Asymmetric cancer cell division regulated by AKT

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Edited by Robert A. Weinberg, Whitehead Institute for Biomedical Research; Massachusetts Institute of Technology; Ludwig MIT Center for Molecular Oncology, Cambridge, MA, and approved June 17, 2011 (received for review March 25, 2011)

Human tumors often contain slowly proliferating cancer cells that resist treatment, but we do not know precisely how these cells arise. We show that rapidly proliferating cancer cells can divide asymmetrically to produce slowly proliferating “G0-like” progeny that are enriched following chemotherapy in breast cancer patients. Asymmetric cancer cell division results from asymmetric suppression of AKT/PKB kinase signaling in one daughter cell during telophase of mitosis. Moreover, inhibition of AKT signaling with small-molecule drugs can induce asymmetric cancer cell division and the production of slow proliferators. Cancer cells therefore appear to continuously flux between symmetric and asymmetric division depending on the precise state of their AKT signaling network. This model may have significant implications for understanding how tumors grow, evade treatment, and recur.

Tumors generally evolve through years of mutation and clonal selection (1). This favors the outgrowth of rapidly proliferating cancer cells over time. However, even advanced tumors contain many cancer cells that appear to be proliferating slowly (2). This proliferative heterogeneity correlates closely with time to clinical detection, growth, metastasis, and treatment response across all tumor types, but we still do not understand clearly how it arises. The rate of mammalian cell proliferation is generally determined by the time spent in G1 of the cell cycle. Critical genetic and epigenetic changes within cancer cells accelerate G1 transit, whereas a suboptimal microenvironment with imbalance of growth factors, nutrients, or oxygen can slow G1 progression (3). Therefore, individual cancer cells within a tumor are thought to vary significantly in their proliferative rate depending on the precise balance of these intrinsic and extrinsic factors. Interestingly, however, many tumor-derived cancer cell lines also produce slowly proliferating cells. These established lines have many acquired mutations that drive cell proliferation. They have also been grown ex vivo for years in a stable microenvironment to promote unbridled proliferation. These factors afforded a strong purifying selection against slow proliferators. We worked to understand how slowly proliferating cells seem to arise paradoxically in cancer cell lines.

Results

G0-Like Cancer Cells in Vitro. We began by studying MCF7, a highly proliferative, aneuploid, ER+/HER2+ human breast cancer cell line. This line displays significant proliferative heterogeneity despite mutations in CDKN2A and PIK3CA that cooperatively drive cell-cycle progression (4). We first hypothesized that slowly proliferating MCF7 cells might produce low levels of reactive oxygen species (ROS). This hypothesis was based on previous observations that slowly cycling hematopoietic, neural, and breast adult stem cells and cancer stem cells produce low levels of ROS (5–7). We stained MCF7 cells with 5-(and-6)chloromethyl 1-2,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA), a live-cell dye for ROS, and used fluorescence-activated cell sorting (FACS) to isolate ROSlow and ROShigh cells (which we defined as the bottom and top 1% of the observed distribution, respectively).

Indeed, cell-cycle analysis confirmed that ROSlow cancer cells were predominantly in G1/G0 of the cell cycle compared with actively cycling ROShigh cells (Fig. L4). ROSlow cells did not alter expression of ESR1 or MYC, which are both necessary for MCF7 proliferation (Fig. 1B and SL4) (4, 8). However, these cells expressed very low levels of critical proliferation proteins (e.g., MKI67low, MCM2low, CDC6low, GMNNlow, AURKAlow, PLK1low) and histone marks (i.e., H3S10phlow, H3K4me2low, H3K9me2low, H3K7me3low, H4K12aclow, H4K16aclow) that are suppressed in noncycling cells (Fig. 1 C–E and SL4) (2, 9–12). Notably, ROSlow cells were not unique to MCF7; we could also find similar slowly cycling cells in the MDA-MB-231 breast and HCT116 colon cancer lines (Fig. SL1B).

Molecular Profiling of G0-Like Cells. We next used two different molecular profiling approaches to further characterize ROSlow cancer cells. Microarray transcriptional profiling revealed that ROSlow MCF7 and HCT116 cells modestly up-regulated the HES1 transcription factor [false discovery rate (FDR) = 0.05, fold change = 2.8] (Fig. 1F and G). This was unexpected because HES1 was recently reported to uniquely mark normal quiescent fibroblasts that have completely withdrawn from the cell cycle into a G0 state (13). We therefore considered the provocative possibility that ROSlow cancer cells might actually be an “out-of-cycle” subpopulation in G0 (rather than prolonged G1). Within 24–48 h of culture in vitro, however, we found that many ROSlow cells up-regulated proliferation proteins and histone marks, divided, and formed colonies normally (Fig. 1H and J). This argued that ROSlow/HES1high cancer cells might express a special G0 marker but that they were, in fact, not quiescent. In addition, ROSlow cells did not express a cancer stem cell transcriptional or marker profile (e.g., CD44high/CD24low or VIMhigh/CDH1low) (Fig. 1C and D) (14, 15). We therefore chose the term “G0-like” to describe these slowly cycling ROSlow cancer cells.

