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Supplementary Information for

Fibroblast-tumor cell signaling limits HER2 kinase therapy response via activation of MTOR and anti-apoptotic pathways

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Supplementary Methods

Cell lines and reagents

Each cell line was grown in the appropriate culture medium formulation (see table below). Cells were grown for less than six passages. Breast cancer cells were engineered to express H2B-GFP using a retroviral plasmid[1] and were FACS sorted to select a GFP+ population. All dose-response and protein expression experiments were performed using 10% HI-FBS. Lapatinib was a gift from GlaxoSmithKline, everolimus, INK128, ABT263 and S63845 were purchased from Sellekchem. Fluorescently tagged antibodies were used for the cyclic immunofluorescence assay, phospho-S6 (CST 4851), phospho-AKT (CST 4075), phospho-EGFR (CST 1456) and phospho-HER2 (MilliporeSigma 16-236).

Cell line	Culture medium
EFM192, HCC1954, HCC1419, HCC202	RPMI + 10% HIFBS + 1% Pen/strep
SUM225	Ham's F12 + 10% HIFBS + 1µg/ml hydrocortisone + 5µg/ml insulin + 1% Pen/strep
BT474	DMEM/F12 + 10% HIFBS + 1% Pen/strep
Fibroblasts	DMEM + 10% HIFBS + 1% Pen/strep

Primary normal and cancer associated fibroblasts

AR22 mammary fibroblasts were derived from reduction mammoplasty tissue. The cells were immortalized by infection with hTERT containing retrovirus and were selected with hygromycin B to make a stable hTERT expressing fibroblast line. Cancer associated fibroblasts were derived by enzymatically digesting primary breast tumors using a mixture of collagenase/hyaluronidase and adherent cultures were established after two passages.

Drug response assays

Cells were seeded in black/clear 96well plates at a density of 2 000 cells/well (100µl) in triplicate wells and allowed to grow for 48hrs prior dosing with increasing drug concentrations (100µl of fresh medium + inhibitor). For all fibroblast co-cultures a 1:1 tumor:fibroblast ratio was used. Endpoint drug response experiments were performed using a laser-scanning plate cytometer (TTP Labtech) to measure the number of viable cells. Live cell counting assays were performed using an Incucyte S3 system. Dead cells were assayed by adding Ethidium bromide (final concentration 200nM) and the number of viable cells (subtracting Ethidium Bromide+ from GFP+) was reported. Area under the curve (AUC) values are reported for all lapatinib dose-response experiments with higher AUC values corresponding to larger number of viable cells. Growth-response metrics were calculated using the viable cell numbers from the incucyte data[2].

Analysis of Cyclic Immunofluorescence, Reverse Phase Protein Arrays, and fibroblast secreted factors

For the cyclic immunofluorescence assay, 2 000 tumor cells or 2 000 tumor cells + 2 000 fibroblasts were plated in 96 well plates. Cells were allowed to grow for 2days prior and then were treated with DMSO or lapatinib (0.1µM) for 48hrs, prior fixing with 4% paraformaldehyde. Cells were permeabilized by 10min incubation with ice-cold method prior, blocking for 1hr with Odyssey blocking buffer. Primary fluorescence-conjugated antibodies were incubated overnight (1/250 dilution). Next day, cells were stained with Hoechst for 10min and washed twice with PBS prior imaging using a GE Cytell Imaging system. Analysis of the fluorescence images was performed in ImageJ and MATLAB using previously published methods[3].

Tumor cells under monoculture and coculture conditions were cultured in 6 well plates or transwell filters to prepare lysates for reverse phase protein arrays. 100 000 breast cells/well were plated in the bottom chamber and 100 000 AR22 cells in the upper transwell compartment. Cells were allowed to grow for 2days prior treatment with lapatinib (0.1µM) or DMSO for 48hours. A two-step wash with ice-cold PBS was performed prior lysing cells. Details on protocols, reagents and have been previously described[4]. Pathway score analysis and heatmap visualization was performed in R.

450 000 fibroblasts were seeded using DMEM in 15cm plates and grew for 5days prior switching the medium to RPMI and allowing the fibroblasts to conditioned the medium. Conditioned medium was filtered using a 0.2µm filter and stored at -80C. Secreted factors were assayed using the Human XL cytokine array (R&D systems Ary022) and the manufacturers recommended protocol. RPMI medium was used as a control, conditioned medium from two normal fibroblasts was assayed in two biological replicates. For all conditioned medium experiments, tumor cells were treated with a final concentration of 33% fibroblast conditioned medium (1 part of pure conditioned medium mixed with 2 parts of RPMI medium) that did not result in significant changes in tumor cell growth rate (Fig 2C). Low- versus high-molecular weight fractions of conditioned medium were prepared using a 3kDa centrifugal filter (EMD Millipore) and were used instead of conditioned medium at a final concentration of 33% fibroblast conditioned medium.

