SUPPLEMENTAL DATA

Aggressiveness of HNSCC tumors depends on expression levels of cortactin, a gene in the 11q13 amplicon.

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Supplemental Methods

Matrigel Transwell Invasion Assay

This assay was adapted from a protocol by Brabek et al. {Brabek, 2004 #203}. Cells were serum-starved overnight prior to plating in the transwell chambers. The invasion chambers (Matrigel-BD Biocoat, 6.5 mm diameter, 0.33 cm^2 surface area, 24-well plates, pore size 8.0 μm) were incubated for 2-3 hours in serum-free DMEM, washed with PBS then 650 μl of DMEM supplemented with 20% FetalClone III (Hyclone) and 10% Nu-Serum (Invitrogen) was added to the lower chamber to act as the attractant. 100 μl of 2 x 10^5 cells/ ml were prepared in serum-free media and added to the top of each chamber. The chambers were incubated for 48 hrs, washed with PBS, and the non-invaded cells were removed with a sterile swab. The chambers were placed in 3.7% paraformaldehyde for 1 hour, washed and stained with crystal violet for 45 minutes. Duplicate chambers were prepared for each cell line and cells from five randomly chosen fields from each membrane were counted.

Cell transfection and FACS analysis

Cells (5 x 10^5) were transfected in 100-mm tissue culture plates with 2 μg MT1-MMP-GFP DNA in 7 ml medium or mock transfected using Effectene (60 μl (Qiagen)). The media was changed following an overnight incubation with the Effectene/DNA mixture and the cells were incubated an additional 24 hrs. The cells were then scraped in PBS + 5 mM EGTA, followed by surface staining with an antibody against MT1-MMP (Chemicon MAB3328) and the appropriate AlexaFluor-633 (APC)-conjugated secondary antibody plus 1:1000 dilution of propidium iodide (PI; Sigma). All staining and washing was done in 4% FBS in PBS. All solutions were kept ice-cold and the cells were kept on ice to ensure surface labeling. FACS was performed using an LSRII Flow Cytometer (BD Biosciences) at the VUMC Flow Cytometry Core Facility and analysis was performed using FlowJo (Tree Star). Only PI-negative cells were analyzed.

Figure Legends

Supplement Figure 1. Cortactin expression correlates with tumor size in SCC25 cells. Immunohistochemistry analysis of tumor size indicates that cortactin expression levels regulate the size of SCC25 non-11q13 amplified HNSCC tumors grown in rat tracheas. **A.** Representative Western blot and densitometry of cortactin expression in SCC25 cells expressing either scrambled cortactin siRNA oligo (scrambled oligo), cortactin knockdown cells (pRS-KD1), overexpression vector control (LZRS) and cortactin overexpressing cells (LZRS-CortFL). **B.** Tumor area measured from IHC-stained tumor sections. Data are represented as mean ± SEM. N=number of trachea implanted for each cell line and is shown for each cell line. Data are from one trial for each cell line with three mice used for each cell line. Asterisks indicates $p<0.05$. **C.** *In vivo* invasion was quantitated by measuring the area of tumor invaded beyond the tracheal cartilaginous ring in Ku70-stained slides of SCC25 tumor sections. Data are represented as mean ± SEM. **D.** Representative images from tumors derived from cortactin-manipulated SCC25 cells. Tumor cells were grown in rat tracheas implanted into the flanks of nude mice. Tracheas were left for one month, removed, fixed and sagitally sectioned and stained. Shown are the hematoxylin and eosin (H&E), Ku70

