Supporting Information

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SI Methods

Patient Samples. Primary human NSCLC tumors and pleural effusion were obtained from Suburban Hospital, Bethesda, MD, and the University of Maryland, Baltimore, MD. The project was approved by the institutional review boards of the National Institutes of Health and the participating hospitals. Informed consent was properly obtained from all participants. Tumors were cut into small pieces, and tissue dissociation was carried out by enzymatic digestion in 1.5 mg/mL of collagenase II (Invitrogen), 20 μg/mL of DNase (Roche), and 4 μg/mL of heparin (Sigma-Aldrich) for 2 h at 37 °C. Pleural effusion cells were collected by centrifugation.

Cell Culture. The human NSCLC cell lines A549, NCI-H441, NCI-H1975, NCI-H23, SK-LU-1 (adenocarcinoma), NCI-H1650 (adenocarcinoma/bronchioalveolar carcinoma), and NCI-H157 (squamous cell carcinoma) were obtained from American Type Culture Collection. Cells were grown in RPMI-1640 supplemented with 100 U/mL of penicillin, 2 mM glutamine, 0.1 mg/mL of streptomycin, and 10% FBS and cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C. Primary tumor cells were grown in DMEM/F12 supplemented with B-27 Supplement Minus Vitamin A (Invitrogen) and 10% FBS. Fibroblasts were removed during passaging by pretreating flasks for 1 min in prewarmed 0.25% trypsin. To establish HBET cells, primary human bronchial epithelial cells were immortalized with pCLXSN-hTERT (1). HBET cells were verified to be cytokeratin-positive by immunocytochemistry and were cultured in fibronectin-coated flasks with serum-free LHC-9 medium (Biosource) under 3.5% CO_2. KnockOut SR (Invitrogen) was used for serum-free conditions, and a 1% O_2 incubator was used for hypoxic conditions. Cell lines were grown as tumorspheres as described previously (2).

BrdU Administration and Chase. For colony assays, BrdU-pulsed cells were labeled with CTR (Invitrogen) and then plated onto poly-l-lysine–coated glass slides with an attached microwell coverslip (Corning) at one cell per well. Coculture cells (the same cell line for H441 and A549, and for A549 cells for primary tumors) were plated at 1,000 cells/well (no membrane barrier) or 10^6 cells on the upper chamber of a 100-mm Transwell dish containing a 0.4 μM polycarbonate membrane insert (Costar) (membrane barrier). Single cells were monitored until they reached a four-cell stage using a Zeiss Axioplan 2 fluorescence microscope. For the time-lapse imaging experiments, BrdU-pulsed cells were plated onto poly-l-lysine–coated glass slides and cultured for two cell divisions.

For assessing SP LRCs, BrdU-pulsed cells were labeled with 5 μM CFDA-SE (Vibrant CFDA-SE Cell Tracker Kit; Invitrogen), trypsinized, and replated in medium without BrdU. Cells were harvested at day 0 of the chase, labeled with CTR for the colony assay or with CFDA-SE, chased for four cell divisions, and then harvested for the LRC assay.

Immunostaining. To reveal BrdU-labeled DNA, cells were fixed in cold 70% ethanol and incubated in 2 N HCl containing 0.5% Triton-X-100 for 1 h. Cells were then washed with Dulbecco's PBS (DPBS) containing 0.1% BSA and 0.5% Triton-X-100 and then in DPBS containing 0.1% BSA. For mitotic shake-offs, cells were incubated with a 1:5 dilution of anti–BrdU-FITC (BD Biosciences) for 1 h at room temperature, washed, cytospun onto glass microscope slides, and then mounted using Vectashield containing DAPI (Vector Laboratories). For colonies, cells were blocked with 10% normal donkey serum in DPBS for 1 h and incubated with a 1:2.5 dilution of anti–BrdU-FITC overnight at 4 °C. For flow cytometry, cells were incubated with a 1:50 dilution of anti–BrdU-APC (BD Biosciences) for 1 h at room temperature.