Supplementary Figures

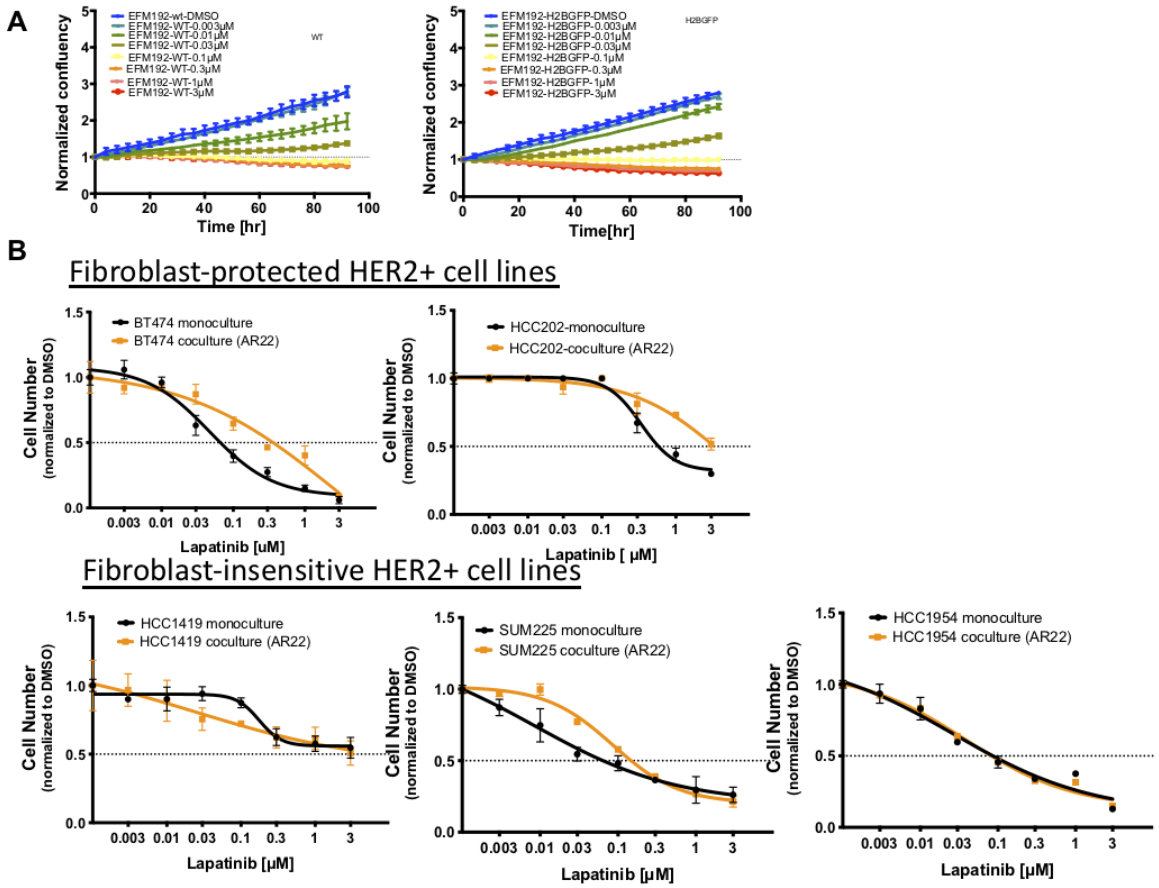


Fig. S1. **Lapatinib sensitivity in fibroblast-protected and fibroblast-insensitive HER2+ breast cancer cells. (A)** Change in tumor cell confluency over time at increasing lapatinib concentrations for wildtype EFM192 (left) and H2BGFP expressing EFM192 cells (right) under monoculture and coculture with AR22 fibroblasts. Data are representative of two independent experiments and error bars are SD for three replicate wells. **(B)** Endpoint lapatinib dose-response for the panel of HER2+ breast cancer cell lines. EFM192 cells are shown in Fig 1B. Cells were incubated with increasing drug concentrations for 96 hours and the number of tumor cells were assayed in monoculture (black) and AR22 coculture (orange). Data are representative of three independent experiments and error bars are SD for three replicate wells.

