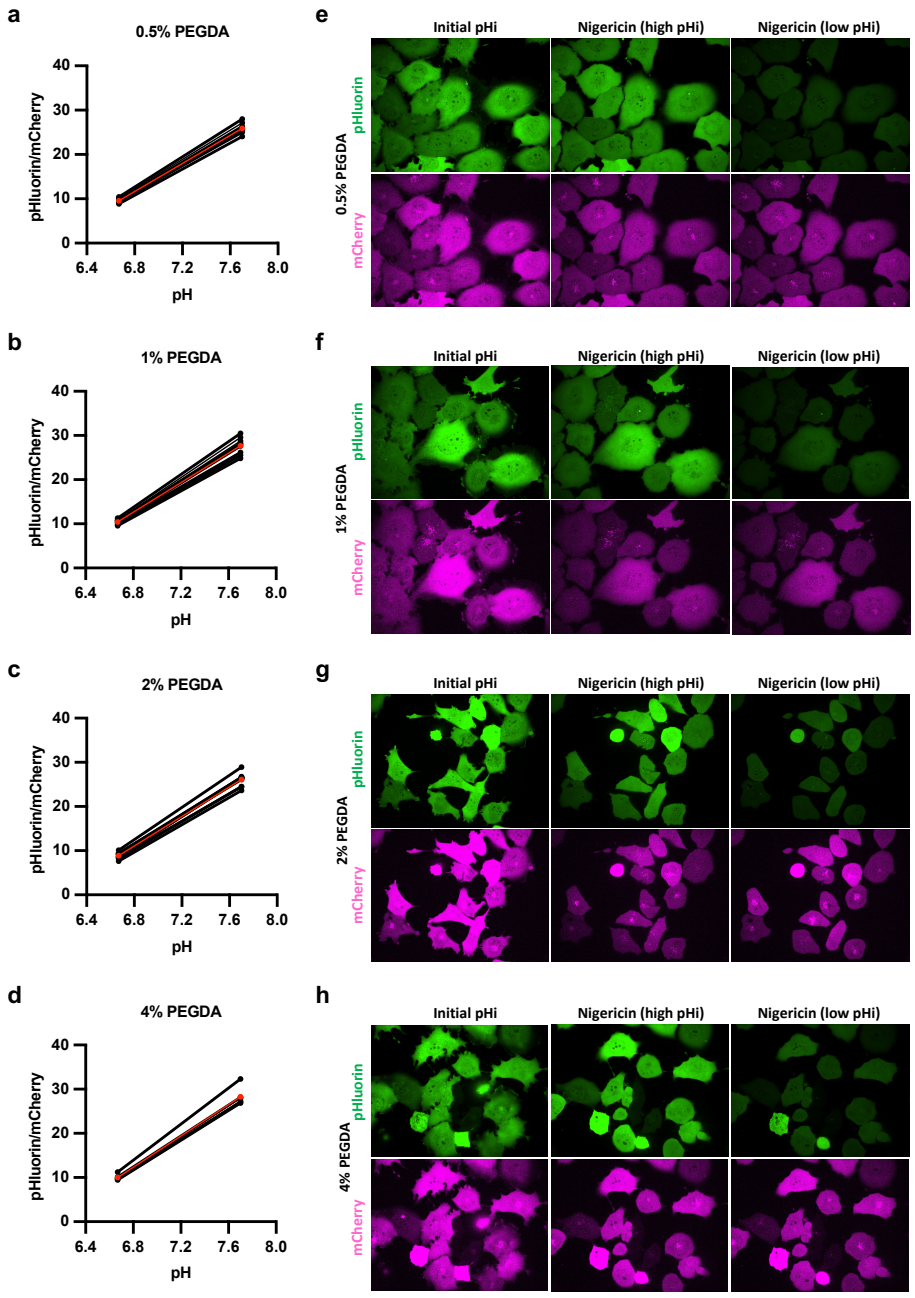
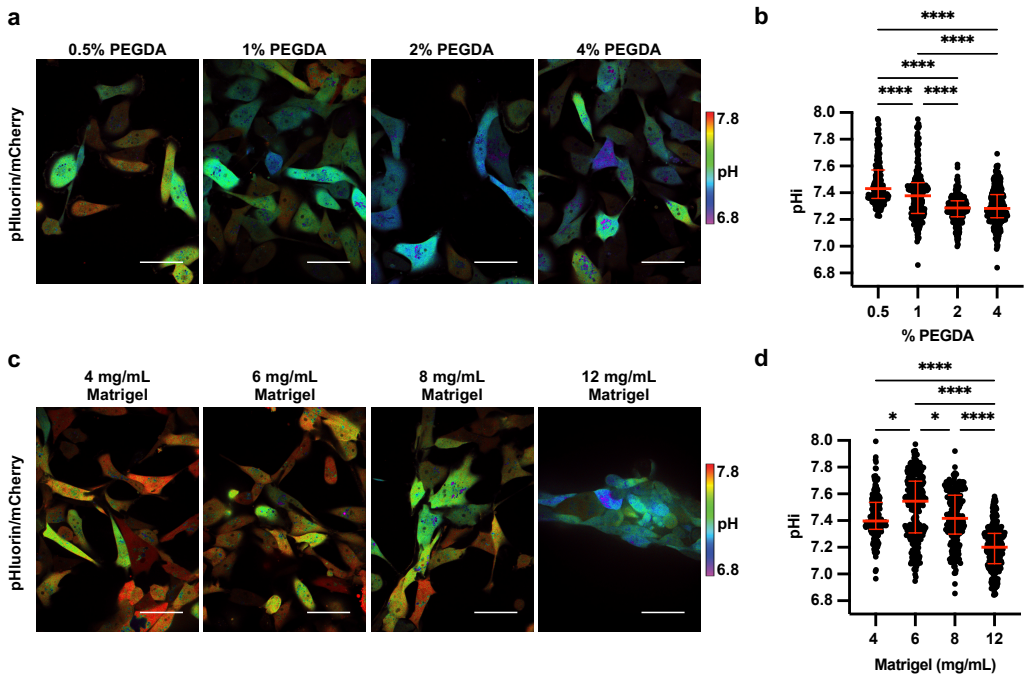
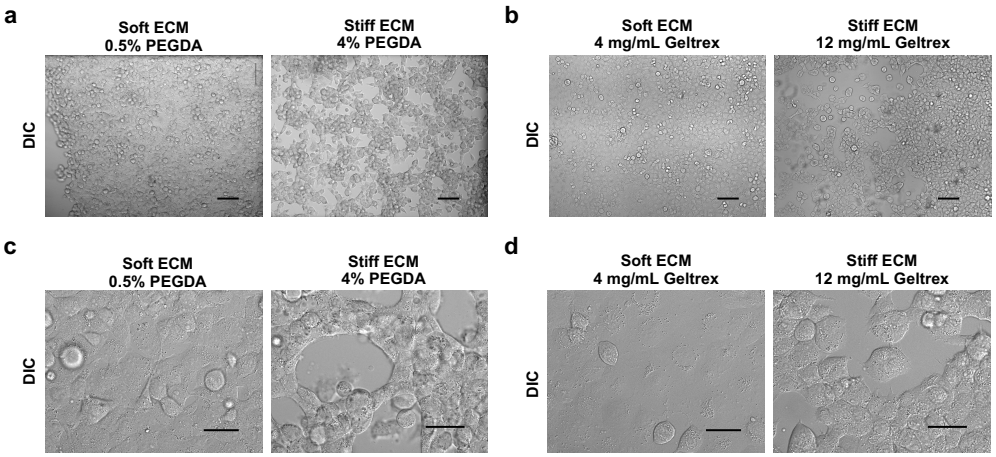


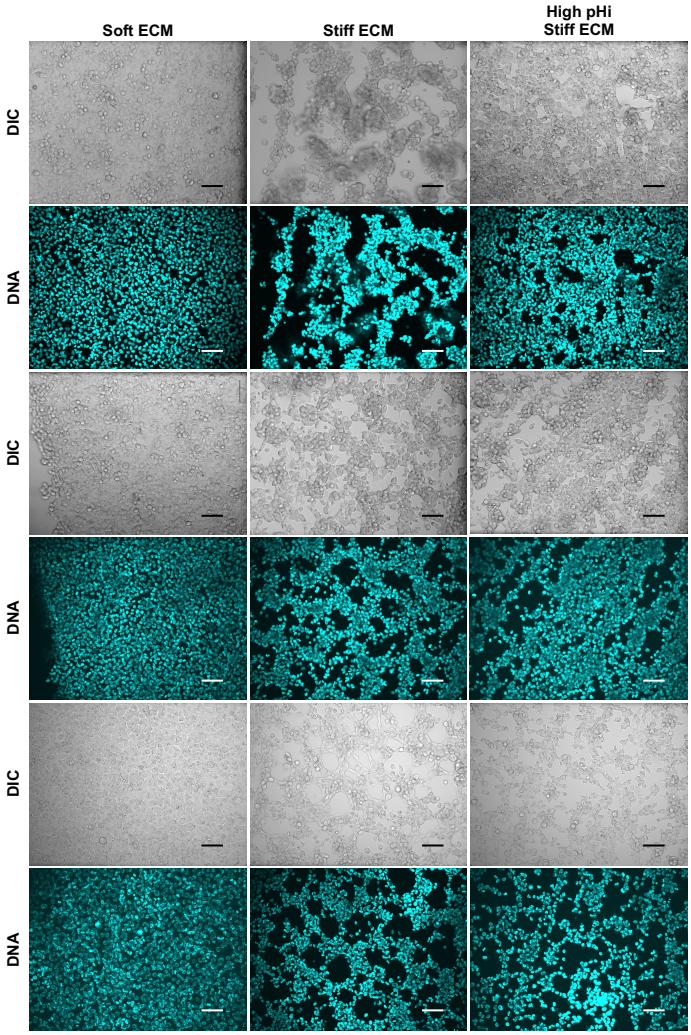
Supplement Figure 1: Nigericin standardization of mCherry-pHluorin biosensor in H1299 cells. Nigericin standard linear regressions across a single replicate for each single cell (black) and mean (red) for H1299-mCherry-pHluorin plated on **a)** 0.5% PEGDA (n=10), **b)** 1% PEGDA (n=10), **c)** 2% PEGDA (n=13), **d)** 4% PEGDA (n=7) HA gels. Single channel images of pHluorin (green) and mCherry (magenta) fluorescence at initial pHi imaging, high nigericin, and low nigericin