To reveal CldU- and IdU-labeled DNA, cells were fixed in 70% cold ethanol and treated with 2 N HCL/0.5% Triton-X-100 for 1 h. After two washes in 0.5% Triton-X-100/0.5% BSA and one wash in 0.5% Triton-X-100/0.1% BSA, cells were blocked in DPBS containing 0.5% Triton-X-100/5% BSA for 1 h, then incubated with a 1:50 dilution of anti–BrdU-FITC (Becton-Dickinson) mouse monoclonal antibody B44, which is known to stain for IdU but not for CldU under optimal staining conditions, and a 1:500 dilution of anti-BrdU (Novus Biologicals) rat monoclonal antibody BU1/75, which is known to stain for CldU but not for IdU, for 1 h at room temp. Cells were then washed and incubated with a 1:2,000 dilution of rat-anti mouse Alexa Fluor 594 (Invitrogen) for 1 h at room temperature. To remove nonspecific cross-staining, cells were incubated for 15 min in DPBS containing 0.4 M NaCl, 0.5% Tween 20, and 0.2% Igepal and then cytospun onto slides, air-dried and mounted with DAPI.

For cell fate markers, cells were fixed in 1% paraformaldehyde (PFA) for 5 min (for CD133 and PCK), permeabilized in 0.1% Triton-X-100 for 10 min, and blocked for 1 h in normal donkey serum (for pro-SP-C). Cells were incubated with a 1:3 dilution of anti-CD133 clone ACC133 (Miltenyi Biotech), a 1:300 dilution of anti-PCK (Abcam), or a 1:300 dilution of anti–pro-SP-C (Abcam) for 1 h at room temprature and then washed and incubated with a 1:200 dilution of goat anti-mouse Alexa Fluor 594 (for CD133 and PCK) or a 1:200 dilution of donkey anti-rabbit Alexa Fluor 594 or 488 (for pro-SP-C) for 1 h at room temperature. Cells were postfixed in 4% PFA for 30 min and in 70% cold ethanol for 30 min and then stained for BrdU as described earlier.

Primary cells were verified using the following antibodies: anti-fibroblast 5B5 (1:25 dilution; Abcam) to detect fibroblasts, anti-M2A oncofetal antigen D2-40 (1:2 dilution; Abcam) to detect mesothelial cells, anti-CD31 WM59 (1:25 dilution; BD Biosciences) to detect endothelial cells, anti-CD45 2D1 (1:5 dilution; BD Biosciences) to detect hematopoietic cells, anti-CD68 Y1/82A (1:25 dilution; BD Biosciences) to detect macrophages, and anti–epithelial-related antigen MOC-31 (1:10 dilution; Dako) to detect NSCLC cells. Cells grown on poly-L-lysine–coated glass slides were fixed in 1% PFA for 5 min, washed, and then incubated in the primary antibody for 1 h at 4 °C. CD68 and fibroblast were costained, and CD31, CD45, and D2-40 were costained. Slides were incubated in a 1:200 dilution of goat anti-mouse Alexa Fluor 488 for 1 h at 4 °C, washed, dried, and then mounted with DAPI.

Immunofluorescence Microscopy and Flow Cytometry. Confocal images were acquired with a Zeiss LSM 510 NLO confocal system with an Axiovert 200M inverted microscope or a Zeiss Axioplan 2 fluorescence microscope, and representative images were obtained using a high-performance CCD imaging system (IP Lab Spectrum). For the single mitotic cell assays, 100–250 anaphase cells were scored per experiment. Cells were scored if both sets of chromosomes were distinctly separated, had parallel and condensed chromatin, and were visually verified by differential interference contrast, visual light, or weak cytoplasmic autofluorescence to be a single anaphase cell (to avoid scoring cell doublets). A cell was determined to have asymmetric partitioning of BrdU if one set of chromosomes was strongly positive and the other set was completely negative, as determined by careful visualization under a

40× or higher magnification. A small percentage of cells (<5%) exhibited no BrdU staining and were not scored. Flow cytometry analysis was performed using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

For SP analysis, cells were incubated in RPMI-1640 containing Hoechst 33342 dye, 2% FBS, and 2 mM Hepes buffer at 1×10^6 cells/mL at 37 °C for 90 min in a shaking water bath. As a control for efflux of the Hoechst dye, a separate set of cells was also incubated with 50 mM verapamil (Sigma-Aldrich). Cells were washed and resuspended in cold HBSS containing 2% FBS and 2 mM Hepes buffer. 7-Amino-actinomycin D (7-AAD) was added to the cells at 20 μg/mL immediately before sorting to exclude dead cells. Sorting was performed on a FACS Vantage SE with DiVa option (BD Biosciences).

