Supplemental Information

A chromatin-mediated reversible drug tolerant state in cancer cell subpopulations

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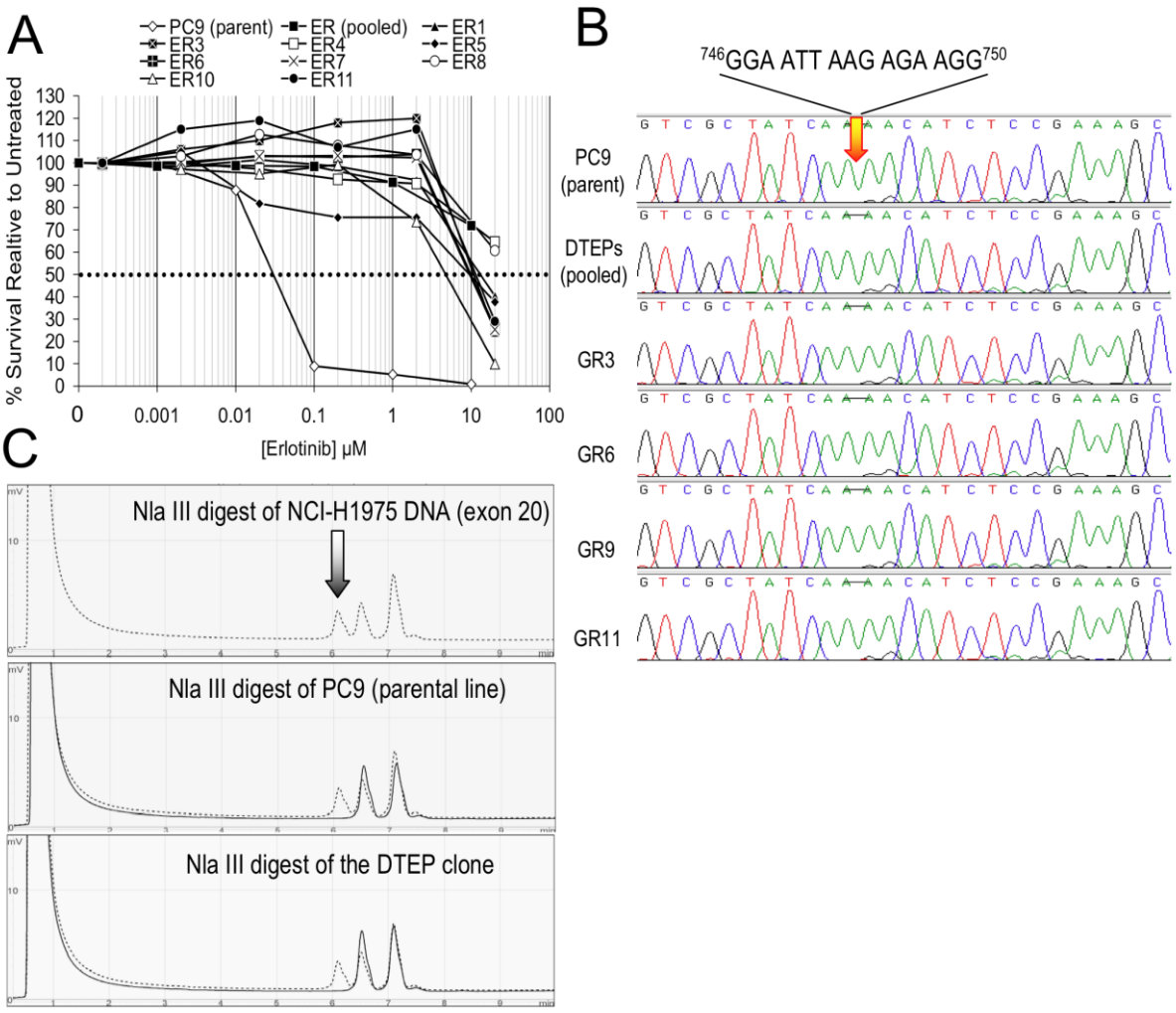


Figure S1, related to Figure 1:

(A) Survival curves of PC9 and several PC9-derived DTEP clones. Cells were treated with the indicated concentrations of erlotinib (ER) for 72 hours. Each data point represents the average value determined from four identically treated samples. The data are expressed as a percentage of surviving cells relative to untreated controls. The dashed line corresponds to 50% cell killing.

(B) DNA sequence analysis of the relevant region of exon 19 of the *EGFR* gene in PC9 and several PC9 derived DTEP clone showing the presence of ΔE746-A750 deletion mutation. Electropherograms showing the sequence of a relevant portion of exon 19 of the *EGFR* gene from PC9 cells and several PC9 derived DTEP clone. PC9 cells harbor an in-frame deletion of 15 bp that results in the removal of 5 amino acids between amino acids 746 to 750 in the kinase domain of the EGFR protein. The nucleotide sequence of the deleted region is also shown. In all the PC9 derived DTEP clones a similar deletion was observed confirming that the DTEP clones derived from PC9 cells.

(C) Absence of *EGFR* T790M mutation in the PC9-derived DTEP clones. HPLC analysis of DNA fragments generated by Nla III digestion of PCR amplified exon 20 region of *EGFR* from PC9 and one PC9-derived DTEP clone. As a positive control, DNA from NCI-H1975 with a known T790M in *cis* was similarly analyzed and shows a third diagnostic Nla III fragment indicating the presence of T790M in these samples. This diagnostic third Nla III fragment is missing from the PC9 or PC9-derived DTEP clone DNA samples. Similar analysis was performed on DNA from several other PC9-derived DTEP clones with identical results.

