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Supplemental Information

High-Dimensional Phenotyping Identifies Age-Emergent Cells in Human Mammary Epithelia

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Supplemental Information

Women <30y			
Sample	Age, y	Source	Known characteristics/ pathology notes
48R	16	RM	African-American
160	16	RM	
407P	19	P	IDC, lymph node+, ER+, PR+
240L	19	RM	Mildly hyperplastic
168R	19	RM	African-American
399E	20	RM	Benign
184	21	RM	
356E	21	RM	Normal
001P	24	P	IDC, lymph node-
123	27	RM	African-American, mammary hyperplasis
195L	27	RM	
97	27	RM	
51L	28	RM	Mild periductal mastitis (R+L) focal microcalcification (R.)
172L	28	RM	Minimal phase of fibrocystic disease
676P	29	P	
124	29	RM	

Women >30y and <50y			
Sample	Age, y	Source	Known characteristics/ pathology notes
42P	30	P	IDC, lymph node-
169L	35	RM	
90P	36	P	BRCA-1 mut (185delAG)
250MK	37	Milk	
100P	39	P	Nonivasive ductal carcinoma, ER-, PR-
6	40	RM	
245AT	41	RM	ATM heterozygote, tissue was clinically normal tissue
173P	45	P	
173T	45	Tumor	IDC, ER-, PR-
208	45	RM	
2	46	RM	
60R	47	RM	
30	49	RM	

Women >50y			
Sample	Age, y	Source	Known characteristics/ pathology notes
178R	51	RM	
191L	56	RM	Slight fibrocystic disease, hypertrophy, stromal fibrosis and adenosis present in mammary parenchyma
117R	56	RM	Patchy stromal fibrosis (R.), fibrocystic disease (L.)
335R	58	P	Infiltrating adenocarcinoma, ER+, PR+/-
153L	60	RM	Benign fibrocystic disease
639P	60	P	
122L	62	RM	Fibrocystic disease, hypertrophy, apocrine metaplasia of ductal epithelium, cystic dilatia of ducts, and focal areas of intraductal hyperplasia
881P	65	P	
96R	66	RM	Slight focal fibrocystic change
29	68	RM	
353P	72	P	Colloid (mucinous) carcinoma, ER+/-, PR-
429ER	72	RM	
464P	80	P	
451P	83	P	
805P	91	P	

Breast Epithelia Samples		
Sample	**Age, y**	**Source**
404EB	19	RM
399E	20	RM
472ER	21	RM
620EL	22	RM
483EHRA	26	RM
400ER	27	RM
437E	29	RM
334ER	55	RM
337ER	57	RM
563HRR	61	RM
395C	62	RM
429EL	72	RM
368E	73	RM

Table S1. HMEC and uncultured breast epithelia samples (Related to Figure 1). HMEC strains and uncultured breast epithelia samples were derived from reduction mammoplasty (RM), peripheral non-tumor regions from mastectomy (P) tissues, milk fluids (Milk) and a tumor (T). The name of the HMEC strain, the age at the time of surgery and the characteristics are indicated.

Isotope	Antigen	Antibody raised against	Pathway	References	Clone	Supplier	Final Concentration ug/mL
ER 170	K14	Total	Myoepithelial marker	(Villadsen et al., 2007)	polyclonal	Thermo	0.25
PR 141	K5/6	Total	Myoepithelial marker	(LaBarge et al., 2007)	D5	Millipore	4
DY 163	K19	Total	Luminal marker	(Villadsen et al., 2007)	TromaIII	DSHB	2
DY164	K7	Total	Luminal marker	(Taylor-Papadimitriou et al., 1989)	RCK105	BD	0.5
YB 174	K8/18	Total	Luminal marker	(Villadsen et al., 2007)	C51	CST	2
YB 173	CD133	Total, epitope 1	Luminal marker	(dos Santos et al., 2013)	AC133	Miltenyi	0.5
GD 161	cKit	Total	Progenitor marker	(Lim et al., 2009; Regan etal., 2012)	104D2	Biolegend	4
ER 168	Axl	Total	Stemness	(Asiedu et al., 2014)	1H12	BergenBio	4
GD 160	CD44	Total, surface	Stemness, migration	(Hebbard et al., 2000; Louderbough et al., 2011)	IM7	BD	0.1
HO 165	HER2	C terminal 1242-1255	Proliferation	(Rubin, Yarden, 2001; Yarden, 2011)	3B5	BD	4
ER 167	YAP	C terminal 379-407	Hippo	(Zhao et al., 2010; Vlug et al., 2013)	H9	Santa Cruz	4
ND148	MST1	aa475-505	Hippo	(Zhao et al., 2010)	polyclonal	LS-Bio	8
SM149	LATS1	N-terminus	Hippo	(Zhao et al., 2010)	polyclonal	LS-Bio	8
DY 162	MST2	Total	Hippo	(Zhao et al., 2010)	polyclonal	LS-Bio	8
LA 139	pCreb	pS133	Survival	(Dietze et al., 2005)	J151-21	BD	4
ND 144	pMEK1/2	pS221	Myoepithelial contractility	(Pasic et al., 2011)	166F8	CST	6
ND 145	pStat3	pY705	Involution	(Haricharan and Li, 2014)	4/pStat3	BD	4
ND 146	pStat5	pY694	Lobuloalveolar development	(Gallego et al., 2001; Barash, 2006)	47	BD	4
ND150	pNFkB	pS529	Mammary gland morphogenesis	(Brantley et al., 2001)	K10-895.12.50	BD	2
EU 151	pEGFR	pY1068	Myoepithelial contractility	(Pasic et al., 2011; Paszek et al., 2005)	Y38	Abcam	2
SM152	pStat1	pY701	Tumor suppressor	(Chan et al., 2012; Haricharan and Li, 2014).	4a	BD	8
EU 153	pAkt	pS473	Survival	(Watson, 2006)	D9E	CST	4
SM 154	pErk1/2	pT202/pY204	Myoepithelial contractility	(Pasic et al., 2011)	20A	BD	4
GD 158	pGsk3	pS9 (Inactivation)	Milk synthesis and cell proliferation	(Dembowy et al., 2015)	D85E12	CST	2
TM169	pPLCgamma2	pY759	Myoepithelial contractility	(Raymond et al., 2011; Reversi et al., 2005)	K86-689.37	BD	1
YB 171	pS6	pS235/pS236	Survival	(Fu et al. 2015; Tumaneng et al. 2012)	N7-548	BD	0.2
GD156	Cyclin B1	Total	Proliferation	(Jin et al., 1998)	GNS-11	BD	2
LU 175	pRb	pS807/811	Proliferation	(Giacinti and Giordano, 2006)	D20B12	CST	0.5
YT 172	Cleaved caspase3	Cleaved@D175	Apoptosis	(Watson, 2006)	5A1E	CST	4

Table S2. Antibody panel (Related to Figure 1). A panel of 29 antibodies is shown, comprising 10 highly informative surface markers, 12 antibody probes against intracellular phosphorylation, 4 antibody probes against the Hippo pathway and 3 antibody probes against cell cycle and apoptosis pathway. Each antibody clone, supplier, epitope and conjugated isotope is indicated. All the antibodies have been previously validated and titrated. References for relevant pathways in the regulation of HMEC are indicated.