Time-Lapse Imaging. BrdU-pulsed cells were plated at high density (1×10^6 per dish) onto 100-mm dishes containing poly-L-lysine–coated microscope slides without BrdU. Between one and two cell divisions, cells were photographed every 10 min for 2 h, using a 10× objective. Filming of cells was carried out with an Olympus 1×51 inverted microscope equipped with an Olympus DP71 CCD camera. Time-lapse sequences were combined using QuickTime Pro-7.0 (Apple, Inc.).

Mitotic Index. Cells were fixed in cold 70% ethanol for at least 30 min, permeabilized with DPBS containing 0.25% Triton-X-100, washed, and then incubated with a 1:10 dilution of anti-PHH3-PE (Cell Signaling). Cells were washed and treated with RNase and then counterstained with 7-AAD. Events (10,000) were acquired on a FACScaliber flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Cells that were in G2/M and positive for PHH3 were gated. To determine whether the frequency of asymmetrically dividing cells was influenced by alterations in the mitotic index, the frequency of asymmetrically dividing cells per total cells in each flask was normalized to the mitotic index in each flask.

Repopulation Assay. A549 cells were detached from the dishes through incubation with prewarmed 10 mM EDTA with 10% FBS for 1–2 min. Cells were stained with a 1:3 dilution of APC-conjugated anti-CD133 clone ACC141 (Miltenyi Biotech), as described earlier. Sorting was performed on a FACS Vantage SE with DiVa option (BD Biosciences). The gate for $CD133^+$ cells was based on a control sample stained with an isotype control. Sorted cells were cultured for 7 days, with the media refreshed on the third day. Population doubling was determined as log (number of cells at the end) − log (number of cells at the start)/log(2).

Calculating the Probability of Asymmetric Segregation of DNA Occurring by Chance. We calculated the probability that our observations could be due to chance. If the segregation of chromosomes is random and independent, then in two cell divisions after removal of BrdU, the probability of there being zero, one, or two BrdU-labeled DNA strands in a given chromosome pair in a specific daughter cell is 1/4, 1/2 and 1/4 respectively. The probability that a specified diploid daughter cell contains no labeled DNA strands and that its sister cell contains all of the labeled stands is then $p_{23} = (1/4)^{23} = 1.4 \times 10^{-14}$, and the probability that the specified daughter cell contains all of the labeled strands and its sister contains none is also p_{23}. Thus, the observation, by random chance, of an asymmetric segregation (as) in any one individual diploid cell during the second cell division is $p_{as} = p_{23} + p_{23} = 2.8 \times 10^{-14}$.

Calculating BrdU Retention. During symmetric divisions, the number of $BrdU^+$ chromosomes will decrease by half, following the formula $x/2^n$, where x is the number of chromosomes and n is the number of cell divisions. A549 cells have a modal chromosome number of 66. From this model, the percentage of positive chromosomes after 15 cell doublings is $66/2^{15} = 0.2\%$.

Statistical Analysis. Quantitative data are presented as mean ± SD. All statistical analyses were performed by ANOVA using Stata version 10 (StataCorp).

1. Sengupta S, et al. (2003) BLM helicase-dependent transport of p53 to sites of stalled DNA replication forks modulates homologous recombination. *EMBO J* 22: 1210–1222.