standardizations for **e)** 0.5% PEGDA, **f)** 1% PEGDA, **g)** 2% PEGDA, **h)** 4% PEGDA. LUTs are identical across each cell line. Note that in all cases, single-cell nigericin standard curves were used to back-calculate single-cell pHi.



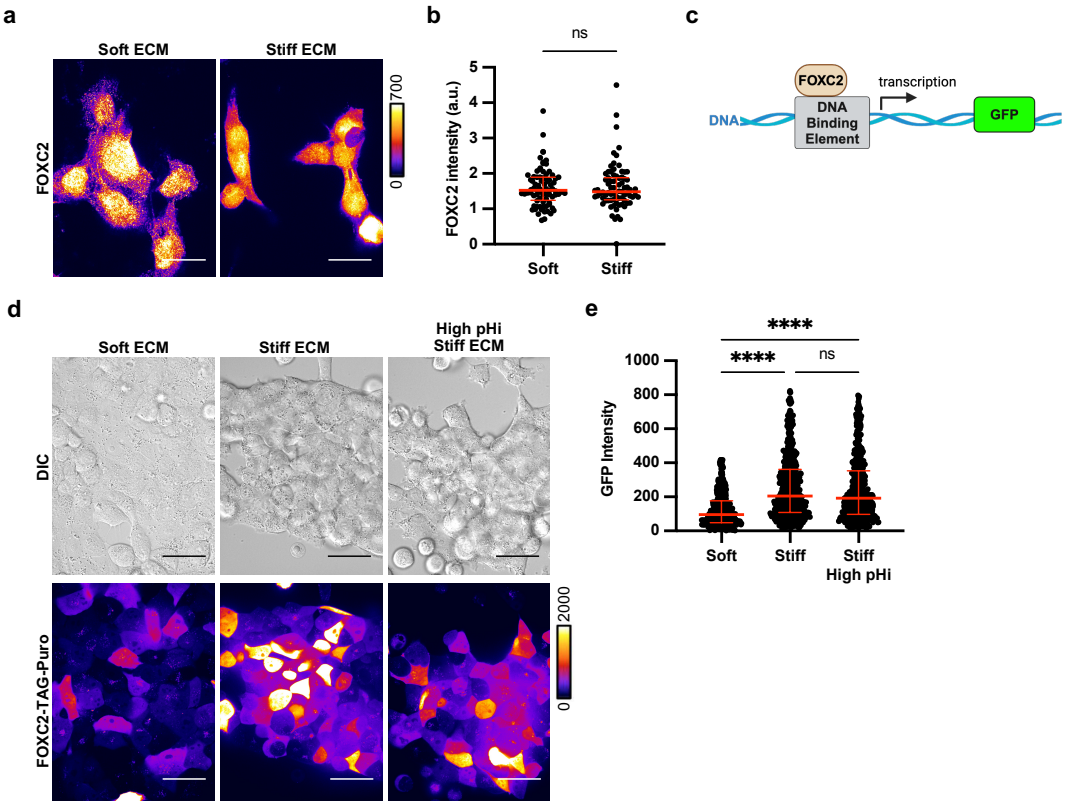
Supplement Figure 2: Stiffening extracellular matrix lowers pHi in metastatic human breast carcinoma (MDA-MB-231). a) Representative images of MDA-MB-231 cells stably expressing mCherry-pHluorin pH biosensor plated on varying HA gel stiffnesses. Images show ratiometric display of pHluorin/mCherry fluorescence. Scale bars: 50 μm. **b)** Quantification of single-cell pHi data collected as shown in (a) (n=3 biological replicates, n=194 0.5% PEGDA, n=228 1% PEGDA, n=265 2% PEGDA, n=334 4% PEGDA. Red lines show medians ± IQR). **c)** Representative images of MDA-MB-231 cells stably expressing mCherry-pHluorin pH biosensor plated on varying Matrigel stiffnesses. Images show ratiometric display of pHluorin/mCherry fluorescence. Scale bars: 50 μm. **d)** Quantification of single-cell pHi data collected as shown in (c) (n=3 biological replicates, n=210 4mg/mL, n=291 6mg/mL, n=222 8mg/mL, n=292 12mg/mL. Red lines show medians ± IQR). For (c) and (d), significance was determined by a Kruskal-Wallis test (****P<0.0001).



