Supporting Information

Szerlip et al. 10.1073/pnas.1114033109

SI Methods

TCGA Data Analysis. ***Focality score determination.*** Genome topography scan (GTS) scores the fraction of the observed copy number change that is shared by neighboring genes while applying a conservative linkage model to account for complex copy number aberration (CNA) topography and/or the potential physical linkage of amplicons mapping to nonadjacent genomic regions. The GTS focality score has an intuitive interpretation: a score of 0.1 for a gene implies that the aberrant log2 ratio spanning that gene is effectively distributed over 10 neighboring genes (1/0.1). In this study, focality scores were determined per gene by dividing the focality-weighted log2 ratio by observed log2 ratios. Scores ≥0.02 were considered significant, implying 20 or fewer genes targeted by the observed log2 ratio shift. Per-sample CNA rates were determined by counting all autosomal segments with mean log2 ratio deviations >0.25. GTS analysis was conducted similarly on array comparative genomic hybridization (aCGH) data from 120 gliomas in the Memorial Sloan-Kettering Cancer Center (MSKCC) Brain Tumor Center Tissue Bank.

Determination of EGFRvIII mutation from aCGH data. Relative deletion of epidermal growth factor receptor (EGFR) exons 2–7 was determined by measuring the difference in mean log2 ratio of probes mapping to the deleted region vs. those mapping 3′ to intron 7. Differences >1 SD (pooled SD) were considered significant for analysis. Expression data (U133A, level 3) for 365 samples were downloaded from the TCGA (The Cancer Genome Atlas) portal and samples assigned transcriptomal class labels as previously described (1, 2).

Tumor Sphere Preparation. Tumor specimen samples were washed in cold PBS twice, then manually dissociated and placed in Accumax (Innovative Cell Technologies) for 15 min under sterile conditions. Cells were subsequently washed and filtered through a 100-μm strainer and plated in NeuroCult NS-A proliferation media (Stemcell Technologies) supplemented with 10 ng/mL rhbFGF for all experimental conditions in the study. Initial cultures were also supplemented with 20 ng/mL rhEGF. Cells were incubated at normal oxygen levels at a temperature 37.0 °C and 5% CO_2. Medium was changed on a Monday/Friday schedule unless cell confluence dictated otherwise.

Fluorescence in Situ Hybridization. ***Cell culture.*** Cells were swollen in 75 mM KCl, fixed with three parts methanol/one part glacial acetic acid, and slides prepared according to standard cytogenetics procedures. Quantification was performed examining 200 cells. Scoring was done by one individual and then checked by a second individual for population scoring of cell cultures. FISH was performed using BAC clones RP11-117E8 and RP11-231C18 (spanning the *PDGFRA* locus in 4q12, both labeled with Green dUTP (Enzo Life Sciences, supplied by Abbott Molecular) together with BAC clone RP11-339F13 and PAC clone RP5-1091E12 spanning the *EGFR* locus in 7p11, both labeled by nick translation with Red dUTP (Enzo Life Sciences, supplied by Abbott Molecular). Clones were obtained from BACPAC Resources, Children's Hospital Research Center at Oakland, CA, or the Wellcome Trust Sanger Institute as part of a 1-Mb clone set (3).

Touch preparation. Slides were fixed in three parts methanol/one part glacial acetic acid and then stored in methanol at −20 °C until processing by standard procedures. Briefly, the slides were rinsed, lightly digested in pepsin-HCl (20 μg/mL, 2 min at 37 °C), rinsed, and postfixed in 1% paraformaldehyde before being dehydrated through ethanol and air dried. Probe and slides were codenatured using a HYBrite automated hybridizer at 73 °C for 4 min, then hybridized overnight at 37 °C.

Paraffin sections. Slides were dewaxed, rinsed, microwaved in 10 mM sodium citrate buffer, then digested in pepsin-HCl (40 μg/mL, 10 min at 37 °C), rinsed, and dehydrated. Probe and slides were codenatured using a HYBrite automated hybridizer at 80 °C for 8 min then hybridized for 2 to 3 d at 37 °C.

Real-Time PCR. Three hundred nanograms of total RNA was reverse-transcribed using the Thermoscript RT-PCR system (Invitrogen) at 52 °C for 1 h. Twenty nanograms of resultant cDNA was used for quantitative PCR reactions using an 7500 Real-Time PCR System (Applied Biosystems). EGFRvIII was detected by custom-designed TaqMan assay (left primer: CGGGCTCTGGAGGAAAAG; right primer: AGGCCCTTCGCACTTCTTAC; hybrid oligo: GTGACAGATCACGGCTCGT). RT-PCR for expression of platelet-derived growth factor receptor α (PDGFRA)$^{\Delta 8,9}$ was performed separately using primers and methods previously described (4). Amplification was carried for 40 cycles (95 °C for 15 s, 60 °C for 1 min). To calculate the efficiency of the PCR, and to assess the sensitivity of each assay, we also performed a six-point standard curve (5, 1.7, 0.56, 0.19, 0.062, and 0.021 ng). Triplicate CT values were averaged, and amounts of target were interpolated from the standard curves and normalized to TBP (TATA box binding protein, assay Hs99999910_m1). Length of vIII product is 129 bp. Length of EGFR WT 925 bp.

Flow Cytometry and Sorting. Cells were prepared as described in *Methods*. For fixation, cells were incubated with Cytofix/Cytoperm solution (BD, #554655) for 15 min on ice, followed by two washes with Perm/Wash Buffer (BD, #554723). This was followed by a 2-h incubation with primary antibody (either anti-PDGFRA or anti-EGFR-APC) either in Perm/Wash Buffer or 1% BSA/PBS. Cells were washed twice with the respective buffers. For primary antibodies requiring secondary antibody labeling, cells were then incubated at a 1:500 dilution of anti-rabbit–FITC to complete the immunostaining. For sorting, FITC-positive and FITC-negative cells were sorted and put back in culture in separate plates.