2. Patrawala L, et al. (2006) Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene* 25:1696–1708.

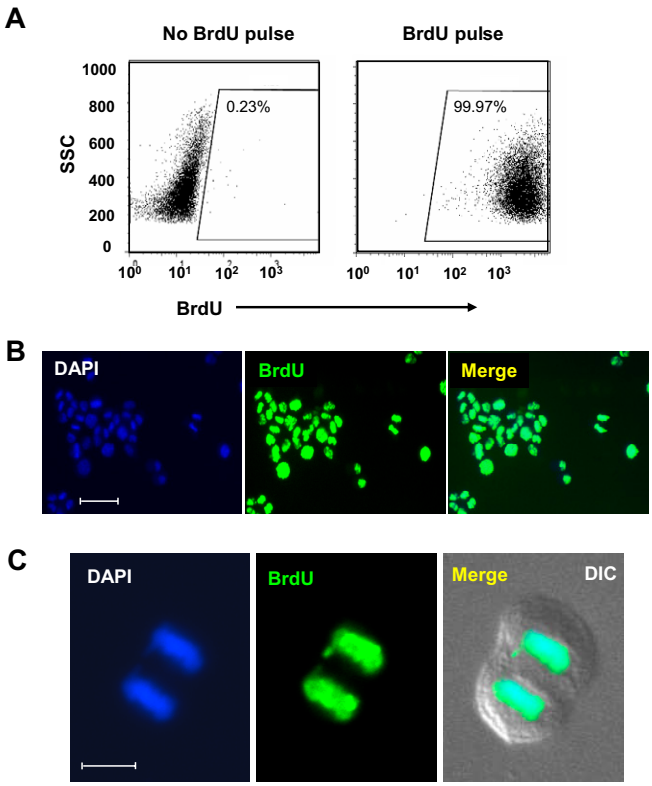


Fig. S1. BrdU staining at the end of the pulse and during the first cell division. (*A*) Cells either not grown in or grown at the end of the BrdU pulse were stained for BrdU label and analyzed by flow cytometry. Gates were made based on the isotype control. Nearly all cells at the end of the BrdU pulse were $BrdU^+$. (*B*) At the end of the BrdU pulse, mitotic cells were stained for BrdU label. A representative image is shown in which all of the cells at various degrees of condensed chromatin were $BrdU^+$ (green). (Scale bar: 50 μm.) (*C*) A representative cell in anaphase during the first cell division after removal of BrdU. Following the model of asymmetric chromosomal segregation, both sets of chromosomes would be expected to be labeled during the first cell division. DNA is stained with DAPI (blue). (Scale bar: 10 μm.)

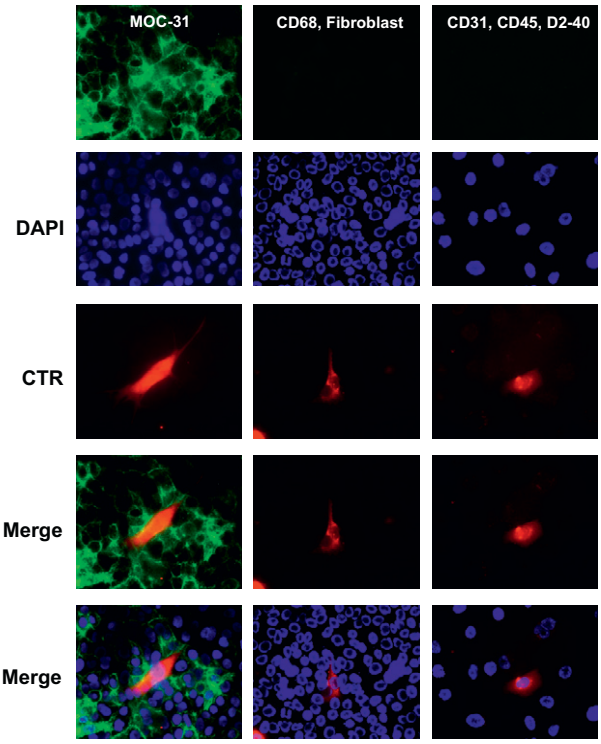


Fig. S2. Lineage identification of primary samples. Short-term cultures of primary human NSCLC samples were examined by immunofluorescence for expression of Moc-31 (NSCLC), CD68 and CD45 (hematopoietic cells), fibroblasts, CD31 (endothelial cells), and D2-40 (mesothelial cells) to verify that the cells examined for asymmetric division were derived from the cancer cells. In these representative images of sample 3, nuclei were stained with DAPI (blue), and lineage markers were labeled green. Primary cells were labeled with CTR (red) and cocultured with A549 cells to mimic conditions of the cells being scored in the asymmetric division colony assay.

