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Supplemental information

Single-cell RNA sequencing identifies a paracrine interaction that may drive oncogenic notch signaling in human adenoid cystic carcinoma

Anuraag S. Parikh, Avishai Wizel, Daniel Davis, Armida Lefranc-Torres, Alejandro I. Rodarte-Rascon, Lauren E. Miller, Kevin S. Emerick, Mark A. Varvares, Daniel G. Deschler, William C. Faquin, Jon C. Aster, Derrick T. Lin, Bradley E. Bernstein, Yotam Drier, and Sidharth V. Puram

SUPPLEMENTAL FIGURES

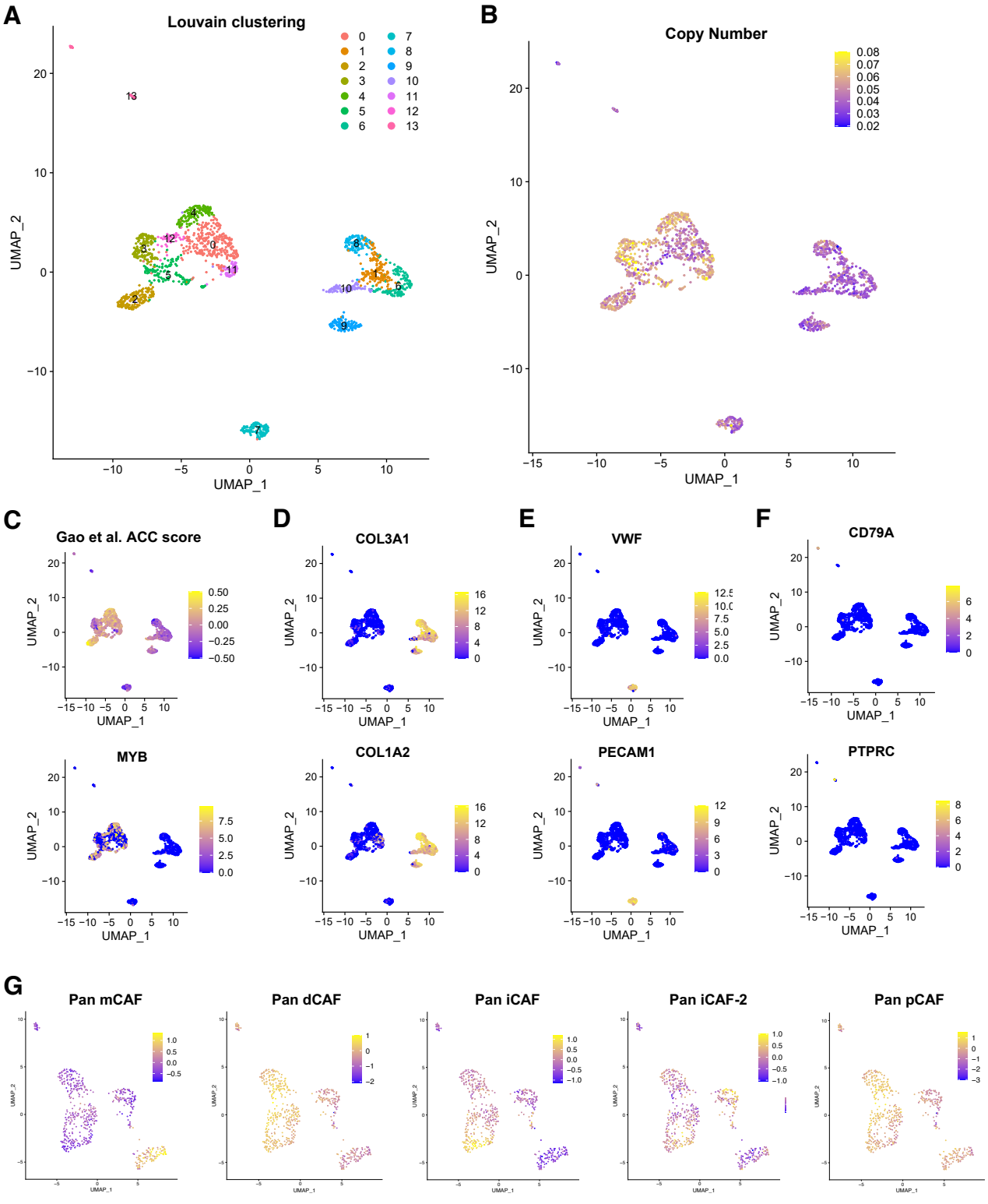

Figure S1. Additional classification of malignant vs. non-malignant cells. Related to Figure 1.