Inhibitor Treatments and Immunoblotting. Cell pellets were flash frozen in liquid nitrogen. Cell pellets were lysed on ice for 20 min in CelLytic MT Mammalian Tissue Lysis/Extraction Reagent (Sigma-Aldrich, C3228) supplemented with protease inhibitor mixture Complete Mini EDTA-free (Roche, 04 693 159 001) and the phosphatase inhibitor mixture PhosSTOP (Roche, 04 906 837 001). Lysates were centrifuged to 15,870 × *g* for 15 min, and supernatants were saved for further analysis. Samples were separated by SDS/PAGE and transferred to nitrocellulose membrane for immunoblotting.

Cell Line Growth Under PI3K, EGFR, and PDGFRA Iinhibition (Fig. S9). For cell viability assays and growth inhibition experiments TS753 original and its derivative lines were plated at 2,000 cells per well in 96-well plates in the presence of indicated amounts of EGF and PDGFB ligands and concentrations of imatinib, gefitinib, or LY0294002. Day 0 is the day when cells are plated. Resazurin (AlamarBlue; Invitrogen, catalog #DAL1025) was added at 1/10th volume directly to the cells in culture medium. Plates were incubated for 5 h at 37 °C and 5% CO_2 previous measurement of resazurin fluorescence at 585 nm with a Molecular Devices luminescence microplate reader. For longitudinal study, resazurin was added at days 4 and 6, following the same procedure for fluorescence reading.

Antibodies. Antibodies used for immunoblotting and immunofluorescence were as follows: phospho-Akt (Cell Signaling Technology, #4060), Akt (Cell Signaling Technology, #9272), phospho-EGFR (Y1068) (Cell Signaling Technology, #3777), EGFR (Cell Signaling Technology, #4267), phospho-PDGFRA (Y720) (Santa Cruz Biotechnology, sc-12910), PDGFRA (Santa Cruz Biotechnology, sc-338), phospho-PDGFRA (Y754) (Cell Signaling Technology, #2992), Phospho-PDGFRA (Y849)/PDGFRB (Y857) (Cell Signaling Technology, #3170), α-tubulin (Sigma-Aldrich, T9026) or (Millipore, 05-829), phospho-PDGFRA (Y742) (Sigma-Aldrich, P8246), ppMAPK p42/44 (T202/Y204) (Cell Signaling, #9101), p44/42 MAPK (Erk1/2) (Cell Signaling, #9107), pp70 S6 kinase (T389) (Cell Signaling, #9234) and β-actin (Sigma-Aldrich, A5441).

Sequence Analysis. For MSK561 and 753, DNA from frozen tumor and cell lines was extracted by the Qiagen QIAamp DNA Mini Kit. *EGFR* and *PDGFRA* were resequenced by the Sanger technique. For MSK753, DNAs from matched blood and from derived cell lines were processed with exome capture (Agilent Sure Select) and paired-end libraries generated for SOLiD sequencing. Each sample was run on a quarter slide yielding at least 30× coverage of at least 70% of target sequence by non-redundant reads. Candidate somatic mutations were identified among single nucleotide variations (SNVs) identified by DiBayes software (Applied Biosystems) and considered only for regions of >20× coverage in all tumor-derived material and >30× coverage in blood. For the purpose of comparing allelic frequencies, SNVs on chromosomes 11, 15, and 20 were excluded after analysis of nucleotide polymorphisms on these chromosomes demonstrated variable loss of heterozygosity among samples.

Simulation of Amplicon Segregation. Initialization of the model is described in *Methods*. On each simulated replication cycle, each cell replicates with a probability based on the selection model. For uniform fitness (no selection), $P = 0.5$. For selection models the probability is a function of L_1 and L_2 (the number of copies of each locus), L_{opt} (an optimal locus count, $L_{opt} = 50$), and W_{sd} (distribution SDs over 0–100 copies); α is the relative weighting of L_1 vs. L_2, and dnorm is the density function for the normal distribution. The following formulas were used for selection:

Selection for sum of loci:

$$p = dnorm\left(\frac{\alpha L_1 + (2-\alpha)L_2 - L_{opt}}{L_{opt}} * W_{sd}\right) \Big/ dnorm(0)$$

Selection for coamplification of loci:

$$p = dnorm\left(\frac{\sqrt{(\alpha L_1 - L_{opt})^2 + ((2-\alpha)L_2 - L_{opt})^2}}{L_{opt}} * W_{sd}\right) \Big/ dnorm(0)$$

The locus counts L_1 and L_2 are multiplied by individual DNA duplication factors between 2 and 2.5 and randomly allotted to daughter cells according to binomial or uniform distribution model. Replication cycles continue until the simulated growth exceeds >1,000,000 cells. Cell positions are tracked, and daughter cells replace the parent cell ± 1/4 cell width at a random orientation. After all cells replicate, positions are recomputed using a circle-packing algorithm. Cells more than 25 cell widths from the center are excluded for further tracking to reduce computational time. Images in Fig. 4 and Fig. S8 represent the center of the expanding mass. For each model, 1,000 iterations are performed.

Coculture Experiment (Fig. S8*E*). EGF/imatinib- and PDGF/gefitinib-selected populations were plated independently and at (1:1) and (1:3) EGF/PDGFB ratios at a final concentration of 32,000 cells/mL in 12-well plates. Cells were grown for 6 d in NeuroCult supplemented with EGF+PDGFB, EGF, PDGFB, and no growth factors. Final concentrations of EGF and PDGFB were 20 ng/mL and 10 ng/mL, respectively. Triplicates of each condition were harvested and counted in a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter) every 3 d.

