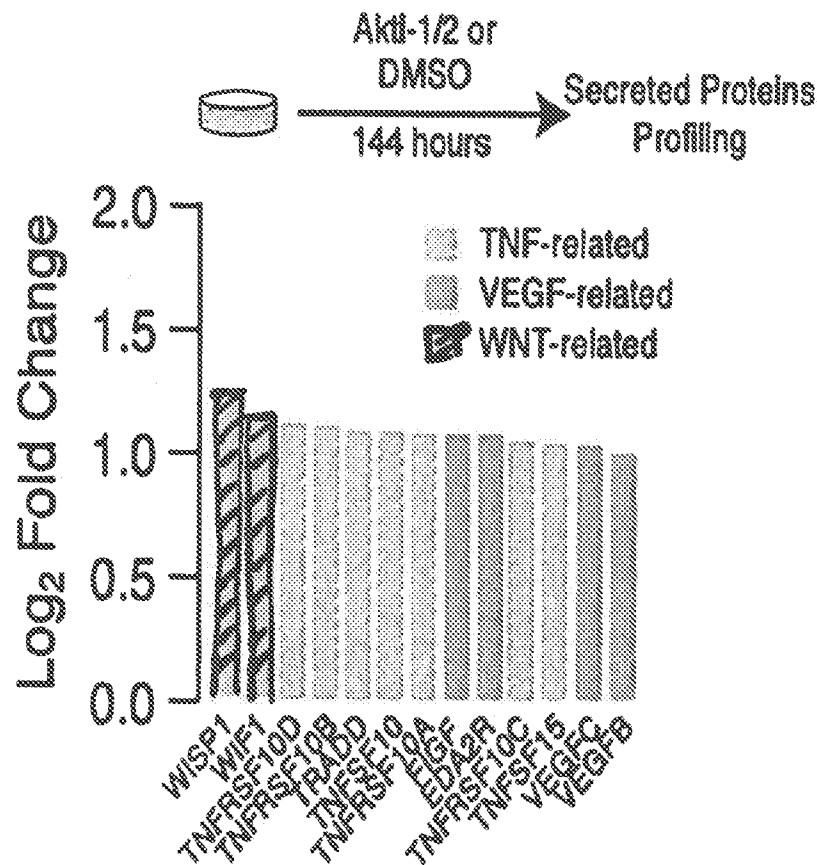


Figure 26B

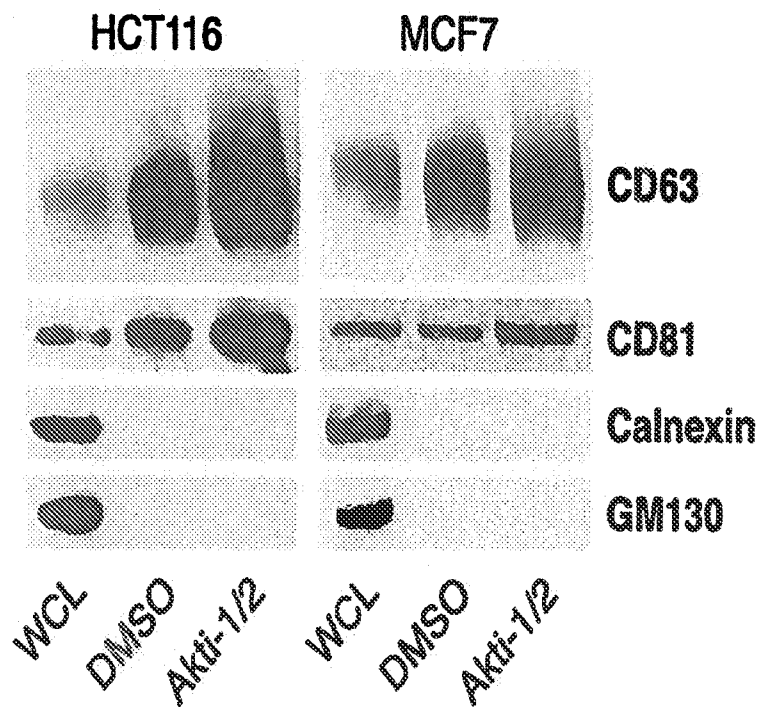


Figure 26C

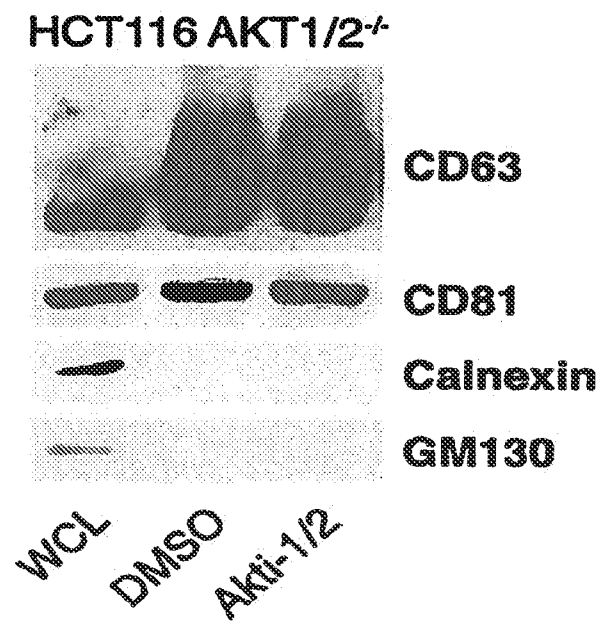