We also used a reverse-phase protein microarray approach to probe ROSlow G0-like cancer cells for alterations in common signaling pathways (16). We made microarrays with whole-cell lysates of ROSlow and ROShigh cells from MCF7 and HCT116. We then interrogated these arrays with 89 different, highly validated antibodies targeting a spectrum of cancer-relevant phospho-proteins and proteins (Table S1) (17). Statistical analysis of the...


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1109632108/-/DCSupplemental.
Fig. 1. Slowly cycling G0-like cancer cells in vitro. (A) FACS analysis of MCF7 cells with gates for sorting ROS\textsuperscript{low} (blue) and ROS\textsuperscript{high} (red) cells. Insets show cell-cycle profiles of ROS\textsuperscript{low} and ROS\textsuperscript{high} populations. Tables indicate percentage of cells with respective DNA contents. (B–F) Cytospin of cells sorted for high or low ROS staining and stained for (B) ESR1, (C) MKI67, (D) MCM2, (E) H3K9me2, and (F) HES1. Merged images represent respective stains merged with underlying DAPI stain. (G) Heatmap of transcriptional profiles of three independent replicates of ROS-sorted MCF7 and HCT116 cells (columns). Rows depict expression of genes with greater than twofold change in expression and FDR < 0.25. Numbers on the right indicate fold change. Colorgram depicts high (red) and low (blue) relative levels of gene expression. (H) ROS-sorted cells stained for MKI67 and H3K9me2 either as cytospins (0 h) or after 24 h of culture. Merged images represent respective stains merged with underlying DAPI stain. (I) Plot shows number of colonies from MCF7, ROS\textsuperscript{low}, or ROS\textsuperscript{high} from three independent experiments (each symbol represents one independent experiment). Error bars indicate SD.
G0-Like Cancer Cells Arise Through Asymmetric Division. AKT signaling plays a major role in promoting cell-cycle progression (18). We therefore wondered whether slowly cycling AKT<sup>low</sup>-G0-like cancer cells arise by suppressing AKT protein levels. To test this hypothesis, we overexpressed a cDNA for an AKT1-mCherry fusion protein in MCF7 cells to ask whether this would inhibit the formation of G0-like cancer cells. However, forced AKT overexpression did not appreciably change the frequency of these cells. Instead, G0-like cancer cells with low endogenous AKT levels also suppressed expression of exogenous AKT1-mCherry protein in both MCF7 and HCT116 cells (Fig. S2 . A and B).

In addition, we noted that actively dividing MCF7 cells occasionally showed striking asymmetric expression of both the exogenous AKT1-mCherry and endogenous AKT protein, in contrast to most dividing cells, which appeared symmetric (Fig. 3 . A–C). This asymmetric expression occurred exclusively in telophase (after formation of the nuclear membrane but before cytokinesis) in the thousands of dividing cancer cells that we examined. In asymmetric mitosis, one daughter was in a proliferative stance (e.g., H3K9me<sup>2</sup><sub>high</sub>/HES1<sub>low</sub>) with diffuse AKT expression in both the nucleus and cytoplasm (Fig. 3 . B and D). Its sibling was in a G0-like posture (e.g., H3K9me<sup>2</sup><sub>low</sub>/HES1<sub>high</sub>) with perinuclear HES1 expression and more intense nuclear localization of AKT (Fig. 3 . B and D). Importantly, G0-like cells in telophase that were AKT-nuclear<sup>high</sup> appeared to suppress AKT protein expression to become AKT<sup>low</sup> after cell division (Fig. 3 . E). G0-like daughters in interphase also had higher levels of nuclear-localized FOXO1, a direct target of AKT signaling that localizes to the nucleus with loss of AKT signaling and can strongly suppress cell-cycle progression (Fig. 3 . F) (18). We did not observe any consistent differences in the size or general appearance of G0-like daughters. G0-like cancer cells therefore appeared to arise through occasional, asymmetric loss of AKT signaling (with nuclear localization and then suppression of AKT protein), resulting in the birth of a slowly cycling cancer cell.