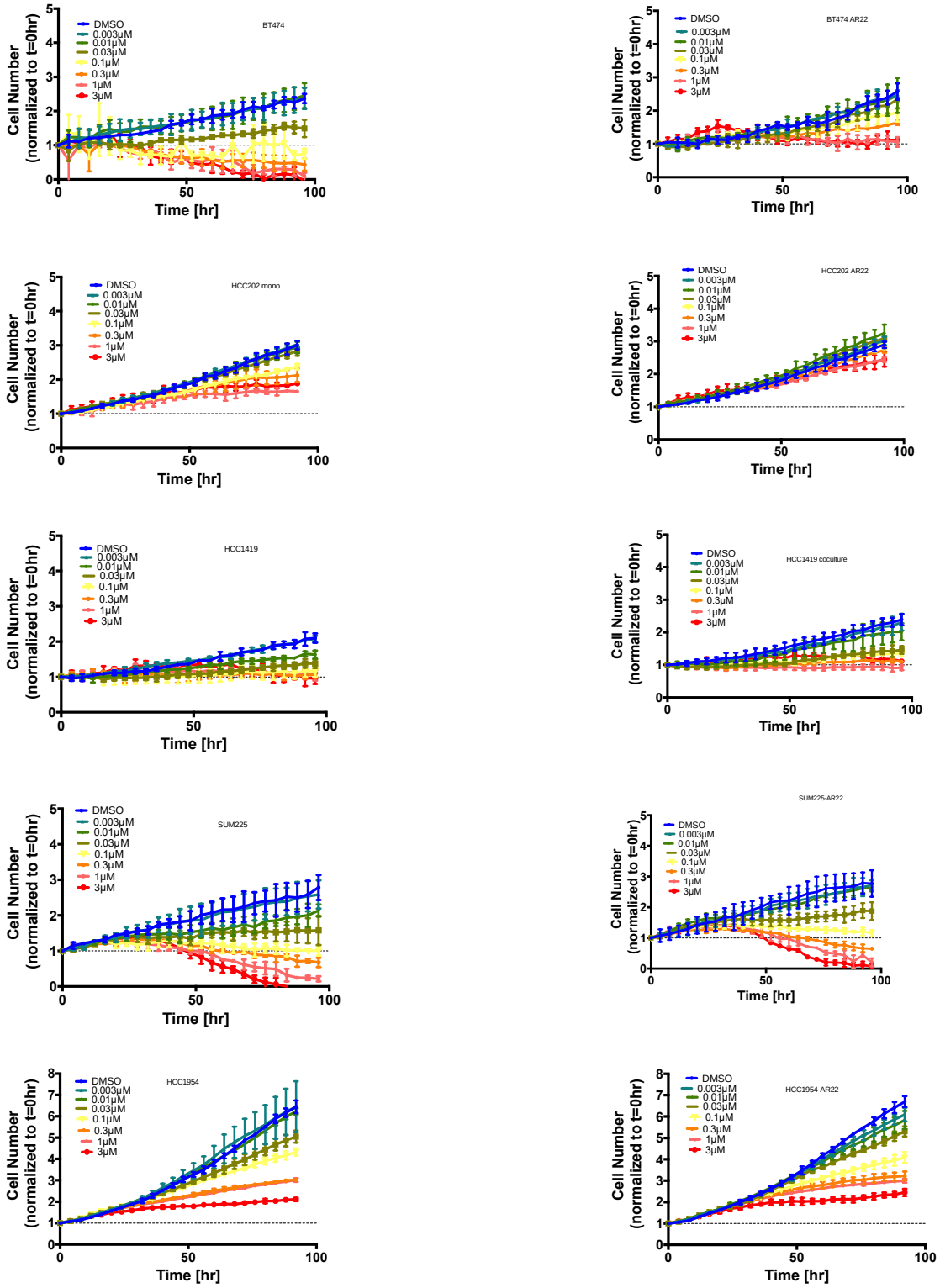


Fig. S2. **Cell growth curves of monoculture and AR22 coculture for the HER2+ breast cancer cell line panel.** Change in tumor cell numbers over time at increasing lapatinib concentrations for conditions of monoculture (left) and AR22 coculture (right). Data are representative of at least two independent experiments and error bars correspond to SD for n=3 replicate wells.

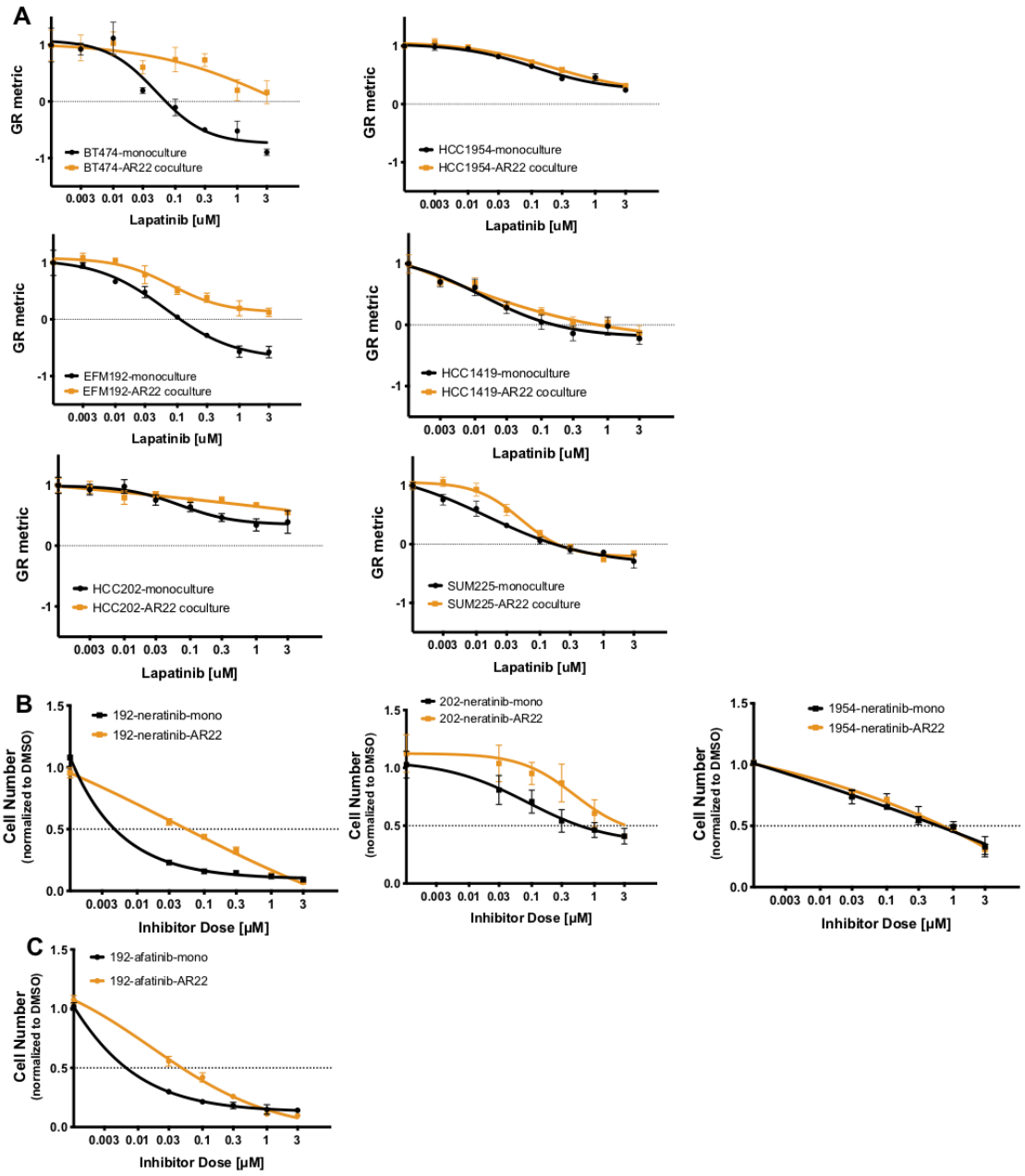


Fig. S3. Fibroblasts alter HER2-targeted therapy sensitivity in fibroblast-protected cell lines for multiple inhibitors. (A) GR-metrics for all HER2+ breast cancer cell lines under monoculture (black) and AR22 coculture (orange) conditions. Data are representative of at least two independent experiments and error bars correspond to SD for n=3 replicate wells. **(B)** EFM192 (left), HCC202 (middle) and HCC1954 (right) cells were incubated with increasing neratinib concentrations for 96 hours and the number of tumor cells were assayed in monoculture (black) and AR22 coculture (orange). Data are representative of two independent experiments and error bars are SD for three replicate wells. **(C)** EFM192 cells were incubated with increasing afatinib concentrations for 96hours and viable cells were counted in monoculture (black) and AR22 coculture (orange). Data are representative of two independent experiments and error bars are SD for three replicate wells.

