Inventory of Supplemental Information

Supplemental Figures and Legends

Figure S1 is related to Figure 2. Contains additional data highlighting the differences in tumor propagating ability of Sca1+ and Sca1- cells from Kras;p53-flox tumors, addressing secondary tumor size, differentiation and self-renewal capacity. Also includes data showing that both Sca1+ and Sca1- cell populations contain similar quantities of tumor cells to further emphasize a true functional difference between the two populations.

Figure S2 is related to Figure 3. Illustrates that the Sca1+ and Sca1- cell populations from Kras tumors are similar to their corresponding populations in the Kras;p53-flox tumor model. Contains additional data showing that both Sca1+ and Sca1- cells from Kras tumors have equal differentiation and self-renewal capacity in serial transplants. Includes data showing that p53 is intact and expressed in Kras tumor cell populations. And incorporates representative data regarding EGFR tumor transplants.

Supplemental Tables

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Supplemental Experimental Procedures

Supplemental References

Supplemental Information

Primary tumor genotype is an important determinant in identification of lung cancer propagating cells

Stephen J. Curtis, Kerstin W. Sinkevicius, Danan Li, Allison N. Lau, Rebecca R. Roach, Raffaella Zamponi, Amber E. Woolfenden, David G. Kirsch, Kwok-Kin Wong, and Carla F. Kim

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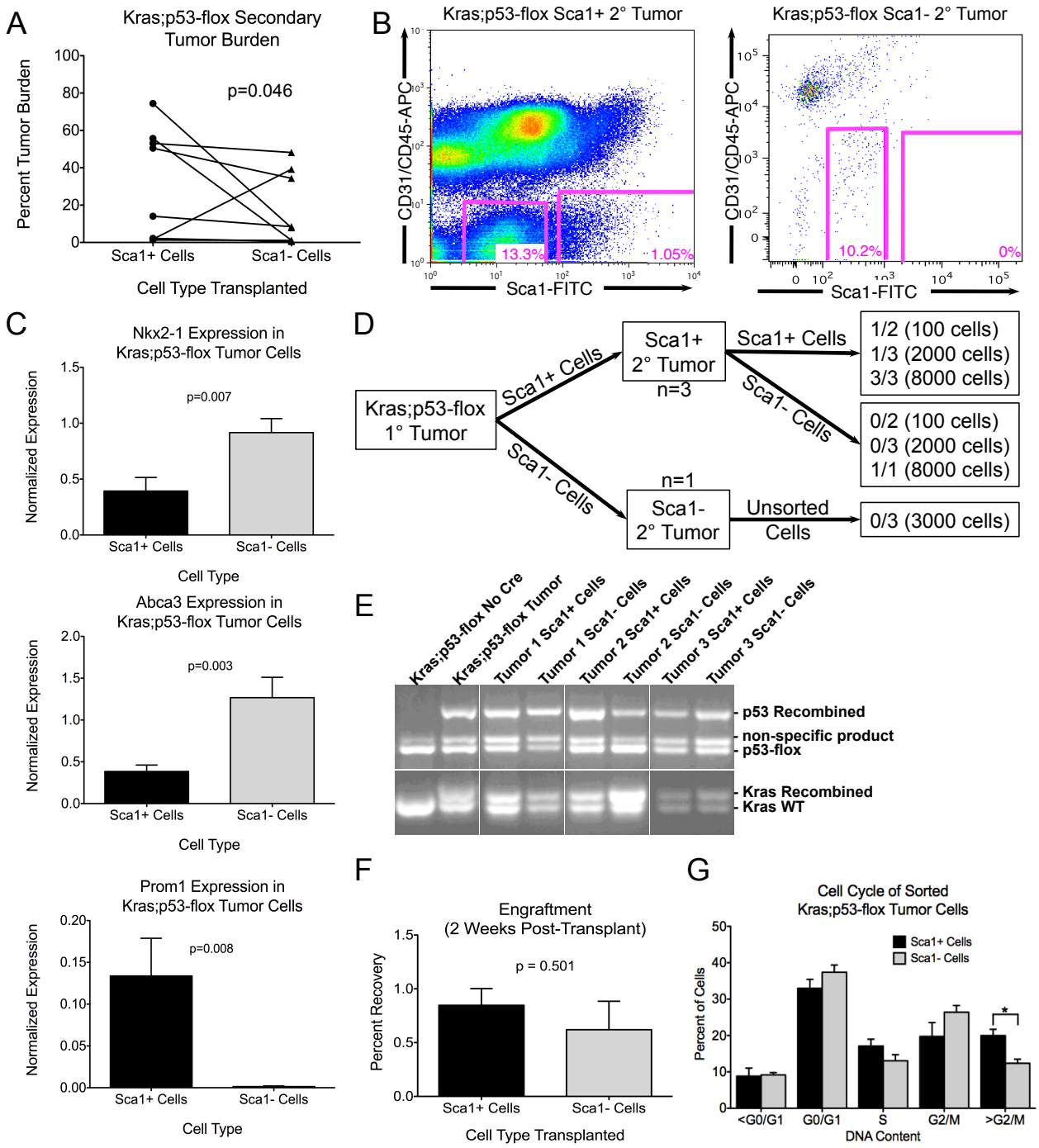