1. Verhaak RG, et al.; Cancer Genome Atlas Research Network (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17:98–110.
2. Huse JT, Phillips HS, Brennan CW (2011) Molecular subclassification of diffuse gliomas: Seeing order in the chaos. *Glia* 59:1190–1199.
3. Fiegler H, et al. (2003) DNA microarrays for comparative genomic hybridization based on DOP-PCR amplification of BAC and PAC clones. *Genes Chromosomes Cancer* 36: 361–374.
4. Ozawa T, et al. (2010) PDGFRA gene rearrangements are frequent genetic events in PDGFRA-amplified glioblastomas. *Genes Dev* 24:2205–2218.

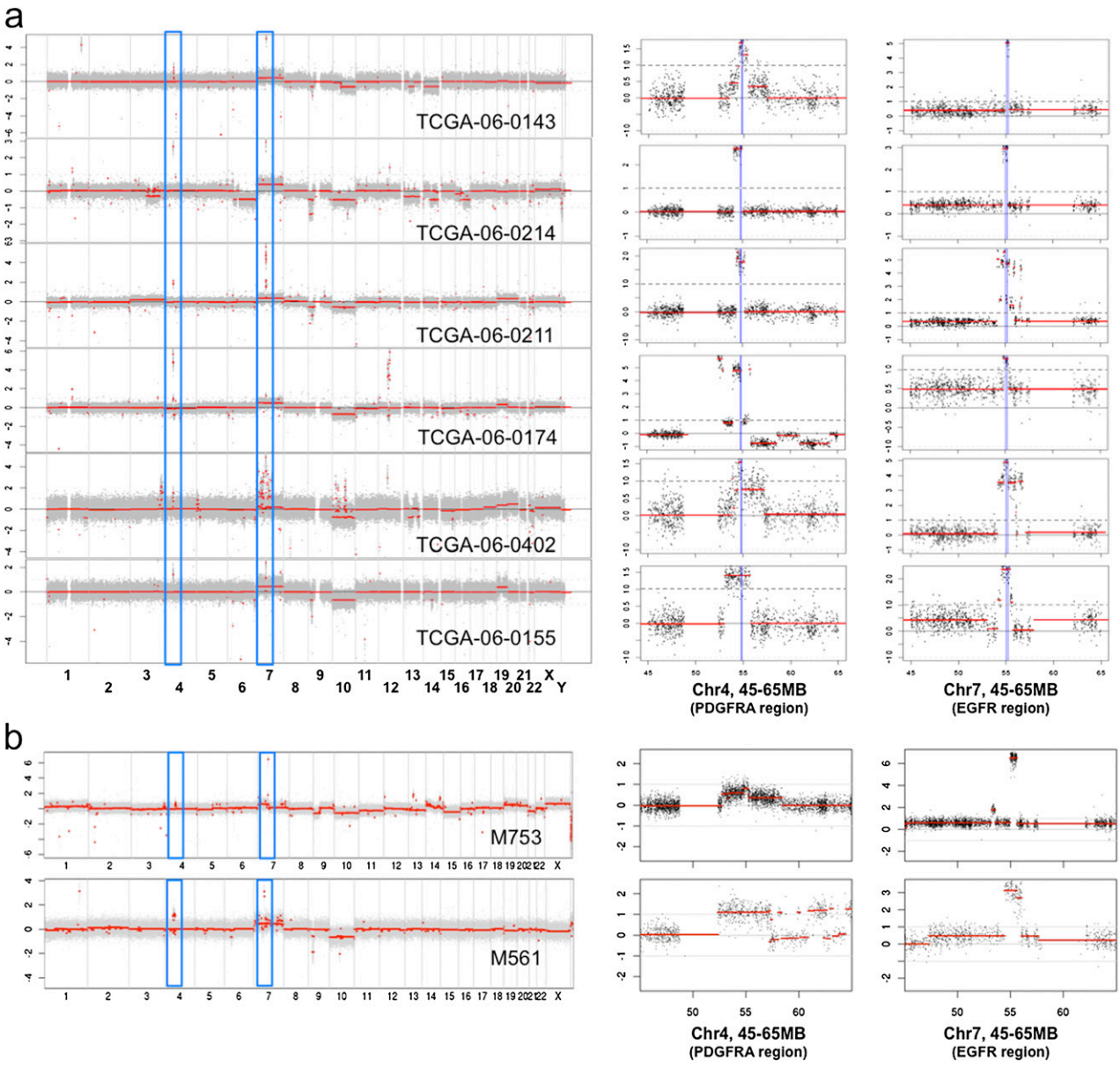

Fig. S1. Simultaneous focal *EGFR* and *PDGFRA* amplified tumors selected for FISH. (*A*) aCGH profiles from TCGA showing six *EGFR* and *PDGFRA* coamplified cases for which matching formalin-fixed, paraffin-embedded (FFPE) specimens were obtained. *Left*: Genome-wide profiles with raw normalized aCGH data (gray) and segmented values (red). Regions marked by blue boxes span *PDGFRA* on chr4 and *EGFR* on chr7. These are enlarged at *Right*. *PDGFRA* and *EGFR* indicated by solid blue lines. (*B*) Additional *PDGFRA/EGFR* coamplified tumors M561 and M753, identified from the Brain Tumor Center at MSKCC, for which frozen tumor and tumor sphere lines were available. aCGH of frozen tumor demonstrates focal amplifications spanning PDGFRA and EGFR on chr4 and chr7, respectively. Amplicon distributions are scored by FISH in Fig. 3.