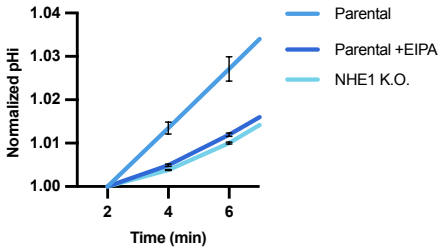
Supplement Figure 3: Representative images of Vasculogenic mimicry phenotype on soft and stiff Geltrex and HA gels. a) Differential interference contrast (DIC) images of H1299 cells plated on soft (0.5% PEGDA) and stiff (4% PEGDA) HA gels. Scale bars: 100 μm. **b)** Differential interference contrast (DIC) images of H1299 cells plated on soft (4mg/mL) and stiff (12 mg/mL) Geltrex gels. Scale bars: 100 μm. **c)** Differential interference contrast (DIC) images of H1299 cells plated on soft (0.5% PEGDA) and stiff (4% PEGDA) HA gels. Scale bars: 50 μm. **b)** Differential interference contrast (DIC) images of H1299 cells plated on soft (4mg/mL) and stiff (12 mg/mL) Geltrex gels. Scale bars: 50 μm.



Supplement Figure 4: Additional representative images of Vasculogenic mimicry phenotype on HA gels. Differential interference contrast (DIC) and Hoechst stain (DNA, cyan) images of H1299 cells plated on soft (0.5% PEGDA), stiff (4% PEGDA), and stiff (4% PEGDA) with high pHi HA gels. Scale bars: 100 μm.



Supplement Figure 5. FOXC2 has stiffness-dependent activity that is insensitive to pHi. a). Representative images of H1299 cells plated on soft (4 mg/mL) and stiff (12 mg/mL) Geltrex fixed and stained for FOXC2. FOXC2 is pseudocolored according to scale. Scale bars: 50 μm. **b)** Quantification of FOXC2 intensity per cell collected as shown in (a) (n=3 biological replicates, n=80 soft and n=77 stiff. Red lines show medians ± IQR). **c)** Schematic of FOXC2-TAG-Puro reporter of FOXC2 transcriptional activity. **d)** Representative images of H1299 cells plated on soft (0.5% PEGDA), stiff (4% PEGDA) and stiff (4% PEGDA) with raised pHi HA gels. Images show Brightfield display (DIC) and FOXC2-TAG-Puro. FOXC2-TAG-Puro is pseudocolored according to scale. Scale bars: 50 μm. **e)** Quantification of FOXC2-TAG-Puro intensity per cell collected as shown in (d) (n=3 biological replicates, n=416 soft, n=478 stiff, n=461 stiff high pHi. Red lines show medians ± IQR).



Supplement Figure 6: NHE1 activity is decreased in H1299 cells with CRISPR knockout of NHE1. Normalized pHi recovery measurements after acid load of parental H1299 cells (see methods) and H1299 cells where NHE1 has been removed via CRISPR (H1299-NHE1 K.O., see methods). Recovery assays were performed with and without the presence of a specific NHE1 inhibitor (EIPA). (n=2 biological replicates; n=6 H1299 parental, n=6 H1299 parental +EIPA, n=6 H1299 NHE1 K.O. -EIPA); (means ± SEM).