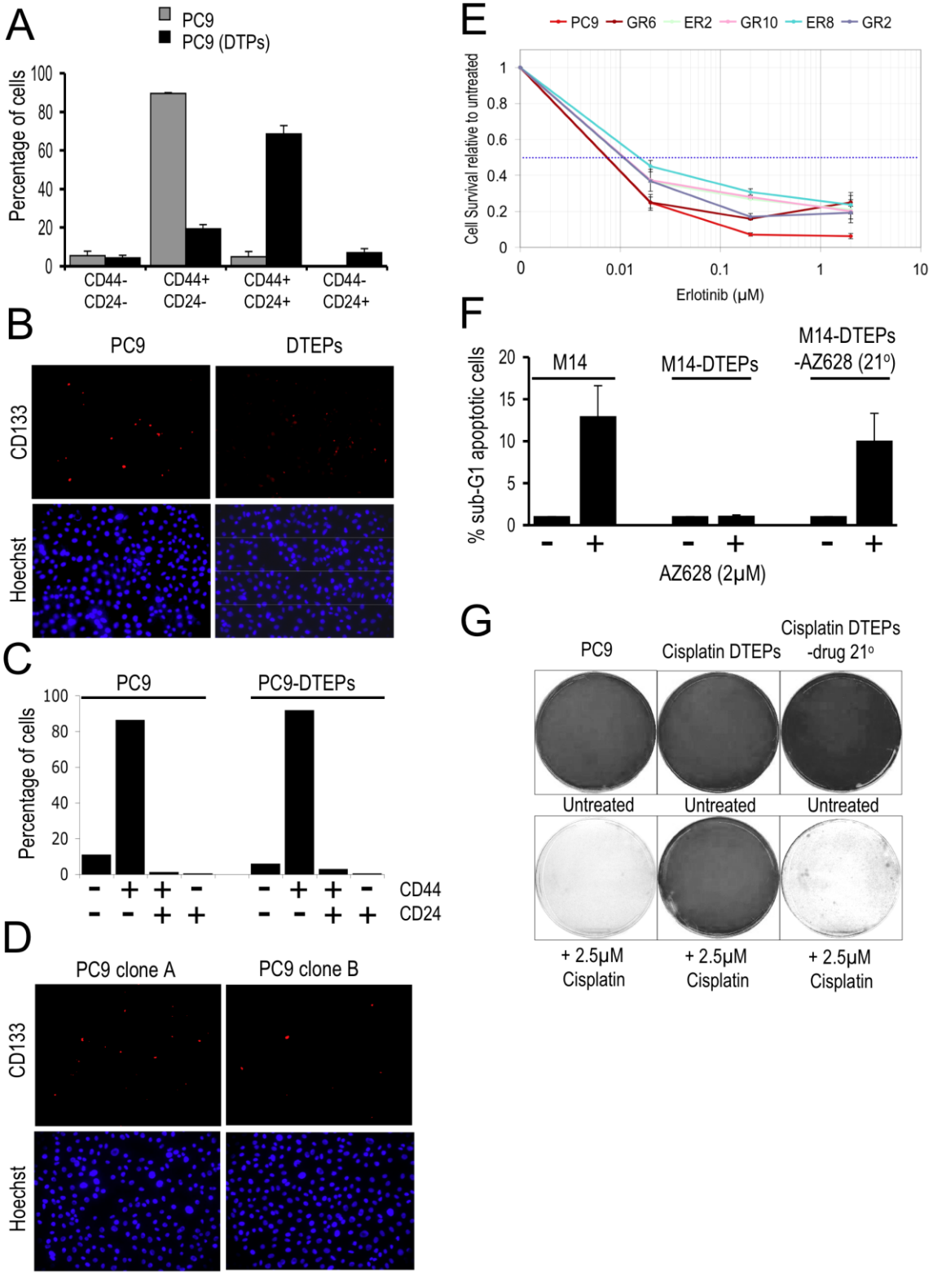

A
PC9
PC9 (DTPs)
Percentage of cells
100
80
60
40
20
0
CD44-
CD24-
CD44+
CD24-
CD44+
CD24+
CD44-
CD24+
B
PC9
DTEPs
CD133
Hoechst
C
PC9
PC9-DTEPs
Percentage of cells
100
80
60
40
20
0
- + + -
- - + +
CD44
CD24
D
PC9 clone A
PC9 clone B
CD133
Hoechst
E
PC9
GR6
ER2
GR10
ER8
GR2
Cell Survival relative to untreated
1
0.8
0.6
0.4
0.2
0
0
0.01
0.1
1
10
Erlotinib (μM)
F
M14
M14-DTEPs
M14-DTEPs
-AZ628 (21°)
% sub-G1 apoptotic cells
20
15
10
5
0
- + - + - +
AZ628 (2μM)
G
PC9
Cisplatin DTEPs
Cisplatin DTEPs
-drug 21°
Untreated
Untreated
Untreated
+ 2.5μM
Cisplatin
+ 2.5μM
Cisplatin
+ 2.5μM
Cisplatin

Figure S2, related to Figure 2:

(A) DTPs are enriched for CD24 expression. Quantitation of FACS data presented in Figure 2C. Graphs reflect the average results from two independent experiments.

(B) DTEPs exhibit a CD133 expression profile resembling parental PC9 cells. PC9 cells or PC9-derived DTEPs (growing in erlotinib) were immunostained with anti-CD133 antibody (red) and counterstained with Hoechst dye (blue), revealing a similar percentage of CD133-positive cells in both cases. Magnification, 20X.

(C) DTEPs exhibit a CD24 and CD44 expression profile resembling parental PC9 cells. PC9 and PC9-derived DTEPs (growing in erlotinib) were dually labeled with anti-CD44 conjugated to APC and anti-CD24 conjugated to PE (phycoerythrin). Cells were subjected to FACS analysis and the different populations were quantified and plotted as percentages in the histogram.

(D) CD133 expression in clonally-derived PC9 cells. Two different single cell-derived PC9 clones were immunostained with anti-CD133 antibody (red) and counterstained with Hoechst dye (blue). Magnification, 20X.

(**E) Reversibility of tolerance to the EGFR TKIs.** Survival curves of PC9 and several PC9-derived DTEP clones after 30 passages in drug-free medium. Drug sensitivity was determined by treating cells with the indicated concentrations of erlotinib. Each data point represents the average value determined from four identically treated samples. The data are expressed as a percentage of surviving cells relative to untreated controls. Error bars represent standard deviation from the mean value. The dashed line corresponds to 50% cell killing.