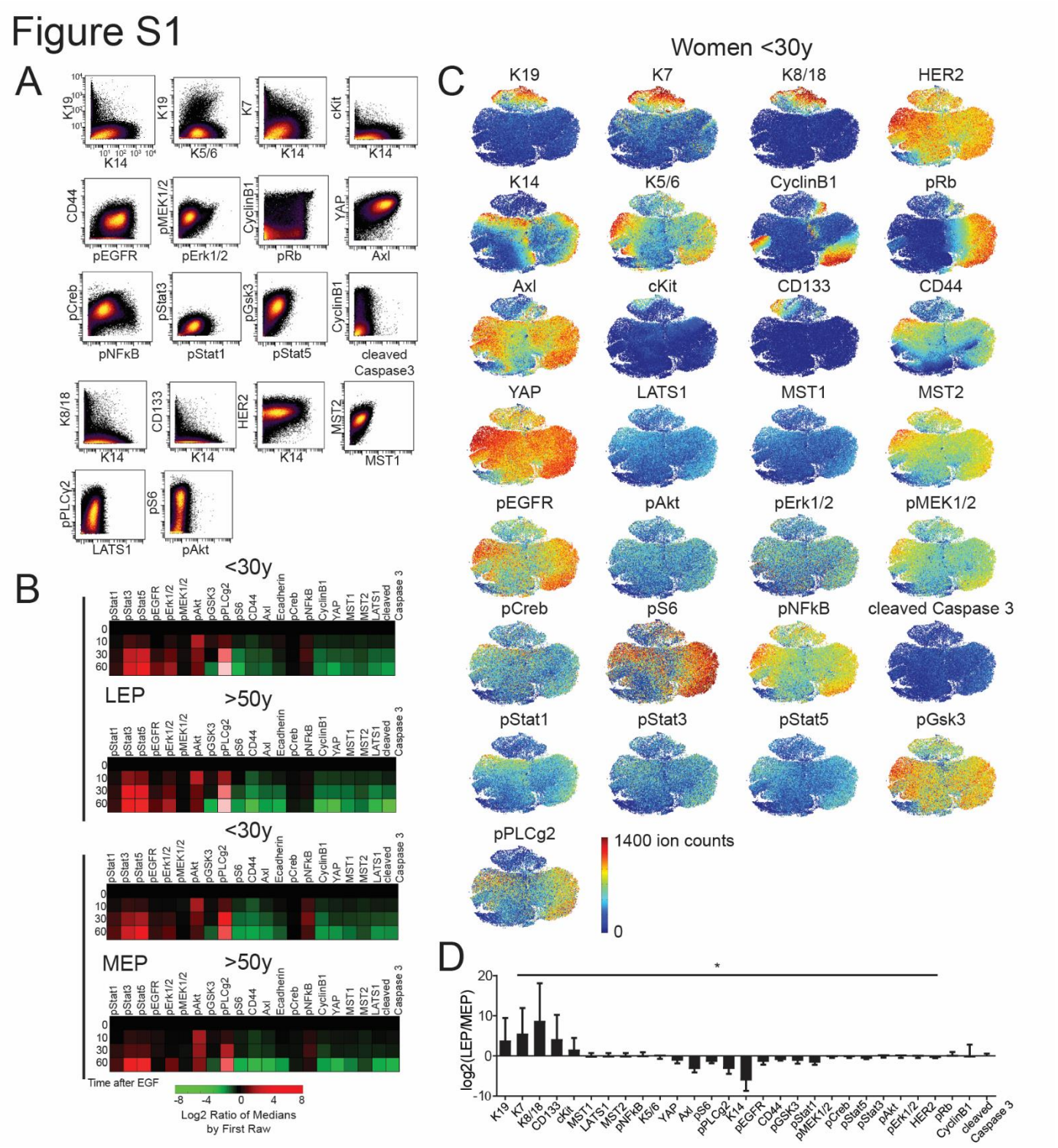

Figure S1. Validation of the antibody panel (Related to Figure 1 and 2). The antibody panel has been extensively validated and titrated (data not shown). The raw data has been transformed with arcsinh with the cofactor of 5. (A) To illustrate the functionality of each metal-conjugated

antibody, representative biaxial plots show staining profiles of the antibodies used in women <30y (merged, N=16), ranging from 10^0 to 10^4 ion counts per cell. (B) Heatmaps of marker expression in HMEC from three <30y and >50y women treated with EGF and vanadate for 60min, manually gated after tSNE projection. At, t=0, 10, 30 and 60min cells were harvested and analyzed with mass cytometry using barcoding and a panel of 23 antibody probes. The fold change of marker expression is ranged from the lowest (green) to the highest (red). This experiment validates the signaling pathway antibodies. (C) tSNE maps from HMEC from women <30y (merged, N=16). Only 50'000 cells are subsampled for display. The marker expression is ranged from 0 (blue) to 1400 ion counts (red). (D) Log_2 fold change in marker expression of LEP over MEP manually gated from tSNE. Paired student t-test was performed on median of protein expression in LEP vs MEP before logarithm transform, * $p<0.05$ N=16.