Figure 26D

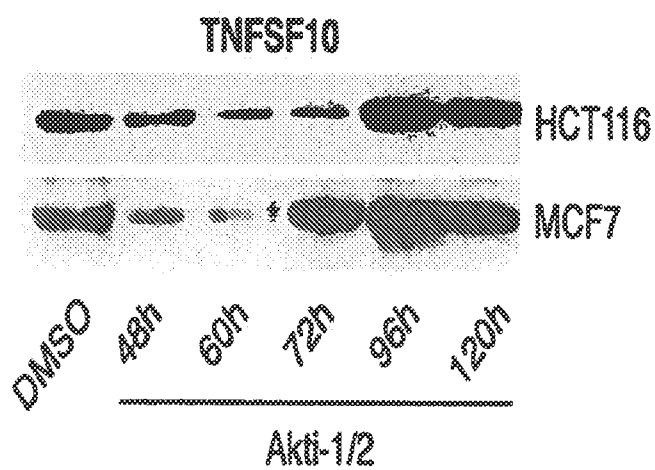


Figure 26E

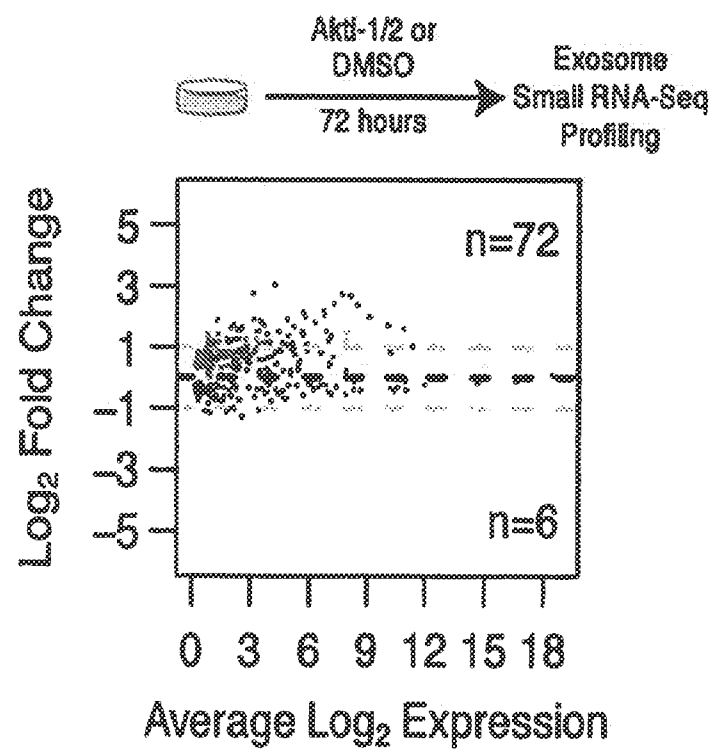


Figure 26F