Figure S1, related to Figure 2.

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(A) Eight pairs of matched secondary recipients from Sca1 sorted Kras;p53-flox tumor cell transplants of varying number were analyzed for tumor burden. Percent tumor burden is reported as area of tumor divided by total lung area in H&E stained sections. Slides with maximum tumor area were used. In 7 out of 8 the matched pairs, the Sca1+ recipient had more tumor burden than the corresponding Sca1- recipient. The Sca1+ recipients had a significantly higher tumor burden than Sca1- recipients (p=0.046, one-sided, Wilcoxon Signed Rank test).

(B) Representative FACS plot of a secondary Kras;p53-flox tumor propagated from a Sca1+ cell transplant (left) and the one FACS plot we were able to obtain from a Sca1- cell transplant (right).

(C) Quantitative RT-PCR analysis of Sca1 fractionated tumor cells from a set of 12 Kras;p53-flox tumors. Expression values were normalized to total unsorted lung cells. Nkx2-1 (or Ttf-1) (top) and Abca3 (middle) are both well characterized markers of differentiated lung cells and are expressed at significantly higher levels in Sca1- Kras;p53-flox tumor cells (p=0.007 and p=0.003, respectively). However, Prom1 (or CD133) (bottom), which is a putative marker of human lung cancer stem cells as well as stem cells and cancer stem cells from many other organs, is expressed more highly in Sca1+ Kras;p53-flox tumor cells (p=0.008).

(D) Schematic of serial transplants performed, showing the number of tumor-bearing secondary recipient mice used for serial transplantation and the resulting number of recipients that developed tertiary tumors over the number of transplants performed for all cell types and numbers shown.

(E) Recombination specific PCR of genomic DNA from Sca1 fractionated tumor cells at the *p53* locus (top) and *Kras* locus (bottom) showing similar amounts of recombination in both cell types. Unfractionated tumor and normal lung from a Cre-naive Kras;p53-flox mouse were used for controls.

(F) 2-weeks post-transplant of GFP marked Sca1+ and Sca1- Kras;p53-flox tumor cells, lungs of recipient mice were harvested and sorted for 7AAD-, CD31-, CD45-, GFP+ cells. Sca1+ and Sca1- cells showed equal ability to transplant short-term (0.84% and 0.62%, respectively, p=0.501, n=3).

(G) Cell cycle analysis of Sca1 fractionated tumor cells from 3 primary Kras;p53-flox tumors, as determined by Hoechst dye staining (in the presence of verapamil), showed that both cell populations were cycling at equal levels. However, the Sca1+ population was found to include more cells with a greater than 4N DNA content (*, p=0.022).

For all pooled data, Mean and SEM are plotted.