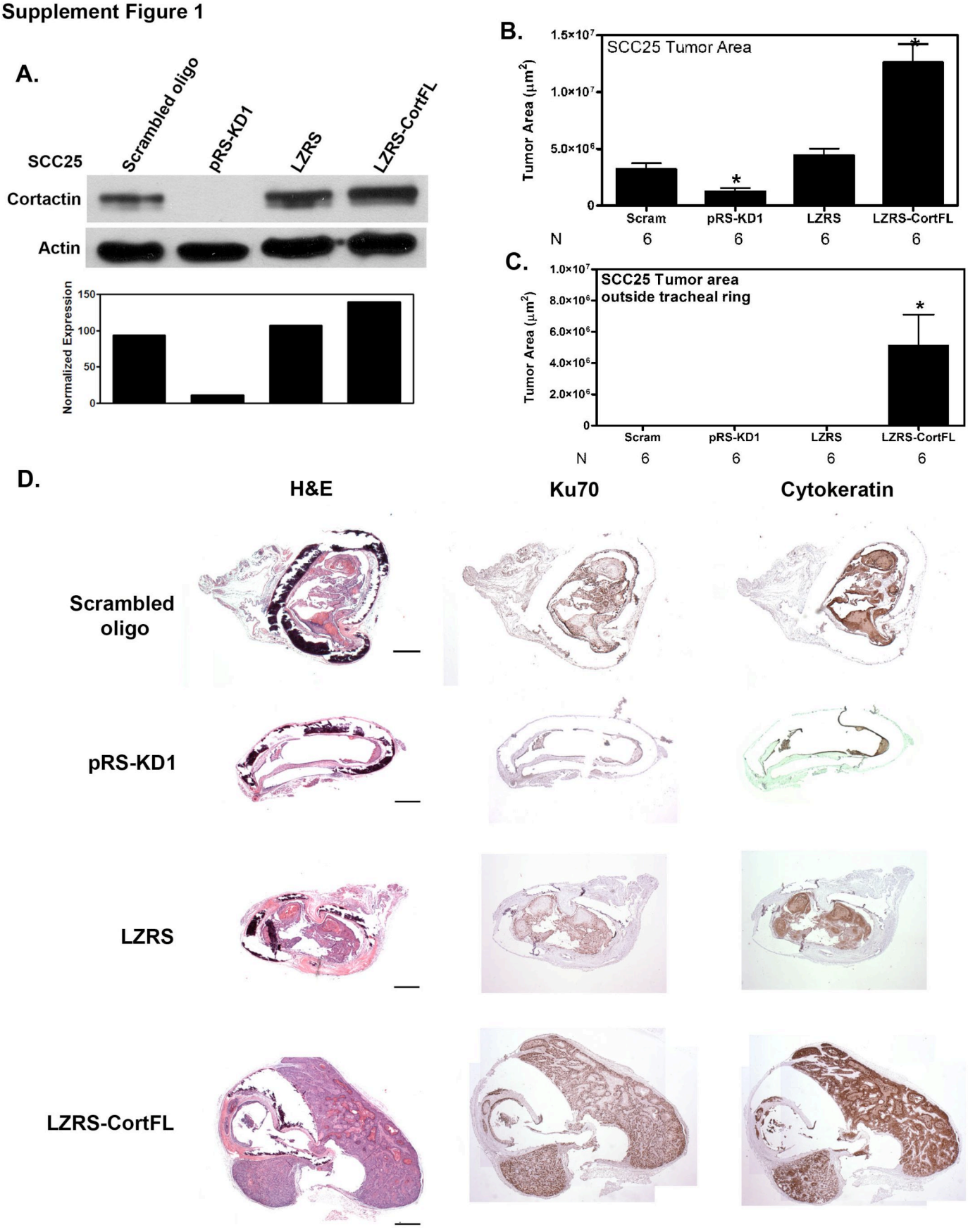
(identifies human cells) and cytokeratin (identifies cells of epithelial origin). Positive staining for Ku70 and cytokeratin is brown. Scale bar = 1 mm.

Supplement Figure 2. Cortactin promotes *in vitro* and *in vivo* tumor cell invasiveness.
A. *In vitro* invasion of SCC61- and FaDu-cortactin manipulated cells through Matrigel-coated transwells demonstrates that cortactin expression profoundly affects the ability of HNSCC cells to degrade and migrate through extracellular matrix. Data are represented as mean $\pm$ SEM percent of cells invaded relative to scrambled oligo (set to 100%). Shown is the combined data for three independent experiments with duplicate transwells for each transwell per cell line per experiment. Five randomly chosen 10x fields per transwell were analyzed. **B.** *In vivo* invasion was quantitated by using the tracheal ring as a fiduciary marker of tissue boundaries that would need to be degraded to prevent tumor crossing. Thus, the area of tumor invaded beyond the tracheal cartilaginous ring was quantitated from Ku70-stained slides of SCC61 tumor sections and plotted in the bar graph. As with *in vitro* invasion, cortactin expression profoundly affects *in vivo* invasion, although this effect may be confounded by effects of cortactin on tumor size. FaDu tumors could not be quantitated due to higher in vivo aggressiveness with full degradation and loss of the tracheal ring fiduciary marker. Data are represented as mean $\pm$ SEM. Asterisks indicate $p<0.05$ compared with scrambled oligo control.