A. UMAP shows all cells that passed QC filters, colored by clusters as defined by Louvain clustering.

B. UMAP of all cells that passed QC filters, colored by copy number aneuploidy score (high aneuploidy, yellow), highlights malignant cells.

C. UMAPs of all cells that passed QC filters, colored by expression of Gao *et al.* ACC signature and MYB (high expression, yellow) highlight cancer cells.

D. UMAPs of all cells that passed QC filters, colored by expression of COL3A1 and COL1A2 (high expression, yellow), define clusters of CAF.

E. UMAPs of all cells that passed QC filters, colored by expression of vWF and PECAM1 (high expression, yellow), define cluster of endothelial cells.

F. UMAPs of all cells that passed QC filters, colored by expression of CD79A and PTPRC (high expression, yellow), define cluster of WBC.

G. UMAPs of all cancer associated fibroblasts (CAFs), colored by expression of signatures of previously reported pan-cancer CAF subtypes,[38] including myofibroblasts (myCAF), desmoplastic CAF (dCAF), inflammatory CAF (iCAF), inflammatory CAF-2 (iCAF-2), and proliferative CAF (pCAF) (high expression, yellow).

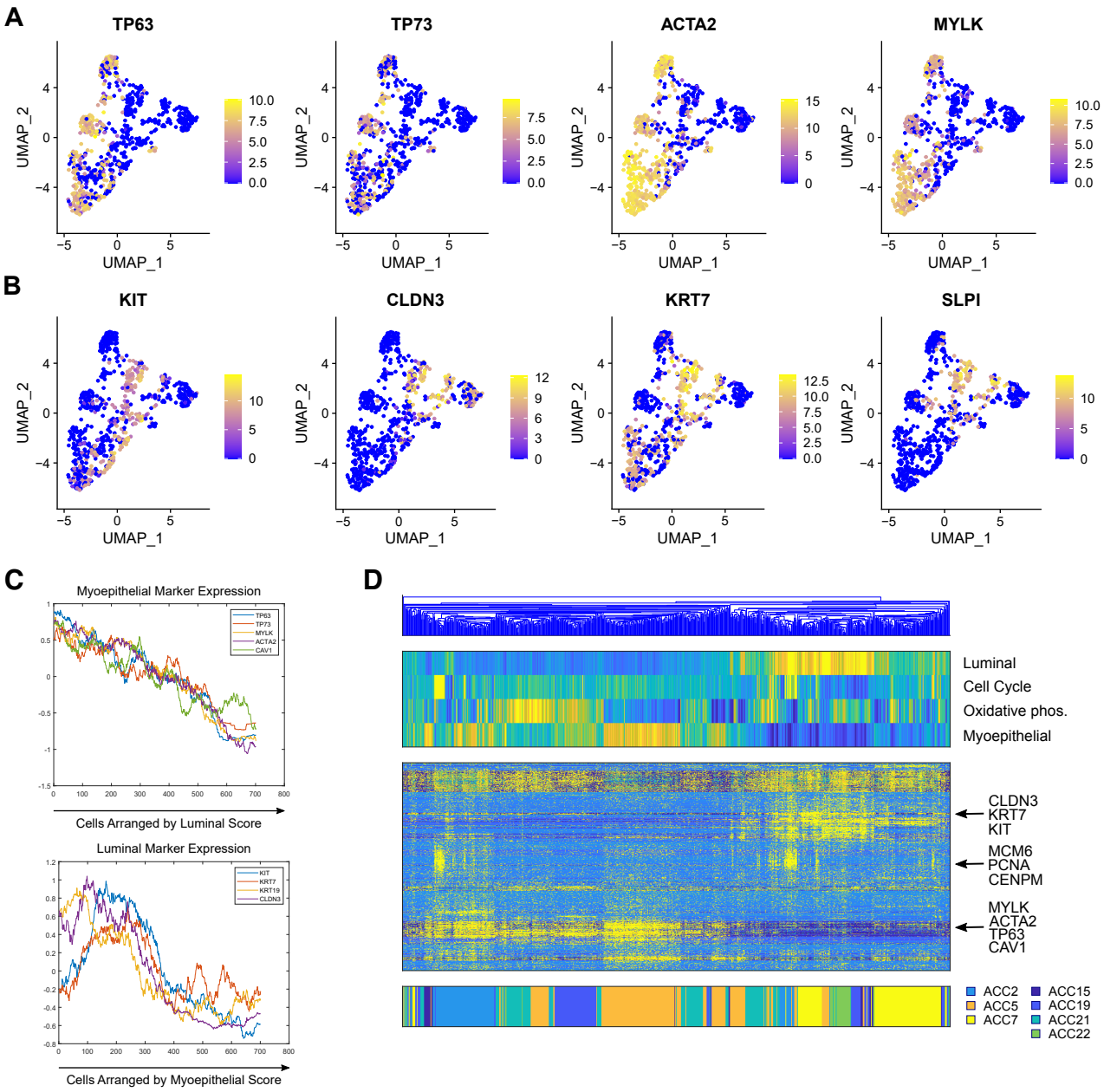

Figure S2. Additional analysis of malignant compartment heterogeneity. Related to Figure 2.

A. UMAPs of all malignant cells colored by expression of four top myoepithelial markers (TP63, TP73, ACTA2, MYLK; high expression, yellow) highlight myoepithelial cancer cells.

B. UMAPs of all malignant cells colored by expression of four top luminal markers (KIT, CLDN3, KRT7, SLPI; high expression, yellow) highlights luminal cancer cells.

C. Line plots show moving average across a sliding window of 50 cells of myoepithelial marker expression in cells arranged by luminal score (top panel) and luminal marker expression in cells arranged by myoepithelial score (bottom

panel). There is a strong negative relationship between myoepithelial and luminal markers, supporting the robustness of the classification of cancer cells into one of these two malignant cell types.

D. Gene expression heatmap shows NMF clustering of all malignant cells from primary tumors. Consistent luminal, myoepithelial, cell cycle, and oxidative phosphorylation programs are observed across tumors in the cohort.