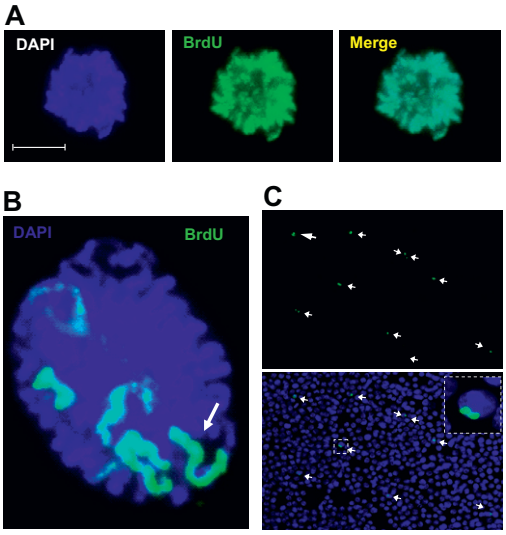


Fig. S3. Sensitivity of detection of BrdU label in NSCLC cells. (*A*) An A549 cell at the end of the BrdU pulse was stained for BrdU label (green). DNA was stained with DAPI. Shown is a representative image of a cell in metaphase. Every chromosome is BrdU-labeled, demonstrating that each chromosome incorporates BrdU during the pulse. (*B*) An A549 cell in metaphase was pulsed with BrdU and then chased for four cell divisions, collected by mitotic shake-off, and stained for BrdU. Entire BrdU-labeled chromosomes are shown in plane (white arrow). (*C*) Cells were chased for 15 cell divisions, then collected at all phases of the cell cycle and stained for BrdU. White arrows point to nuclei with $BrdU^+$ foci. *Inset* in the lower panel is a magnification of the boxed cell.

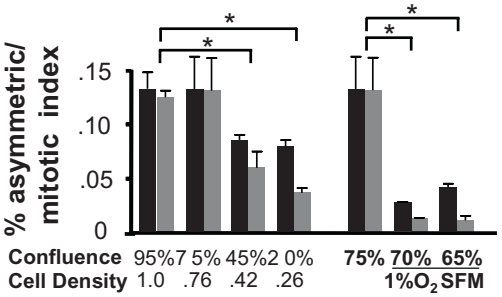


Fig. S4. Asymmetric segregation of template DNA is modulated by environmental changes. Cosegregation of template DNA was decreased at lower cell densities, and the frequency of asymmetric template DNA division was decrease under hypoxic conditions and serum deprivation. Frequencies of asymmetric division of template DNA were normalized to the mitotic index. A549 and H441 cells are indicated by black and gray bars, respectively. Values are mean ± SD from three independent experiments. *$P < 0.001$, one-way ANOVA. Cell density is reported as million/cm^2.

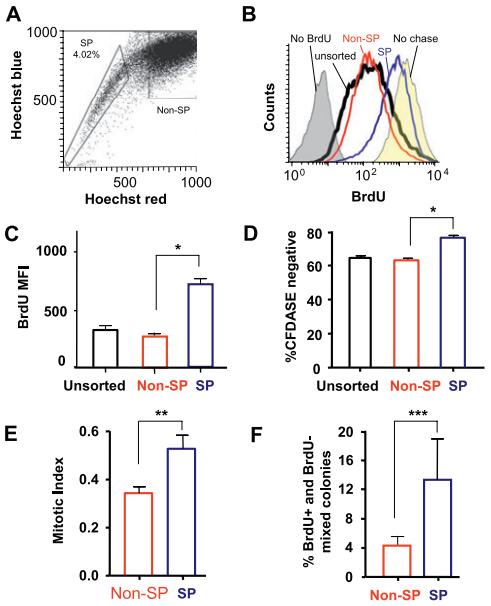


Fig. S5. The SP fraction in lung cancer is enriched for cells that asymmetrically divide their template DNA. (*A*) BrdU-pulsed NSCLC cells were labeled with CFDA-SE, chased for four cell divisions, and sorted into SP and non-SP fractions. (*B*) The sorted SP, non-SP, and unsorted fractions were examined for BrdU retention by flow cytometry. Controls included cells that were never exposed to BrdU (no BrdU) and BrdU-pulsed cells with no chase (no chase). (*C*) Histograms showing the quantitative data from *B* in three independent experiments. SP cells were significantly more likely than non-SP cells to retain BrdU label. *$P <$ 0.001. (*D*) There were significantly more CFDA-SE-negative/dim cells in the SP fraction compared with the non-SP fraction, indicating that SP cells divided more frequently than non-SP cells during the chase period. (*E*) SP cells had a significantly higher mitotic index than non-SP cells. **$P = 0.04$. (*F*) Sorted SP and non-SP cells were labeled with CTR and plated as single cells in a coculture with unlabeled A549 cells, and were allowed to grow to four-cell colonies. The percentage of colonies harboring mixed BrdU$^+$ and BrdU$^-$ cells was scored. SP cells were 3-fold more likely to give rise to mixed colonies. ***$P = 0.05$.

