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Supplementary Materials for

Cooperative pro-tumorigenic adaptation to oncogenic RAS through epithelial-to-mesenchymal plasticity

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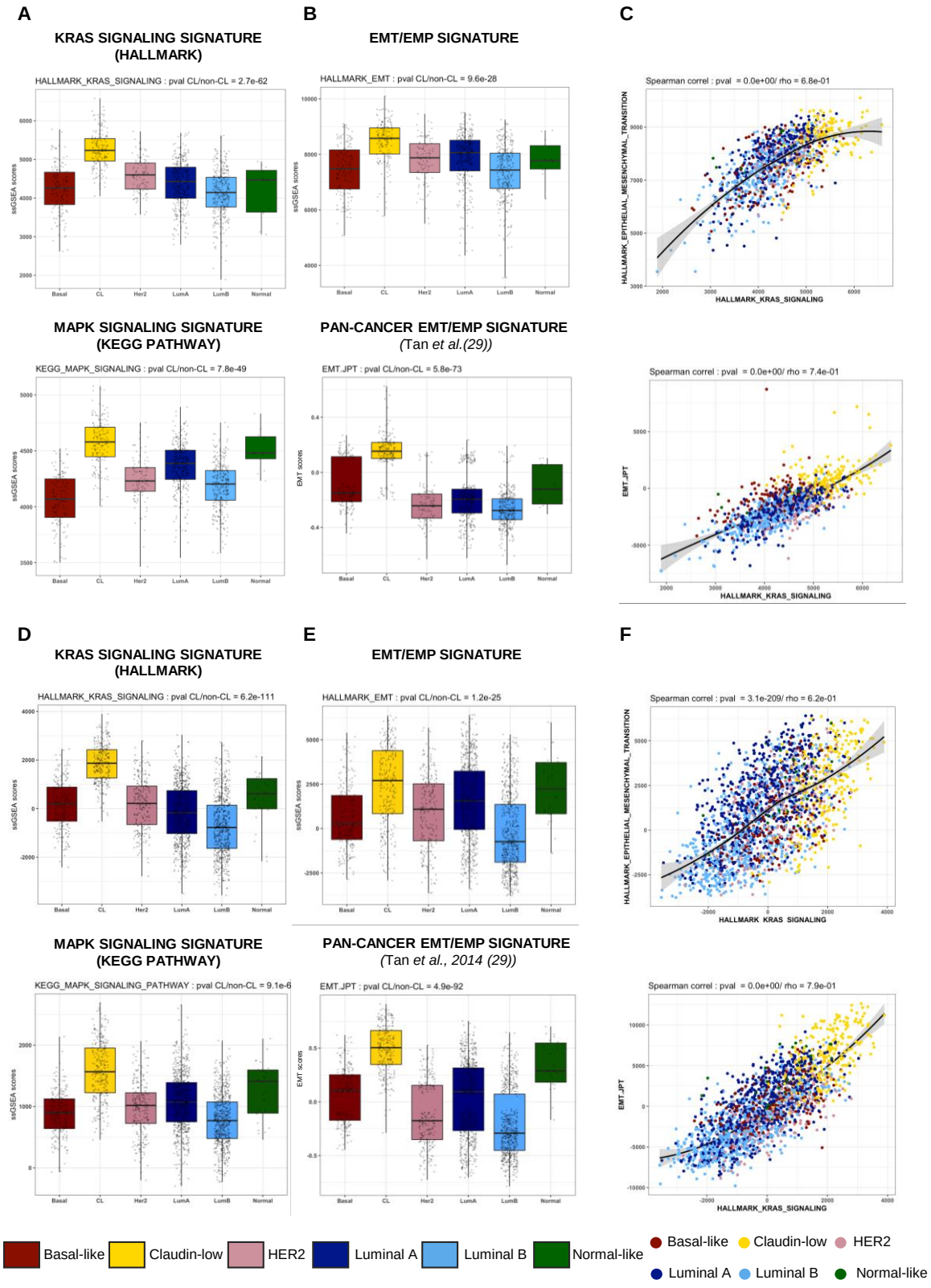
The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

