Supplementary Methods

Murine mammary stem cell isolation. Mammary glands from 6-12 week old female C57Bl/6J or 129S1/SvImJ mice were dissociated as described[1] with minor modifications. Specifically, mammary fat pads were harvested and placed directly into Medium 199 (Gibco BRL) supplemented with 20mM HEPES and Penicillin, Streptomycin, and Actinomycin (PSA). Tissue was minced using sterile razor blades and 4 Wünsch units of Liberase Blendzyme 4 (Roche 1988476) and 100 Kunitz units of DNase I (Sigma D4263) were added. Tissue was incubated for 60-90 minutes in a 37°C/5% CO_2 incubator, during which the cells were mechanically aspirated every 30 minutes. Cells were pelleted by centrifugation for 5 min at 4°C and 350 x *g*. After lysis of the red blood cells with ACK lysis buffer (Gibco), a single cell suspension was obtained by further enzymatic digestion for ~2 min in 0.25% trypsin, followed by another ~2 min in 5 mg/ml dispase II (StemCell Technologies) plus 200 Kunitz units DNase I (Sigma). Cells were then filtered through 40-μm nylon mesh, pelleted, and resuspended in staining media (HBSS + 2% HICS). Cells were counted using trypan blue dye exclusion.

Tumor dissociation. Human and mouse tumors were dissociated as previously described[2] with minor modifications. Depending on the time of the surgical case, some of the human tumors were kept over night at 4°C prior to dissociation. Tumors from patients or MMTV-*Wnt-1* tumor-bearing FVB/NJ female mice were minced with a razor blade and suspended in 20 ml of Medium 199 (Gibco BRL) supplemented with 20mM Hepes. The dissociation enzyme cocktail consisted of 100 Kunitz units of DNAse I (Sigma D4263), 8 Wünsch units of Liberase Blendzyme 2 (Roche 1998433), and 8 Wünsch units Liberase Blendzyme 4 (Roche 1988476). Tumors were digested to completion (1.5-2.5 hours at 37°C/5% CO_2) with pipetting every 30 minutes for manual dissociation. Once digested, 30 ml of RPMI (BioWhittaker) with 10% calf serum (HICS) was added to the digestion solution to inactivate the collagenases. A 40 μm nylon filter was

used to filter the sample. After pelleting, cells were resuspended in 5 ml of ACK buffer for red blood cell lysis. HBSS (BioWhittaker) with 2% heat-inactivated calf serum (HICS) was used to dilute the ACK buffer and the cells were again filtered through a 40 μm nylon filter. The filtered cells were spun down and resuspended in HBSS with 2% HICS.

Cell staining and flow cytometry. Cells were stained at a concentration of 1 x 10^6 cells per 100μL of HBSS with 2% HICS (staining media). Cells were blocked with rabbit or mouse IgG (1 mg/ml) at 1:100 dilution and antibodies were added at appropriate dilutions determined from titering experiments. For the normal mammary stem cell experiments, antibodies included CD49f, CD31, CD45, Ter119 (BD Pharmingen), CD24, Thy1.2, and CD140a (eBioscience). For the murine MMTV-Wnt-1 breast cancer experiments, antibodies included CD24, Thy1.1, CD140a (eBioscience), CD45 and CD31 (BD Pharmingen). For the human breast cancer experiments, antibodies included CD44, CD24, CD45, CD3, CD20, CD10, Glycophorin A (BD Pharmingen), CD31 (eBioscience) and CD64 (Dako). For the primary human head and neck cancer experiment, antibodies included CD44, CD45 (BD Pharmingen), and CD31 (eBioscience) and for the xenograft experiments, antibodies included CD44, mCD45 (BD Pharmingen), mCD31 (Abcam), and H-$2K^d$ (eBioscience). Cells were stained for 20 minutes on ice and washed with staining media. When biotinylated primary antibodies were used, cells were additionally stained with streptavidin-conjugated fluorophores and washed. Ultimately, cells were resuspended in staining media containing 7-aminoactinomycin D (7-AAD, 1 μg/ml final concentration) or 4'-6-Diamidino-2-phenylindole (DAPI, 1 μg/ml final concentration) to stain dead cells.