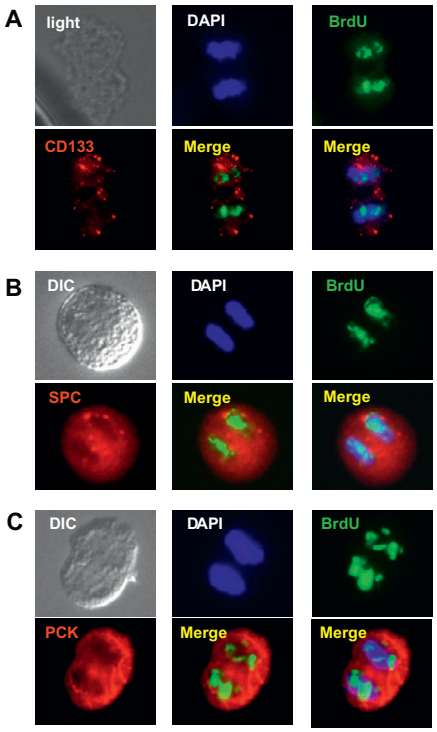


Fig. S6. Segregation patterns of cell fate markers during random segregation of template DNA. Respective images are shown of CD133 (*A*), Pro-SP-C (*B*), and PCK (*C*) (all in red) segregating symmetrically to both daughter cells during symmetric chromosomal segregation. BrdU-labeled template DNA is randomly segregated to both sides of the metaphase plate (green). DNA is stained with DAPI (blue).

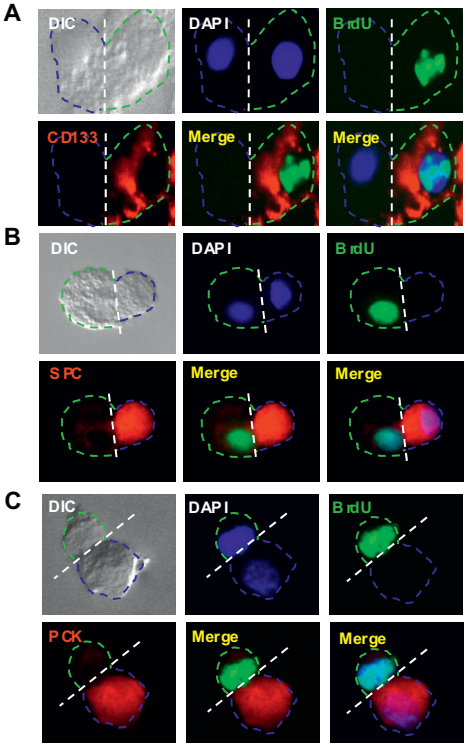


Fig. S7. Segregation patterns of cell fate markers during asymmetric division of template DNA in primary NSCLC tumor cells. Primary tumor cells were BrdU-pulsed, chased, and then treated with nocodazole. Shown are CD133 (*A*), pro-SP-C (*B*), and PCK (*C*) cells (all in red) in late telophase/early cytokinesis segregating asymmetrically to the daughter cell receiving BrdU (*A*) or to the daughter cell not receiving BrdU (*B* and *C*) during asymmetric chromosomal segregation. BrdU-labeled template DNA is segregated exclusively to one set of sister chromatids (green). DNA is stained with DAPI (blue). Each daughter cell is outlined, and the cleavage furrow is indicated by a dotted white line.