(F) Partial re-sensitization of M14-derived DTEPs after 21 passages out of drug (AZ628). M14 or M14-derived DTEPs and M14-derived DTEPs that have been propagated without drug for 21 passages (M14-DTEPs-AZ628 (21^{o}) were plated at equal density and left un-treated (-) or treated with 2mM AZ628. 48 hours post-plating cells were labeled with BrdU for 1 hour and subjected to FACS analysis. Presented in the figure is the percentage of cells with a sub-G1 DNA content indicative of apoptotic cells. The values presented are the average of 2 separate experiments. Error bars represent standard deviations from the mean.

(G) Reversibility of cisplatin tolerance in PC9 cells. PC9 cells, PC9-derived DTEPs selected in 2.5 mM cisplatin, and PC9-derived DTEPs selected in 2.5 mM cisplatin and then re-sensitized to cisplatin by 21 passages in drug-free medium were either untreated or treated with 2.5 mM cisplatin for 15 days and plates were stained with Giemsa.

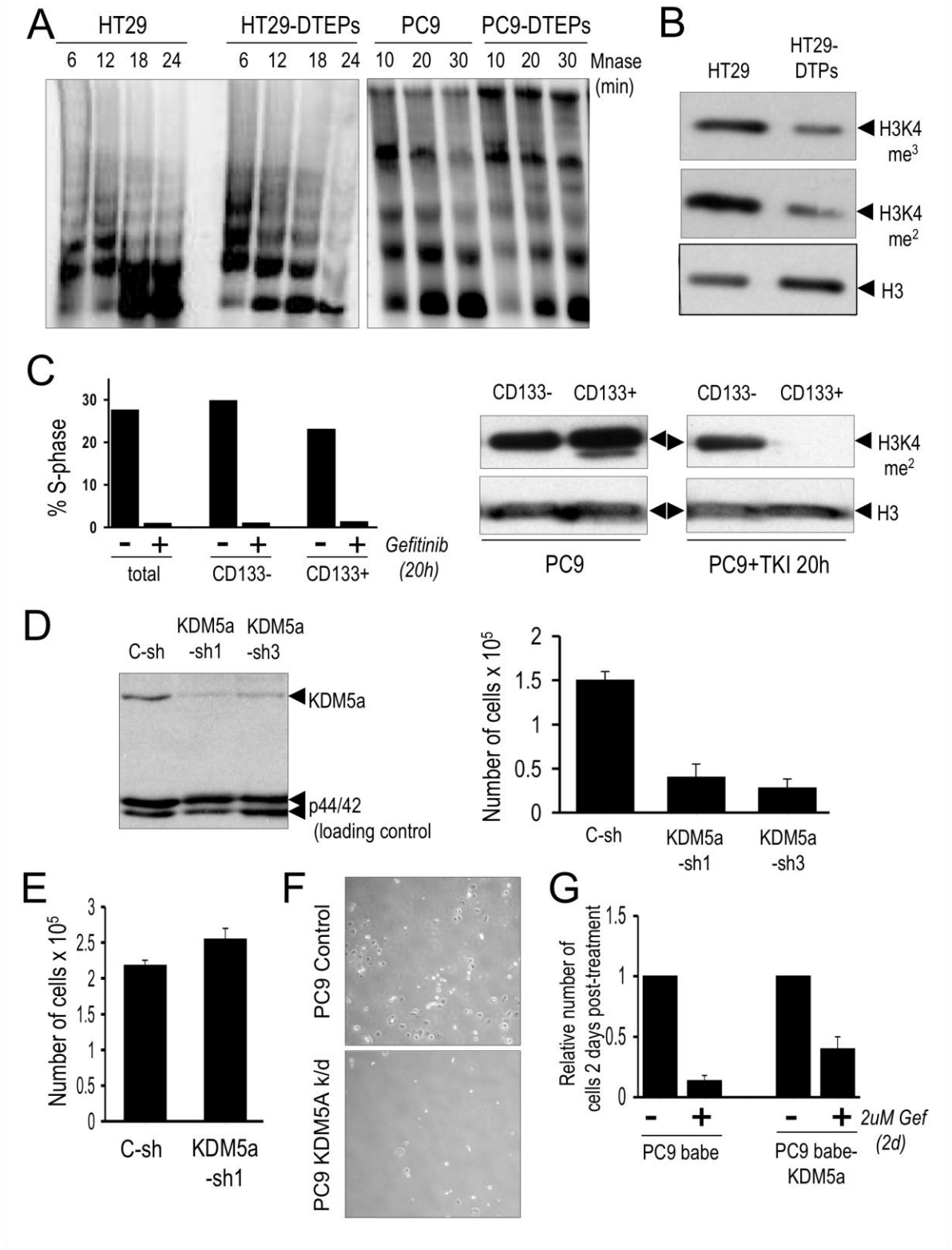

A
HT29
HT29-DTEPs
PC9
PC9-DTEPs
6 12 18 24
6 12 18 24
10 20 30
10 20 30
Mnase (min)
B
HT29
HT29-DTPs
H3K4 me3
H3K4 me2
H3
C
% S-phase
30
20
10
0
- + - + - +
total
CD133-
CD133+
Gefitinib (20h)
CD133- CD133+ CD133- CD133+
H3K4 me2
H3
PC9
PC9+TKI 20h
D
C-sh
KDM5a -sh1
KDM5a -sh3
KDM5a
p44/42 (loading control
Number of cells x 10^5
2
1.5
1
0.5
0
C-sh
KDM5a -sh1
KDM5a -sh3
E
Number of cells x 10^5
3
2.5
2
1.5
1
0.5
0
C-sh
KDM5a -sh1
F
PC9 Control
PC9 KDM5A k/d
G
Relative number of cells 2 days post-treatment
1.5
1
0.5
0
- + - +
2uM Gef (2d)
PC9 babe
PC9 babe-KDM5a

Figure S3, related to Figure 3:

(A) Reduced micrococcal nuclease sensitivity of chromatin extracted from DTEPs. Nuclei were isolated from HT29 colorectal cells, PC9 NSCLC cells, and from HT29-derived from AZ628-treated HT29 cells and erlotinib-treated PC9 cells and treated with micrococcal nuclease for the indicated times. DNA was isolated and resolved by agarose gel electrophoresis and visualized with ethidium bromide stain.