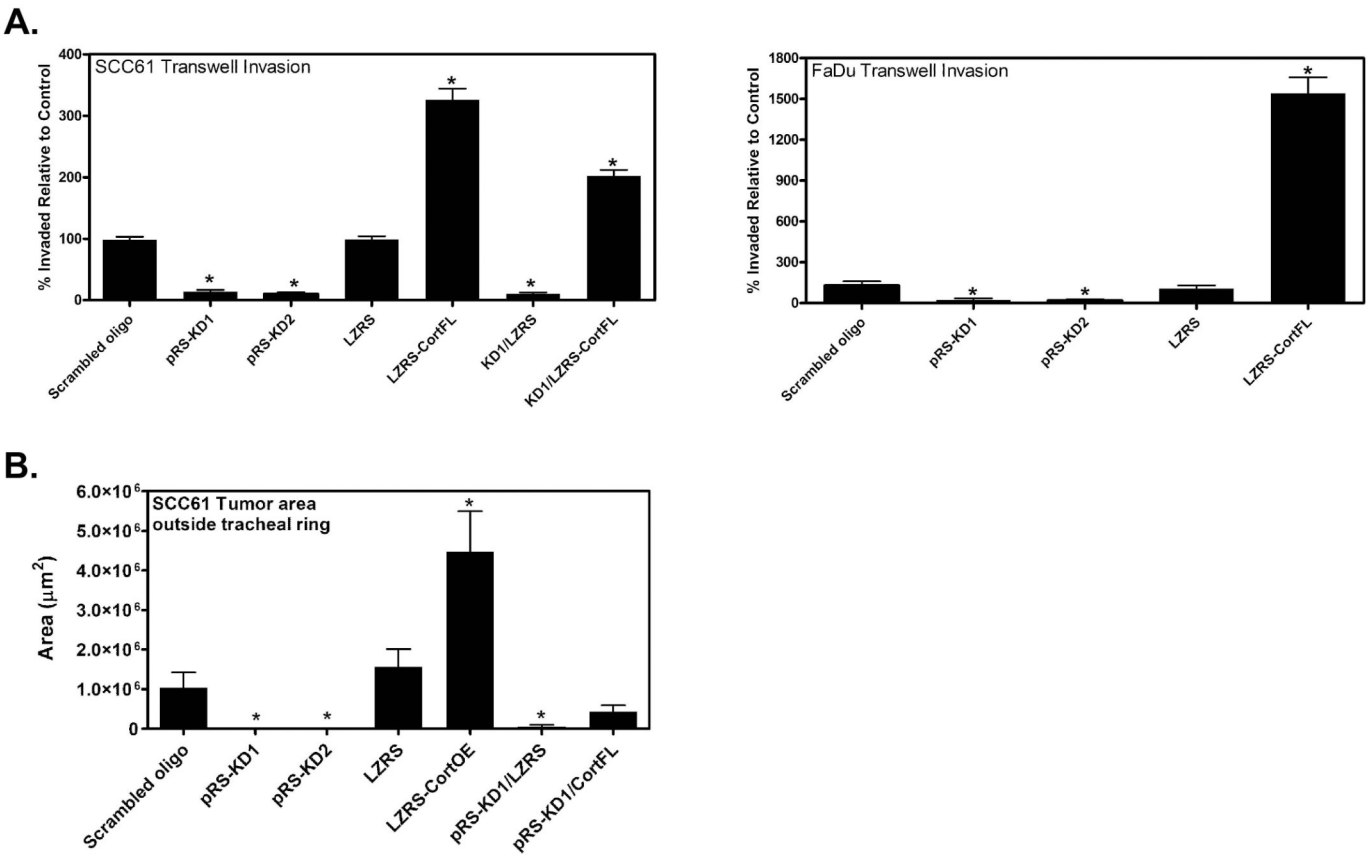
Supplement Figure 3. Cortactin levels have no effect on growth in "fluid" Matrigel culture.
SCC61 and FaDu scrambled cortactin oligo (scrambled oligo) and cortactin-knockdown (pRS-KD1) cell lines were cultured for 16 days under "fluid" conditions in growth media with 2% Matrigel, sitting on top of a 100% Matrigel bed. Duplicate wells were used for each cell line and the experiment was performed twice. Cells were imaged every two days. For each, eight randomly chosen 10x fields were imaged and the diameter of the "in-focus" structures were measured using Metamorph software. **A.** Representative images from Day 12. Scale bar = 100 μm. **B.** Combined data from two independent experiments for SCC61 and FaDu cells. Data are represented as mean $\pm$ SEM.