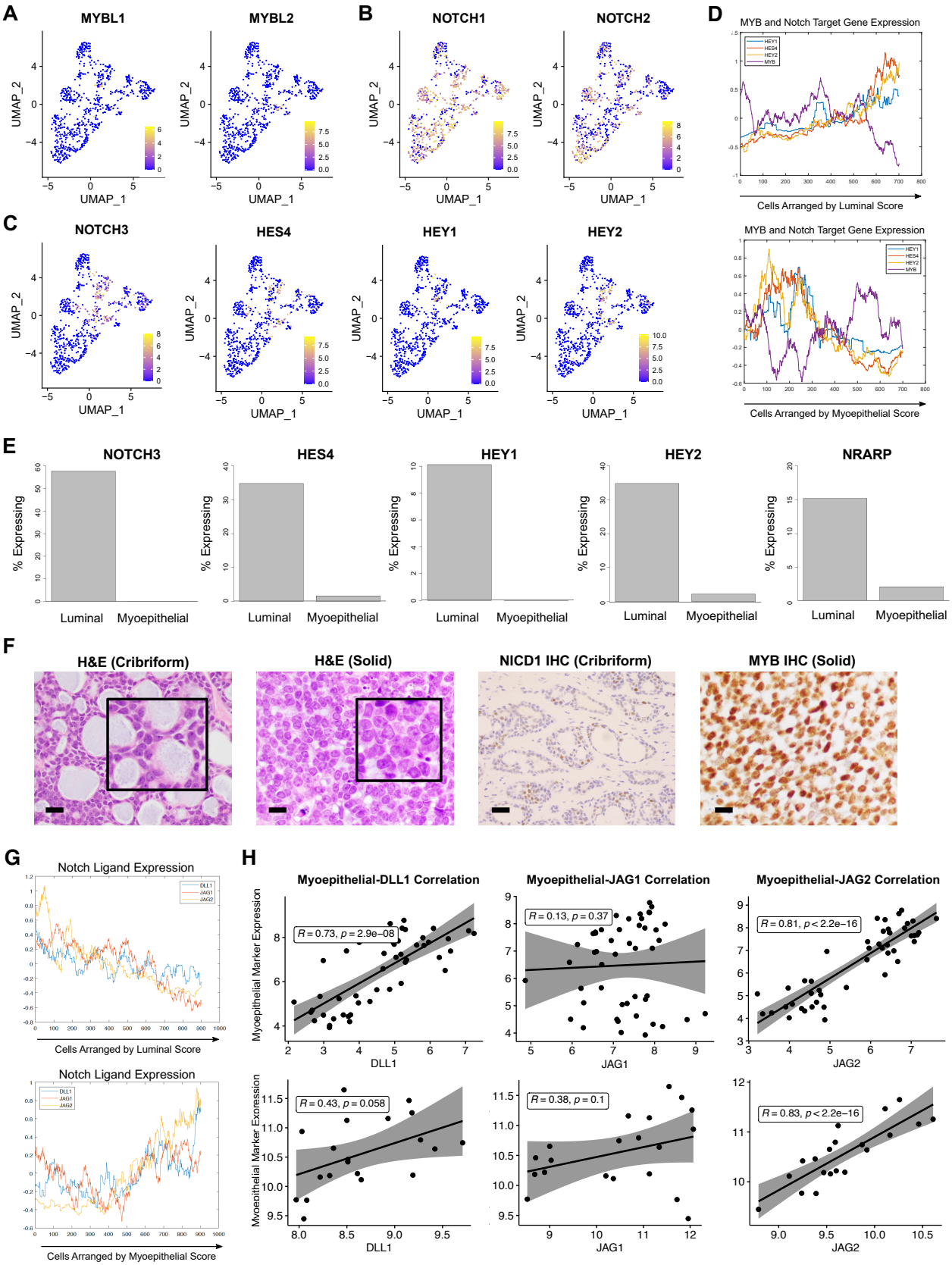
A
MYBL1
MYBL2
B
NOTCH1
NOTCH2
C
NOTCH3
HES4
HEY1
HEY2
UMAP_1
UMAP_2
D
MYB and Notch Target Gene Expression
HEY1
HES4
HEY2
MYB
Cells Arranged by Luminal Score
MYB and Notch Target Gene Expression
Cells Arranged by Myoepithelial Score
E
NOTCH3
HES4
HEY1
HEY2
NRARP
% Expressing
Luminal
Myoepithelial
F
H&E (Cribriform)
H&E (Solid)
NICD1 IHC (Cribriform)
MYB IHC (Solid)
G
Notch Ligand Expression
DLL1
JAG1
JAG2
Cells Arranged by Luminal Score
Notch Ligand Expression
Cells Arranged by Myoepithelial Score
H
Myoepithelial-DLL1 Correlation
Myoepithelial-JAG1 Correlation
Myoepithelial-JAG2 Correlation
Myoepithelial Marker Expression
R = 0.73, p = 2.9e−08
R = 0.13, p = 0.37
R = 0.81, p < 2.2e−16
R = 0.43, p = 0.058
R = 0.38, p = 0.1
R = 0.83, p < 2.2e−16
DLL1
JAG1
JAG2

Figure S3. MYB and Notch expression heterogeneity in the malignant compartment. Related to Figure 3.

A. UMAPs show malignant cells colored by MYBL1 (left panel) and MYBL2 (right panel) expression (high expression, yellow).

