SUPPLEMENTARY INFORMATION

Cell growth density modulates cancer cell vascular invasion via Hippo pathway activity and CXCR2 signaling

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SUPPLEMENTARY FIGURES

Figure S1. MDA-MB-231 cell viability in vitro and lung metastases in mice after growth at low or high density. (A) Proliferation was monitored using an ECIS readout to track different numbers of cells seeded in a 96-well format plate (Rosenfield *et al*, 2012; Wellstein, 2010). (B) Apoptosis analysis using FACS analysis for PI and Annexin V staining. (C) The number of zebrafish embryos that have detectable, intravasated MDA-MB-231 cells appearing in the tail region. The yolk was injected with either low or high density cells, labeled with the same Q-tracker. * $p<0.05$. (D) Lung colonization in athymic nude mice after tail vein injection of MDA-MB-231 cells that were previously grown at low or high density. *, $p<0.05$. (E) Number of low or high density MDA-MB-231 cells attached to an endothelial monolayer. ***, $p<0.001$.

Figure S2. Analysis of signaling pathways and YAP localization in cancer cells propagated at low or high density. (A, B) Equal amounts of protein were blotted to assess changes in protein or phosphoprotein in cells propagated at different densities. Chemokine signalling: Stat3, NFkB, and IκB; EMT: vimentin, β-catenin and E-cadherin; Hippo: YAP; Actin served as a loading control. (C) Immunofluorescence of total YAP (green, left panels), DAPI-labeled nuclei (middle panels), and the merged images (right panels) of MDA-MB-231, E0771 mouse mammary, and Colo357 pancreatic cancer cells. (D to F) qRT-PCR for YAP downstream targets CTGF and CYR61 in MDA-MB-231 cells grown at low or high density (D), MDA-MB-231 cells treated with 0.1 μM Verteporfin (VP) for 24 h (E) and E0771 cells treated with 0.1 μM VP for 6 h (F). Primer sequences for the RT-PCR are provided in Supplementary Table S1.

Figure S3. Time series of the YAP status in MDA-MB-231 cells grown at low or high density and switched to the opposing cell density. (A) Immunofluorescence of YAP protein (green, left panels), DAPI-labeled nuclei (middle panels), and the merged images (right panels) of MDA-MB-231. The switch of nuclear vs cytoplasmic localization of YAP when cells are switched to high or at low density respectively. (B) Western blot for YAP phosphorylation status at different time points when MDA-MB-231 cells are switched to either high or low density respectively. GAPDH was used as

a loading control.

Figure S4. The effect of YAP knockdown on transendothelial invasion and transwell migration. (A, B) MDA-MB-231 cells invasion when YAP is knocked down ± exogenous CXCL1 and IL8. (C) E0771 cells invasion when YAP is knocked down. (D) Western blot for YAP in infected MDA-MB-231 and E0771 cells with shRNA targeting YAP or Luciferase or a nonsilencing shRNA. Actin was used as a loading control. Migration of MDA-MB-231 (E) or E0771 (F) cells when YAP is knocked down ± exogenous CXCL1 and IL8.

Figure S5. CGH Array for MDA-MB-231 cells grown at different densities. (A) Array-based CGH ideograms of chromosomal gains and losses for high-density cells (red tracings) and low-density cells (green tracings). The aberrations are summarized by the colored bars (red for high-density cells and green for low-density cells). DNA gain is indicated by bars on the right of the tracings and DNA loss by bars on the left. The length of the bar delineates the region of the chromosome involved in the gain or loss. (B) A table listing the chromosomal loci of the genes showing differential expression in the cDNA array from MDA-MB-231 cells grown at different densities. Loci are also illustrated in panel (A).

Figure S6. Cytokine expression and function dependent on cell growth density and YAP activity. (A) Upstream regulator pathways in MDA-MB-231 cells grown at low vs. high density. Z-scores and p-values from an Ingenuity Pathway Analysis. (B) Cytokine array showing the effect of VP treatment (inhibitor of YAP activity) on the abundance of cytokines present in the MDA-MB-231 conditioned media (CM). (C) A heat map of the fold change in cytokine levels in untreated and VP-treated MDA-MB-231 CM.

Figure S7. Effect of cancer cell conditioned media (CM) on endothelial monolayer wounding and repair. (A) Carton to illustrate the approach. A confluent endothelial monolayer on ECIS electrodes (a) is wounded by a brief high current sent through the electrodes (b) and monitored for closure (c) by electrical impedance sensing. (B) Treatment with CM from high density MDA-MB-231 cells showed no significant effect on endothelial monolayer closure. (C) CM from low density MDA-MB-

231 cells delayed monolayer closure. The lines are the average of two replicates. (D) Time needed for 50% wound closure from panel C. The relative amounts of CM added with the growth media are indicated. (E) Anti-CXCR2 effect on wounded monolayer closure. The lines are the average of two replicates. (F) Time needed for 50% wound closure from panel E.

Table S1. Primer sequences for mouse and human CTGF and CYR61.

Gene	Primer
hCTGF -fwd	TGGCGAAGCTGACCTGGAA
hCTGF-rev	GATTTTGGGAGTACGGATGCA
hCYR61 -fwd	GAATCATGGAGAAAATGGGCG
hCYR61-rev	CAATCGTGGCTGCATTAGTG
mCTGF-fwd	AGCTGACCTGGAGGAAAACA
mCTGF-rev	GACAGGCTTGGCGATTTTAG
mCyr61-fwd	CTGCGCTAAACAACTCAACGA
mCyr61-rev	GCAGATCCCTTTCAGAGCGG

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Table S2. Fold enrichment and sequence of shRNA targeting LATS1 from the kinome screen.

Gene name	Fold	shRNA sequence
LATS 1	57.3	TGAGGAGGATCATCTGTCAGTAGCCTG
	56.0	GAGGATCATCTGTCAGTAGCCTGTTCA
	16.6	GATCATCTGTCAGTAGCCTGTTCACCC

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SUPPLEMENTARY MATERIALS AND METHODS

Cell growth and apoptosis. Cells were plated at different densities on E-plates from xCELLigence (Roche (Abassi *et al*, 2009)) and monitored in real time for 60 h (Rosenfield *et al*, 2012). The rate of apoptosis was determined by Annexin V-FITC staining and FACS analysis of 1×10^6 cells grown at high or low density using a commercially available assay (Trevigen, Gaithersburg, MD).

Comparative Genomic Hybridization (CGH) array. An Agilent SurePrint G3 Human CGH array was used for comparative genomic hybridisation of paired DNA samples.

SUPPLEMENTARY REFERENCES

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