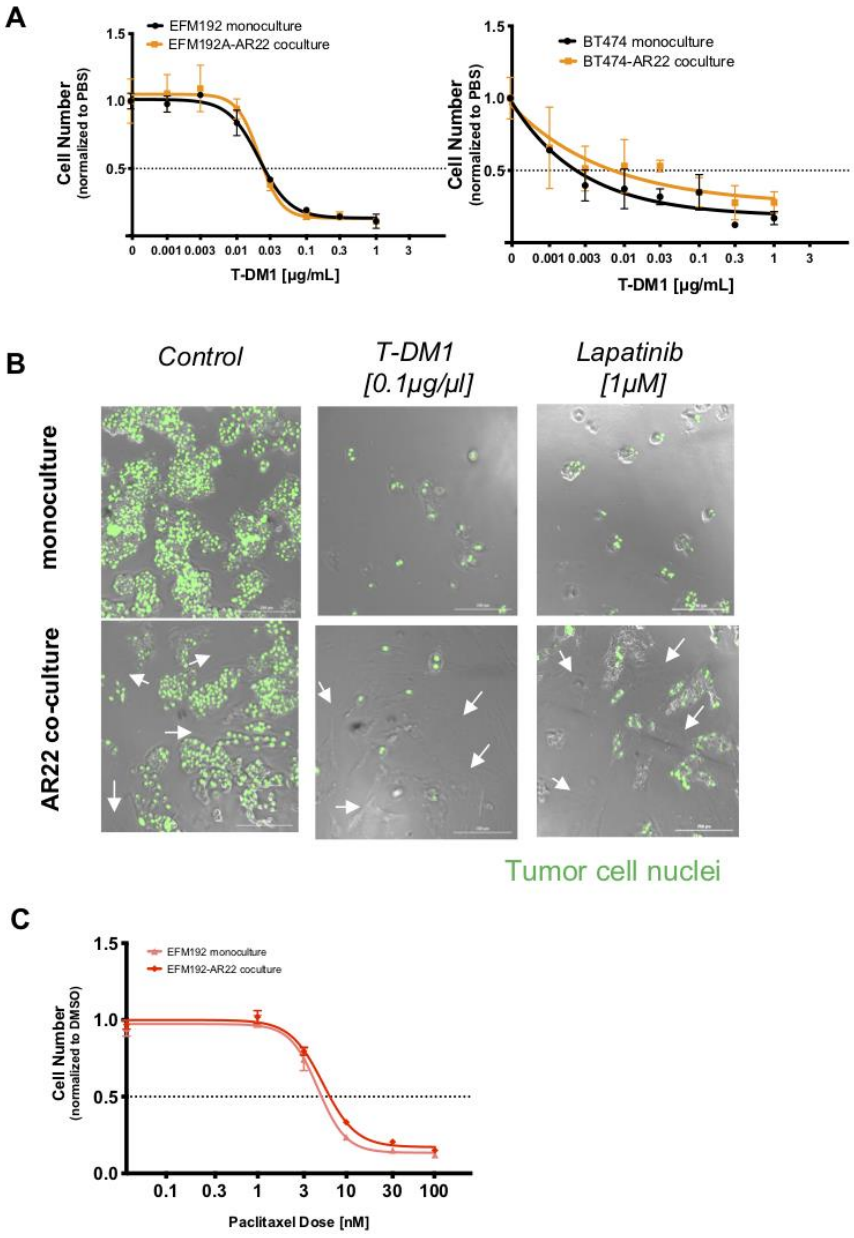
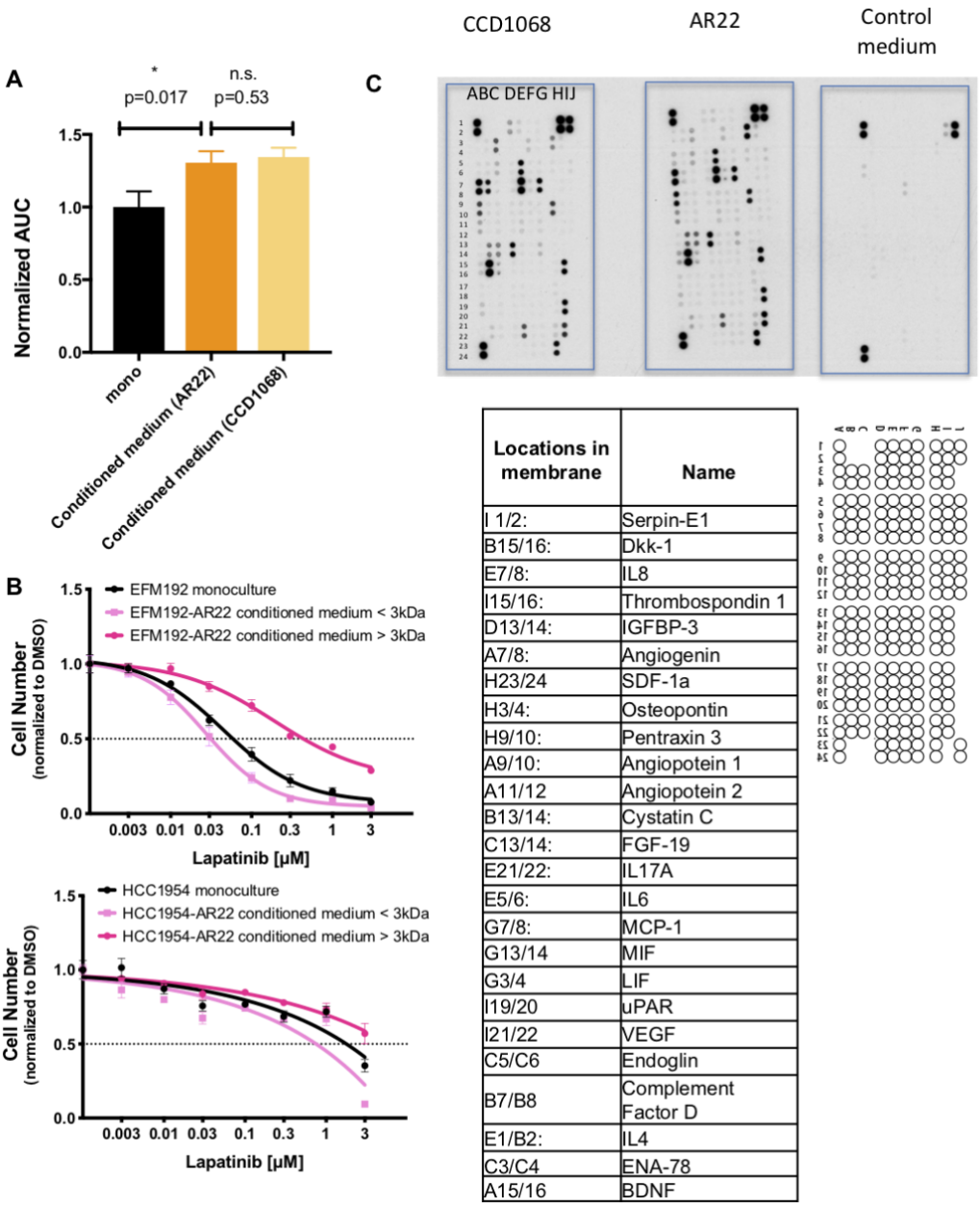