For all experiments, cells were analyzed and sorted using a FACSAria cell sorter (BD Bioscience). Side scatter and forward scatter profiles were used to eliminate debris and cell doublets. Dead cells were eliminated by excluding $DAPI^+$ cells, whereas contaminating human or mouse Lin^+ cells were eliminated by excluding cells labeled with the fluorophore used for the lineage antibody cocktail. In cell-sorting experiments, cell populations underwent two consecutive

rounds of purification (double sorting) when the initial purity was not deemed high enough and a sufficient number of cells were available. Final purities ranged from ~60% to >95%.

MMTV-*Wnt-1* breast tumor radiation experiments. For *in vivo* irradiation experiments, tumor-bearing animals were irradiated on consecutive days with the indicated doses of ionizing radiation using a 160 kVp cabinet irradiator (Faxitron). Tumors were grown on the ventral surface of mice in the vicinity of the second mammary fat pad and the delivered dose was adjusted to compensate for attenuation by overlying tissues. Seventy-two hours after the final fraction was delivered, tumors were harvested, dissociated, and analyzed by flow cytometry as above. For each experiment, at least one untreated control tumor was analyzed in parallel. When multiple control tumors were available, the percentage of $Thy1^+CD24^+Lin^-$ cells in these tumors were averaged to define the baseline percentage of $Thy1^+CD24^+Lin^-$ cells for that experiment. In other experiments, tumors were irradiated and these tumors along with controls were harvested 15 minutes after irradiation. Tumors were dissociated as above and cells were stained with a phospho-specific (Ser 139) histone H2AX antibody (Cell Signaling Technology) as described in the main methods section.

Human breast and head and neck cancer primary specimens. Primary tumor specimens were obtained with informed consent after approval of protocols by the Stanford University and City of Hope Institutional Review Boards. Tumors were from untreated patients, except for the two breast cancer tumors shown in Supplementary Figure S2a-d, which had been treated with neoadjuvant chemotherapy prior to resection.

Human head and neck cancer xenograft radiation experiments. Tumors were grown subcutaneously on the backs of Rag2γDKO mice as previously described[3]. Mice were irradiated as above, except that for each fraction half of the dose was delivered from the left side of the animal and half from the right.

The non-tumor-bearing portions of each animal were shielded using custom-made lead chambers.

MMTV-*Wnt-1* DCF-DA transplant experiments. Lin^- or $Thy1^+CD24^+Lin^-$ cells from tumors were sorted as described above. Cells were loaded with 10 μM DCF-DA (Invitrogen), incubated at 37°C for 30 min, and sorted into "ROS-low" and "ROS-high" sub-populations based on their DCF-DA staining profile. Sorted cells were injected into FVB female mice in Matrigel (BD Bioscience) in the vicinity of the second mammary fat pad at the indicated cell numbers.

Normal mammary stem cell DCF-DA transplant experiments. $CD24^{med}CD49f^{high}Lin^-$ mammary cells (enriched for mammary repopulating units) were isolated from mammary fat pads from C57Bl/6J female mice as described above. Cells were loaded with 10 μM DCF-DA (Invitrogen), incubated at 37°C for 30 min, and sorted into "ROS-low" and "ROS-mid" sub-populations based on their DCF-DA staining profile (in comparison to that of $CD24^{high}CD49f^{low}Lin^-$ progenitor cells, which displayed a "ROS-high" profile). Mammary glands of 21-day-old female C57Bl/6J mice were cleared of endogenous epithelium as previously described[1], and sorted cells were injected into each cleared fat pad using a Hamilton syringe. Injected glands were removed for wholemount analysis after 5–6 weeks. Transplants were scored as positive if epithelial structures consisting of ducts with lobules and/or terminal end buds and that arose from a central point were present.