(B) Reduced H3K4 methylation in DTPs. Immunoblots demonstrating reduced methylation of histone H3K4 (tri- and di-methylation) in HT29-derived DTPs (following treatment with the RAF kinase inhibitor AZ628). Total histone H3 serves as a loading control.

(C) Demethylation of H3K4 is only observed in the CD133+ population. (Left) PC9 cells untreated or treated with EGFR TKI-treated PC9 cells (for 20 hours) were BrdU labeled for 45 minutes followed by fractionation on the basis of CD133 surface expression using magnetic beads, and then subjected to FACS to determine the percentage of cells in S-Phase. The experiment shows that both CD133-negative and -positive populations become quiescent in response to drug treatment. (Right) Immunoblots of H3K4 methylation in lysates from PC9 cells (untreated or EGFR TKI-treated) fractionated on the basis of CD133 surface expression. The experiment shows that demethylation of H3K4 is only observed in the CD133+ population; i.e., the subpopulation that survives drug exposure and becomes DTPs. Total histone H3 levels (H3) are also shown as a loading control.

(D) KDM5A knockdown reduces the number of DTPs. (Left) Immunoblot demonstrating reduced expression of KDM5A in PC9 cells stably expressing lentivirally-expressed short hairpin RNAs directed against KDM5A with two different short hairpin constructs targeting KDM5A (sh1-used in all the other experiments, and sh3) compared to PC9 cells infected with a control short hairpin (C-sh). (Right) The number of cells 9 days post treatment with EGFR-TKI in cells infected with the control short hairpin or short hairpin 1 and 3 that target KDM5A. Error bars are based on 2 different experiments, each performed in duplicate. The cells were plated at 10^6/dish the day before drug addition.

(E) KDM5A knockdown does not affect overall survival of PC9 cells. PC9 cells stably transduced with a control or KDM5A-specific short hairpin were counted two days following plating (FACS shown in Figure 3C). This experiment shows that KDM5A knockdown has no significant effect on the proliferation or survival of the bulk population.

(**F) KDM5A knockdown reduces the number of DTPs.** PC9 cells (PC9 control) and PC9 cells in which KDM5A was depleted by shRNA (PC9 KDM5A k/d) were either untreated or treated with gefitinib for 9 days, at which time photomicrographs were taken.

(G) Exogenous expression of KDM5A increases the survival of PC9 cells treated with EGFR-TKI. Quantitation of PC9 cells 2 days following infection with a control vector (babe) or a KDM5A-expressing vector in the presence or absence of 2μM EGFR TKI gefitinib.