B. UMAPs show malignant cells colored by Notch1 (left) and Notch2 (right) expression (high expression, yellow).

C. UMAPs show malignant cells colored by expression of Notch target genes (Notch3, HES4, HEY1, HEY2; high expression, yellow).

D. Line plots show moving average across a sliding window of 50 cells of MYB and Notch target gene expression in cells arranged by luminal (top panel) and myoepithelial (bottom panel) score. Notch targets show high expression in more luminal cells and low expression in more myoepithelial cells.

E. Bar plots show percent of cells expressing Notch targets Notch3, HES4, HEY1, NEY2, and NRARP. All Notch targets are more highly expressed in luminal than myoepithelial cells.

F. 600X images show hematoxylin and eosin (H&E) sections of classic cribriform (first panel) and solid (second panel) type ACC tumors, NICD1 immunohistochemistry (brown) in a representative cribriform tumor (third panel), and MYB immunohistochemistry (brown) in a representative solid type tumor (fourth panel).

G. Line plots show moving average across a sliding window of 50 cells of expression of Notch ligands DLL1, JAG1, and JAG2 in cells arranged by luminal score (top panel) and myoepithelial score (bottom panel). Notch ligands show high expression in more myoepithelial cells and low expression in more luminal cells.

H. Scatter plots show Spearman correlations between myoepithelial markers and DLL1, JAG1, or JAG2 in Gao *et al.*[12] (top panels) and Ferrarotto *et al.*[26] (bottom panels) cohorts.