Fig. S4. Fibroblasts do not alter sensitivity to HER2 antibody-drug conjugate T-DM1 or an anti-mitotic chemotherapy drug (A) EFM192 (left) and BT474 (right) cells were incubated with increasing T-DM1 concentrations for 96 hours and the number of tumor cells were assayed in monoculture (black) and AR22 coculture (orange). Data are representative of two independent experiments and error bars are SD for three replicate wells. **(B)** H2B-GFP (green) expressing EFM192 tumor cells cocultured with AR22 fibroblasts for 96 hours under control (DMSO), T-DM1 (0.1 µg/ml) and lapatinib (1µM) treatment. Representative images from three biological replicates of monoculture and coculture of tumor cells (white arrows represent fibroblasts). Scalebar is 200µm. **(C)** EFM192 cells were incubated with increasing paclitaxel concentrations for 96 hours and the number of tumor cells were assayed in monoculture (pink) and AR22 coculture (red). Data are representative of two independent experiments and error bars are SD for three replicate wells.



Locations in membrane	Name
I 1/2:	Serpin-E1
B15/16:	Dkk-1
E7/8:	IL8
I15/16:	Thrombospondin 1
D13/14:	IGFBP-3
A7/8:	Angiogenin
H23/24	SDF-1a
H3/4:	Osteopontin
H9/10:	Pentraxin 3
A9/10:	Angiopotein 1
A11/12	Angiopotein 2
B13/14:	Cystatin C
C13/14:	FGF-19
E21/22:	IL17A
E5/6:	IL6
G7/8:	MCP-1
G13/14	MIF
G3/4	LIF
I19/20	uPAR
I21/22	VEGF
C5/C6	Endoglin
B7/B8	Complement Factor D
E1/B2:	IL4
C3/C4	ENA-78
A15/16	BDNF

Fig. S5. **Conditioned medium from different fibroblast cell lines can reduce lapatinib sensitivity. (A)** Lapatinib AUC for EFM192 cancer cells under conditions of monoculture or treatment with conditioned medium from different fibroblasts. Error bars are SEM from three biological replicates. **(B)** EFM192 (top) and HCC1954 (bottom) cells were incubated with increasing lapatinib concentrations in fibroblast conditioned medium (light pink: fraction <3kDa; dark pink: fraction >3kDa) for 96 hours and the number of tumor cells were assayed (orange). Data are representative of two independent experiments and error bars are SD for three replicate wells. (**C**) Analysis of secreted factors in fibroblast conditioned medium. Base medium is RPMI + 10% HI-FBS, fibroblasts were cultured for 96hr and supernatant medium was collected.