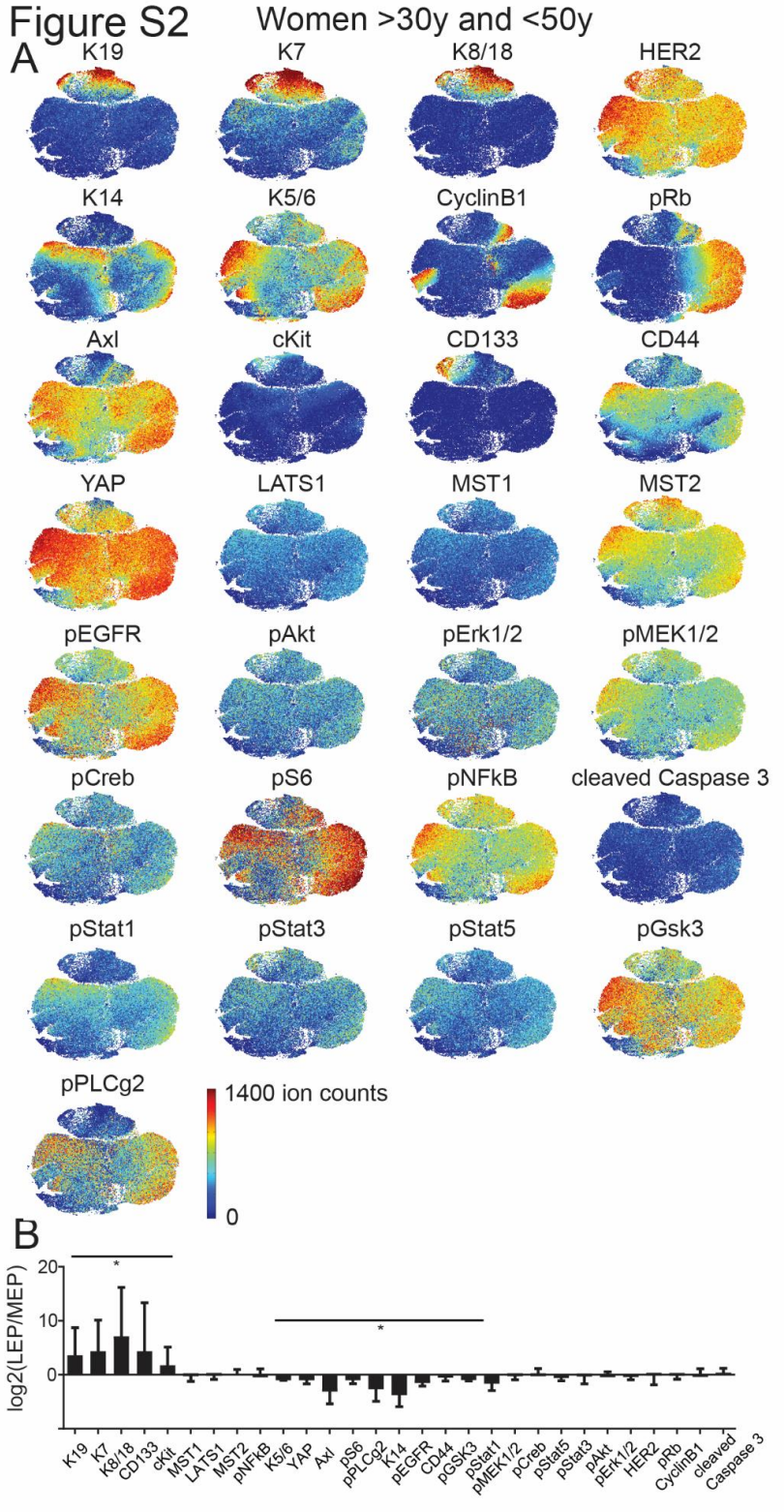

Figure S2. Luminal and myoepithelial lineages exhibit a phenotypic divergence in women >30 and <50y (Related to Figure 2). (A) tSNE maps from HMEC from women >30y and <50y (merged and subsampled at 50'000 cells, N=9). The marker expression is ranged from 0 (blue) to 1400 ion counts (red). (B) Log_2 fold change in marker expression of LEP over MEP manually gated from tSNE. Four abnormal samples were excluded: a sample from milk fluid (250MK), two samples bearing BRCA1 or ATM mutation (90P and 245AT), and one sample from a tumor (173T). Paired student t-test was performed on median of protein expression in LEP vs MEP before logarithm transform. The non-significance was due to a lower sample number (N=9) in addition to age-related changes. Student t-test * $p<0.05$ N=9.