SUPPLEMENTARY FIGURES


A
KRAS SIGNALING SIGNATURE (HALLMARK)
HALLMARK_KRAS_SIGNALING : pval CL/non-CL = 2.7e-62
ssGSEA scores
Basal
CL
Her2
LumA
LumB
Normal
B
EMT/EMP SIGNATURE
HALLMARK_EMT : pval CL/non-CL = 9.6e-28
ssGSEA scores
C
Spearman correl : pval = 0.0e+00/ rho = 6.8e-01
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_KRAS_SIGNALING
MAPK SIGNALING SIGNATURE (KEGG PATHWAY)
KEGG_MAPK_SIGNALING : pval CL/non-CL = 7.8e-49
ssGSEA scores
PAN-CANCER EMT/EMP SIGNATURE
(Tan et al.(29))
EMT.JPT : pval CL/non-CL = 5.8e-73
EMT scores
Spearman correl : pval = 0.0e+00/ rho = 7.4e-01
EMT.JPT
HALLMARK_KRAS_SIGNALING
D
KRAS SIGNALING SIGNATURE (HALLMARK)
HALLMARK_KRAS_SIGNALING : pval CL/non-CL = 6.2e-111
ssGSEA scores
E
EMT/EMP SIGNATURE
HALLMARK_EMT : pval CL/non-CL = 1.2e-25
ssGSEA scores
F
Spearman correl : pval = 3.1e-209/ rho = 6.2e-01
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION
HALLMARK_KRAS_SIGNALING
MAPK SIGNALING SIGNATURE (KEGG PATHWAY)
KEGG_MAPK_SIGNALING_PATHWAY : pval CL/non-CL = 9.1e-6
ssGSEA scores
PAN-CANCER EMT/EMP SIGNATURE
(Tan et al., 2014 (29))
EMT.JPT : pval CL/non-CL = 4.9e-92
EMT scores
Spearman correl : pval = 0.0e+00/ rho = 7.9e-01
EMT.JPT
HALLMARK_KRAS_SIGNALING
Basal-like
Claudin-low
HER2
Luminal A
Luminal B
Normal-like
Basal-like
Claudin-low
HER2
Luminal A
Luminal B
Normal-like