Fig. 2. Molecular profiling of G0-like cells. (A) Heatmap of proteomic profiles of two independent replicates of ROS-sorted MCF7 and HCT116 cells (columns). Rows represent proteins with significant change in expression (FDR < 0.25). Numbers on the right indicate FDR. Colorgram depicts high (yellow) and low (blue) relative levels of proteins. (B and C) Cytospins of ROS-sorted cells stained for (B) pAKT (Ser473), pAKT (Thr308), pS6RP (235/236), and pS6RP (240/244) and (C) pan-AKT. (D) Western blot for pan-AKT on bulk and ROS-sorted MCF7 cells. p, phosphorylated.

resulting dataset identified 9 up- and 14 down-regulated protein markers in ROS<sup>low</sup> compared with ROS<sup>high</sup> cells (FDR < 0.25) (Fig. 2A). Interestingly, these differentially expressed proteins included important members of the AKT/PKB signaling pathway that suggested a down-regulation of AKT signaling in slowly cycling ROS<sup>low</sup> cells (i.e., pS6RP, p70S6K, pFOXO1, pGSK3<sub>αβ</sub>, PRAS40) (18). Consistent with this inference, we found that ROS<sup>low</sup> cells had low levels of both phospho-AKT (pAKT-T308 and pAKT-S473) and its downstream target phospho-S6RP (pS6RP-S235/236 and pS6RP-S240/244) (Fig. 2B and Fig. S1E). Furthermore, ROS<sup>low</sup> cells also expressed low levels of total AKT protein itself (Fig. 2C and D).

Fig. 3. G0-like cancer cells arise through asymmetric division. (A–D) MCF7 cells in telophase stained for DAPI, β-tubulin (TUBB). (A) AKT1-mCherry cells, asymmetric, (B) pan-AKT, asymmetric; (C) pan-AKT, symmetric, and (D) HES1, asymmetric. (E and F) MCF7 cells in interphase stained for (E) DAPI, β-tubulin, H3K9me2, and pan-AKT and (F) DAPI, HES1, H3K9me2, and FOXO1.
Induction of Asymmetric Cancer Cell Division with AKT Inhibition.

Both MCF7 and HCT116 have activating mutations in PIK3CA that drive constitutive AKT signaling on which these cells depend for their survival (19). It was thus curious that G0-like cells found in MCF7 and HCT116 could tolerate suppression of AKT signaling. We therefore asked whether experimental suppression of AKT signaling could actually induce G0-like cancer cells rather than killing them. We treated MCF7 cells with a small-molecule, allosteric AKT-1/2 inhibitor (AKT-1/2i) for 72 h and looked for G0-like cells (20). As expected, low-dose AKT-1/2i (0.1 μM) had no appreciable effect on these cells, whereas a high dose (5 μM) induced significant apoptosis (Fig. 4A). Surprisingly, however, an intermediate dose of AKT-1/2i (2 μM) (which only partially inhibited AKT signaling; Fig. 4B) increased the number of MCM2\textsuperscript{low}/H3K9me2\textsuperscript{low}/HES1\textsuperscript{high} G0-like cells from an estimated 1% at baseline to about 50% of the population (Fig. 4C and D). Furthermore, 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). We also obtained similar results in HCT116 cells and with a second, clinical-grade allosteric AKT-1/2 inhibitor (MK-2206) (Fig. S3A–Q) (21). In contrast, we could not induce a similar G0-like marker profile in cells treated with 20 μM rapamycin (a specific inhibitor of the mTOR kinase, which is a direct downstream target of AKT), 10 nM staurosporine (a general protein kinase inhibitor), or 50 μM N-[3,5-di-fluorophenyl]glycine-1,1-dimethylhydrazine (DAPT) (an inhibitor of the γ-secretase/NOTCH pathway that regulates HES1), which inhibited cell proliferation but did not induce G0-like cells.

We also performed live-cell imaging experiments to examine the dynamics of cancer cell division with and without AKT-1/2i treatment. We first obtained serial images of MCF7 cells dividing over time. We then analyzed these images to identify individual cells, created lineage traces of these individual cells and their progeny to identify sibling pairs, and measured intermitotic times for each cell. We found that AKT inhibition slowed the average cell-cycle time of MCF7 cells from 26 to 49 h and dramatically increased the fraction of slowly cycling cells. For example, 1% of cells had a cell-cycle time of >100 h at baseline, whereas AKT-1/2i treatment increased this fraction to about 10% (Fig. 4G). Furthermore, this increased proliferative heterogeneity was associated with an increase in asymmetric division. Sibling–sibling differences in time to next mitosis increased from about 6.2 to 16.9 h on average. Whereas 2% of sibling cells had a difference in time to next mitosis of >60 h at baseline, AKT-1/2i treatment increased this fraction to about 10% (Fig. 4H and Movie S1). In addition, AKT-1/2i induced asymmetric division more potently when first delivered to cells just before mitosis rather than at other points in the cell cycle (Fig. 4I). These results suggested that quantitative inhibition of AKT signaling induced asymmetric division with respect to the proliferative potential of daughter DMSO or 2 μM AKT-1/2i at t = 0, with washout at t = 5 d. Events were recorded by tracking the length of the first two complete cell-cycle divisions after t = 0. Cell events in the AKT-1/2i group were discounted if exposure to AKT-1/2i was <24 h before washout. n = 483 and 825 for DMSO- and AKT-1/2i-treated cells, respectively. (H) Percentage of sibling pairs with cell-cycle times <t for DMSO (red) versus 2 μM AKT-1/2i (blue) -treated cells. Treatment protocol is the same as in G. n = 221 and 326 for DMSO- and AKT-1/2i-treated cells, respectively. (I) Percentage of sibling pairs with cell-cycle time differences <t for cells initially exposed to 2 μM AKT-1/2i within 6 h after mitosis (red; n = 41), between 6 and 12 h after mitosis (light red; n = 55), between 6 and 12 h after mitosis (light blue; n = 46), and within 6 h before mitosis (blue; n = 34).