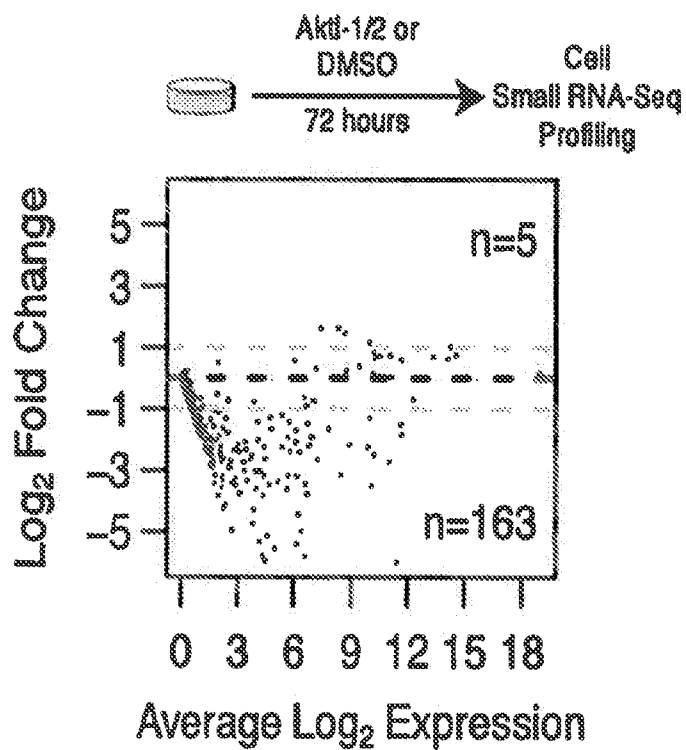


Figure 26G

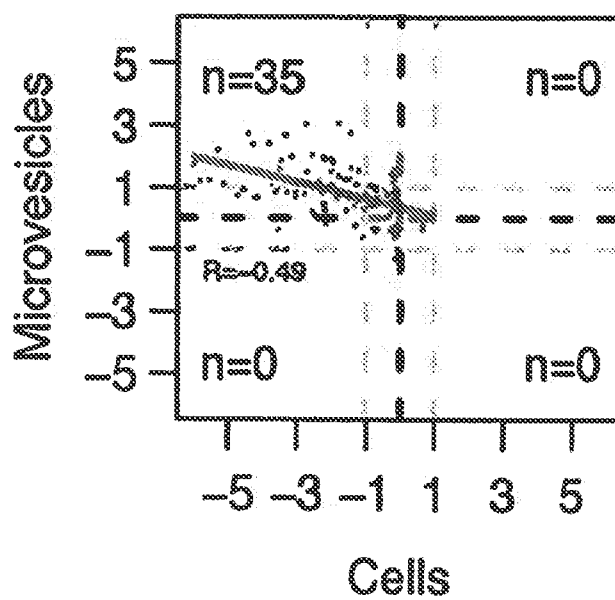
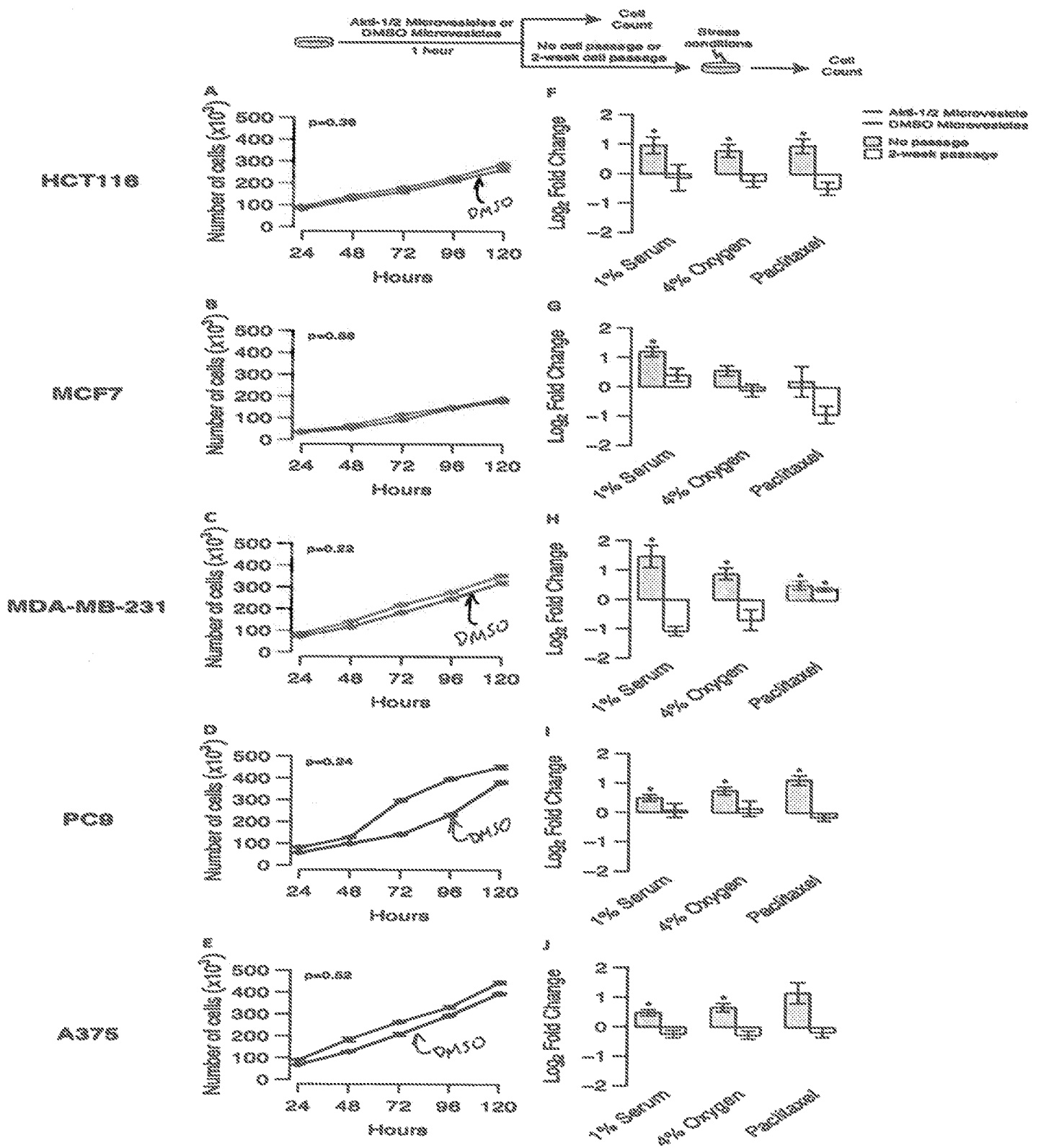