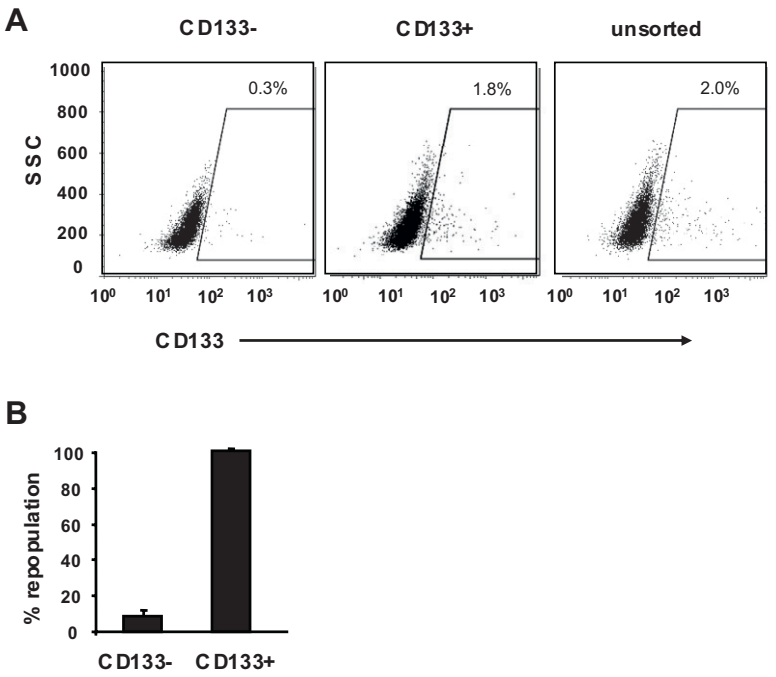


Fig. S8. Repopulation capabilities of CD133-sorted NECLC cell fractions. (*A*) A549 cells were sorted into $CD133^+$ and $CD133^-$ cell fractions, cultured for 7 days, and then examined for CD133 expression by flow cytometry. Gates were made based on the isotope control. (*B*) Bar graphs showing a significantly greater repopulation capability of $CD133^+$ cells compared with $CD133^-$ cells.

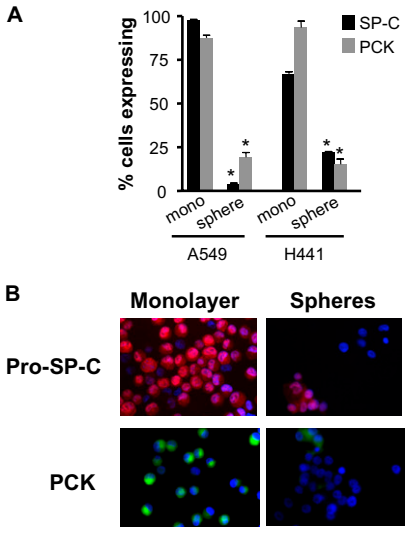
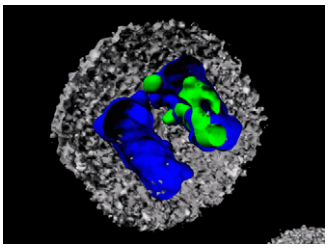
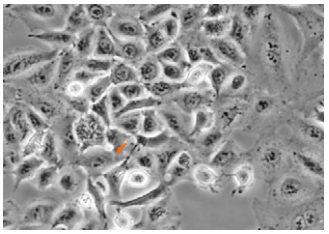


Fig. S9. Determination of markers expressed by differentiated lung cancer cells. (*A*) A549 and H441 cells were grown either in a monolayer with 10% FCS or as tumorspheres in lung cancer stem cell media without serum in the presence of EGF/FGF. Cells were collected, dissociated, stained for pro-SP-C and PCK, and then examined by flow cytometry. In both cell lines, expression of pro-SP-C and PCK was significantly reduced in lung tumorspheres compared with monolayer cells. $*P < 0.001$. (*B*) Representative images of A549 monolayer or tumorsphere cell cytospins stained for pro-SP-C (red) or PCK (green). Images show decreased staining of pro-SP-C and PCK in tumorsphere cells compared with monolayer cells. DNA was stained with DAPI (blue).



Movie S1. Three-dimensional movie of an H441 NSCLC cell in anaphase during the second division of the BrdU chase. The upper set of sister chromosomes inherited all of the template BrdU-labeled DNA, whereas the lower set was devoid of BrdU stain. BrdU is shown in green, and DNA is stained with DAPI (blue). Cytoplasm is shown in gray.

Movie S1



Movie S2. Time-lapse imaging of an LRC in mitosis. This movie shows a single A549 NSCLC cell undergoing mitosis, indicated by arrows. The daughter cells were subsequently stained for BrdU (Fig. 1*F*).

Movie S2