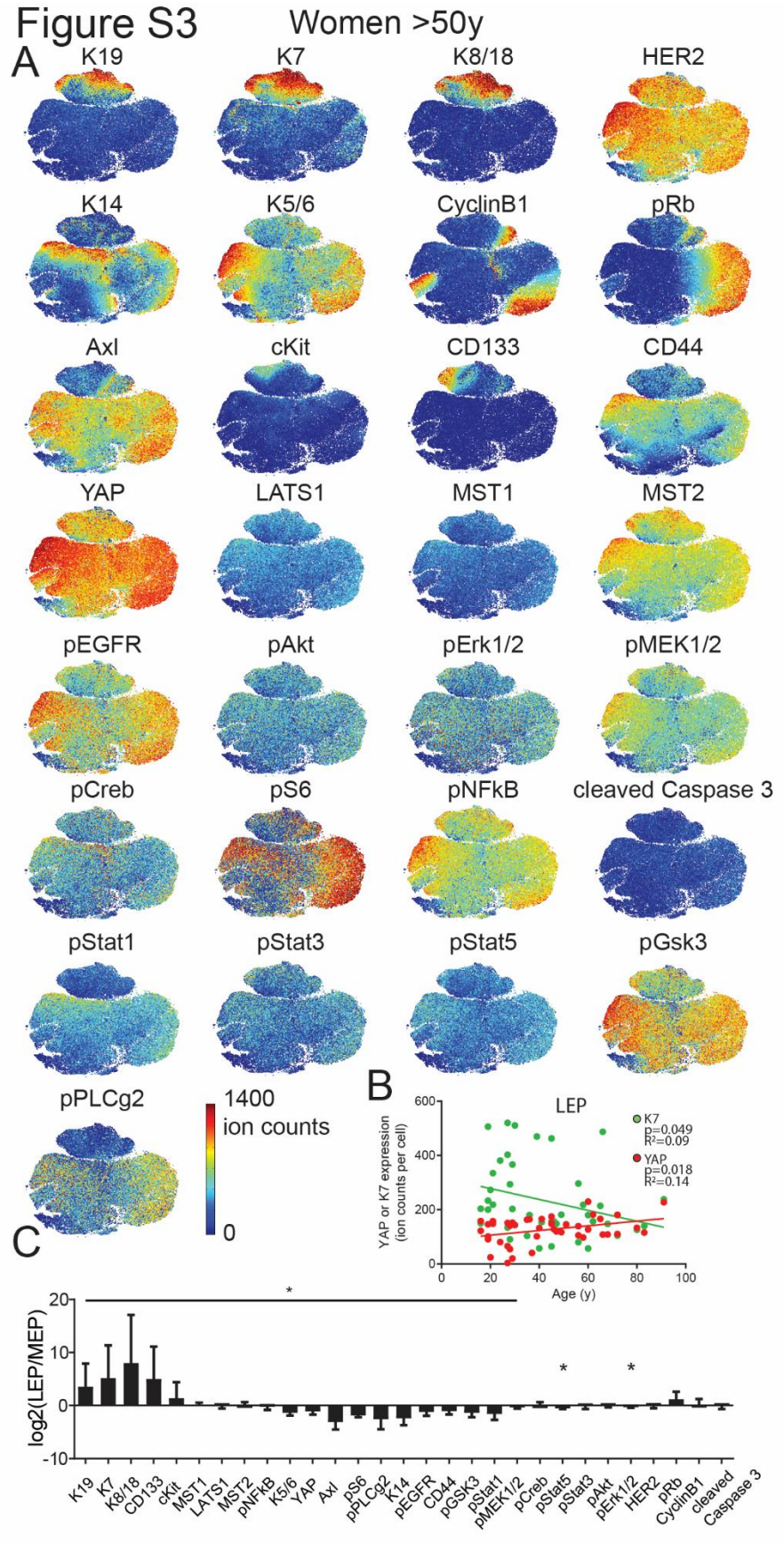

Figure S3. Luminal and myoepithelial lineages exhibit a phenotypic divergence in women >50y (Related to Figure 2). (A) tSNE maps from HMEC from women >50y (merged and subsampled at 50'000 cells, N=15). The marker expression is ranged from 0 (blue) to 1400 ion counts (red). (B) Linear regression of K7 and YAP expression in LEP as a function of age. (C) Log_2 fold change in marker expression of LEP over MEP manually gated from tSNE. Paired student t-test was performed on median of protein expression in LEP vs MEP before logarithm transform, * $p<0.05$ N=15.

Figure S4

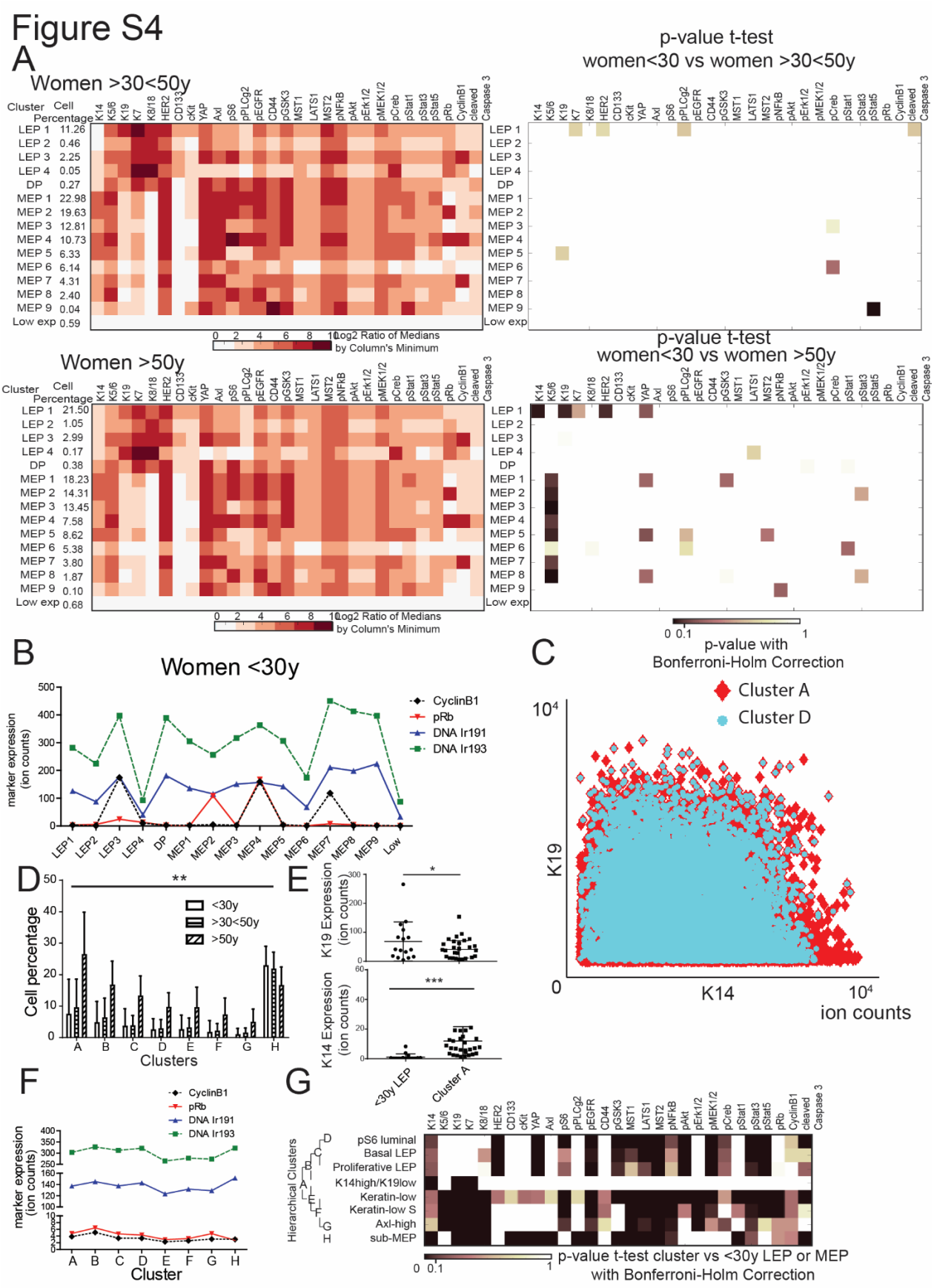
A
Women >30<50y
Women >50y
p-value t-test
women<30 vs women >30<50y
p-value t-test
women<30 vs women >50y
Log2 Ratio of Medians
by Column's Minimum
p-value with
Bonferroni-Holm Correction
B
Women <30y
marker expression
(ion counts)
CyclinB1
pRb
DNA Ir191
DNA Ir193
C
Cluster A
Cluster D
K19
K14
ion counts
D
Cell percentage
<30y
>30<50y
>50y
Clusters
E
K19 Expression
(ion counts)
K14 Expression
(ion counts)
<30y LEP
Cluster A
F
Cluster
G
Hierarchical Clusters
pS6 luminal
Basal LEP
Proliferative LEP
K14high/K19low
Keratin-low
Keratin-low S
Axl-high
sub-MEP
p-value t-test cluster vs <30y LEP or MEP
with Bonferroni-Holm Correction