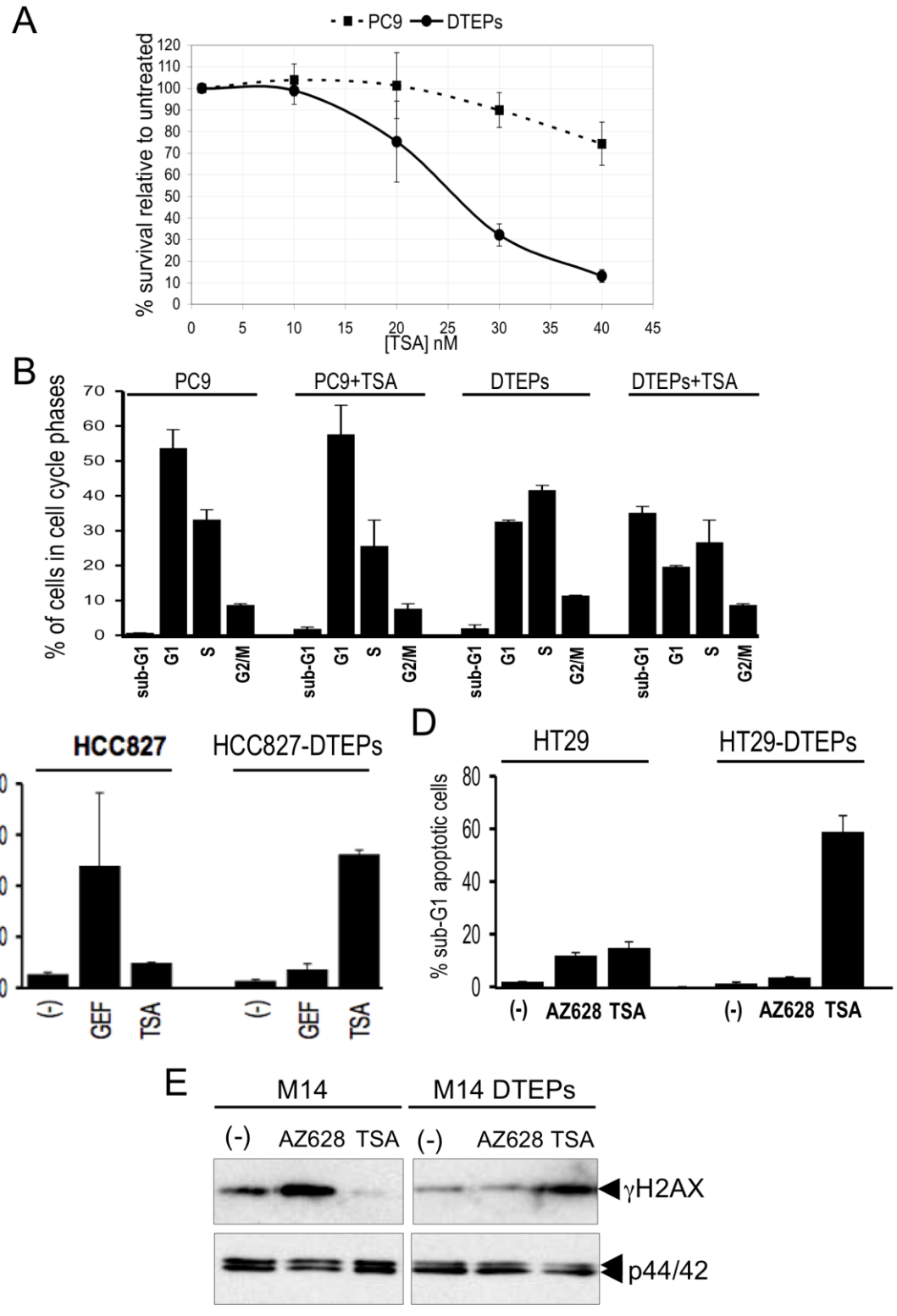

A
PC9
DTEPs
% survival relative to untreated
[TSA] nM
B
PC9
PC9+TSA
DTEPs
DTEPs+TSA
% of cells in cell cycle phases
sub-G1
G1
S
G2/M
C
HCC827
HCC827-DTEPs
% sub-G1 apoptotic cells
(-)
GEF
TSA
D
HT29
HT29-DTEPs
% sub-G1 apoptotic cells
(-)
AZ628
TSA
E
M14
M14 DTEPs
(-)
AZ628
TSA
γH2AX
p44/42

Figure S4, related to figure 4:

(A) Effect of the chromatin-modifying agent TSA on drug-tolerant cells established in PC9 cells. Survival curves of PC9 and a PC9-derived DTEP clone treated with the indicated concentrations of TSA for 72 hours. Each data point represents the average value determined from four identically treated samples. The data are expressed as a percentage of surviving cells relative to untreated controls.

(B) Effect of TSA on drug-tolerant cells established in PC9 cells. PC9 cells and PC9-derived DTEPs were either untreated or treated with 75 nM TSA (+TSA) for 48h, after which cells were labeled with BrdU for 1hour, fixed, stained and subjected to FACS analysis. Indicated in the graph is the percentage of cells in various stages of the cell cycle including the percentage of cells with a sub-G1 DNA content (fragmented DNA) indicative of apoptotic cells. Error bars represent standard deviations from the mean.

(C) Effect of TSA on drug-tolerant cells established in HCC827 cells. HCC827 cells or HCC827-derived DTEPs (EGFR TKI-tolerant; demonstrating *MET* gene amplification) were plated at equal density and were either untreated (-), treated with 2mM gefitinib or 100nM TSA as indicated. 36 hours post-plating cells were labeled with BrdU for 1 hour, fixed and subjected to FACS analysis. Indicated in the graph is the percentage of cell with a sub-G1 DNA content indicative of apoptosis. The values presented are the average of 2 separate experiments. Error bars represent standard deviations from the mean.

(D) Effect of TSA on drug-tolerant cells established in HT29 cells. HT29 colorectal cells or AZ628-tolerant HT29 cells were plated at equal density and were either untreated (-), treated with 50 nM TSA as indicated. 36 hours post-plating cells were labeled with BrdU and subjected to FACS analysis. Indicated in the graph is the percentage of cell with a sub-G1 DNA content indicative of apoptosis. The values presented are the average of 2 separate experiments. Error bars represent standard deviations from the mean.

(E) Altered DNA damage response in drug-tolerant cancer cells. Cell Lysates from M14 cells or M14-derived DTEPs that were either untreated (-), treated with 2μM AZ628 or 50nM TSA for 20 hours were analyzed on SDS-PAGE followed by immunoblotting using antibodies directed against γH2AX and ERK1/2 as a loading control.