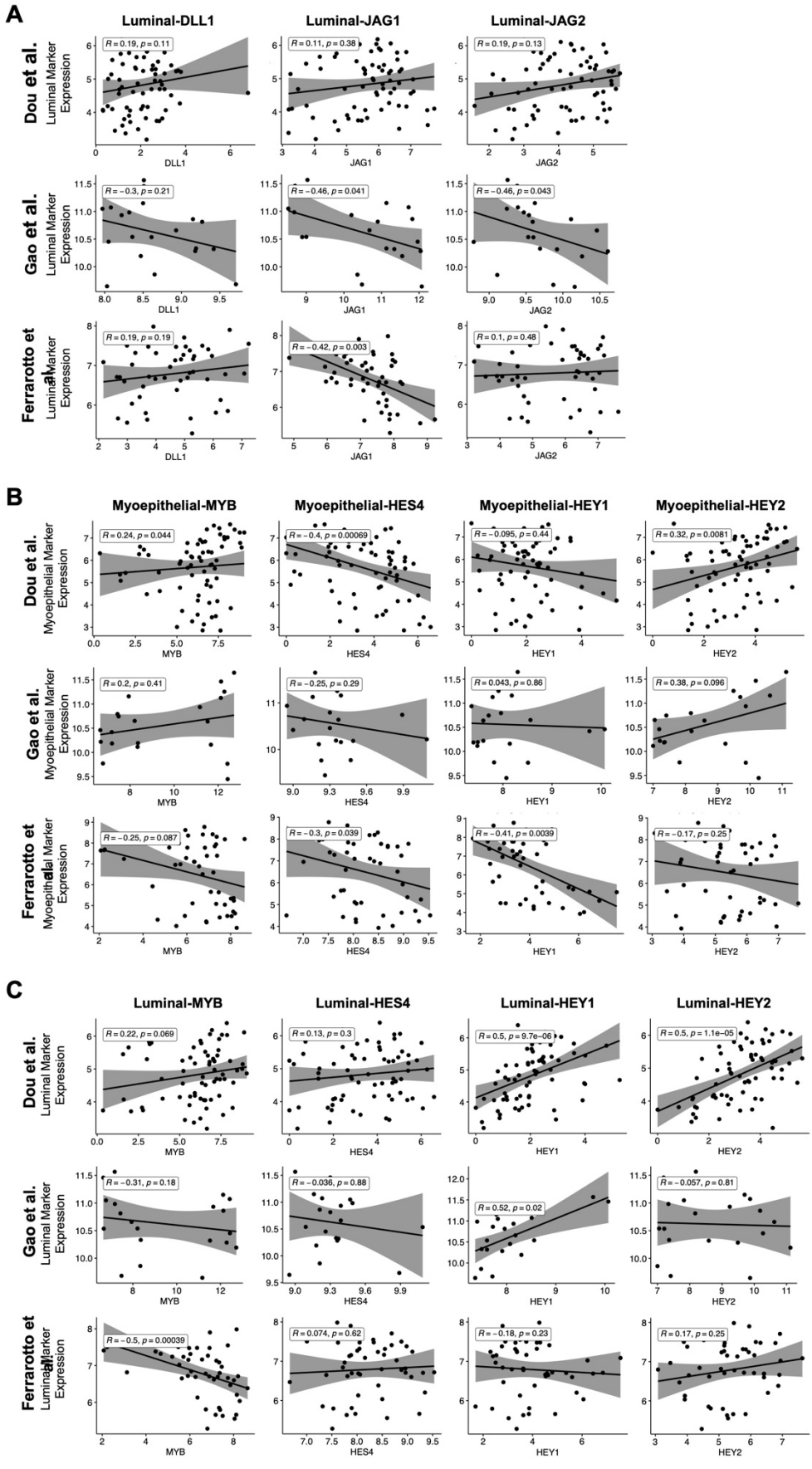
A
Luminal-DLL1
Luminal-JAG1
Luminal-JAG2
Dou et al.
Gao et al.
Ferrarotto et al.
Luminal Marker Expression
R = 0.19, p = 0.11
R = 0.11, p = 0.38
R = 0.19, p = 0.13
R = −0.3, p = 0.21
R = −0.46, p = 0.041
R = −0.46, p = 0.043
R = 0.19, p = 0.19
R = −0.42, p = 0.003
R = 0.1, p = 0.48
DLL1
JAG1
JAG2
B
Myoepithelial-MYB
Myoepithelial-HES4
Myoepithelial-HEY1
Myoepithelial-HEY2
Myoepithelial Marker Expression
R = 0.24, p = 0.044
R = −0.4, p = 0.00069
R = −0.095, p = 0.44
R = 0.32, p = 0.0081
R = 0.2, p = 0.41
R = −0.25, p = 0.29
R = 0.043, p = 0.86
R = 0.38, p = 0.096
R = −0.25, p = 0.087
R = −0.3, p = 0.039
R = −0.41, p = 0.0039
R = −0.17, p = 0.25
MYB
HES4
HEY1
HEY2
C
Luminal-MYB
Luminal-HES4
Luminal-HEY1
Luminal-HEY2
R = 0.22, p = 0.069
R = 0.13, p = 0.3
R = 0.5, p = 9.7e−06
R = 0.5, p = 1.1e−05
R = −0.31, p = 0.18
R = −0.036, p = 0.88
R = 0.52, p = 0.02
R = −0.057, p = 0.81
R = −0.5, p = 0.00039
R = 0.074, p = 0.62
R = −0.18, p = 0.23
R = 0.17, p = 0.25

Figure S4. Correlation of Notch ligand expression and Notch signaling with myoepithelial and luminal markers in bulk RNA-seq cohorts. Related to Figure 3.

A. Scatter plots show Spearman correlations between luminal markers and DLL1, JAG1, or JAG2 in Dou *et al.*,[37] (top panels), Gao *et al*.[12] (middle panels), and Ferrarotto *et al.*[26] (bottom panels) cohorts.

B. Scatter plots show Spearman correlations between myoepithelial markers and MYB, HES4, HEY1, or HEY2 in Dou *et al.*,[37] (top panels), Gao *et al*.[12] (middle panels), and Ferrarotto *et al.*[26] (bottom panels) cohorts.

C. Scatter plots show Spearman correlations between luminal markers and MYB, HES4, HEY1, or HEY2 in Dou *et al.*,[37] (top panels), Gao *et al*.[12] (middle panels), and Ferrarotto *et al.*[26] (bottom panels) cohorts.