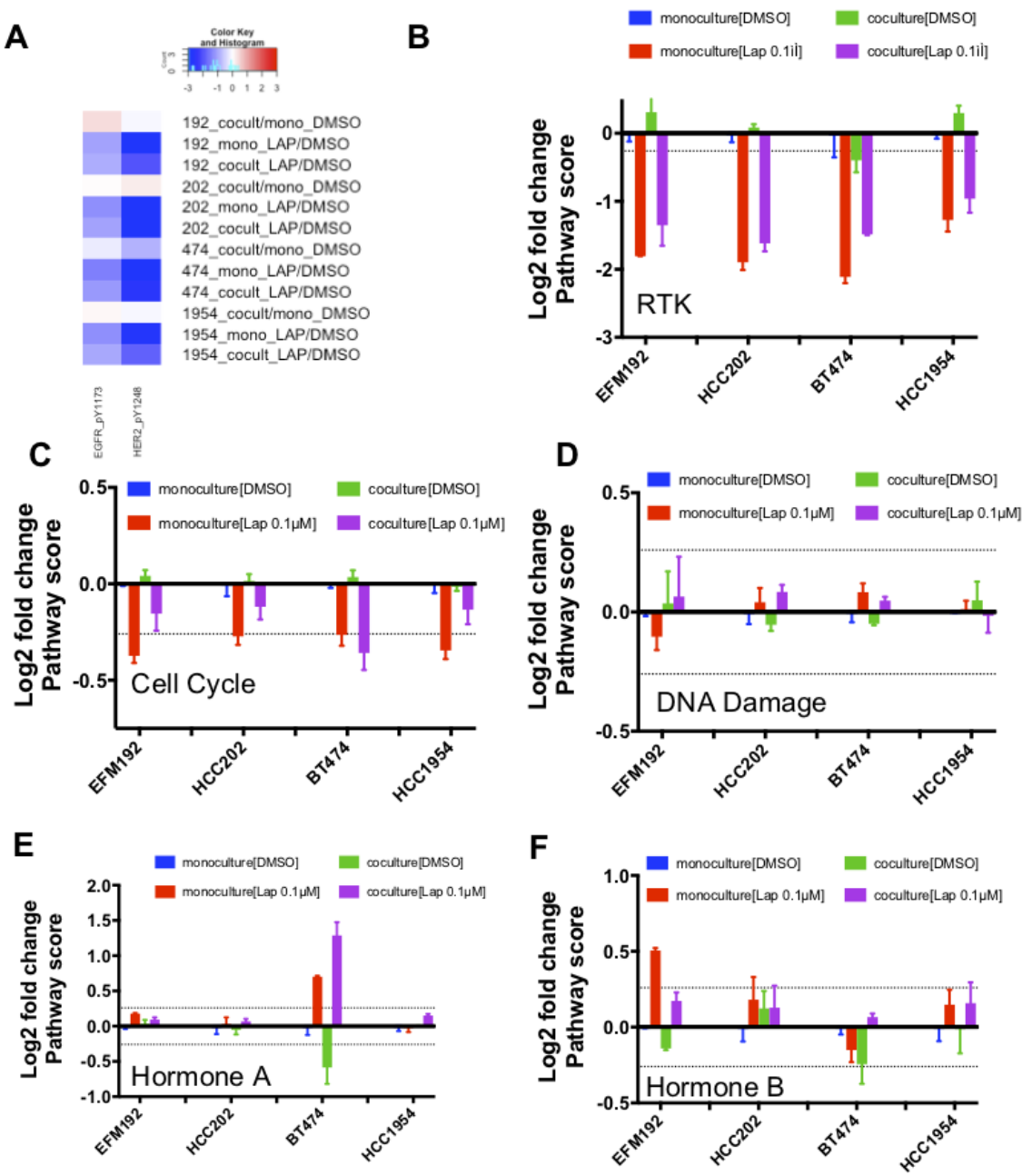


Fig. S6. **Paracrine coculture with fibroblasts results in sustained MTOR signaling in tumor cells. (A)** Lapatinib targets are blocked in monoculture and coculture (0.1µM lapatinib). Heatmap of phospho-EGFR and phosho-HER2 protein expression changes due to coculture(cocult/mono) under untreated (DMSO) and lapatinib treated (LAP/DMSO) conditions (both monoculture and coculture conditions are shown). Values are average log2-ratios from at least two biological replicates. Red indicates protein increase, while blue indicates protein decrease. **(B)** RTK, **(C)** cell cycle, **(D)** DNA damage, **(E)** hormone A and **(F)** hormone B pathway score changes induced by lapatinib (0.1µM) treatment in monoculture and coculture conditions. Values are average log2-transformed ratios of each sample normalized to the DMSO monoculture control in each cell line of at least two biological replicates. Error bars are SEM.