Figure S4. PhenoGraph analysis identified lineage-specific subsets (Related to Figure 3). (A) Heatmaps of marker expression of each cluster identified with PhenoGraph of HMEC from women >30 and <50y and women >50y with their associated p-values with Bonferroni-Holm correction of student t-tests. (B) Plots show CyclinB1 (black dashed line), pRb (red plain line), Iridium 191 labeling DNA (blue plain line) and Iridium 193 labeling DNA (green dashed line) intensity (ion counts) in each cluster identified with PhenoGraph. LEP3, MEP4 and MEP7 had the highest Cyclin B1 expression which correlated with higher Iridium intensity, thus DNA content, as compared to the other clusters. (C) Scatter plot shows the expression of K14 and K19 in Citrus cluster A (red diamonds) and D (blue dots) to illustrate that cells from cluster D belong to cluster A by hierarchical clustering and thus are superposed onto cells from cluster A. (D) Plots of cell percentage in each Citrus cluster. Four abnormal samples were excluded: 250MK, 90P and 245AT, 173T. (E) Plots show the median expression of K19 and K14 in <30y LEP (n=16) and the cluster A of the Citrus tree (N=40). t-test *** $p<0.0001$, * $p=0.0433$. (F) Plots show CyclinB1 (black dashed line), pRb (red plain line), Iridium 191 labeling DNA (blue plain line) and Iridium 193 labeling DNA (green dashed line) intensity (ion counts) in each cluster identified with Citrus. (G) Heatmaps show the p-values with Bonferroni-Holm correction from student t-tests of each marker expression of Citrus clusters vs LEP or MEP <30y.

Figure S5

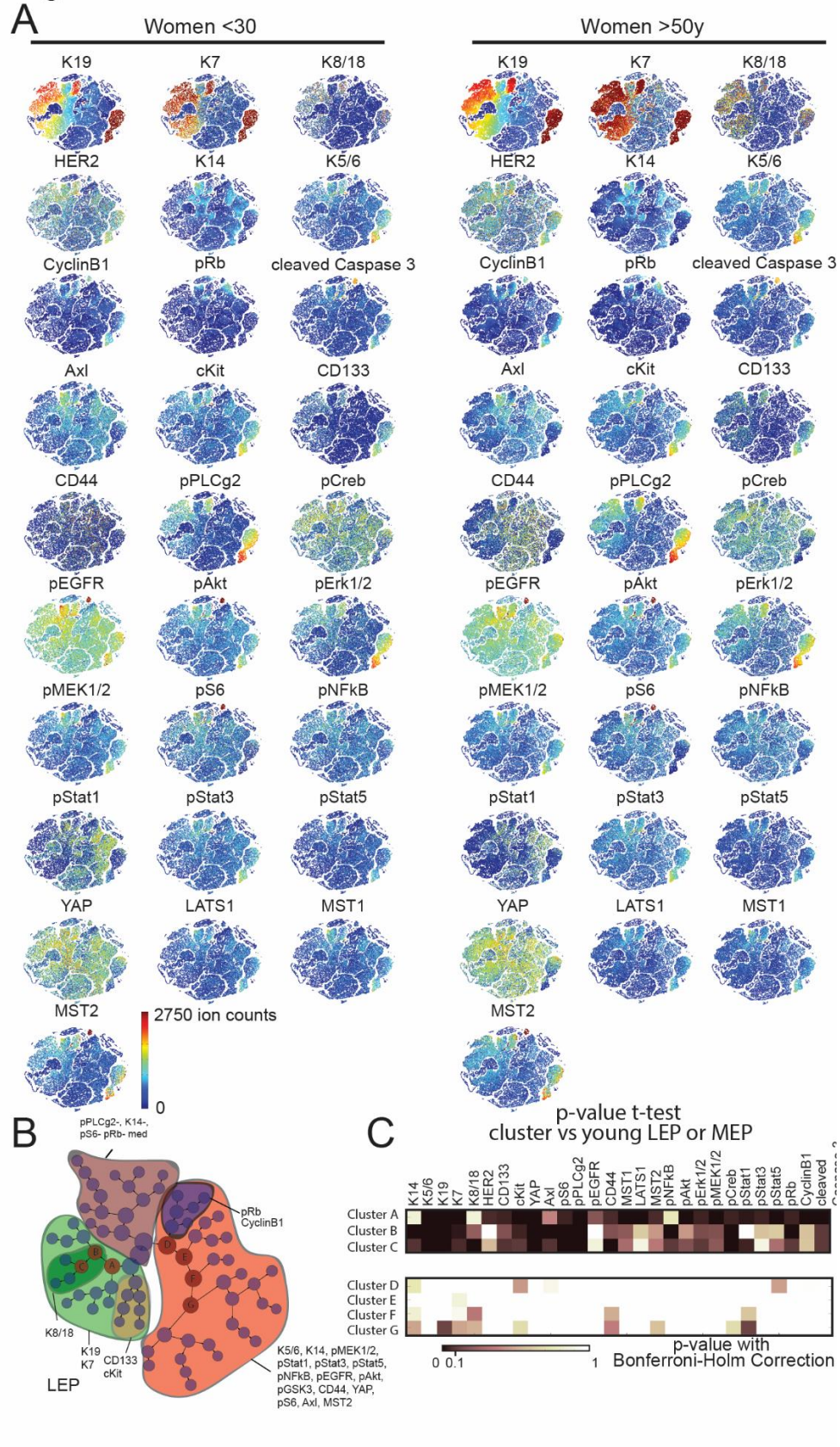
A
Women <30
Women >50y
K19
K7
K8/18
HER2
K14
K5/6
CyclinB1
pRb
cleaved Caspase 3
Axl
cKit
CD133
CD44
pPLCg2
pCreb
pEGFR
pAkt
pErk1/2
pMEK1/2
pS6
pNFkB
pStat1
pStat3
pStat5
YAP
LATS1
MST1
MST2
2750 ion counts
0
B
pPLCg2-, K14-, pS6- pRb- med
pRb CyclinB1
K8/18
K19 K7
CD133 cKit
LEP
K5/6, K14, pMEK1/2, pStat1, pStat3, pStat5, pNFkB, pEGFR, pAkt, pGSK3, CD44, YAP, pS6, Axl, MST2
C
p-value t-test
cluster vs young LEP or MEP
Cluster A
Cluster B
Cluster C
Cluster D
Cluster E
Cluster F
Cluster G
0 0.1
1
p-value with Bonferroni-Holm Correction

Figure S5. High dimensional analysis of cellular heterogeneity within primary human breast epithelia (Related to Figure 5). (A) tSNE maps of breast epithelia from women <30y (merged, and subsampled at 50’000 cells, N=7) and women >50y (merged, and subsampled at 50’000 cells, N=6). The marker expression is ranged from 0 (blue) to 2750 ion counts (red). (B) Hierarchical tree of agglomerative clusters obtained with the Citrus analysis. Node sizes are scaled on the basis of frequency of cells in each cluster. Major cell compartments are contoured on the basis of expression of canonical markers. (C) Heatmaps show the p-values with Bonferroni-Holm correction from student t-tests of each marker expression of Citrus clusters vs LEP or MEP <30y.