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cells, and that the precise timing of AKT signaling loss in mitosis was important for this effect.

**G0-Like Cells Are Enriched After Cytotoxic Treatment in Vivo.** Our findings demonstrated that rare, slowly cycling G0-like cells were present in established human cancer cell lines. Moreover, these G0-like cells appeared to down-regulate AKT protein and signaling. We therefore asked whether we could find similar AKTlow G0-like cancer cells in actual human tumors in vivo. We focused on patients with newly diagnosed breast tumors who were given neoadjuvant chemotherapy before definitive surgical resection of their tumor. This scenario enabled us to examine matched tumor biopsies that were obtained from individual patients before and after treatment with multiple cycles of adriamycin, cyclophosphamide, and paclitaxel chemotherapy (Table S2).

Interestingly, we found occasional MCM2low/H3K9me2low/HES1 high G0-like cancer cells that expressed low levels of total AKT protein in the pretreatment biopsies from each of the five patients we examined (Fig. 5A and C–E and Fig. S4 A and B). These cells were not localized in any appreciable histopathologic pattern but were present across all three molecularly distinct subtypes of breast cancer (ER+, ERBB2+, and ER/ERBB2+). Furthermore, we found a striking enrichment in these AKTlow/MCM2low/H3K9me2low/HES1 high cells in matched biopsies obtained after treatment from all five patients (Fig. 5B and C–E and Fig. S4 A and B). This clinical experiment suggested that slowly cycling G0-like cancer cells could indeed be visualized using the AKTlow/MCM2low/H3K9me2low/HES1 high molecular profile in patients with breast cancer, where they appeared to survive intensive exposure to combination chemotherapy. Additional experiments in vitro further suggested that G0-like cancer cells indeed appeared to survive exposure to cytotoxic insult (Fig. S5).

**Discussion**

Our results suggest that rapidly proliferating cancer cells occasionally suppress AKT signaling asymmetrically during mitosis to produce a slowly cycling G0-like daughter cell with a ROSlow/MKI67low/MCM2low/H3K9me2low/HES1 high/ AKTlow profile (Fig. S6). Asymmetric inhibition of AKT signaling in one emerging daughter cell is associated with nuclear localization followed by suppression of AKT protein and proliferative arrest of this newborn cell. Furthermore, inhibition of AKT signaling modulates the MKI67, MCM2, H3K9me2, and HES1 marker profile while also having a well-described role in regulating cellular ROS levels that both coordinately mark G0-like cancer cells (22–24). G0-like cancer cells are not stably quiescent in cell culture, but rather tend to re-enter the cell cycle within days. Nevertheless, the G0-like cells that we find in breast cancer patients appear to be highly enriched after 6 mo of exposure to combination chemotherapy. This suggests that G0-like cells might be able to maintain a stable “out of cycle” state for a longer period of time in vivo. We do not yet know whether G0-like cells in vivo are indeed regulated by AKT suppression as in vitro. Nor do we know whether G0-like cancer cells are enriched in vivo primarily through selection or induction by chemotherapy. However, many factors in the complex tumor microenvironment, including exposure to chemotherapy, modulate AKT signaling, leading us to speculate that AKT modulation (either naturally occurring or pharmacologically induced) may in fact regulate the proportion of G0-like cancer cells within actual human tumors (25, 26). Interestingly, two recent reports have identified rare, slowly cycling subpopulations in lung and melanoma cancer cell lines that are drug resistant, but it is unclear whether these cells similarly arise through asymmetric division (27, 28).

The asymmetric cancer cell division that we observe is not a special property of a discrete subpopulation. Rather, it appears to be a latent but general property of any cancer cell that can be dynamically unmasked depending on the precise state of its AKT signaling network. Presumably, both intrinsic and extrinsic factors that modulate AKT signaling can shift the dynamic between symmetric and asymmetric division. These observations may therefore open previously unappreciated experimental opportunities for studying asymmetric division in mammalian cell culture, a long sought after but unrealized goal (29). It is critical to note that this asymmetric cancer cell division which we describe does not relate to the generation of cells with a different size or fate, as

![Fig. 5.](image_url) G0-like cells enriched after treatment in vivo. Human breast tumor samples from five different patients were stained for H3K9me2, MCM2, HES1, pan-AKT, and cytokeratin. G0-like cytokeratin-positive cells (defined as H3K9me2low/MCM2low, H3K9me2low/HES1high, or H3K9me2low/pan-AKTlow) before and after chemotherapy were counted. (A and B) Human breast tumor from patient 1 stained for DAPI (blue), dimethyl-H3K9 (green), human cytokeratin (yellow), and pan-AKT (red) (A) before (pretreatment) and (B) after (posttreatment) chemotherapy. The arrow points to G0-like cells. (C–E) Bar graph of percentages of H3K9me2low/MCM2low, H3K9me2low/HES1high, and H3K9me2low/pan-AKTlow cytokeratin-positive cells before (red) and after (blue) chemotherapy for the five patients.