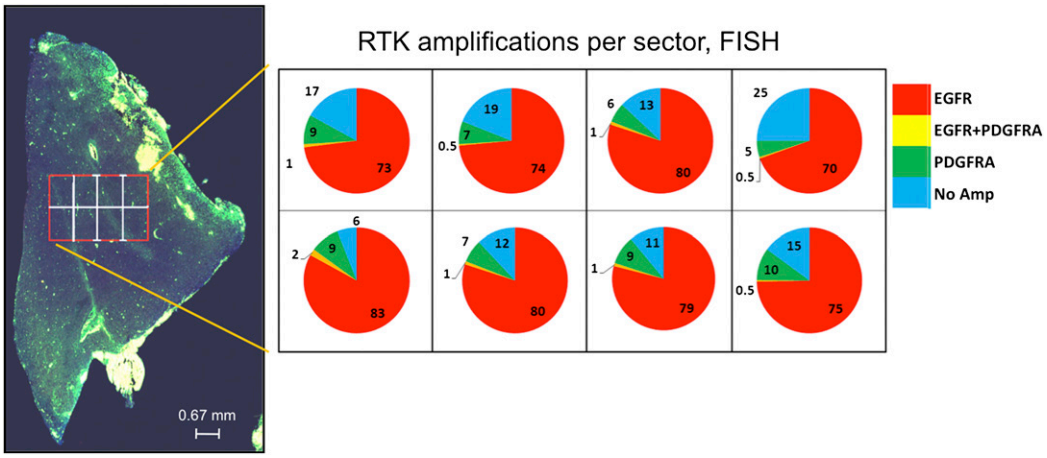

Fig. S2. Regional analysis of *EGFR*- and *PDGFRA*-amplified tumor cell subpopulations reveals populations to be broadly interspersed. *Left*: FISH image map for paraffin section from T143 with (green) *PDGFRA* probe, (red) *EGFR* probe, and (blue) DAPI scanned at 1.5×. Eight adjacent HPF regions were selected from the interior of the section that was free of artifact. *Inset*: Pie chart shows the distribution of the subpopulations in the different regions Red-*EGFR* amplification. Blue, no amplification; green, *PDGFR*A amplification; yellow, *EGFR* and *PDGFRA* amplification in same cell.

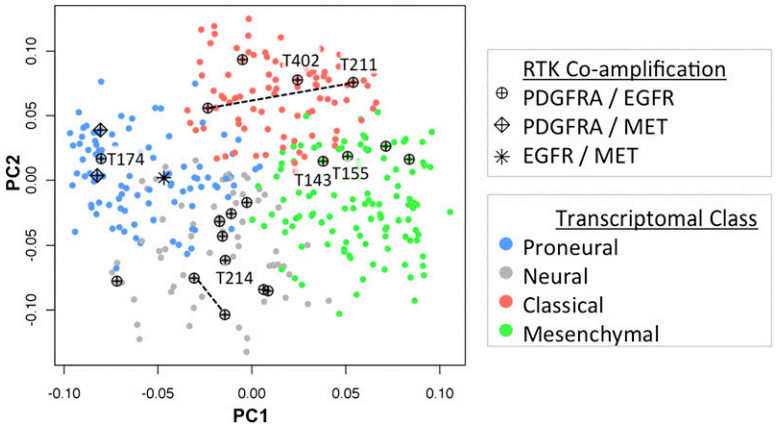

Fig. S3. Receptor tyrosine kinase (RTK) coamplified tumors are transcriptomally diverse. Schematic representation of transcriptomal signatures for 365 glioblastoma (GBM) in the TCGA dataset. Samples were assigned to one of four previously defined transcriptomal subclasses according to nearest-centroid (1). The relative distances are schematically represented by plotting the first two principal components of four distance-to-centroid measures. Transcriptomal class is indicated by color (see key). Relative distances to the four centroids are summarized by principal components analysis. The coamplified specimens are indicated according to the figure key. There were two coamplified specimens for which independent portions of frozen tumor were profiled, and these are connected by the dotted line. The coamplified tumors are distributed in all subclasses, although the mesenchymal class is underrepresented. This may reflect the known paucity of RTK amplifications in this class (1).

1. Verhaak RG, et al.; Cancer Genome Atlas Research Network (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* 17:98–110.