Figure 26H



Figures 27A-27J

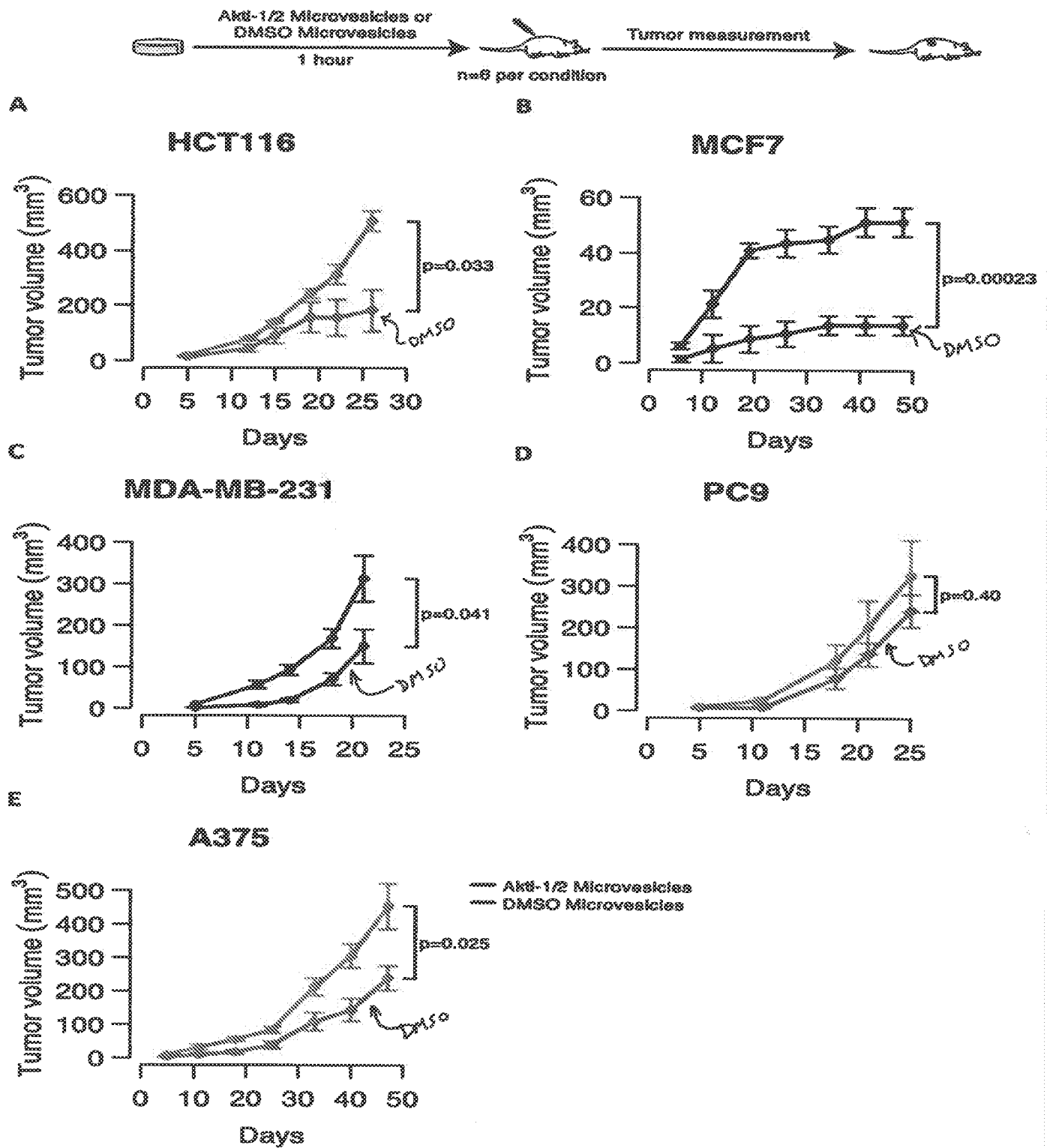


Figure 28A-28E