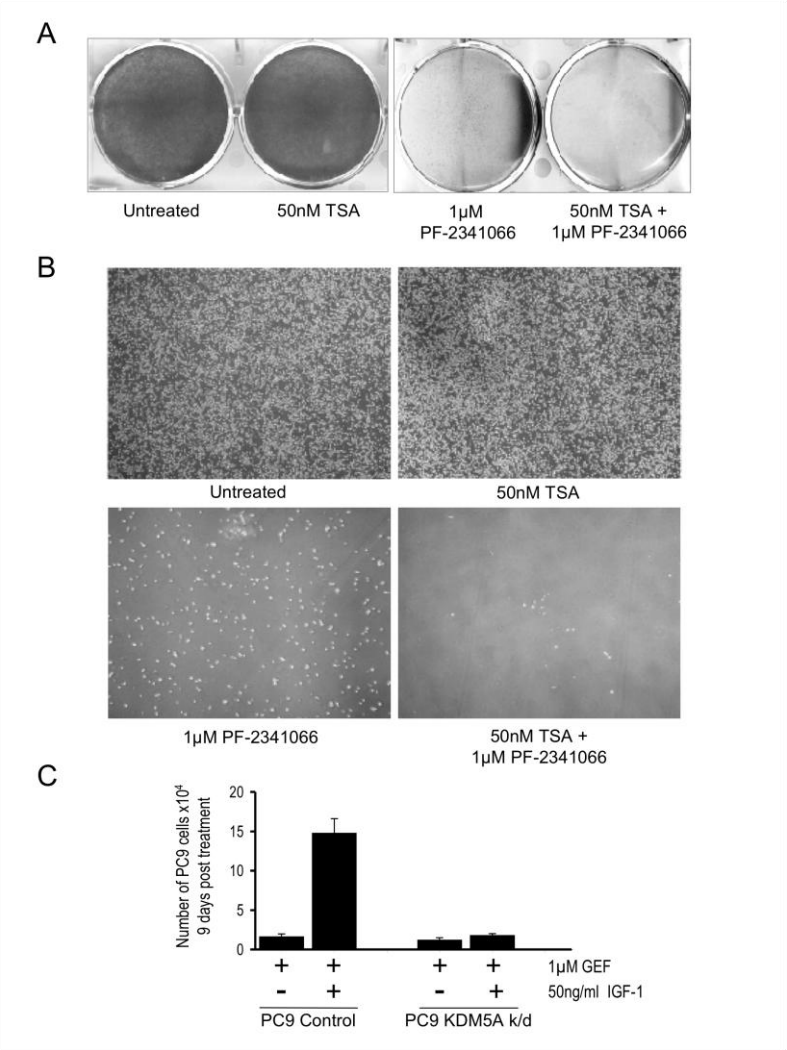
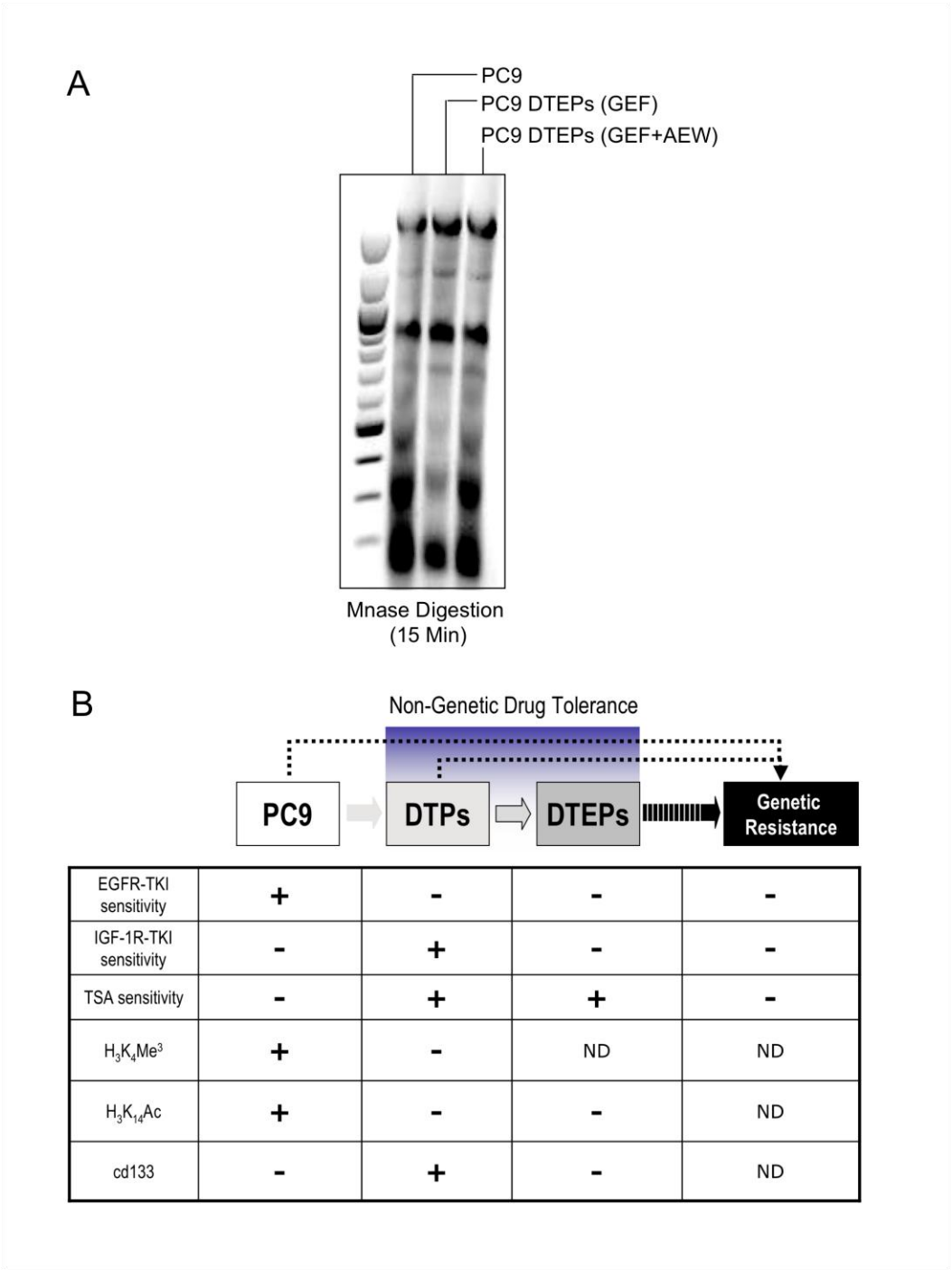


Figure S5, related to Figure 6:

(A) DTPs arise during treatment of MET-amplified Kato II gastric cancer cells with a MET kinase inhibitor. The PF-2341066-sensitive gastric cancer cell line KATO II (MET-amplified) was either untreated, treated with TSA (50nM TSA), PF-2341066 (1μM PF-2341066), or PF-2341066 and TSA (1μM PF-2341066 + 50nM TSA). After 6 days, the untreated and TSA treated plates had reached confluence and were fixed and stained with Giemsa. The PF-2341066 + TSA treated plates were similarly fixed and stained after 90 days of treatment. Drug treatments were repeated every 6 days. The experiment was performed in triplicate and representative stained plates are shown (left).

(B) DTPs arise during treatment of MET-amplified Kato II gastric cancer cells with a MET kinase inhibitor. Photomicrographs of untreated KATO II cells or KATO II cells treated with 50nM TSA after 6 days of treatment (top two photomicrographs). The bottom two photomicrographs show the KATO II derived DTPs after 3 months of treatment with 1mM of PF-2341066 (left) and the result of combining 50nM TSA with 1mM of PF-2341066 on KATO II derived DTPs after 3 months (right).

(C) Effect of KDM5A on IGF-1-mediated expansion of drug tolerant cells. PC9 cells and PC9 cells in which KDM5A was depleted by shRNA (k/d) were either treated with gefitinib (GEF) alone, or gefitinib plus IGF-1 for 9 days, at which time the remaining viable cells were counted. The graph reflects the average of two independent experiments performed in duplicate. Error bars represent standard deviations from the mean.