Figure S6

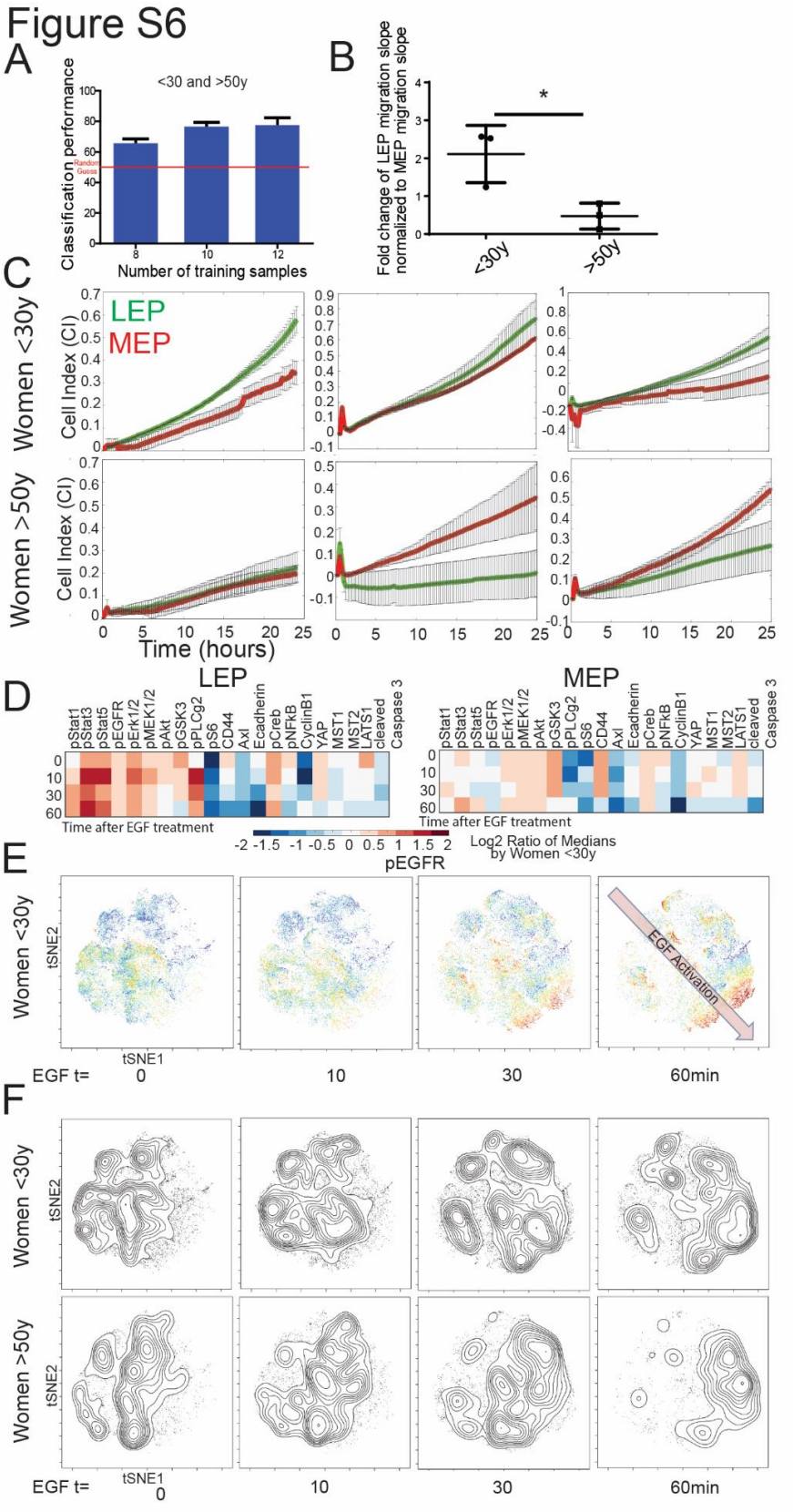
A
<30 and >50y
Classification performance
Random Guess
Number of training samples
B
Fold change of LEP migration slope normalized to MEP migration slope
*
<30y
>50y
C
Women <30y
Women >50y
LEP
MEP
Cell Index (CI)
Time (hours)
D
LEP
MEP
pStat1
pStat3
pStat5
pEGFR
pErk1/2
pMEK1/2
pAkt
pGSK3
pPLCg2
pS6
CD44
Axl
Ecadherin
pCreb
pNFkB
CyclinB1
YAP
MST1
MST2
LATS1
cleaved
Caspase 3
Time after EGF treatment
Log2 Ratio of Medians by Women <30y
pEGFR
E
Women <30y
tSNE2
tSNE1
EGF Activation
EGF t=
0
10
30
60min
F
Women <30y
Women >50y
tSNE2
tSNE1
EGF t=
0
10
30
60min

Figure S6. Evidence of newly acquired functional properties in luminal epithelial cells with age (Related to Figure 6; experimental procedures; data analysis). (A) Citrus classification performance using 8, 10 or 12 training samples. (B) Plots show the fold change of LEP cell migration index (CI) slope normalized to MEP CI migration slope in women <30y and >50y (N=3, p=0.0269). (C) Graphs show CI measured with xCELLigence instrument every 15min for 25h in FACS sorted LEP and MEP from HMEC from three women <30y and from three women >50y. (D) HMEC from three <30y and >50y women were treated with EGF and vanadate for 60min. Heatmaps of marker expression in LEP and MEP manually gated after tSNE projection. Data was normalized to values from <30y women to highlight age-related changes. The $\log_2$ fold change is ranged from the lowest (blue) to the highest (red). (E) tSNE maps of HMEC from women <30y at t=0, 10, 30 and 60min. pEGFR expression from the lowest (blue) to the highest (red) is shown to highlight the movement of HMEC in the phenotypical space upon EGF activation. (F) Density plots in the tSNE phenotypic space exhibited a stronger response in women >50y upon EGF activation. Data are means +/- SEM.