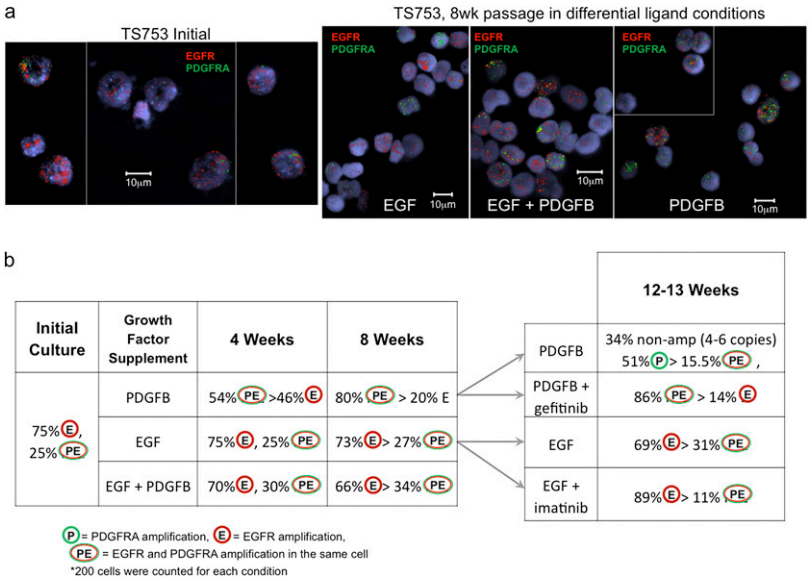

Fig. S4. *EGFR*- and *PDGFRA*-amplified tumor cell subpopulations can be selected for in vitro by exposure to EGF and PDGFB ligand. Tumor sphere line TS753 was maintained in culture with neural stem cell media supplemented with EGF, PDGFB, or both. All media was additionally supplemented with FGF. (*A*) At 4-wk intervals, two-color FISH was used to score the fractions of cells amplified for *EGFR*, *PDGFRA*, or both. (*B*) PDGFB alone led to expansion of the *PDGFRA*/*EGFR* coamplified population from 25% in the initial culture to 80% by 8 wk, and further selection led to heterogeneous loss of *EGFR* amplification at 12 to 13 wk. Growth in EGF led to a relatively stable proportion of *EGFR* and *PDGFRA*/*EGFR*-amplified cells. EGF and PDGFB together led to partial selection. Inhibition of the non–ligand-stimulated receptor seemed to enhance selection of ligand-stimulated cells.

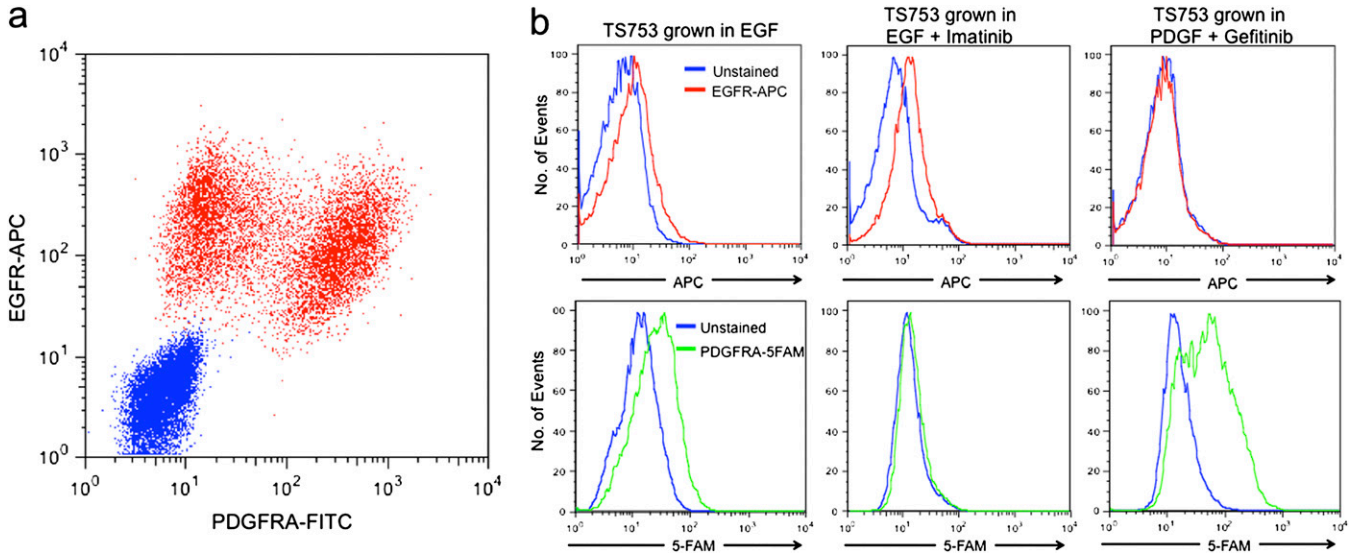

Fig. S5. PDGFRA protein expression is bimodal and EGFR expression ubiquitous in TS753. (*A*) Dual immunofluorescence for total PDGFRA (FITC) and EGFR (APC) was performed in cell lines derived from M753 in which ≈30% of cells harbor *PDGFRA* amplification. FISH confirms PDGFRA amplified cells are in the high-expressing fraction; however, high expression is not exclusive to cells with amplified genotype. (*B*) Single fluorophore FACS analysis of TS753 as well as derived ligand-selected subpopulations. Growth of TS753 in EGF ligand in the presence of the PDGFRA inhibitor imatinib results in selective enrichment for EGFR expression (*Center*). Conversely, growth in PDGF ligand in the presence of EGFR inhibitor gefitinib shows selective enrichment for PDGFRA expression (*Right*).