EGFR-TKI sensitivity	+	-	-	-
IGF-1R-TKI sensitivity	-	+	-	-
TSA sensitivity	-	+	+	-
$H_3K_4Me^3$	+	-	ND	ND
$H_3K_{14}Ac$	+	-	-	ND
cd133	-	+	-	ND

Figure S6, related to figure 7:
(A) IGF-1R inhibition prevents chromatin changes in DTEPs. Nuclei were isolated from PC9 cells, PC9-derived DTEPs (gefitinib-treated; GEF), and from PC9-derived DTEPs (gefitinib- and AEW541-treated; GEF+AEW) and treated with micrococcal nuclease (Mnase) for 15 minutes and DNA was isolated and resolved by agarose gel electrophoresis and visualized with ethidium bromide stain.
(B) Reversible drug-tolerant states can give rise to stable genetically-conferred drug resistance. Summary table describing the identified properties of erlotinib-sensitive PC9 cells, and the DTPs and DTEPs derived from them. ND=Not determined.

Supplemental Experimental Procedures

Antibodies: Antibodies directed against pEGFR, total p44/42 MAPK, and CD133 were from Cell Signaling Technology. The γH2AX antibody was from Upstate Biotechnology. Anti-mouse pIGF-1R antibodies were from Abcam and anti-human pIGF-1R antibodies were from GenScript. KDM5A antibodies were from Bethyl Laboratories. Conjugated anti-human CD44(APC), CD24(PE), and BrdU antibodies were from BD Pharmigen and FITC-conjugated antibodies for FACS analysis were from Vector Laboratories. Antibodies directed against histone H3K4, di-methylated and tri-methylated, $H3K14^{Ac}$ and total histone H3 were from Active Motif. Antibodies directed against total EGFR and glyceraldehyde 3-phosphate dehydrogenase were from Santa Cruz Biotechnology and Biosource International, respectively. Anti-IGFBP3 antibodies were from Genescript. Secondary antibodies including HRP-conjugated anti-mouse and anti-rabbit antibodies, were from Cell Signaling Technology. Texas Red X-conjugated secondary antibody was from Molecular Probes.

Pharmacologic agents: Gefitinib and AZ628 were kindly provided by Astra-Zeneca Pharmaceuticals. Erlotinib, sorafenib, and lapatinib were obtained from the MGH pharmacy. TSA, SAHA, Scriptaid and MS275 were from Biomol. The DAGK inhibitor, JAK inhibitor II, SB203580 and SU6656 were from Calbiochem. Caffeine was from Sigma Aldrich Chemical Company. AEW541 was kindly provided by Novartis Pharmaceuticals. The MET kinase inhibitor PF-2341066 was kindly provided by Pfizer Pharmaceuticals.

Allele-Specific mutation analysis: *EGFR* exon 20 was amplified from genomic DNA of PC9 and several of the PCP-derived DTEP clones. A positive control consisted of genomic DNA from the NSCLC cell line NCI-H1975 known to harbor a T790M mutation. The amplified DNA was digested with Nla III and the digestion products were analyzed by HPLC. The presence of a T790M mutation creates a novel Nla III restriction site and results in the appearance of a novel peak that was readily detected in DNA from NCI-H1975 cells. These results were independently confirmed using allele specific PCR analyses using the T790M mutation test kit following instructions provided by the manufacturer (DxS Diagnostics).

Differential gene expression analysis: RNA extraction was performed using the Qiagen RNA easy kit (P/N 74106). One round of RNA amplification was performed using Arcturus' RiboAmp RNA Amplification Kit using biotinylated ribonucleotides (Perkin Elmer PN Biotin-11-UTP, NEL543001EA / Biotin-11-CTP, NEL542001EA) during the in vitro transcription reaction. The labeled RNA was processed using protocols described within Affymetrix's GeneChip Expression Analysis Technical Manual (PN701021 Rev. 3) under Eukaryotic Sample and Array Processing and was hybridized to Affymetrix Human X3P arrays. Scan data was acquired using the Affymetrix GeneChip 3000 Scanner with autoloader and 7G upgrade. The software used to run the scanner and CEL files was Affymetrix's GCOS version 1.4. Expression values were computed from the CEL files using Affymetrix's mas5 algorithm as implemented by the call.exprs (algorithm="mas5", do.log=FALSE) function of version 2.14.05 of the Simpleaffy package of BioConductor (www.bioconductor.org), which sets the trimmed mean of the expression values for each array to 100. Expression values were obtained from 10 samples of untreated PC9 cells, 5 samples of PC9-derived DTEPs following gefitinib treatment, and 5 samples of PC9-derived DTEPs following erlotinib treatment. Probe sets with 75^{th} percentile of expression less than 20 were considered unexpressed and discarded. For each remaining probe set a p-value was computed for the difference between the untreated cells and DTEPs using a two-sided equal-variance t-test. The p-values were converted to q-values using the method of Benjamini and Hochberg. Probe sets with a q-value less than or equal to 0.05 (corresponding to a false discovery rate of 5%) and with a fold-change greater than or equal to 2 were considered to be differentially expressed.

Cell lines: PC9 cells expressing the *EGFR* exon 19 deletion mutation (ΔE746-A750) were kindly provided by Dr. Kazuto Nishio (National Cancer Center Hospital, Tokyo). The M14 melanoma cell line and the Colo-205 and HT-29 colorectal cancer cell lines harboring the BRAF V600E mutation, and the gastric cancer-derived cell line KATO II harboring amplified MET were obtained from the ATCC and maintained in RPMI 1640 containing 5% fetal-bovine serum. The NSCLC cell line NCI-HCC827 harboring an *EGFR* exon 19 deletion mutation (ΔE746-A750) was kindly provided by Dr. Jeff Engelman (MGH Cancer Center). Unless otherwise stated, PC9- or HCC827-derived DTEPs were maintained in 1μM gefitinib or 2.5μM erlotinib. M14-derived DTEPs were maintained in media containing 2μM AZ628. Colo205-derived cisplatin-tolerant DTEPs were maintained in 2.5 μM cisplatin.