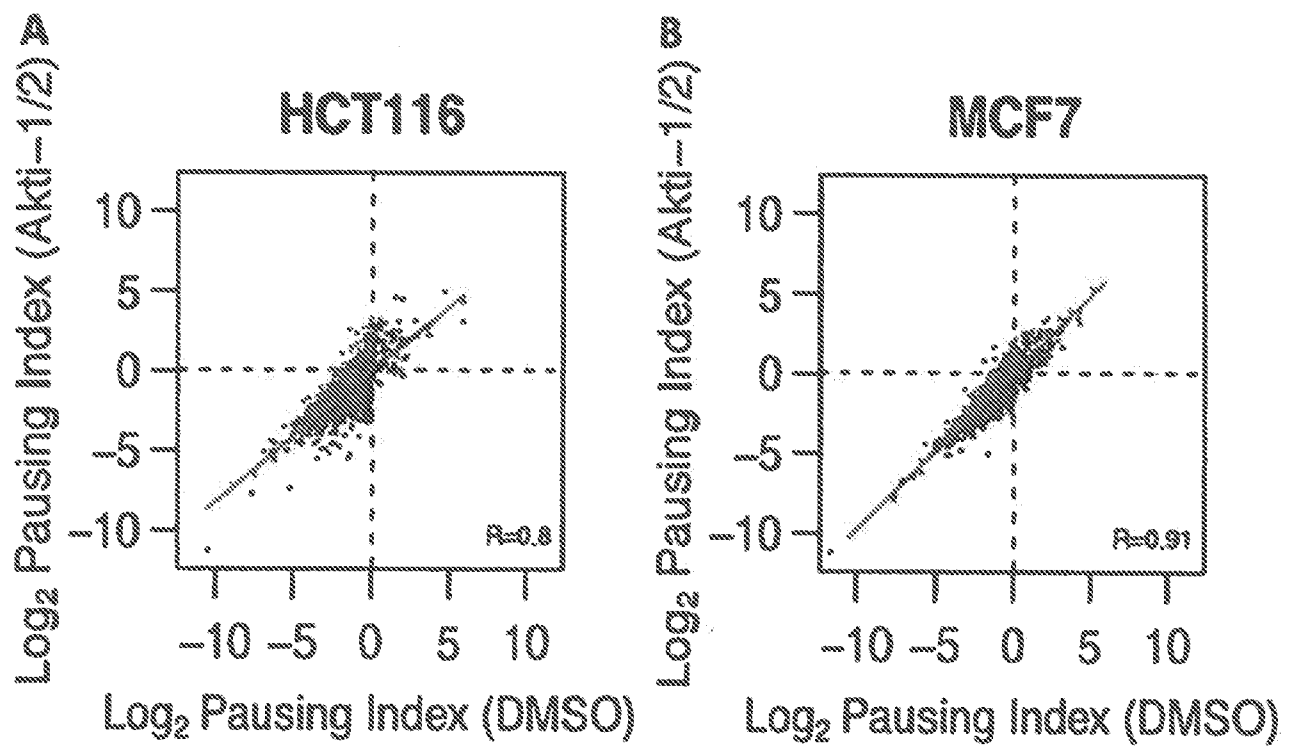
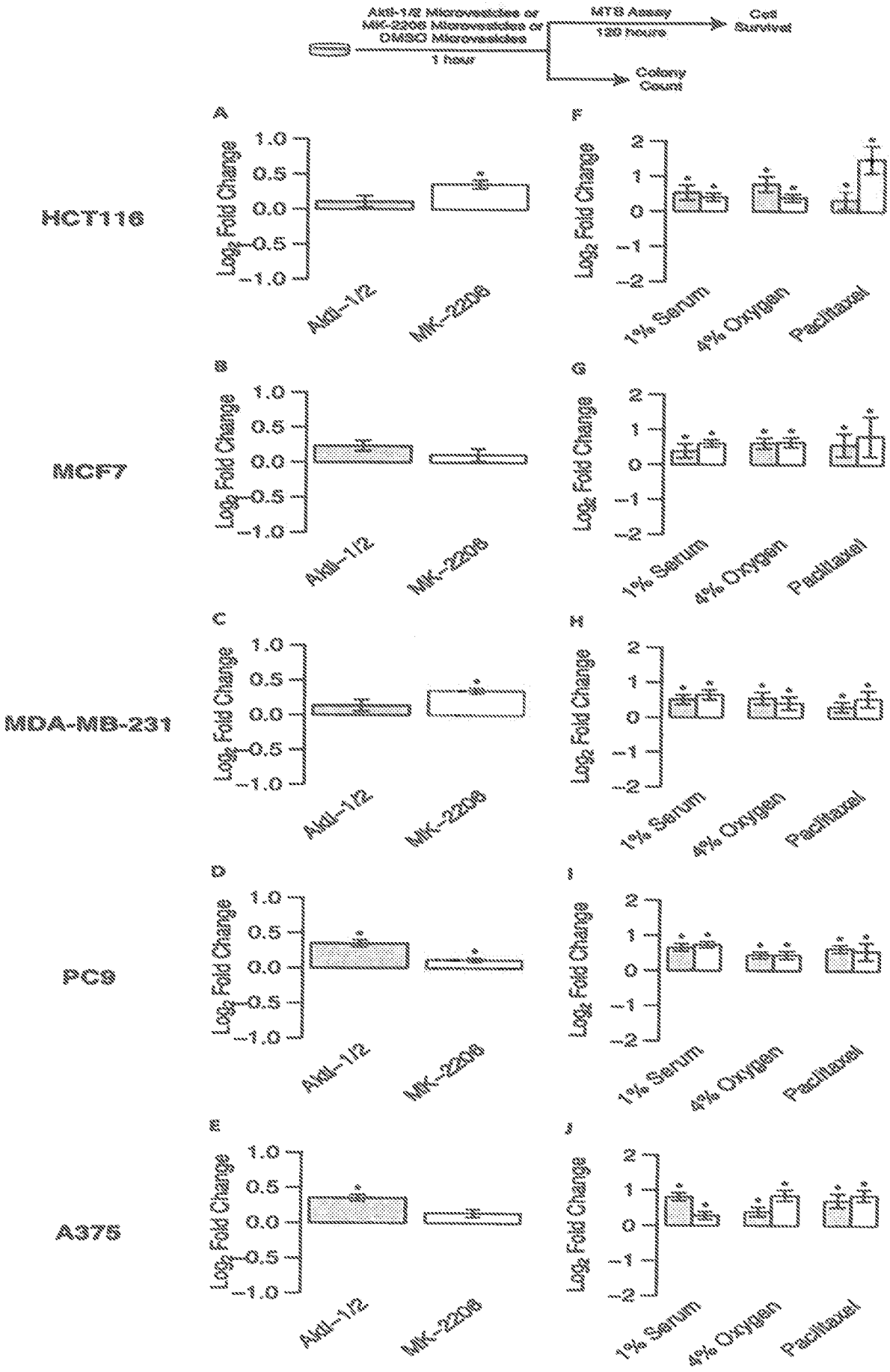