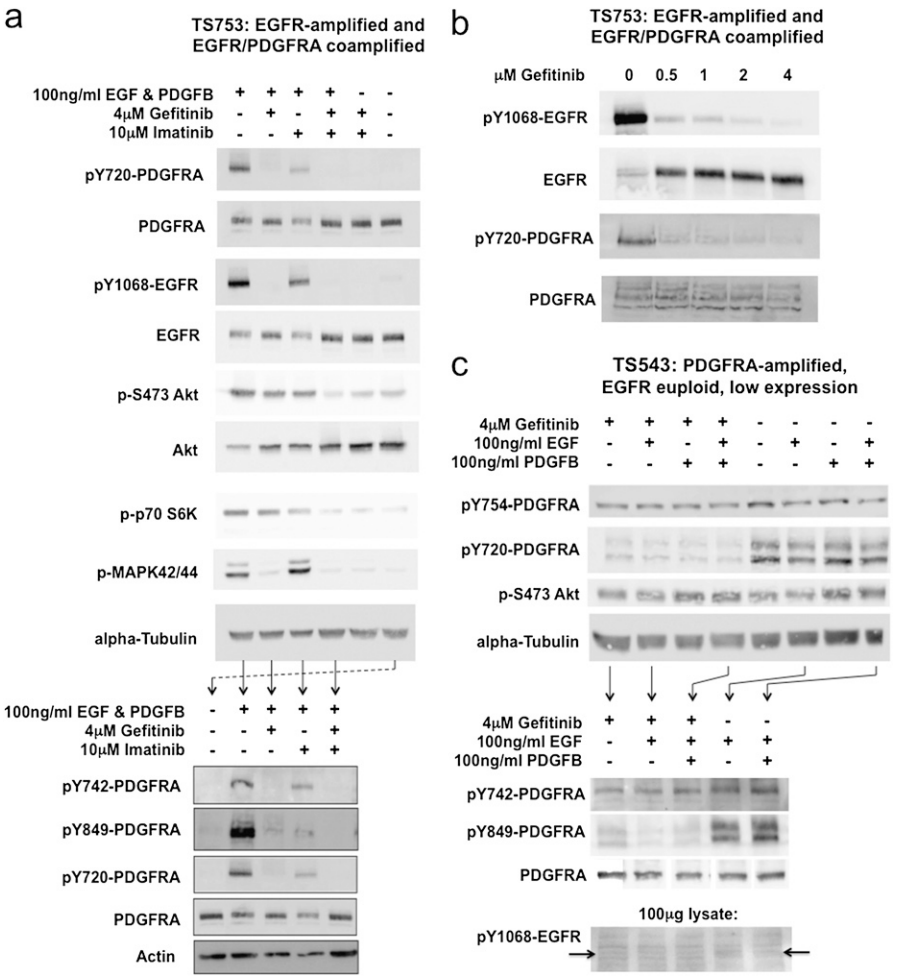

Fig. S6. (*A*) Selective and dual RTK inhibition on ligand-activated TS-753 cells with gefitinib and imatinib. Western blot of TS753 whole-cell lysates treated with either gefitinib (4 μM) and/or imatinib (10 μM) 4 h before activation by indicated ligands. *Upper*: The membrane was probed with antibodies to phospho-PDGFRA (Y720), phospho-EGFR(Y1068), phospho-AKT (S473), phospho-p70 S6 kinase, phospho-ERK1/2, and the indicated total antibodies. *Lower*: The same whole-cell lysates were run on a second membrane and immunoblotted for different PDGFRA phospho-sites, including pY742 [PI (3) kinase docking site], pY720 (Shc docking site), and pY849 (kinase domain phospho site). (*B*) PDGFRA Y720 is dephosphorylated at low doses of gefitinib. Western blot of whole-cell lysates from TS753 treated with indicated concentrations of gefitinib in the presence of 100 ng/mL PDGF and 100 ng/mL EGF was probed with phospho-EGFR (Y1068) and phospho-PDGFRA (Y720) and their respective total RTK antibodies. (*C*) PDGFRA Y720 dephosphorylation by gefitinib is not dependent on EGFR amplification (high-level EGFR expression). *Upper*: Western blot of whole-cell lysates from TS543 (a line with PDGFRA amplification and low EGFR protein expression) treated with 4 μM gefitinib 4 h before activation by indicated ligands. The membrane was probed with antibodies to phospho-PDGFRA at Y720 or Y754, phospho-AKT (S473), and α-tubulin. *Lower*: Thirty micrograms of select lysates were run on a new blot and probed for phospho-PDGFRA at Y742 and Y849. One hundred micrograms of lysate run for phospho-EGFR (Y1068).