Figure S7

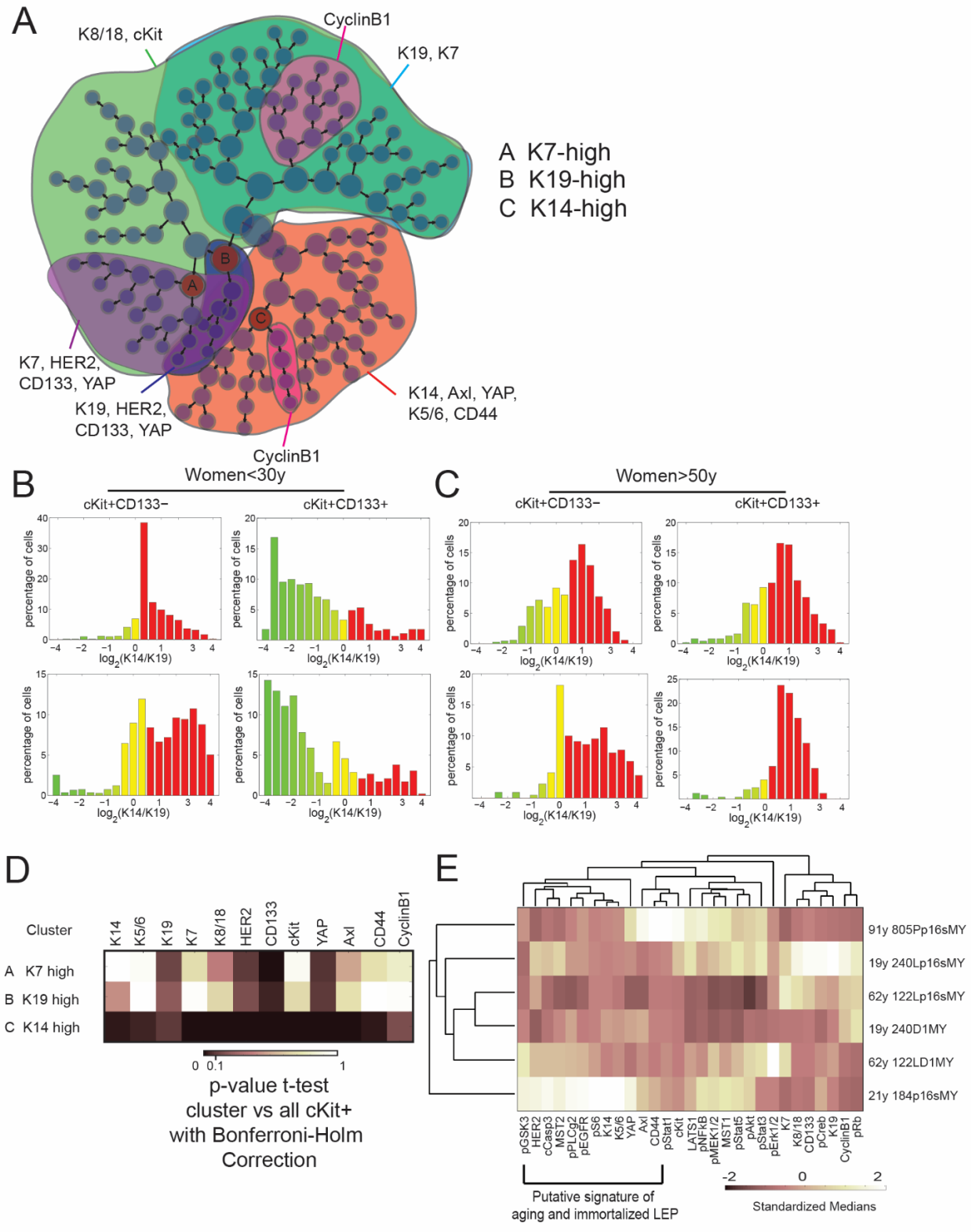
A
K8/18, cKit
CyclinB1
K19, K7
A K7-high
B K19-high
C K14-high
K7, HER2, CD133, YAP
K19, HER2, CD133, YAP
K14, Axl, YAP, K5/6, CD44
CyclinB1
B
Women<30y
cKit+CD133−
cKit+CD133+
C
Women>50y
cKit+CD133−
cKit+CD133+
percentage of cells
log2(K14/K19)
D
Cluster
K14
K5/6
K19
K7
K8/18
HER2
CD133
cKit
YAP
Axl
CD44
CyclinB1
A K7 high
B K19 high
C K14 high
0 0.1 1
p-value t-test cluster vs all cKit+ with Bonferroni-Holm Correction
E
91y 805Pp16sMY
19y 240Lp16sMY
62y 122Lp16sMY
19y 240D1MY
62y 122LD1MY
21y 184p16sMY
pGSK3
HER2
cCasp3
MST2
pPLCg2
pEGFR
pS6
K14
K5/6
YAP
Axl
CD44
pStat1
cKit
LATS1
pNFkB
pMEK1/2
MST1
pStat5
pAkt
pStat3
pErk1/2
K7
K8/18
CD133
pCreb
K19
CyclinB1
pRb
Putative signature of aging and immortalized LEP
-2 0 2
Standardized Medians

Figure S7. Evidence of phenotypic divergence in luminal progenitors with age (Related to Figure 6). (A) Visual representation of unsupervised hierarchical clustering of cKit+ progenitors HMEC with Citrus (N=6). Node sizes are scaled on the basis of frequency of cells in each cluster. Major cell compartments are contoured on the basis of expression of canonical markers. The three clusters changing in abundance with age are shown. (B) Histograms represent log_2-transformed ratios of K14 to K19 protein expression in single cells of acini from two women <30y (124, 29y and 160, 16y) and (C) from two women >50y (335R, 58y and 353P, 72y), histograms are heat-mapped to indicate cells with the phenotypes of K14-/K19+ LEP (green), K14+/K19+ progenitors (yellow), and K14+/K19- MEP (red). (D) Heatmap shows the p-values with Bonferroni-Holm correction from student t-tests of each marker expression of Citrus clusters vs LEP or MEP <30y. (E) Heatmap of marker expression of each immortalized strain, z-score normalized and hierarchically ordered. The fold change of marker expression is ranged from the lowest (black) to the highest (white).

Supplemental Experimental Procedures

Generation of immortal cell lines – Finite lifespan HMEC from specimens 184, 240L, 122L, and 805P were obtained from reduction mammoplasty tissues or peripheral to mastectomy tissues (i.e. 805P). HMEC were grown in M87A supplemented with CT at 0.5 ng/ml, and X (Bachem) at 0.1 nM. Retroviral vectors: The p16 shRNA was in the MSCV vector, c-Myc wsa in the pBabe–hygro (BH2) or LXSN vector. Retroviral stocks were generated from supernatants collected in M87A medium. Strains 240L, 122L, and 805P at passage 3 or and 184 at passage 4 were transduced with MSCV-p16sh or MSCV control and selected with puromycin. At the next passage, after puromycin selection, the p16sh transduced cells were transduced with c-Myc pBabe–hygro (c-myc LXSN for 184) and selected with hygromycin. Vector only control pre-stasis cells entered stasis at passage 12-15, whereas the immortalized lines continued to grow.

