SUPPLEMENTARY METHODS

Generation of NQO1 knockout lines

To generate *NQO1* knockout lines, parental cells were transfected with ribonucleoprotein (RNP) complex composed of guide RNA (designed with Desktop Genetics Inc., Cambridge, Massachusetts; synthesized by Integrated DNA Technologies, Inc., Coralville, Iowa) and Cas9NLS protein using Lipofectamine™ CRISPRMAX™ transfection reagent (Thermo Fisher Scientific) per manufacturer's instructions. In brief, functional guide RNA was generated by annealing tracrRNA and crRNA prior to RNP complex formation. A 1:1 ratio of guide RNA and Cas9NLS protein was mixed with LipoCas9 plus reagent and incubated for 5 minutes at room temperature to produce RNP complex. RNP complex was then mixed with transfection regent and added to parental cell cultures. After overnight incubation, the culture medium was replaced, and cells were expanded until a sufficient quantity of genomic DNA could be extracted. Potential *NQO1* knockout single clones were selected and verified by Western blot.

Measurement of pSTAT3

To examine STAT3 phosphorylation in 3D spheroid co-cultures, 48 spheroids at the desired tumor-to-fibroblast ratios were collected, fixed, and sectioned for pSTAT3 immunofluorescence staining. Harvested spheroids were incubated overnight at 4°C in 10% neutral-buffered formalin, washed in 1x PBS, and resuspended in 20–30 µL of 2% agarose. Spheroids were then dehydrated, embedded in paraffin, and sectioned into 4 µm slices. Spheroids were then baked for 1 hour at 60°C and deparaffinized. Antigens were retrieved by incubating the spheroids for 30 minutes at 98°C in citrate buffer (pH 6.0). After washing with water and permeabilizing with TBS containing 0.3% Triton-X, samples were blocked with 10% normal goat serum for 1 hour at

room temperature and probed overnight at 4°C with pSTAT3 (Tyr705) (D3A7) XP® rabbit monoclonal antibody (Cell Signaling Technologies, Danvers, Massachusetts; diluted 1:100), NQO1 (A180) mouse monoclonal antibody (Cell Signaling Technologies, diluted 1:400), and human/mouse/rat vimentin antibody (R&D Systems, Minneapolis, Minnesota, diluted 1:50). Samples were then incubated with secondary antibody for 1 hour at room temperature and mounted with ProLong Gold Antifade Mountant plus DAPI (Thermo Fisher Scientific) prior to imaging on a Zeiss Axio imager M2.

Fluorescent spheroid images were analyzed and quantified using CellProfiler™ (Broad Institute, Cambridge, Massachusetts). The image analysis workflow was as follows: (1) each individual cell was identified by vimentin signal; (2) the NQO1 image was then used to verify the cell identified in the first step, with nuclei identified using the DAPI image; (3) CellProfiler was then used to measure NQO1- and vimentin-staining intensities in the cytoplasm of all cells and pSTAT3-staining intensity in nuclei; (4) all cells were then divided into two groups, tumor cells (FaDu) and fibroblasts, using the K-means clustering algorithm (classification method) from JMP® statistical software (SAS; Cary, North Carolina); and (5) pSTAT3 signal intensities across all cells in the two groups created in the fourth step were then calculated and compared using unpaired *t* test.