Figure 29A-29B

Exosomes derived from MCF-2206 treated cells
Exosomes derived from MCF-2206 treated cells



Figures 30A-30J

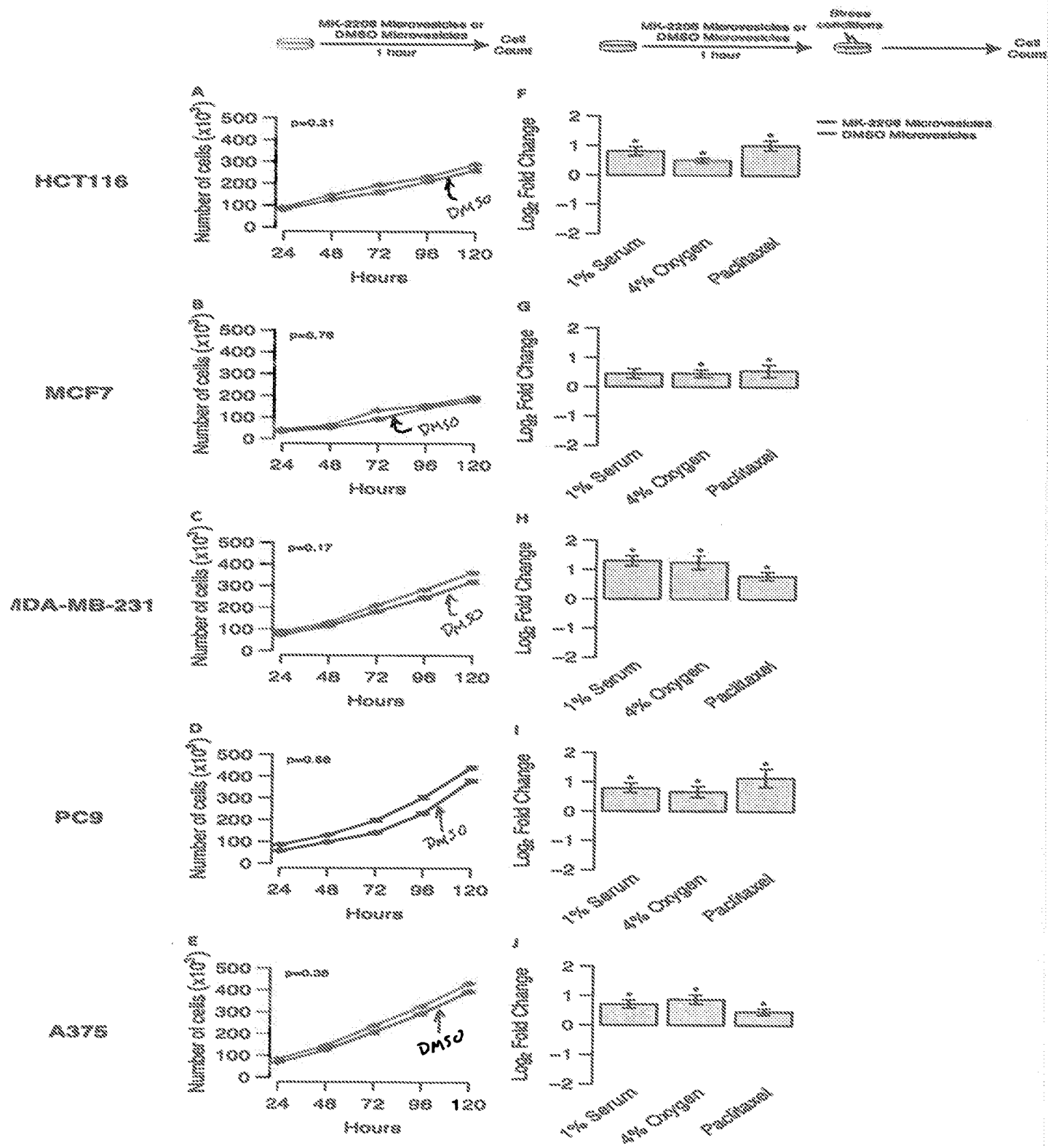


Figure 31A-31J