Mass cytometry analysis. The age of the strains were not known at the time of the experiment. Cells were analyzed on a CyTOF mass cytometer (DVS Sciences) at an event rate of ~500 cells per second. The settings of the instrument and the initial post-processing parameters were described previously. For each barcoded sample several data files were recorded. The files were concatenated using the Cytobank concatenation tool, normalized and debarcoded.

Flow Cytometry – HMEC at fourth passage were trypsinized and resuspended in their media. For enrichment of progenitor, luminal or myoepithelial lineages, anti -CD133-PE-Vio615 (Myltenyi clone AC133, 1:50) anti -CD117-PE (BioLegend, clone 104D2, 1:200), or anti-CD227-FITC (BD; cloneHMPV;1:50), anti-CD10-phycoerythrin (BioLegend; clone HI10a; 1:100), respectively, were added to the media for 25 minutes on ice, washed in PBS, and sorted using FACS Vantage DIVA (Becton Dickinson).

Matrigel/collagen assay – 24-well plates were coated with 50µL of Matrigel as a bottom layer. To create the matrigel/collagen mixture, 50'000 cells in 70µL of media were mixed with 15µL of neutralization solution (100mM Hepes, pH 7.3 in 2X PBS), and 15µL of collagen solution (Corning 354249, 8.69mg/mL), and 100µL of Matrigel for a final concentration of 0.67mg/mL of collagen I. After 3 weeks, gel smears were fixed in methanol:acetone.

Immunofluorescence – Matrigel smears were fixed in methanol:acetone (1:1) at -20°C for 20 minutes, blocked with PBS, 5% normal goat serum, 0.1% Triton X-100, and incubated with anti-K14 (1:1000, Covance, polyclonal) and anti-K19 (1:10, Developmental Studies Hybridoma Bank, clone Troma-III) overnight at 4°C, then visualized with fluorescent secondary antibodies (Invitrogen) incubated with sections for 2 hours at room temperature. EdU was added to culture media 4h prior to fixing cells, and was imaged with A647 click reagents (Invitrogen). Cells were imaged with LSM510 confocal microscope (Carl Zeiss). Image analyses were conducted using a modified watershed method in Matlab software (Mathworks).

Immunostaining of tissue sections- Healthy breast tissue sections were obtained through University of California Davis in accordance with all IRB procedures. Paraffin-embedded sections were deparaffinized and antigen retrieved (Vector Laboratories) and stained with primary antibodies to K14 (1:1'000; Covance; PRB-155P; visualized with A647 Zenon probes from Invitrogen), and K19 (1:100; Abcam; AAH07628). Cells were imaged with LSM710 confocal microscope (Carl Zeiss).

Classification using morphometric context- Each image was represented as its Cellular Morphometric Context, which was constructed as the histogram of cellular morphometric subtypes derived from the cellular morphometric features (K14/K19 signals) through K-Means (dictionary size =1024). Homogeneous kernel map was then applied on the Cellular Morphometric Context representation, so that linear support vector machine (SVM) could be adopted for efficient and effective differentiation among age groups.

xCELLigence analysis- The lower xCELLigence chambers were filled with M87A media with 10% FBS and the upper chamber were filled with $4x10^5$ cells in serum-free M87 media. Cell Index (CI) and slopes were measured using the RTCA-DP instrument.

R code for Citrus classification-

```
library("citrus")
# Where the data lives
dataDirectory = "C:/Users/Fanny/Documents/Cytof/The ladies/"

# List of files to be clustered= your training set
fileList1 = data.frame(c("30_Ladies01_B10_123.fcs","20_Ladies01_E10_160.fcs", "30_Ladies01_C5_195L.fcs", "30_Ladies01_B7_184.fcs", "60_Ladies01_C3_191L.fcs", "70_Ladies01_F4_29.fcs", "70_Ladies01_E8_122L.fcs","80_Ladies01_F5_429ER.fcs"))

# List of files to be mapped= your entire test set
fileList2 = data.frame(c("20_Ladies01_B11_407P.fcs", "20_Ladies01_C9_399E.fcs", "20_Ladies01_E10_160.fcs", … include all files… "100_Ladies01_B2_805P.fcs")

# Read the data
citrus.combinedFCSSet1 = citrus.readFCSSet(dataDirectory,fileList1,fileSampleSize = 5000)
citrus.combinedFCSSet2 = citrus.readFCSSet(dataDirectory,fileList2,fileSampleSize = 5000)

# List of columns to be used for clustering
clusteringColumns = c(surface markers)

# Cluster first dataset
citrus.clustering = citrus.cluster( citrus.combinedFCSSet1, clusteringColumns, minimumClusterSizePercent = 0.1)

# Map new data to exsting clustering
```

```
citrus.mapping = citrus.mapToClusterSpace(citrus.combinedFCSSet.new = citrus.combinedFCSSet2, citrus.combinedFCSSet.old = citrus.combinedFCSSet1, citrus.clustering)

# Large Enough Clusters
largeEnoughClusters = citrus.selectClusters(citrus.clustering)

# Clustered Features and mapped features
clusteredFeatures = citrus.calculateFeatures(citrus.combinedFCSSet1, clusterAssignments = citrus.clustering$clusterMembership, clusterIds = largeEnoughClusters)
mappedFeatures = citrus.calculateFeatures(citrus.combinedFCSSet2 ,clusterAssignments = citrus.mapping$clusterMembership, clusterIds= largeEnoughClusters)

# Labels
# Labels for training set
labels = as.factor(c("Young","Young","Young","Young","Old","Old","Old","Old"))
trainingLabels = as.factor(c("Young","Young","Young","Young","Old","Old","Old","Old"))
#Labels for test set
testingLabels  = as.factor(c(rep("<30",16),rep(">30<50",13),rep(">50",15)))



# Build Endpoint Model
citrus.endpointModel = citrus.buildEndpointModel(clusteredFeatures, trainingLabels)

# Calculate regularization thresholds
regularizationThresholds = citrus.generateRegularizationThresholds( features= clusteredFeatures, labels=trainingLabels, modelType="pamr",family="classification")

# Calculate CV Error rates
thresholdCVRates = citrus.thresholdCVs.quick( modelType="pamr", features=clusteredFeatures, labels=trainingLabels, regularizationThresholds,family="classification")
```

```
# Get pre-selected CV Minima
cvMinima = citrus.getCVMinima("pamr",thresholdCVRates)

# Predict lables of testing data at CV.1se
predictions = citrus.predict( citrus.endpointModel, newFeatures=mappedFeatures)
[,cvMinima$cv.1se.index]

# Contingency Table of results
table(predictions,testingLabels)
```