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classically found in normal stem cells (30, 31). Rather, it relates to differences in proliferative potential between siblings arising from the same cell division event. It is intriguing to speculate, however, that we might be observing a modified “stem-like” behavior of molecularly deranged cancer cells. In this view, cancer cells divide asymmetrically like normal stem cells, but the G0-like progeny that this type of cell division produces are unable to differentiate, do not die, and therefore eventually reenter the cell cycle. Recent findings suggest that adult epithelial stem cells appear to suppress AKT signaling as they switch from actively cycling to quiescent modes, which may be consistent with this model (32, 33).

Finally, these findings may have important clinical implications. Many cancer drugs target AKT signaling either directly or indirectly (34, 35). Suboptimal dose, schedule, or delivery of these drugs in patients may therefore result in partial suppression of AKT signaling, thus promoting asymmetric division, proliferative heterogeneity, and drug resistance rather than apoptosis as intended. In addition, this model of cancer cell behavior has the power to explain many clinical phenomena that relate to asynchronous tumor growth, disease recurrence, and treatment response and are not easily understood with current paradigms.

Materials and Methods

MCF7-BOS breast cancer cells were a gift from Dr. Toshi Shioda (MGH Cancer Center, Boston, MA). MDA-MB-231 breast and HCT116 colon cancer cells were purchased from the American Type Culture Collection (ATCC). Cell culture, clonogenic assays, and drug treatment were performed using established protocols. All experiments were performed using standard cell culture conditions. Fluorescence activated cell sorting (FACS) (on BD FACSCalibur, LSRII, FACSARia, and FACSARia II cell sorters), immunofluorescence imaging (on a Nikon Eclipse Ti A1R-A1 confocal microscope), and live cell imaging (on the Nikon BioStation CT platform) were performed using established methods and standard protocols. Transcriptional profiling was performed using the Affymetrix IVT express kit and Human Genome U133 Plus 2.0 microarrays (Affymetrix). Reverse-phase protein microarray profiling and Western blotting were performed using antibodies listed in Table 1. Full details can be found in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Nick Dyson, Daniel Haber, Raul Mostoslavsky, Shobha Vasudevan, and key anonymous reviewers for valuable advice, criticism, and discussion. We thank Dennis Sgroi for expert pathology advice. We thank Kat Folz-Donahue, Supriya Gupta, Laura Prickett-Rice, and Elizabeth Richardson for expert technical advice and assistance. The human studies described were performed under a Partners Human Research Committee Institutional Review Board-approved protocol (2009-P-002302). This work was supported by the Massachusetts General Hospital Cancer Center, the National Cancer Institute (K08 CA100339, P50 CA89393, P50 CA127003), Department of Defense (W81XWH-02-2-0033), Howard Hughes Medical Institute (Physician-Scientist Early Career Award), Sidney Kimmel Foundation (Translational Science Award), Smith Family Foundation (New Investigator Award), Swiss National Science Foundation (postdoctoral award; to A.W.), the Emma Muschamp Foundation (postdoctoral award; to A.W.), and a Howard Hughes Medical Institute Medical Student Research Training Fellowship (to A.C.Y.).
Supporting Information

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SI Materials and Methods

Cell Culture. MCF7-BOS cells were a gift from Toshi Shioda (MGH Cancer Center, Boston, MA). MDA-MB-231 and HCT116 cells were purchased from the American Type Culture Collection. MCF7-BOS and MDA-MB-231 cells were maintained in DMEM, 10% FCS, 40 mM glucose, 100 U/mL penicillin, and 100 μg/mL streptomycin. HCT116 cells were maintained in McCoy’s 5a medium supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified atmosphere at 37 °C and 5% CO2.

Fluorescence-Activated Cell Sorting. For detection of reactive oxygen species (ROS), cells were harvested using trypsin, counted, and washed in PBS. They were resuspended in PBS, 2% FCS at 10⁶ cells/mL and 2.5 μM CM-H2DCFDA (Invitrogen) and incubated for 30 min at 37 °C. Cells were harvested, resuspended in fresh PBS, 2% FCS at 10⁷ cells/mL, and incubated 1 h at 37 °C. Cells were concentrated to 10⁶ cells/mL for sorting on a Becton Dickinson FACSaria II cell sorter. For concurrent cell-cycle analysis, cells were incubated with CM-H2DCFDA for 30 min, and then incubated with 5 μM reserpine (Sigma) for 5 min before adding Hoechst 33342 (Invitrogen) to 5 μg/mL and incubated 1 h at 37 °C. Cells were washed with PBS, 2% FCS containing 5 μM reserpine, resuspended in PBS, 2% FCS, 5 μM reserpine, and analyzed on a Becton Dickinson LSRII flow cytometer. Staining for CD24 and CD44 was performed after ROS staining by incubating the cells directly conjugated anti-CD24-PE and anti-CD44-APC antibodies (BD Pharmingen). Cells were washed and analyzed on a Becton Dickinson FACSCalibur flow cytometer. For all FACS experiments, propidium iodide was added immediately before analysis to exclude dead cells. Results were analyzed using FlowJo software (Tree Star).