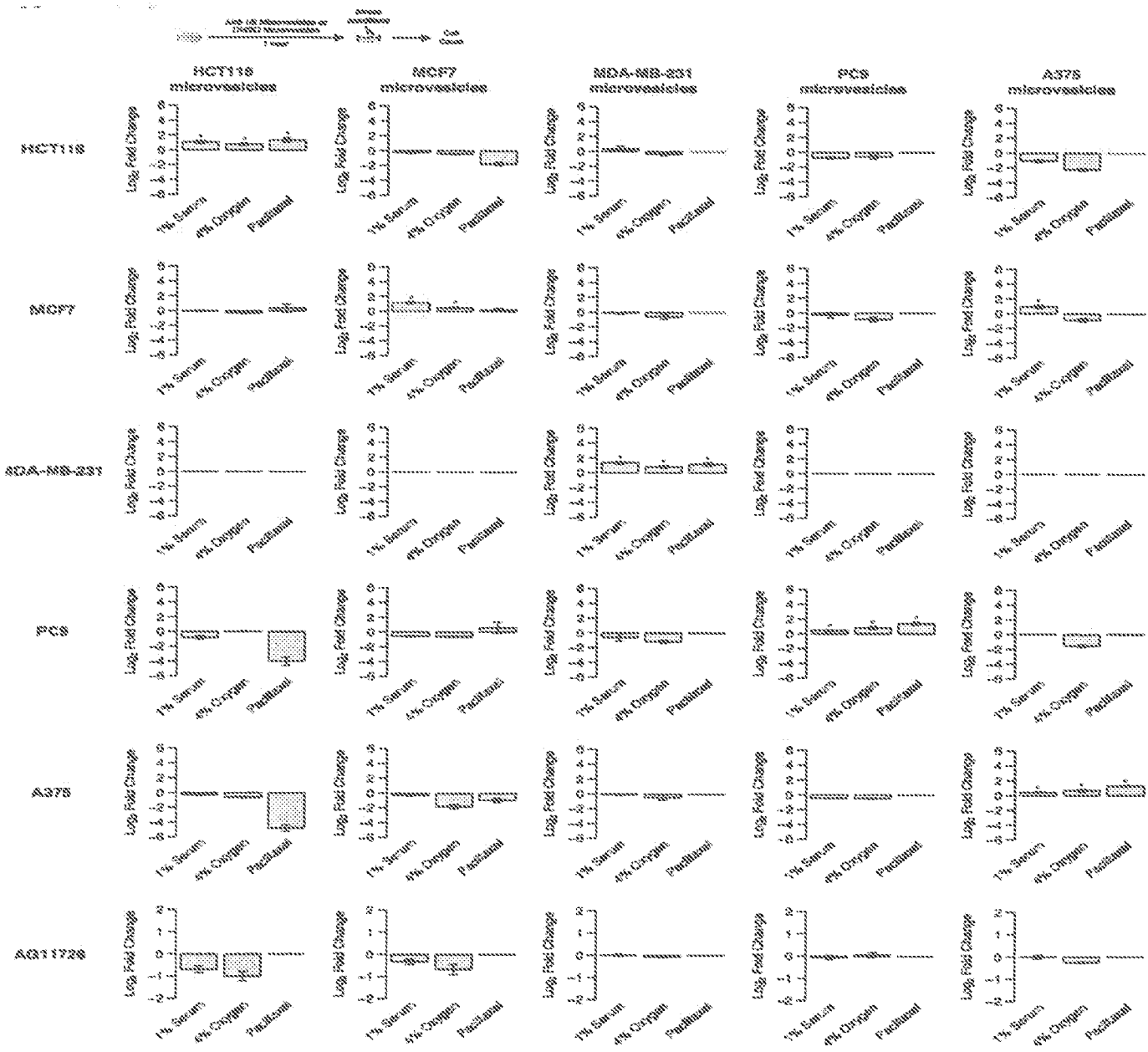


Figure 32

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/12597

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 5/00, C12N 5/02 (2016.01)