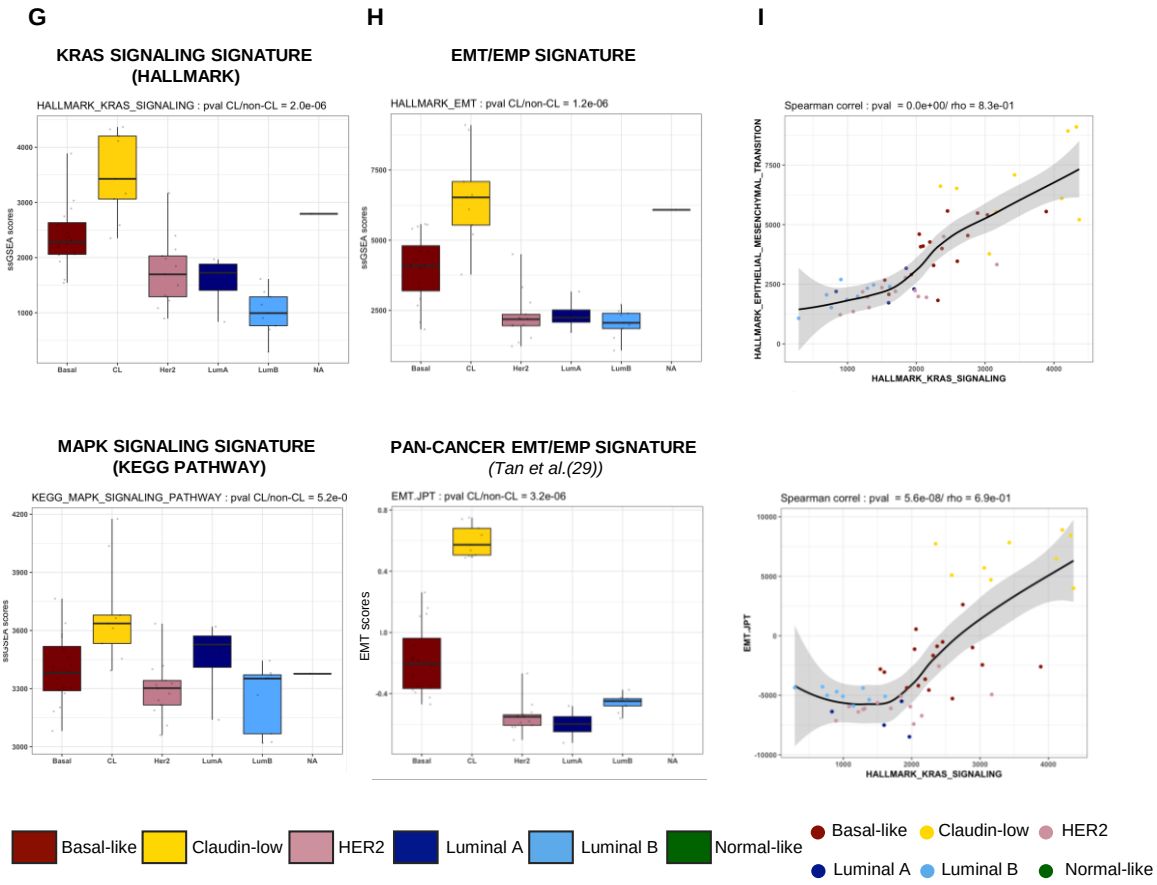


Fig. S1. RAS/MAPK and EMT/EMP pathways are highly correlated and strongly activated in claudin-low breast cancer subtype. (**A** and **B**) Distribution of ssGSEA or EMT scores for each molecular subtype of breast cancer in TCGA cohort, corresponding to (**A**) RAS or MAPK pathway (ssGSEA score) and (**B**) EMT/EMP pathway (ssGSEA or EMT score (*29*)). (**C**) Spearman correlations between KRAS signaling pathway (ssGSEA score) and EMT/EMP pathway (ssGSEA score or EMT score (*29*)) in breast tumors from TCGA dataset. (**D** and **E**) Distribution of ssGSEA or EMT scores for each molecular subtype of breast cancer in METABRIC cohort, related to (**D**) RAS or MAPK pathway (ssGSEA score) and (**E**) EMT/EMP pathway (ssGSEA or EMT score (*29*)). (**F**) Spearman correlations between KRAS signaling pathway (ssGSEA score) and EMT/EMP pathway (ssGSEA or EMT score (*29*)) in breast tumors from the METABRIC dataset. (**G** and **H**) Distribution of ssGSEA or EMT scores for each molecular subtype of breast cancer in CCLE cohort, related to (**G**) RAS or MAPK pathway (ssGSEA score) and (**H**) EMT/EMP pathway (ssGSEA or EMT score (*29*)). (**I**) Spearman correlations between KRAS signaling pathway (ssGSEA score) and EMT/EMP pathway (ssGSEA or EMT score (*29*)) in breast tumors from the CCLE dataset.