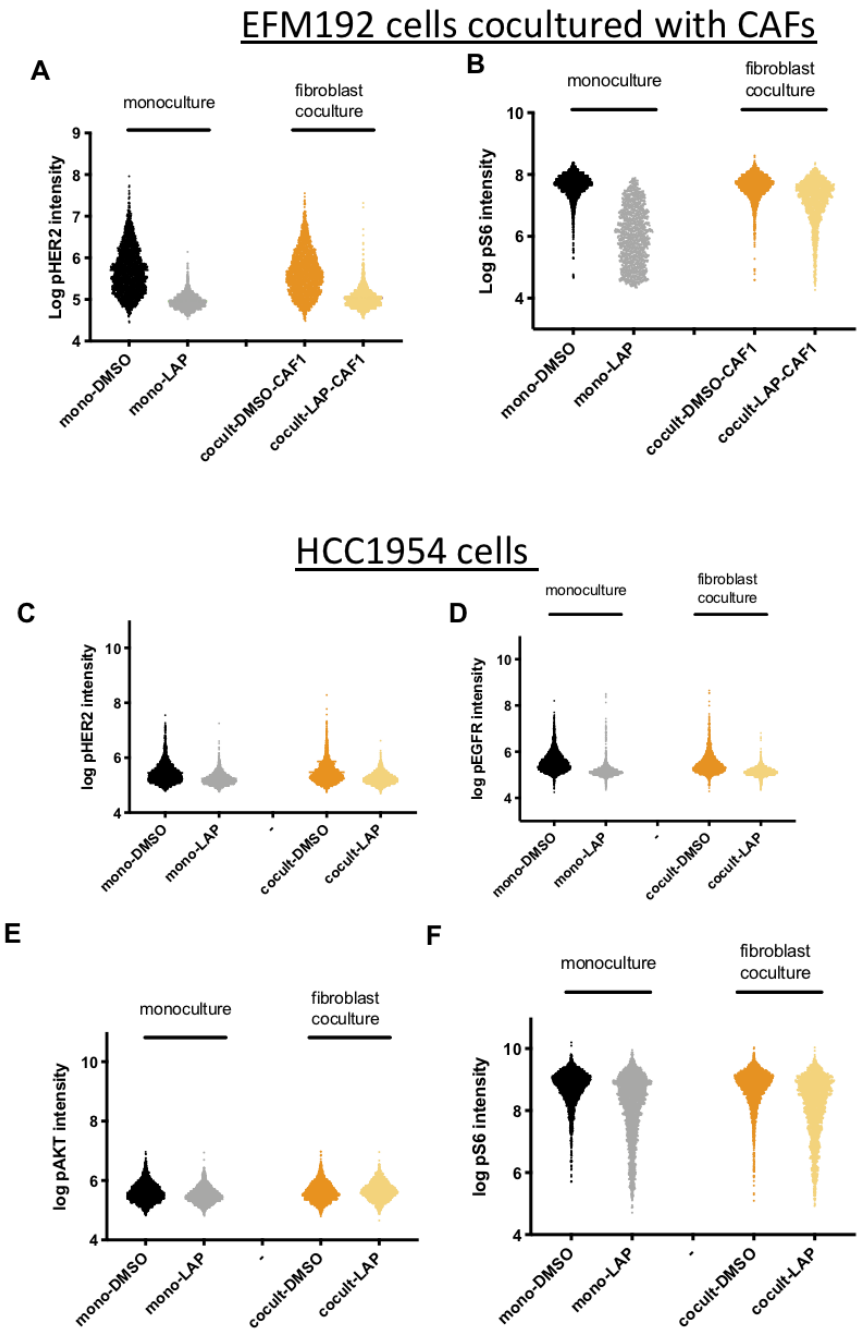


Fig. S7. **Direct coculture with fibroblasts results in sustained MTOR signaling only in fibroblast-protected tumor cells. (A-B)** Fibroblast-protected EFM192 cells: analysis of phospho-HER2 (Tyr1248) and phospho-S6 (Ser235/236) protein expression changes in direct EFM192-CAF (cancer associated fibroblast) cocultures using immunofluorescence. **(C-F)** Fibroblast-insensitive HCC1954 cells: Analysis of phospho-HER2 (Tyr1248), phospho-EGFR (Tyr1068), phospho-AKT (Ser473) and phospho-S6 (Ser235/236) protein expression changes in direct HCC1954-AR22 cocultures using immunofluorescence. Cells were dosed with 0.1μM of lapatinib for 48 hours. Data are representative for two biological replicates.

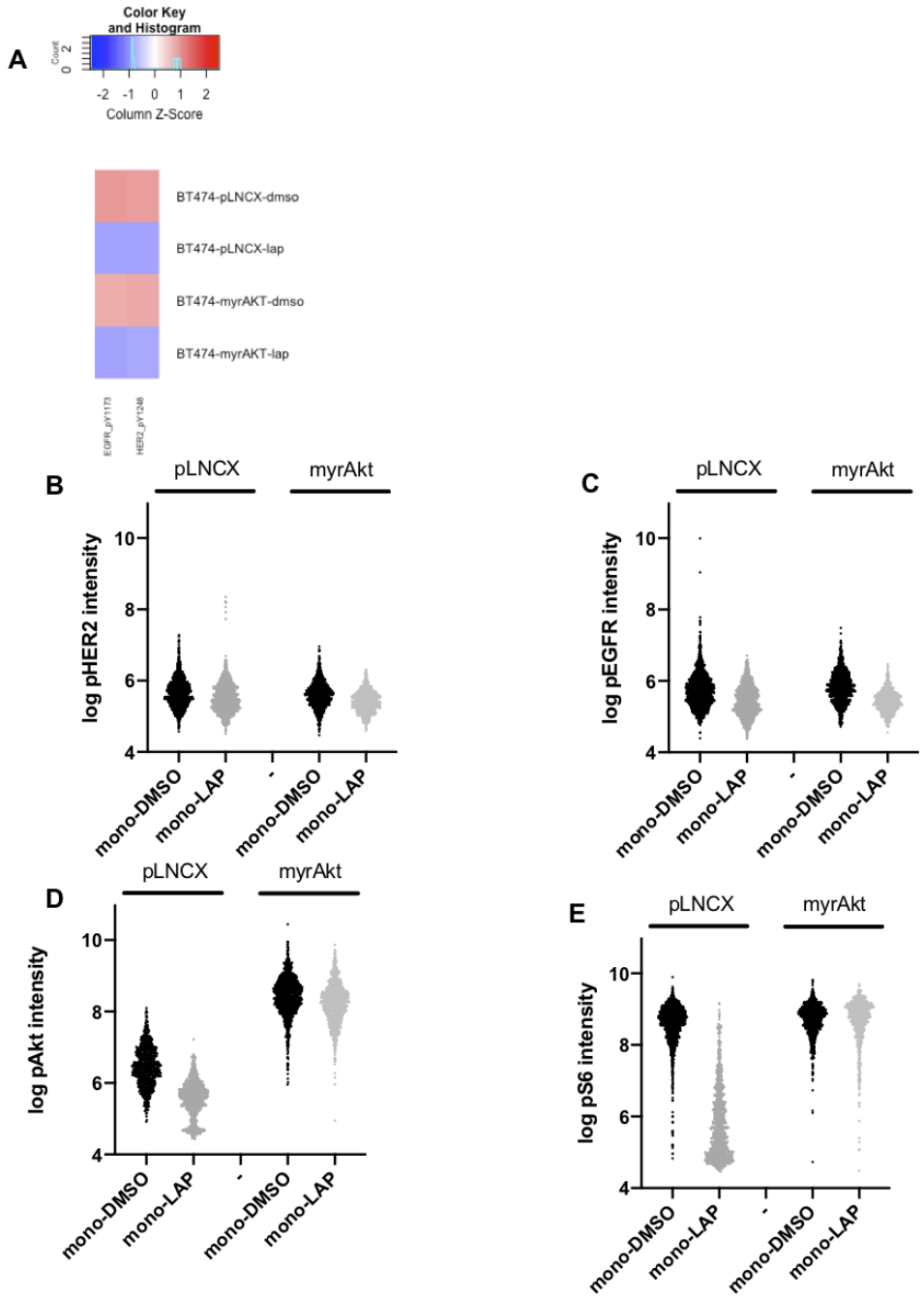


Fig. S8. **AKT overexpression does not affect HER2 target inhibition by lapatinib, but suppresses AKT/MTOR pathway blockade. (A)** Heatmap of absolute protein expression levels of phospho-EGFR and phospho-HER2 in control BT474 cells (pLNCX) and AKT-overexpressing BT474 (myrAKT) under no-treatment (DMSO) and lapatinib-treatment (0.1μM) conditions. Data are average median-normalized values for at least two biological replicates. Red indicates protein increase compared to median, while blue indicates protein decrease. **(B-E)** Analysis of phospho-HER2 (Tyr1248), phospho-EGFR (Tyr1068), phospho-AKT (Ser473) and phospho-S6 (Ser235/236) protein expression changes in control and myrAKT BT474 cells using immunofluorescence. Cells were dosed with 0.1μM of lapatinib for 48hr. Data are representative of two biological replicates.