Supplement Figure 4. Cortactin affects the cell-surface expression of MT1-MMP in FaDu and SCC61 cells.
FACS analysis of cell surface expression of MT1-MMP from three independent experiments for SCC61 (**A**) or FaDu (**B**) cells. Control (scrambled oligo) or cortactin knockdown (pRS-KD1) cells were either transfected (trx) or mock transfected (mock) with MT1-MMP-GFP and then surface stained for MT1-MMP. Cells were gated for negative propidium iodine staining and analysis for transfected cells was performed on GFP-positive cells (transfection efficiency ~70%; data not shown). A representative histogram from three independent experiments is shown. Note that in the FaDu cortactin-KD (pRS-KD1-mock and pRS-KD1-trx) cells, there are two peaks of MT1-MMP fluorescence. One of the peaks has approximately the same level of MT1-MMP surface expression as control cells and likely represents cells with incomplete knockdown (note incomplete knockdown by Western blot analysis in Fig 2A). The other peak has essentially no MT1-MMP surface expression and there is no shift with transfection of MT1-MMP-GFP (compare lower peaks of pRS-KD1-mock and pRS-KD1-trx), which would be consistent with an inability of MT1-MMP to traffick to the cell surface in cells with complete cortactin knockdown.

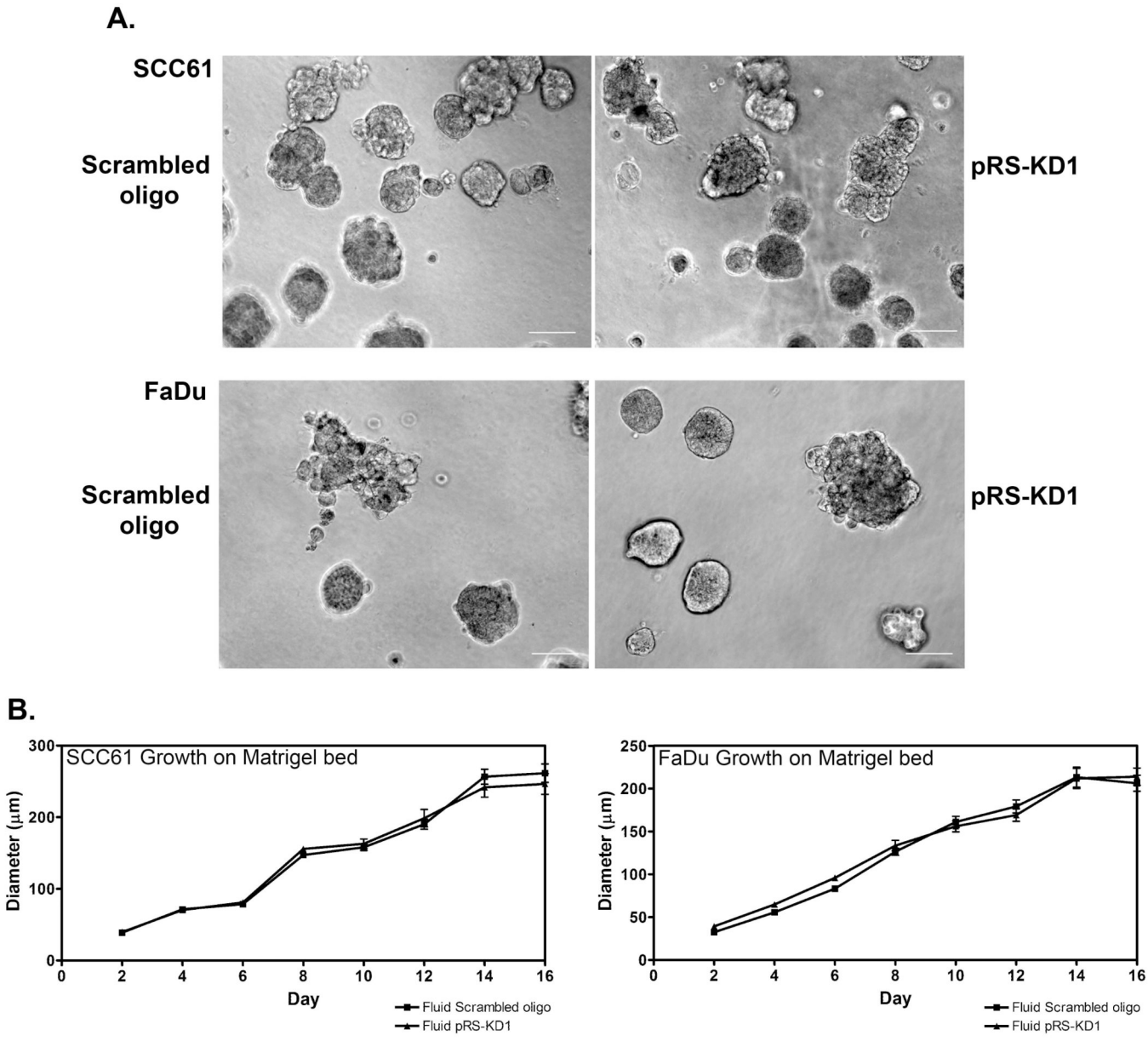
Supplement Figure 1



Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4