CPC - A61K 35/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - C12N 5/00, C12N 5/02 (2016.01)

CPC - A61K 35/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/377, 514/1.1, 424/158.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

pubWEST; PatBase; Google Scholar

search terms - Asymmetric, proliferation, division, slow, cancer, proliferat\$, Focal adhesion factors, Focal adhesion factor, FAK, beta1-integrin, inhib\$, AKT-1, akt1, akt, degradation, PF562271, PF-562271, PF-562,271, NVP-TAE226

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/047398 A1 (THE GENERAL HOSPITAL CORPORATION) 27 March 2014 (27.03.2014) para [0019]; [0055]-[0061].	1, 2
A	Dey-Guha, Ipsita, et al. "Asymmetric cancer cell division regulated by AKT." Proceedings of the National Academy of Sciences 108.31 (2011): 12845-12850. entire document, especially abstract; p. 12848.	1
A	Solit et al., entitled "Inhibition of Heat Shock Protein 90 Function Down-Regulates Akt Kinase and Sensitizes Tumors to Taxol" CANCER RESEARCH 63, 2139-2144, May 1, 2003. entire document, especially abstract.	1

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

19 April 2016

Date of mailing of the international search report

05 MAY 2016

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/12597

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13*ter*. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*. 1(a)).
 - ☐ on paper or in the form of an image file (Rule 13*ter*. 1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/12597

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 14, 15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1 and 2, directed to a method of increasing the rate of asymmetric proliferation in a cancer cell.

Group II, claims 3-13, directed to a method of decreasing the rate of asymmetric proliferation in a cancer cell/a method of treating cancer in a subject in need thereof.

The inventions listed as Groups I and II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

--continued on first extra sheet attached hereto--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 2

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

--continuation of Box No. III--

Special technical features

Group I has the special technical feature of an agonist of AKT1 degradation, that is not required by Group II.

Group II has the special technical feature of an inhibitor of AKT1 degradation, that is not required by Group I.

Common technical features:

Groups I-II share the common technical feature of a method of modifying the rate of asymmetric proliferation in a cancer cell, the method comprising: contacting the cancer cell with a modifier of AKT1 degradation, wherein a modification in AKT1 degradation changes the rate of asymmetric proliferation in the cancer cell. However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is made obvious by the article by Dey-Guha et al., entitled "Asymmetric cancer cell division regulated by AKT." (Proceedings of the National Academy of Sciences 108.31 (2011): 12845-12850), (hereinafter Dey-Guha), in view of the article by Solit et al., entitled "Inhibition of Heat Shock Protein 90 Function Down-Regulates Akt Kinase and Sensitizes Tumors to Taxol" (CANCER RESEARCH 63, 213972144, May 1, 2003), (hereinafter 'Solit').

Dey-Guha teaches a method of modifying the rate of asymmetric proliferation in a cancer cell, the method comprising: contacting the cancer cell with a modifier of AKT1 activity, wherein a change in AKT1 activity changes the rate of asymmetric proliferation in the cancer cell (Abstract 'inhibition of AKT signaling with small molecule drugs can induce asymmetric cancer cell division and the production of slow proliferators'; p. 12848, col 2, para 1 'AKT-1/2i treatment also increased the frequency of asymmetric mitotic cells by approximately threefold (Fig. 4E). These increases were associated with a profound slowing of proliferation, which was completely reversible with inhibitor washout, but no appreciable cell death (Fig. 4F)').

Solit teaches the use of a modulator of AKT1 degradation (abstract 'Inhibitors of Hsp90 cause Akt degradation. 17-allylamino-17-demethoxygeldanamycin (17-AGG) is an Hsp90 inhibitor currently in Phase I clinical trial'), wherein said modulator also suppressed AKT1 activity (abstract 'Enhancement was schedule-dependent and maximal when Taxol and 17-AAG were administered on the same day. These results suggest that Hsp90 inhibitors can effectively suppress Akt activity in animal models of human cancer at nontoxic doses, thus sensitizing tumor cells to proapoptotic stimuli'). It would have been obvious to one of ordinary skill in the art to use a modulator of AKT1 degradation and activity, such as the one taught by Solit, when practicing the methods of Dey-Guha, in view of Solit teaching said modulators for 'sensitizing tumor cells to proapoptotic stimuli' (abstract), in order to further modify the effect of Akt signaling on cell proliferation.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Groups I-II lack unity of invention under PCT Rule 13.

Note: Claims 14 and 15 are improper multiple dependent claims because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).