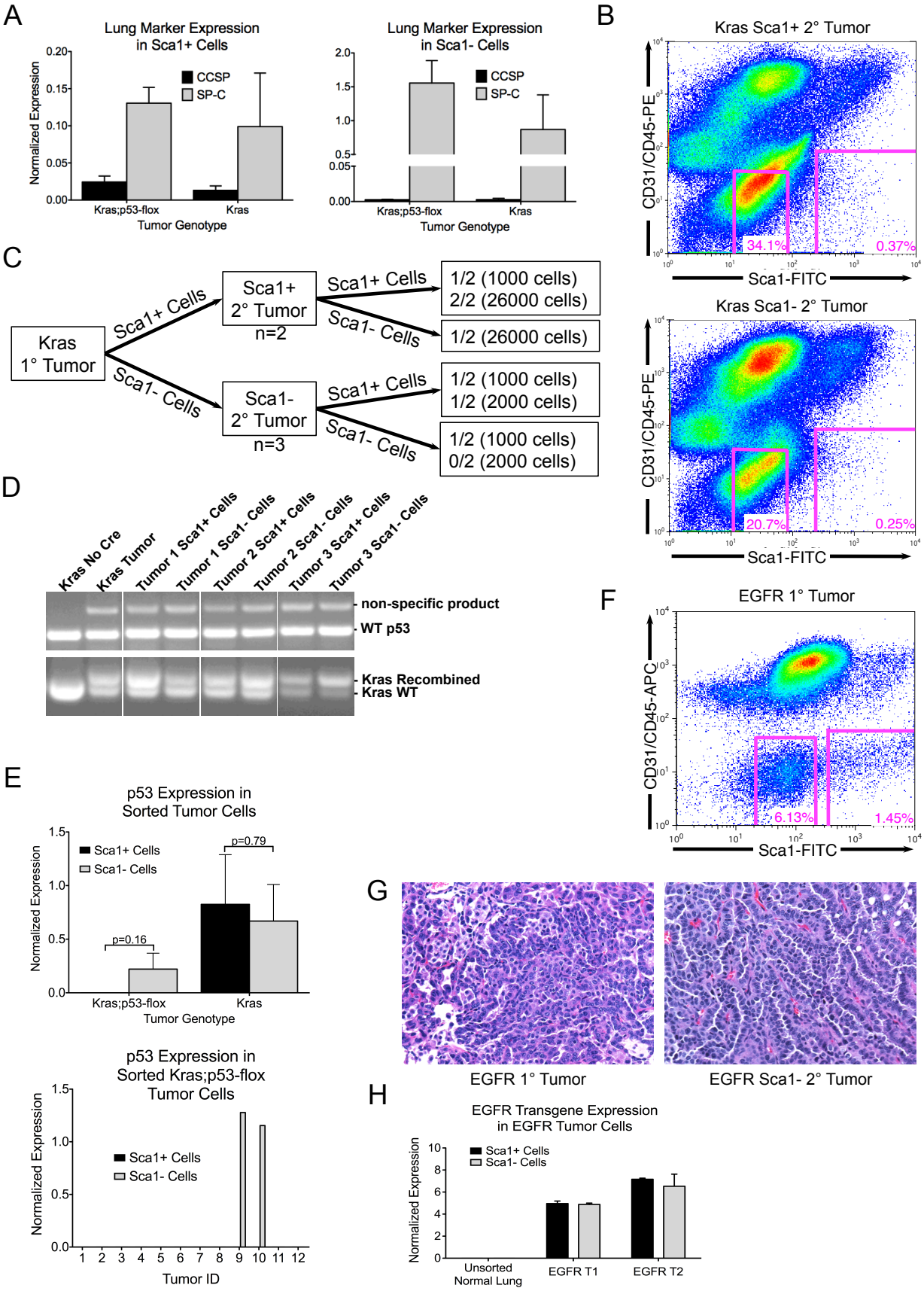

Figure S2, related to Figure 3.

Figure S2, related to Figure 3.

(A) Quantitative RT-PCR expression analysis to determine the expression of Clara cell secretory protein (CCSP) and surfactant protein-C (SP-C) in Sca1 fractionated tumor cells from a set of 12 Kras;p53-flox tumors and 8 Kras tumors. Expression in Sca1 fractionated cells was normalized to the level of each gene's expression in unfractionated normal lung. Sca1+ cells (left) from both tumor models showed low levels of SP-C and CCSP as expected, and Sca1- cells (right) from both models showed virtually no CCSP expression, yet high levels of SP-C expression as expected.

(B) Representative FACS plots of a secondary Kras tumor propagated from a Sca1+ cell transplant (top) and a Sca1- cell transplant (bottom).

(C) Schematic of serial transplants performed, showing the number of tumor-bearing secondary recipient mice used for serial transplantation and the resulting number of recipients that developed tertiary tumors over the number of transplants performed for all cell types and numbers shown.

(D) Recombination specific PCR of genomic DNA from Sca1 fractionated tumor cells at the *p53* locus (top) and *Kras* locus (bottom) showing intact *p53* and similar amounts of *Kras* recombination in both cell types. Unfractionated tumor and normal lung from a Cre-naive Kras mouse were used for controls.