Immunofluorescence Staining. Cells sorted for high and low ROS staining were cytopun onto slides. For bulk populations and for colonies, cells were grown directly on collagen IV (Sigma)-coated coverslips. Cells were fixed in 3.7% formalin, permeabilized with 0.1% Triton X-100, and treated with 0.1% SDS. They were blocked in 1% BSA and then incubated with primary antibody diluted in blocking solution, washed, and incubated with the respective secondary antibody (for primary antibodies, see Table S3). Cells were mounted using hardset mounting media containing DAPI (Vector Laboratories). All secondary antibodies were Alexa Fluor conjugates (488, 546, 555, 568, 633, and 647) (Invitrogen).

Drug Treatment in Vitro. For immunofluorescence staining, cells were seeded onto collagen IV-coated coverslips, allowed to attach overnight, and treated with vehicle (DMSO) or 2 or 3 μM AKT-1/2i (SignaGen Labs) or 10 or 20 μM MK-2206 (Selleck Chemicals) in MCF7 and HCT116, respectively, for 3 d. For washout experiments, cells were treated for 3 d with AKT-1/2i or MK-2206, washed twice with PBS before adding fresh culture medium, and incubated for an additional 6 d before staining. For colony formation assays, cells were seeded at a density of 300 cells per well in six-well plates, allowed to attach overnight, and treated with AKT-1/2i or MK-2206 or vehicle for 3 d. Cells were then washed and incubated with fresh medium for an additional 6 or 12 d. Cells were fixed and stained with Coomassie blue and imaged.

Cytotoxic Treatment for Imaging Analysis in Vitro. For imaging analysis of cytotoxicity, MCF7 cells were grown as colonies by very low density seeding on collagen IV-coated glass coverslips and treated with either 1 μM paclitaxel (Sigma) for 24 h, 100 nM fulvestrant for 5 d, or irradiated with 2 Gy and incubated for 48 h. Cells were fixed and stained as above. Using CellProfiler software (Broad Institute), nuclei were identified based on DAPI staining, and staining intensities were measured for all channels (MKI67, CASP7, H3K9me2). Cells were classified as H3K9me2high or H3K9me2low using a threshold of 15% of maximum intensity and as CASP7high or CASP7low using a threshold of 5% of maximum intensity. We then used a two-sided Fisher's exact test of the null hypothesis of independence between being H3K9me2high and CASP7low.

Immuno Staining. Immunofluorescence-stained samples were obtained from the institutional review board-approved Massachusetts General Hospital (MGH) Breast Tumor Bank (courtesy of Dennis Sgroi, MGH Cancer Center). Tumor samples were cut from formalin-fixed paraffin-embedded sections using the method of Benjamini and Hochberg (3). Protein data are not deposited in GEO; we provide antibodies in Table S1.

Western Blotting. Western blotting was performed according to standard protocol using antibodies against pan-AKT, pAKT (Thr308), and pS6RP (Ser235/236) (Cell Signaling) and secondary HRP-conjugated anti-mouse or anti-rabbit antibodies (Vector Laboratories).

Reverse-Phase Protein Microarray Profiling. MCF7 and HCT116 cells were sorted for high and low ROS staining using the Becton Dickinson FACSaria II cell sorter. Antibodies used in the experiments are listed in Table S3. P values for the difference between ROSlow and ROShigh cells were obtained after correcting for cell line using an analysis of covariance (ANCOVA) model. FDR (Q) values were computed from P values using the method of Benjamini and Hochberg (3). Protein data are not deposited in GEO; we provide antibodies in Table S1.

Transcriptional Profiling. RNA was extracted from MCF7 and HCT116 cells sorted for high and low ROS staining using the RNeasy Micro Kit (QIAGEN). Microarray profiling was performed using the Affymetrix IVT Express Kit and the Affymetrix Human Genome U133 Plus 2.0 Array. Data were normalized using the MAS5 algorithm (Affymetrix) with updated probe set definitions (ENSG 12.1) (1, 2). The data were then unlog-transformed. P values for the differences between ROSlow and ROShigh cells were obtained after controlling for cell line using an analysis of covariance (ANCOVA) model. FDR (Q) values were computed from P values using the method of Benjamini and Hochberg (3). Fold change was calculated for each cell line and the geometric mean of those fold changes was used as a single combined fold change. Raw data have been submitted to the Gene Expression Omnibus (GEO) repository (GSE30465).