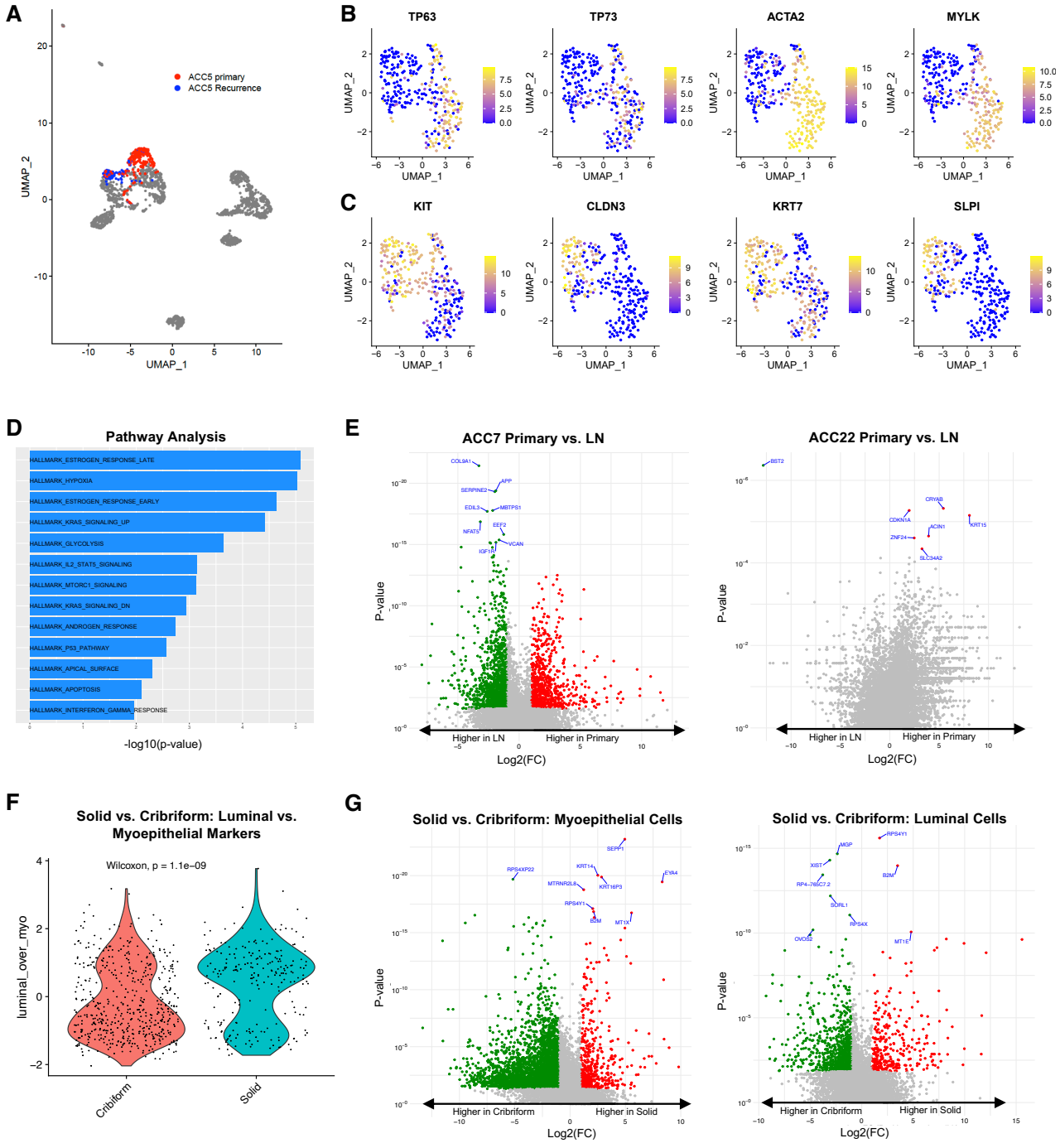

Figure S5. Recurrent tumors may be more luminal and less myoepithelial. Related to Figure 4.

A. UMAP shows all cells that passed QC filters and highlights cells from ACC5 primary tumor (red) and ACC5 recurrence (blue).