(E) Quantitative RT-PCR analysis of p53 expression in Sca1 fractionated tumor cells from a set of 8 Kras and 12 Kras;p53-flox tumors. Expression values were normalized to CD31-/CD45- normal lung cells. Although Sca1- cells from Kras;p53-flox tumors showed a small amount of p53 expression, this was limited to only 2 out 12 tumors (see lower panel, data from individual tumor sets) and was not significantly different than expression in Sca1+ cells ($p=0.16$).

(F) Representative FACS analysis of EGFR tumors showing a very similar pattern to both Kras and Kras;p53-flox tumors.

(G) Histological analysis showing a primary EGFR tumor (left) and a corresponding secondary tumor from transplanted Sca1- cells (right).

(H) Quantitative RT-PCR specific to the human *EGFR* gene from two EGFR primary tumors (EGFR T1 and EGFR T2) showing that both Sca1+ and Sca1- cells express the mutant *EGFR* transgene at equal levels. Expression was normalized to the level of expression in the H1975 human lung cancer cell line, which harbors the same $EGFR^{T790M-L858R}$ mutant allele used in the EGFR mouse model. Technical triplicates are plotted for each sample. Total unsorted normal mouse lung was used a negative control.

For all pooled data, Mean and SEM are plotted.

All images, 200X magnification. Scale bar = 100μM.

Supplemental Tables

Kras;p53-flox Tumor Cells			
		Sca1+ Tumor Cells	Sca1- Tumor Cells
Donor ID(s)	**Number of Cells Injected**	**No. Mice with Tumors / No. Transplanted**	
114*	5	2/2	ND
114*	100	1/3	1/3
1937+1972+1973	300	ND	0/3
1975	790	2/3	1/3
1021+1024	2000	1/2	0/2
2129+2092+2128	2000	ND	0/2
114*	2000	1/3	1/3
5001	2500	3/4	1/3
308	3000	2/2	3/3
2477+2524	3333	3/3	1/3
270+286	8000	2/2	1/2
114*	10000	ND	0/2
Total Donor Mice = 16		Total Recipients = 24	Total Recipients = 29

Table S1, related to Figure 2 and Table 1.Transplant data from individual experiments with Kras;p53-flox mice.
Donor (primary) mice are listed with the number of cells transplanted and the results from transplanting Sca1+ and Sca1- tumor cells.
On days where multiple mice were pooled prior to sorting and transplant, the donor IDs are joined by a “+” sign.
* Denotes the use of a single tumor instead of the more common pool of 3-5 tumors from the donor mouse.
An individual experiment is defined as one day of primary tumor isolation, FACS and transplantation.

Kras Tumor Cells			
		Sca1+ Tumor Cells	Sca1- Tumor Cells
Donor ID(s)	Number of Cells Injected	No. Mice with Tumors / No. Transplanted	
134*	672	2/2	1/2
135*	872	1/1	1/2
2578*	1000	0/3	3/3
1258	1000	1/4	1/3
4001	2880	2/3	1/3
2041	3000	1/3	ND
2150	4700	0/1	1/2
4002	7322	1/3	ND
100	8000	1/3	3/3
1111+1071+1138	8400	1/3	2/3
4002	8516	ND	1/3
184	9800	2/2	3/3
2041	10000	ND	5/6
1908+1909	12500	ND	2/3
1112	22500	2/3	2/3
2166+2161	36000	2/2	ND
2150	150000	ND	3/3
1112	225000	ND	2/3
2166+2161	230000	ND	2/2
Total Donor Mice = 18		Total Recipients = 33	Total Recipients = 47

Table S2, related to Figure 3 and Table 1. Transplant data from individual experiments with Kras mice.
Donor (primary) mice are listed with the number of cells transplanted and the results from transplanting Sca1+ and Sca1- tumor cells.
On days where multiple mice were pooled prior to sorting and transplant, the donor IDs are joined by a "+" sign.
* Denotes the use of a single tumor instead of the more common pool of 3-5 tumors from the donor mouse.
An individual experiment is defined as one day of primary tumor isolation, FACS and transplantation.

Supplemental Experimental Procedures

Tumor Burden Analysis

Hematoxylin and Eosin (H&E) stained sections of lungs of recipient mice were imaged at 40X on a Nikon 90i microscope and stitched together using Photoshop CS3. Lung area and tumor area were determined using ImageJ. Of the step sections described in the "Mice and Tissues" method, the slide with the highest degree of tumor burden was used for this comparison.