Clonogenicity Assay. ROShigh, ROSlow, or un gated MCF7 and HCT116 cells were directly sorted into 10-cm tissue culture plates (2 × 10⁴ per plate) in triplicate and incubated until small colonies were visible by eye. Cells were then washed and stained with Coomassie blue. Plates were imaged and colonies were counted.

References. Dey-Guha et al. 10.1073/pnas.1109632108.
**Generation of MCF7/NLS-mCerulean Cell Line.** pMSCV-CMV-NLS-mCerulean was a gift from Joan Brugge (Harvard Medical School, Boston, MA). Virus carrying this construct was produced by transfecting 293-T cells plated at 500,000 cells per well in a six-well plate at t = 24 h later with 1 μg target vector pMSCV-CMV-NLS-mCerulean, 1 μg packaging vector pCL-Ampho, and 3 μL FuGENE HD mixed with 100 μL reduced serum solution (Opti-MEM; Invitrogen). Virus was collected 24 h following transfection. Before infection, 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 μL 1,000x polybrene per well. A media change was performed the following day, and cells were allowed to grow to confluence before splitting into a 10-cm dish and selection with 2 μM puromycin. Following selection, cells were allowed to grow to confluence 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 d.

**Time-Lapse Analysis.** To follow the fate of MCF7 cells in vitro, we plated MCF7/NLS-mCerulean cells in tissue culture-treated 96-well plates (Corning) at a density of 1,000 cells per well along with unlabeled MCF7 cells at a density of 4,000 cells per well. Serial imaging was performed using an inverted microscope fitted in a standard-sized tissue culture incubator (Nikon Biostation CT) every 20 min at 10x magnification for 291 h. Both phase and fluorescence images were captured. Cells were excited with a light emitting diode (LED) (CREE XR-EROY-L1-00A02) and passed through a filter series (Chroma). All cells were initially grown in DMEM, 10% FCS at t = 0. We performed two media changes, one at t = 24 h, where cells were subjected to either 1:1,000 DMSO (control) or 2 μM AKT-1/2i, and one at t = 145 h as a washout step.

All cell-division events were tracked manually using the CFP images by recording the following characteristics for each cell: identification based on initial frame of appearance and x/y coordinate, first frame, last frame, origin ID, progenitor IDs, x/y coordinates for first and last frame, and end method (cell division, lost in tracking due to overlap, lost to washout, or lost in tracking due to disappearance of fluorescent nuclei). Statistical analysis of the resulting dataset was performed using R (v2.8.0) (The R Foundation for Statistical Computing). To control for the influence of density on cell-division rates, only the first two complete divisions in both the DMSO control and 2 μM AKT-1/2i-treated cells after the first media change were counted.

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Slowly cycling G0-like cancer cells in vitro. (A) Micrographs of ROS\textsuperscript{high} and ROS\textsuperscript{low}-sorted MCF7 cells stained for MYC, CDC6, polo-like kinase 1 (PLK1), aurora kinase A (AURKA), geminin (GMNN), H3S10ph, H3K4me2, H3K27me3, acetyl-H4K12, acetyl-H4K16ac, or H3 core. Shown are the proteins of interest and merged images. Merged images represent respective stains merged with underlying DAPI stain. (B) Micrographs of ROS\textsuperscript{high} and ROS\textsuperscript{low}-sorted MCF7 cells, HCT116 cells, and MDA-MB-231 cells stained for MCM2 and H3K9me2. (C) ROS\textsuperscript{high} and ROS\textsuperscript{low}-sorted MCF7 cells stained for E-cadherin (CDH1; green) and vimentin (VIM; red). (D) FACS dot plot for ROS\textsuperscript{high} (red) and ROS\textsuperscript{low} (blue) MCF7 cells stained for CD24 and CD44. (E) Cytospins of HCT116 ROS-sorted cells stained for pS6RP (235/236) and pS6RP (240/244). me2, dimethyl; me3, trimethyl; p, phosphorylated.
Fig. S2. G0-like cancer cells arise through asymmetric division. Cytospin of (A) MCF7 cells and (B) HCT116 cells sorted by FACS for high- or low-AKT1-mCherry staining and stained for (A) pan-AKT, MCM2, H3K9me2, and HES1 and (B) MCM2, H3K9me2, and HES1.
Fig. S3. Induction of asymmetric cancer cell division with AKT inhibition. (A) Survival curve of MCF7 cells treated with different doses of MK-2206. (B) Colony formation after 6 d of MK-2206 or DMSO followed by 12 d of washout. White and gray columns indicate control (DMSO) and MK-2206-treated, respectively. (C) Cells treated with DMSO or MK-2206 for 3 d at 2 μM concentration or treated with MK-2206 for 3 d followed by 6 d of washout (MK-2206 6dwash) and stained for MCM2, H3K9me2, and HES1. Error bars indicate mean ± SD. (D and E) MCF 7 cells treated with DMSO or MK-2206 for 3 d at 2 μM concentration and counted for (D) G0-like cells or (E) asymmetric cells. Red and blue columns indicate control (DMSO) and MK-2206-treated, respectively. Error bars indicate mean ± SD. (F) Survival curve of HCT116 cells treated with different doses of AKT-1/2i. (G) Colony formation after 6 d of AKT-1/2i or DMSO followed by 6 d of washout. Error