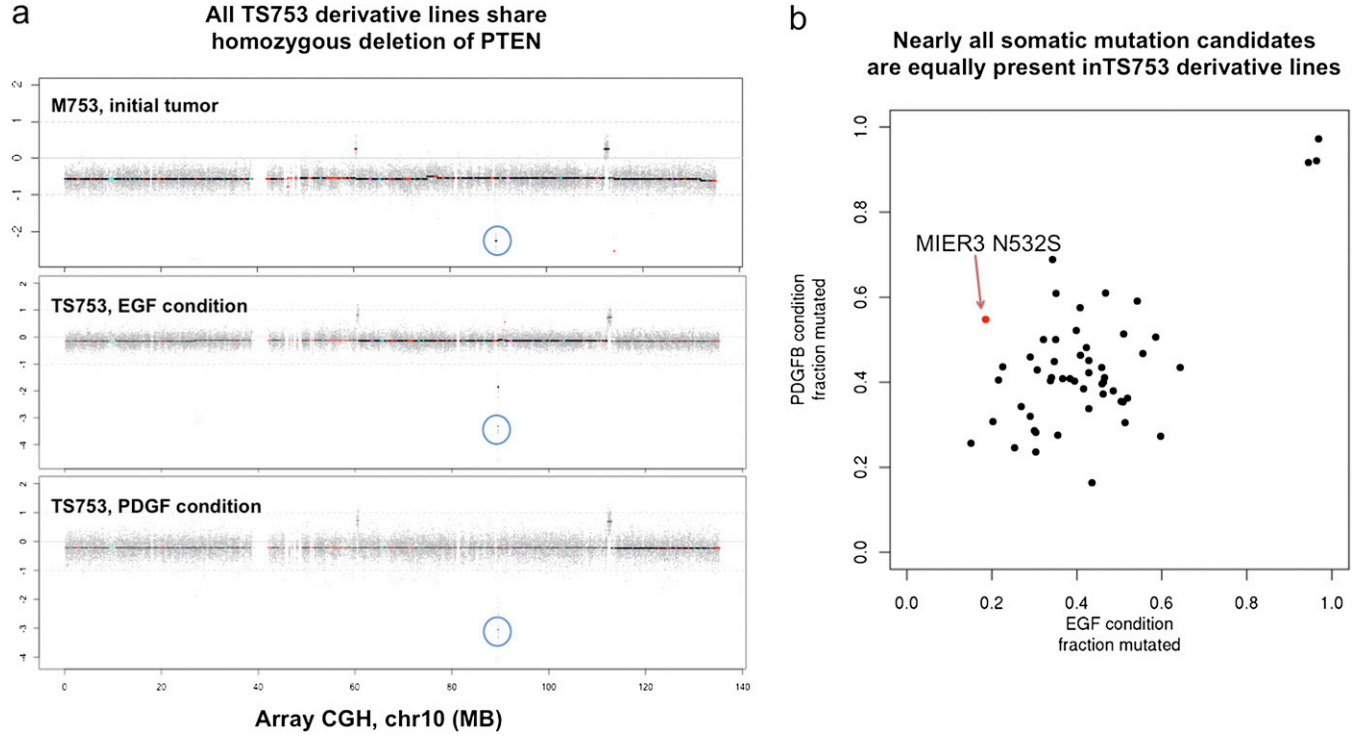

Fig. S7. (*A*) Highly focal deletion spanning PTEN is present in all major subpopulations of M753 and derived cell lines. *Top*: aCGH of initial frozen banked tumor M753 show homozygous PTEN deletion at locus indicated in blue. *Middle*: Tumor sphere line generated from primary GBM tissue, TS753, grown in EGF for 8 wk (100 ng/mL). *Bottom*: TS753 grown in PDGF ligand (100 ng/mL) for 8 wk. (*B*) Analysis of mutation allelic frequency between genotype-divergent tumor sphere subcultures. For M753, 53 candidate somatic mutations were determined from exome capture sequencing, and their allelic frequencies (fraction of reads showing the mutation) are plotted for tumor sphere line TS753. TS753 aliquots are grown in PDGF ligand (*y* axis) and EGF ligand (*x* axis) until 80% and 25% of cells, respectively, of *PDGFRA*-amplified cells are selected. Among three homozygous mutations and 50 heterozygous, all but one (red point, MIER3 N532S) are found at similar prevalence in both conditions (no significant skew in prevalence by Fisher exact test). Table S1 is a full descriptive table of these candidate mutations.