Gene Expression Analysis

RNA from sorted cell populations was extracted using the Stratagene Absolutely RNA Nanoprep Kit. cDNA was made using the SuperScript III kit (Invitrogen). Quantitative PCR was performed using TaqMan Assays (Applied Biosystems) for *Nkx2-1* (*Ttf-1*) (Mm00447558_m1), *Abca3* (Mm00550501_m1), *Prom1* (*CD133*) (Mm00477115_m1), *Scgb1a1* (*CCSP*) (Mm01230908_m1), *Sftpc* (*SP-C*) (Mm00488144_m1), *Trp53* (*p53*) (Mm01337166_mH), and *EGFR* (Hs01076092_m1) with a BioRad iQ5 iCycler and software as per the manufacturer's recommendations. Mouse ACTB (B-actin, 4352341E) or Human ACTB (for the H1975 cell line) (B-actin, 4326315E) was used as an endogenous control for normalization.

Recombination Specific PCR

Genomic DNA was isolated from Sca1 fractionated tumor cell populations from both Kras and Kras;p53-flox tumors. PCR for 1-lox *Kras* (Forward Primer: 5'-GGG TAG GTG TTG GGA TAG CTG-3', Reverse Primer: 5'-TCC GAA TCC AGT GAC TAC AGA TGT ACA GAG-3') was completed using the Advantage-GC cDNA PCR Kit (Clonetech). Expected bands: 285 bp for Wild-type allele and 315 bp for 1-lox (recombined) allele. PCR for 1-lox *p53* (Primer A: 5'-CAC AAA AAC AGG TTA AAC CCA G-3', Primer B: 5'-AGC ACA TAG GAG GCA GAG AC-3', Primer C: 5'-GAA GAC AGA AAA GGG GAG GG-3') was completed using GoTaq (Promega). Expected bands: 288 bp for the Wild-type allele, 370 bp for the floxed (non-recombined) allele, and 612 bp for the 1-lox (recombined) allele. Note: a non-specific band usually appears around 400-420 bp.

Engraftment Analysis

Kras;p53-flox mice were bred with mice harboring *Rosa26-rtTA* (Hochedlinger et al. 2005) and *tetO-H2B-GFP* (Tumbar et al. 2004) alleles. After tumor induction with Adeno-Cre the mice were given doxycycline in their water to activate H2B-GFP expression. Tumors were isolated, sorted, and transplanted as described. Recipient mice were put on doxycycline containing water 1 week prior to transplant and were maintained on doxycline containing water for the duration of the experiment. Two weeks post-transplant, the lungs of recipient mice were harvested, dissociated and prepared as described previously (Kim et al. 2005). The single cell suspension from each recipient mouse was stained for 7AAD, CD31, and CD45 and analyzed on a BD LSRII flow cytometer. The percent recovery was determined by the number of GFP+ (7AAD-, CD31-, CD45-) cells recorded, divided by the number of cells transplanted.

Cell Cycle Analysis

Tumor cells were fractionated by Sca1 expression as described. Live tumor cells were incubated for 45 min at 37°C with 20 μg/ml HOECHST 33342 (Invitrogen) in HBSS medium containing 10% FBS, 20 mM Hepes (pH .2), 1 g/liter glucose, and 5 μg/ml Verapamil as described previously (Passegué et al. 2005). Cells were washed and stained with 1 μg/ml propidium iodide (PI) just prior to analysis. Flow cytometry was performed on a BD LSRII. Only live (PI-negative) cells were included in the analysis. Data were analyzed with FloJo software (Tree Star, Inc.).

Mice and Tissues

EGFR-mutant mice harbored a *TetO-EGFR*$^{T790M-L858R}$ allele in addition to a *CCSP* promoter driven *rtTA* and tumors were initiated with doxycycline as described (Li et al., 2007). Mice were maintained on a doxycycline containing diet for 8-10 weeks prior to harvesting lungs with tumors. Recipient mice were also maintained on doxycycline from 24 hours prior to the transplant, until they were sacrificed. Mice were maintained in viral-free conditions, and the protocol for the EGFR animal work was approved by Dana-Farber Cancer Institute Institutional Animal Care and Use Committee and the CHB Animal Care and Use Committee, both accredited by AAALAC, and were performed in accordance with relevant institutional and national guidelines and regulations.

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