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bars indicate mean ± SD. (H) Cells treated with DMSO or AKT-1/2i for 3 d at 20 μM concentration or treated with AKT-1/2i for 3 d followed by 3 d of washout (AKT-1/2i 3dwash) and stained for the markers. (I and J) HCT116 cells treated with DMSO or AKT-1/2i for 3 d at 20 μM concentration and counted for (I) G0-like cells or (J) asymmetric cells. Red and blue columns indicate control (DMSO) and AKT-1/2i-treated, respectively. Scale bars indicate mean ± SD. (K) Survival curve of HCT116 cells treated with different doses of MK-2206. (L) Colony formation after 6 d of MK-2206 or DMSO followed by 6 d of washout. Error bars indicate mean ± SD. (M) Cells treated with DMSO or MK-2206 for 3 d at 10 μM concentration or treated with MK-2206 for 3 d followed by 3 d of washout (MK-2206 3dwash) and stained for the markers. (N and O) HCT116 cells treated with DMSO or MK-2206 for 3 d at 10 μM concentration and counted for (N) G0-like cells or (O) asymmetric cells. Red and blue columns indicate control (DMSO) and MK-2206-treated, respectively. Scale bars indicate mean ± SD. (P and Q) Structures for (P) AKT-1/2i and (Q) MK-2206.

**Fig. S4.** G0-like cells enriched after treatment in vivo. Human breast tumor of patient 1 stained for DAPI (blue), dimethyl-H3K9 (green), human cytokeratin (yellow), and (A) MCM2 (red). (B) HES1 (red) before (pretreatment) and after (posttreatment) chemotherapy. Arrows point to G0-like cells.
Fig. S5. G0-like cells are treatment-resistant in vitro. G0-like cancer cells are functionally resistant to cytotoxic treatment. (A) Micrograph of an MCF7 colony stained with DAPI, MKI67, cleaved caspase 7 (CASP7), and H3K9me2. (Left) DAPI channel used to demarcate cells using CellProfiler. (Right) These outlines are overlaid onto the merged image for the remaining three channels MKI67 (red), CASP7 (green), and dimethyl-H3K9 (blue). Arrows point to cells showing absence of staining for MKI67, CASP7, and dimethyl-H3K9 markers. (B) Graphs show relative staining intensities of individual cells shown in A for dimethyl-H3K9 versus MKI67 and dimethyl-H3K9 versus CASP7. G0-like cells are located in the lower left quadrant as demarcated by the two red lines and encompass the cells indicated by arrows in A. (C) Micrograph of MCF7 cells treated with paclitaxel. (D) Graphs of marker intensities for cells treated with paclitaxel. (E) Micrograph of cells treated with radiation. (F) Graphs of marker intensities for cells treated with radiation. (G) Micrograph of cells treated with fulvestrant. (H) Graphs of marker intensities for cells treated with fulvestrant. P values represent two-sided Fisher’s exact test of the null hypothesis of independence between being H3K9me2low and CASP7low.
Asymmetric cancer cell division: a model.

Movie S1. Time-lapse imaging of MCF7/NLS-mCerulean cells treated with 2 μM AKT-1/2i. An asymmetrically dividing cell is marked in cyan. Progeny cell-cycle times are 161.8 h (cyan) and 47.4 h (yellow). Details of the protocol can be found in SI Materials and Methods. Imaging shown in the figure begins at t = 24 h of the described protocol. Time counter in the upper left corner is displayed as h:mm:ss. Combined phase and fluorescent images are shown, with a gradual fadeout of the phase channel to allow for better visualization of cell migration.

Table S1. A panel of antibodies targeting a spectrum of cancer-relevant phospho-proteins and proteins

A reverse-phase microarray was carried out with the antibodies in this panel for ROS^{high}- and ROS^{low}-sorted MCF7 cells and HCT116 cells.
Table S2. Five human tumor samples were stained for H3K9me2, MCM2, HES1, and cytokeratin

Table S2

The number of G0-like cytokeratin-positive cells, defined as H3K9me2<low>/MCM2<low>, H3K9me2<low>/HES1<high>, or H3K9me2<low>/pan-AKT<low> before and after chemotherapy, was counted. The table shows the clinicopathological characteristics of each tumor, the percentages of G0-like cells before and after chemotherapy, treatment (tx) regimen, duration of treatment, tumor response to treatment (→, tumor size unchanged; ↓, tumor shrinkage), as well as lag time (time from end of chemotherapy to surgical tumor resection).

Table S3. Primary antibodies

Table S3