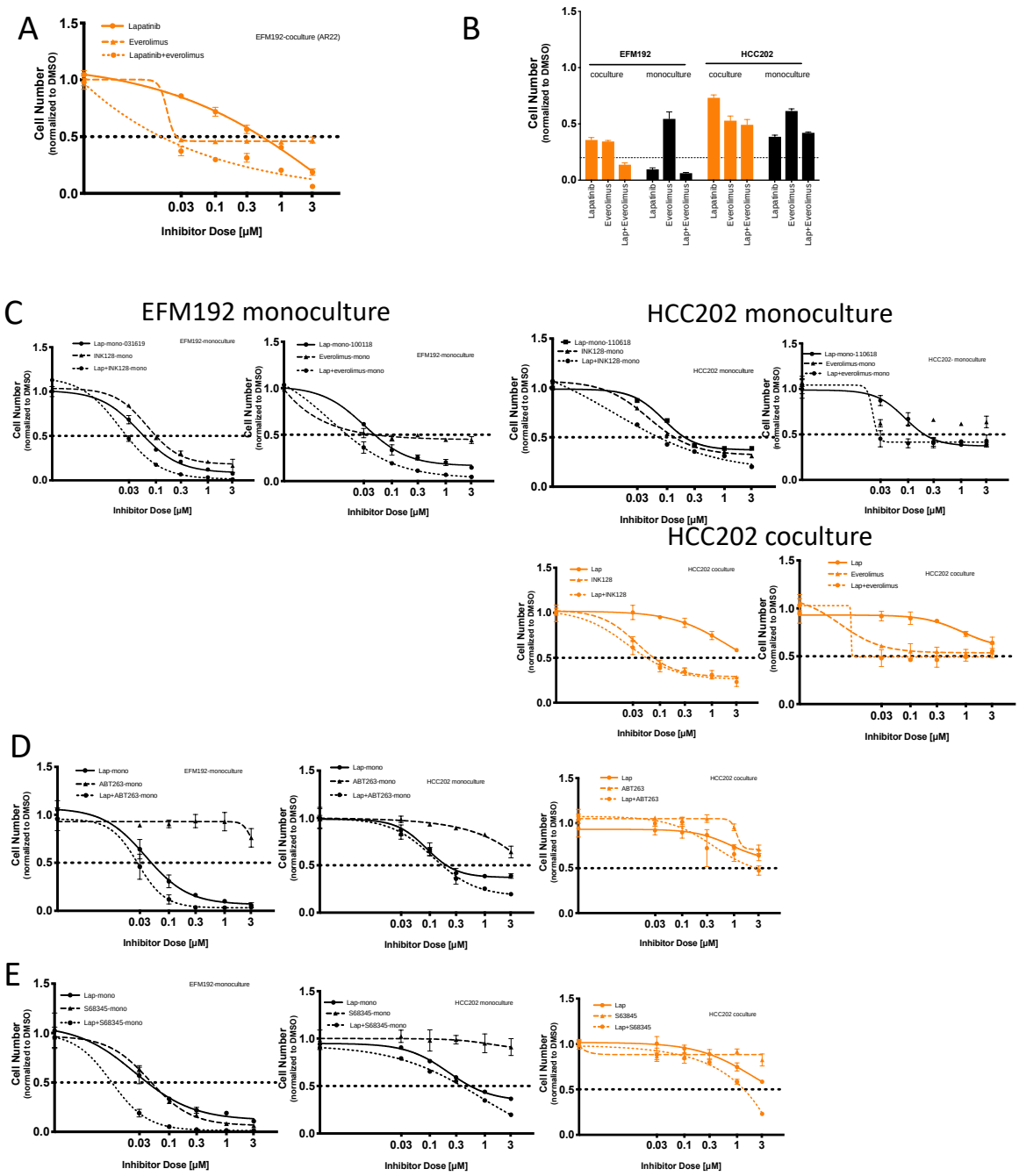


Fig. S9. **Fibroblast-protected tumor cells can be eliminated by targeting downstream survival programs. (A)** Combination dose-response curves for everolimus in EFM192 coculture with AR22. **(B)** Comparison of cell numbers for EFM192 cell line for single agent treatment and combination for monoculture and coculture. Error bars are SD for three replicate wells and results are representative of at least two biological replicates. **(C-E)** Single-agent and combination dose-response curves for MTOR, BCL-2/XL and MCL-1 inhibitors in monoculture and AR22 coculture for EFM192 and HCC202. Error bars are SD for three replicate wells and results are representative of at least two biological replicates.

No	Cell Line	ER[1]	PR[1]	HER2[1]	PAM50 subtype[1]
1	EFM192A	+	+	+	Luminal B
2	BT474	+	+	+	Luminal B
3	HCC202	-	-	+	HER2-enriched
4	HCC1954	-	-	+	HER2-enriched
5	HCC1419	+	-	+	HER2-enriched/Luminal B
6	SUM225	-	-	+	HER2-enriched

[1] Dai, Xiaofeng, et al. "Breast cancer cell line classification and its relevance with breast tumor subtyping." *Journal of Cancer* 8.16 (2017): 3131.

Table S1. List of HER2+ cell lines and their characteristics

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4. R. Tibes *et al.*, Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. *Mol Cancer Ther* **5**, 2512-2521 (2006).