DNA Sequence Analysis: Genomic DNA was isolated from cell lines using the Gentra purification system according to the manufacturer's protocol. The EGFR kinase domain (Exons 18-24) was amplified from genomic DNA by PCR. PCR products were digested with exonuclease I and shrimp alkaline phosphatase (United States Biochemical, Cleveland, OH) followed by bidirectional sequencing using BigDye v1.1 (Applied Biosystems, Foster City, CA) in combination with an ABI3100 sequencer (Applied Biosystems). Electropherograms were analyzed using Sequence Navigator software (Applied Biosystems).

Cell cycle stage determination by FACS analysis: The analysis of cells in the G1, S and G2/ M phases of the cell cycle as well as cells containing a sub-G1 content of DNA indicative of apoptosis was measured using fluorescence-activated cell sorting (FACS) according to the manufacturer's protocol (Becton Dickinson). Briefly, cells were incubated with Cell Labeling Reagent (BrdU, Amersham Pharmacia) at 37^0 C for 1 hour. Before analysis, cells were fixed and stained according to the manufacturers recommendations (Becton Dickinson). All cells (adherent and floating) were washed with phosphate-buffered saline (PBS) and fixed in 80% ethanol. DNA was denatured for 30 minutes with 2 M HCl/0.5% Triton X-100 and neutralized with 0.1 M $NaB_4O_7 \bullet 10H_2O$ (pH 8.5), before incubation with anti-BrdU antibody (1:500, Becton-Dickinson) and a FITC-conjugated goat anti-mouse secondary antibody (1:50, Vector Laboratories). Cells were stained with 5 μg/mL propidium iodide (Sigma) and treated with RNAse A (Sigma) prior to two-dimensional FACS analysis using CELLQUEST software (Becton Dickinson). The number of gated cells in the various phases of the cell cycle including the sub-G1 population is presented.

Chromososomal arrangement of differentially expressed genes: Differentially expressed probe sets were mapped to Entrez GeneIDs using the following two-step procedure: Step 1: Mapping probe set IDs to UniGene IDs. If the Affymetrix annotation file provided a UniGene ID, that was used. Otherwise, if the Affymetrix annotation file provided an Entrez Gene ID and that was found in a record of the UniGene file Hs.data, the UniGene ID of that record was used. Otherwise, if the Representative Public ID from the Affymetrix annotation file was present in a record of the UniGene file Hs.data, the UniGene ID of that record was used. Otherwise, if the Representative Public ID from the Affymetrix annotation file was present in the Entrez Gene file gene2accession.gz and the associated Entrez Gene ID was found in a record of the UniGene file Hs.data, the UniGene ID of that record was used. Step 2: Mapping probe set IDs to Entrez GeneIDs. If the Affymetrix annotation file provided an Entrez GeneID, that was used. Otherwise, if the Representative Public ID from the Affymetrix annotation file was present in the Entrez Gene file gene2accession.gz, the associated Entrez Gene ID was used. Otherwise, if the Affymetrix annotation file provided a UniGene ID and that was found in a record of the UniGene file Hs.data and that record provided an Entrez GeneID that was used. Otherwise, if a UniGene ID was determined in Step 1 above and that ID was found in a record of the UniGene file Hs.data and that record provided an Entrez GeneID that was used.

To determine genomic locations for the Entrez GeneIDs that resulted from mapping the differentially express probe sets, the refseq table corresponding to build Hg18 available from the Table Browser at genome.ucsc.edu was used. That refseq table has multiple entries for some Entrez GeneIDs, however, so

we employed the following procedure. Entrez GeneIDs located on multiple chromosomes were discarded.For each remaining Entrez GeneID in the table, we defined the combined span to be the min of the starts and the max of the ends of all entries in the table associated with the Entrez GeneID. If the combined span was more than four times the length of the maximum of the lengths of the spans of the individual entries corresponding to that Entrez GeneID, we discarded that Entrez GeneID. The location of the remaining Entrez GeneIDs was defined as the midpoint of the combined span. Up (resp. down) genes were defined as Entrez GeneIDs resulting from the procedure above that correspond to probe sets more (resp. less) expressed in DTEPs. Non-random chromosomal arrangement of up- and down-regulated genes was demonstrated by counting the number of neighboring differentially expressed genes that are either both up- or both down-regulated. The count for the actual up- and down-regulated genes was compared with 10,000 counts generated by randomly permuting the up and down designations.

Micrococcal nuclease digestion: For the Micrococcal nuclease (MNase) digestion experiments, 1-2x10^7 cells/sample were permeabilized in 0.3M sucrose in 60 mM KCl, 15mM NaCl, 5mM $MgCl_2$, 0.1mM EGTA, 15mM Tris-HCl (pH 7.5), and 0.4% (v/v) IGEPAL (adopted from D. Umlauf, Institute of Molecular Genetics, CNRS). The resulting nuclei were isolated by spinning at 10,000 g, for 20 minutes at 4°C on a 1.2M sucrose cushion, and subsequently digested (~ 6x10^6 nuclei/sample) with 2 units of micrococcal nuclease (Worthington Biochemical) in digestion buffer (.32M sucrose, 50mM Tris-HCl (pH 7.5), 4mM $MgCl_2$, and 1mM $CaCl_2$) for the indicated times at room temperature. The DNA was subsequently phenol-chloroform extracted, resuspended in TE. The DNA concentration of each individual sample was measured and equivalent amounts of DNA from each timepoint were subjected to electrophoresis in 0.5xTBE on 1% agarose gels and stained with ethidium bromide.

Analysis of CD24 and CD44 surface expression by FACS: PC9, PC9-derived DTPs, and PC9-derived DTEPs were washed with PBS and detached using Accutase. Suspended cells were washed in PBS and resuspended in PBS containing 1% FBS at 1X10^6 cells/ml. 1ml of cell suspension was resuspended in 100µL HEPES-buffered saline (+2% FBS, 10mM HEPES). Following a 10-minute incubation on ice, 5µl of each antibody was added and the cells were incubated an additional 30 min on ice and washed twice in 500µL PBS with 1% FBS. Cell pellets were resuspended in PBS+propidium iodide (PI) to exclude dead cells from FACS analysis. Cells labeled with an isotype control IgG conjugated to PE were used as a negative control in the analysis.