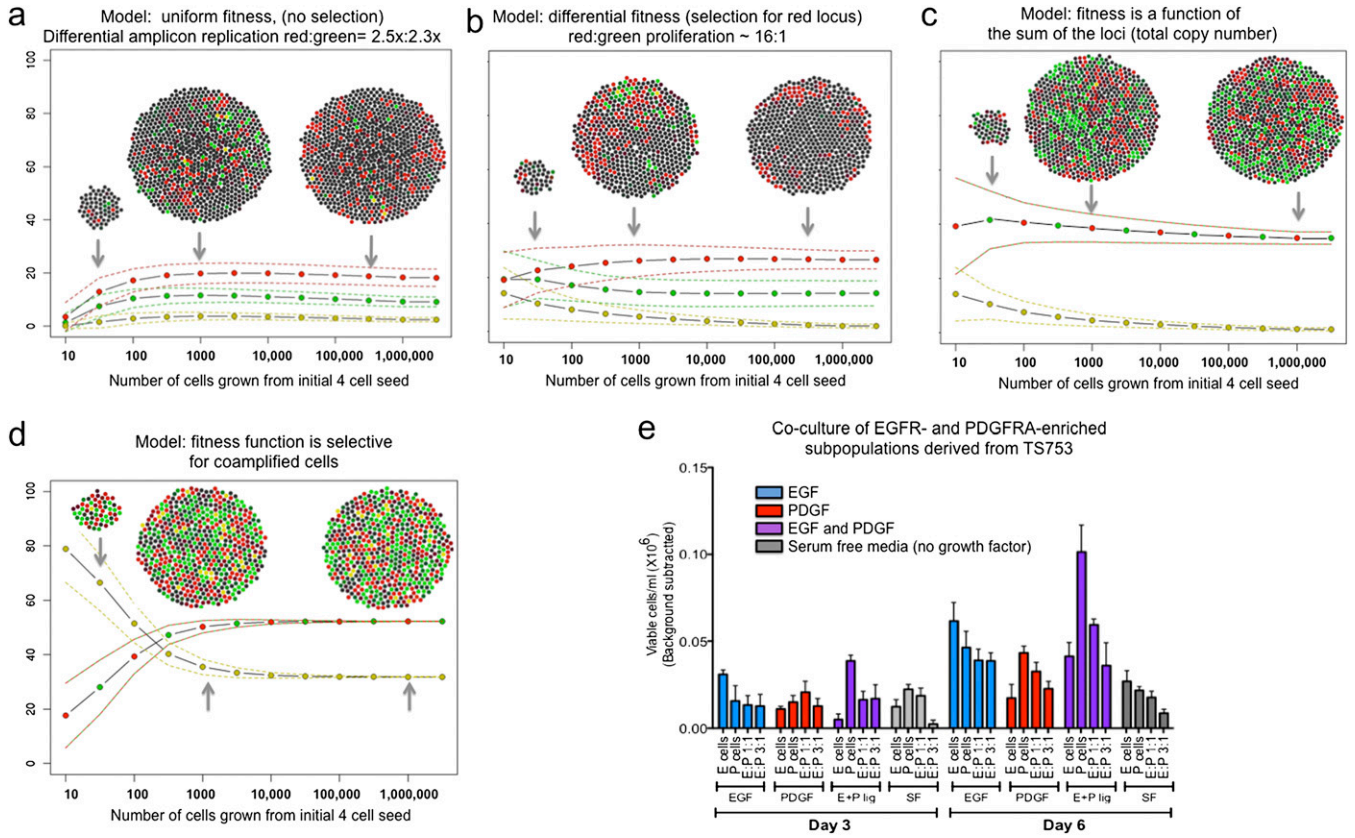

Fig. S8. Example simulations of tumor cell genotypes resulting from unequal segregation of two unstable loci (for color legend see Fig. 4). (*A*) Uniform fitness model (no selection) with differential DNA replication rates for L1 and L2 loci (red 2.5× and green 2.3×, respectively). This model demonstrates enrichment of L1 genotype mimicking selection. (*B*) Enrichment of L1 genotype by selection (16:1 fitness advantage over L2). DNA replication is 2× for both loci. (*C*) Selection function for amplified cells without preference for genotype (fitness is a function of the sum of the loci). Single-locus genotypes replicate with the same probability as dual-locus genotypes, and the latter are lost through unequal segregation (sum of single-locus genotypes denoted by alternating red–green curve in *C* and *D*). (*D*) Selection for coamplified cells (≈20-fold fitness vs. single locus amplification) leads to higher prevalence, but the majority of cells remain with single-RTK genotypes due to repeated rounds of unequal segregation. Amplicon segregation was by uniform distribution in these simulations. (*E*) Co-culture of cells selected under EGF/imatinib and PDGF/gefitinib conditions demonstrates no synergistic growth when mixed in proportions 1:1 or 3:1, respectively. Cells are grown as tumor spheres with the majority of cells in contact.

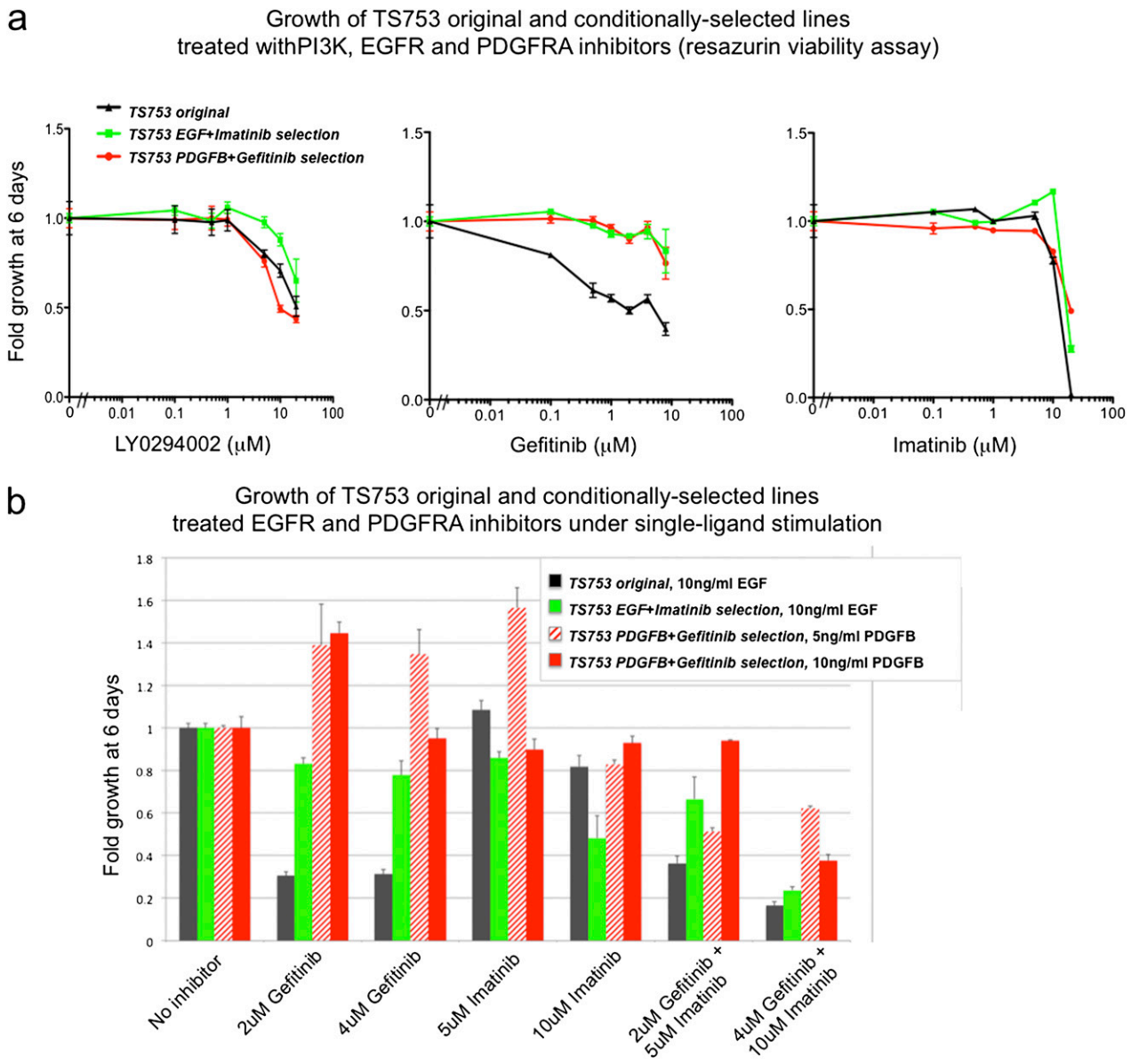

Fig. S9. Assessment of differential sensitivity to PI3K, EGFR, and PDGFRA inhibition among TS753 original and selected lines. (*A*) Cell viability assays for gefitinib, imatinib, and LY0294002 in parental TS753 and subpopulations selected for PDGFRA amplification/expression ("PDGFB+Gefitinib selection") or EGFR amplification/expression ("EGFR+Imatinib selection"). Readout is after 6 d of inhibition with cells in serum-free media with supplement, EGF, and PDGFB ligand. (*B*) Growth inhibition from gefitinib, imatinib, and both inhibitors tested in the same lines but under single ligand (EGFr or PDGFB) supplementation of serum-free media.

Other Supporting Information Files

Dataset S1