***In vitro* colony assay**. Sorted tumor cells were cultured in Epicult B medium (StemCell Technologies) with 5% serum in the presence of ~13,000 cm^{-2} irradiated NIH-3T3 cells. After 24-48 hrs, the media was replaced with serum-free Epicult B, and ~7 days later, colonies were fixed with acetone:methanol (1:1), stained with Giemsa, and counted. Colonies were counted if they contained >= ~30 cells and the number of colonies in control wells was in the range of 30-100, depending on how many cells were sorted and the plating efficiency of a given

tumor. For the L-*S,R*-Buthionine Sulfoximine (BSO) experiments, cells were cultured for 24 hours in modified mammosphere medium[4] in the presence or absence of 1 mM BSO and the drug was removed immediately prior to irradiation. For the tempol experiments, cells were treated with 10 mM tempol for 15 minutes prior to irradiation, after which the drug was removed. For all drug experiments, "drug only" controls were run in parallel to adjust for effects of each drug on baseline colony counts.

Gene Set Enrichment Analysis. To define a list of genes involved in ROS metabolism and regulation, we began with a previously generated list from a recently published study by Tothova et al[5]. Briefly, this list was initially generated by these authors using the Gene Ontology GO:TERMFINDER program (http://search.cpan.org/dist/GO-TermFinder/) to classify genes by biological process, molecular function, or cellular component. The biological process terms included were: response to oxidative stress; response to reactive oxygen species; response to hydrogen peroxide; response to oxygen radical; and response to superoxide. We manually curated this list by performing PubMed-based literature searches for each gene and only retaining those that had published evidence of involvement in ROS metabolism or regulation (i.e. removing genes that appeared to be included solely due to electronic curation with inferred evidence). This trimmed the ROS gene list from 55 to 36 unique symbols (Supplementary Table 4). Gene Set Enrichment Analysis (GSEA) was then applied as previously described[6]. The "core enriched genes" shown in Figure 4 were defined by the algorithm.

Single cell gene expression analysis. For the single cell gene expression experiments we used qPCR DynamicArray microfluidic chips (Fluidigm). Single MMTV-*Wnt-1* $Thy1^+CD24^+Lin^-$ CSC-enriched cells (TG) and "Not $Thy1^+CD24^+$" Lin^- non-tumorigenic cells (NTG) cells were sorted by FACS into 96 well plates containing PCR mix (CellsDirect, Invitrogen) and RNase Inhibitor (SuperaseIn, Invitrogen). After hypotonic lysis we added RT-qPCR enzymes (SuperScript III

RT/Platinum Taq, Invitrogen), and a mixture containing a pool of low-concentration assays (primers/probes) for the genes of interest (Gclm-Mm00514996_m1, Gss-Mm00515065_m1, Foxo1-Mm00490672_m1, Foxo4-Mm00840140_g1, Hif1a-Mm00468875_m1 , Epas1- Mm00438717_m1). Reverse transcription (15 minutes at 50°C, 2 minutes of 95°C) was followed by pre-amplification for 22 PCR cycles (each cycle: 15sec at 95°C, 4 minutes at 60°C). Total RNA controls were run in parallel. The resulting amplified cDNA from each one of the cells was inserted into the chip sample inlets with Taqman qPCR mix (Applied Biosystems). Individual assays (primers/probes) were inserted into the chip assay inlets (2 replicates for each). The chip was loaded for one hour in a chip loader (Nanoflex, Fluidigm) and then transferred to a reader (Biomark, Fluidigm) for thermocycling and fluorescent quantification.

To remove low quality gene assays, we discarded gene assays whose qPCR curves showed non-exponential increases. To remove low quality cells (e.g. dead cells) we discarded cells that did not express the housekeeping genes Actb (beta-actin) and Hprt1 (hypoxanthine guanine phosphoribosyl transferase 1). This resulted in a single cell gene expression dataset consisting of 248 cells (109 tumorigenic and 139 non-tumorigenic) from a total of 7 chip-runs. A two-sample Kolmogorov-Smirnov (K-S) statistic was calculated to test if genes were differentially expressed in the two populations. We generated *p* values by permuting the sample labels (i.e. TG vs NTG) and comparing the actual K-S statistic to those in the permutation-derived null distribution. The *p* values were further corrected by Bonferroni correction to adjust for multiple hypothesis testing.

References

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