B. UMAPs show malignant cells in ACC5 colored by expression of four top myoepithelial markers (TP63, TP73, ACTA2, MYLK; high expression, yellow), highlighting myoepithelial cancer cells, primarily in ACC5 primary.

C. UMAPs show malignant cells in ACC5 colored by expression of four top luminal markers (KIT, CLDN3, KRT7, SLPI; high expression, yellow), highlighting luminal cancer cells, primarily in ACC5 recurrence.

D. Bar plot shows MSigDB hallmark gene sets significantly enriched (FDR < 10%) with genes significantly upregulated in ACC5 recurrence relative to ACC5 primary tumor.

E. Volcano plots show differentially expressed genes across the primary tumor and lymph node settings in ACC7 (left panel) and ACC22 (right panel). Genes colored in green had significantly higher expression (FDR < 10%, > 2 fold change) in the lymph node, while genes colored in red had significantly higher expression (FDR < 10%, > 2 fold change) in the primary tumor.

F. Violin plot shows expression of markers along a scale from luminal to myoepithelial in tumors in the cohort with a significant solid component (ACC7, ACC19) and cribriform tumors without a solid component (ACC2, ACC5, ACC15, ACC21, ACC22). Y-axis represents luminal score minus myoepithelial score, calculated from known markers, as described in the methods.

G. Volcano plots show differentially expressed genes in myoepithelial cells (left panel) and luminal cells (right panel) between tumors in the cohort with a significant solid component (ACC7, ACC19) and cribriform tumors without a solid component (ACC2, ACC5, ACC15, ACC21, ACC22). Genes colored in green had significantly higher expression (FDR < 10%, > 2 fold change) in the cribriform tumors, while genes colored in red had significantly higher expression (FDR < 10%, > 2 fold change) in the solid tumors.

SUPPLEMENTAL TABLES

Table S1. Pathologic characteristics of patient samples profiled. Related to STAR Methods.

Designation	Age/Sex	Primary Site	Other specimen	Histology	PNI	LVI	IHC Profile
ACC 2	64/F	Right preauricular	--	Tubular/cribriform	Absent	Absent	p63/MYB/CD117+
ACC 5	81/F	Left parotid gland	Local recurrence	Cribriform	Present	Absent	MYB+
ACC 7	91/M	Right buccal mucosa	Right level 1 LN	Tubular/solid	Present	Present	p63/MYB/CD117+
ACC 15	81/M	Right orbit	--	Tubular/cribriform	Present	Absent	p63/MYB/CD117+
ACC 19	52/M	Left parotid gland	--	Tubular/solid	Present	Absent	MYB+
ACC 21	57/M	Right base of tongue	--	Cribriform	Present	Absent	MYB+
ACC 22	50/F	Left floor of mouth	Left level 3 LN	Cribriform	Present	Absent	MYB+

LN = lymph node; PNI = perineural invasion; LVI = lymphovascular invasion; IHC = immunohistochemical

Table S2. Clinical characteristics of patient samples profiled. Related to STAR Methods.

Designation	TNM Stage	Adjuvant	OS (m)	DFS (m)	Clinical Course	Vital Status
ACC 2	T1Nx	CRT	72	25	Multiple local recurrences, on chemotherapy trial.	AWD
ACC 5	T1Nx	'--	63	11	Multiple local recurrences treated surgically.	AWD
ACC 7	T3N2b	'--	9	5	Distant metastasis treated with palliative RT.	DOD
ACC 15	T3N0	RT	10	10	No recurrence.	DNED
ACC 19	T4aN0	CRT	47	47	No recurrence.	ANED
ACC 21	T3N1	RT	49	47	Distant metastasis, treated surgically.	ANED
ACC 22	T2N2a	RT	46	19	Distant metastasis, stable.	AWD

TNM = tumor/node/metastasis; CRT = chemoradiotherapy; RT = radiotherapy; OS = overall survival; DFS = disease free survival; m = months; AWD = alive with disease; DOD = died of disease; DNED = died with no evidence of disease; ANED = alive with no evidence of disease.