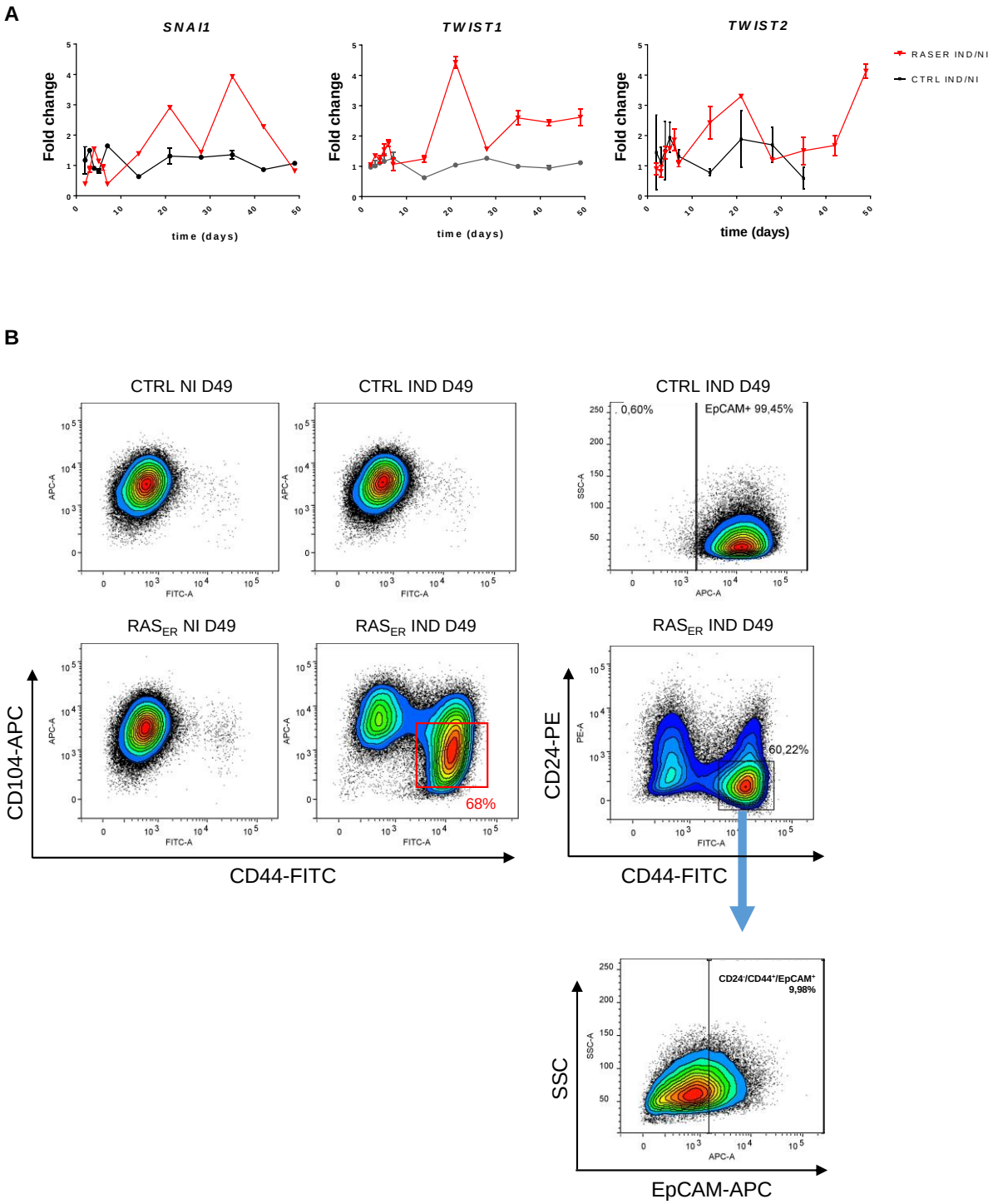


Fig. S2. RAS-activation in HME-RAS$_{ER}$ cells induces expression of EMT-TFs and a shift in CD24/CD44/CD104/EpCAM phenotype. (**A**) Fold change in expression of mRNA across time for EMT-TFs *SNAI1*, *TWIST1*, and *TWIST2* in HME-RAS$_{ER}$ or HME-CTRL cells induced with 4-OHT (IND) or not (NI). Median± range (*n*=2 independent experiments in duplicate); (**B**) Representative flow cytometry analysis of CD24, CD44, CD104, and EpCAM markers in HME-RAS$_{ER}$ (RAS$_{ER}$) and HME-CTRL (CTRL) cells after 49 days of 4-OHT treatment (IND) or not (NI).

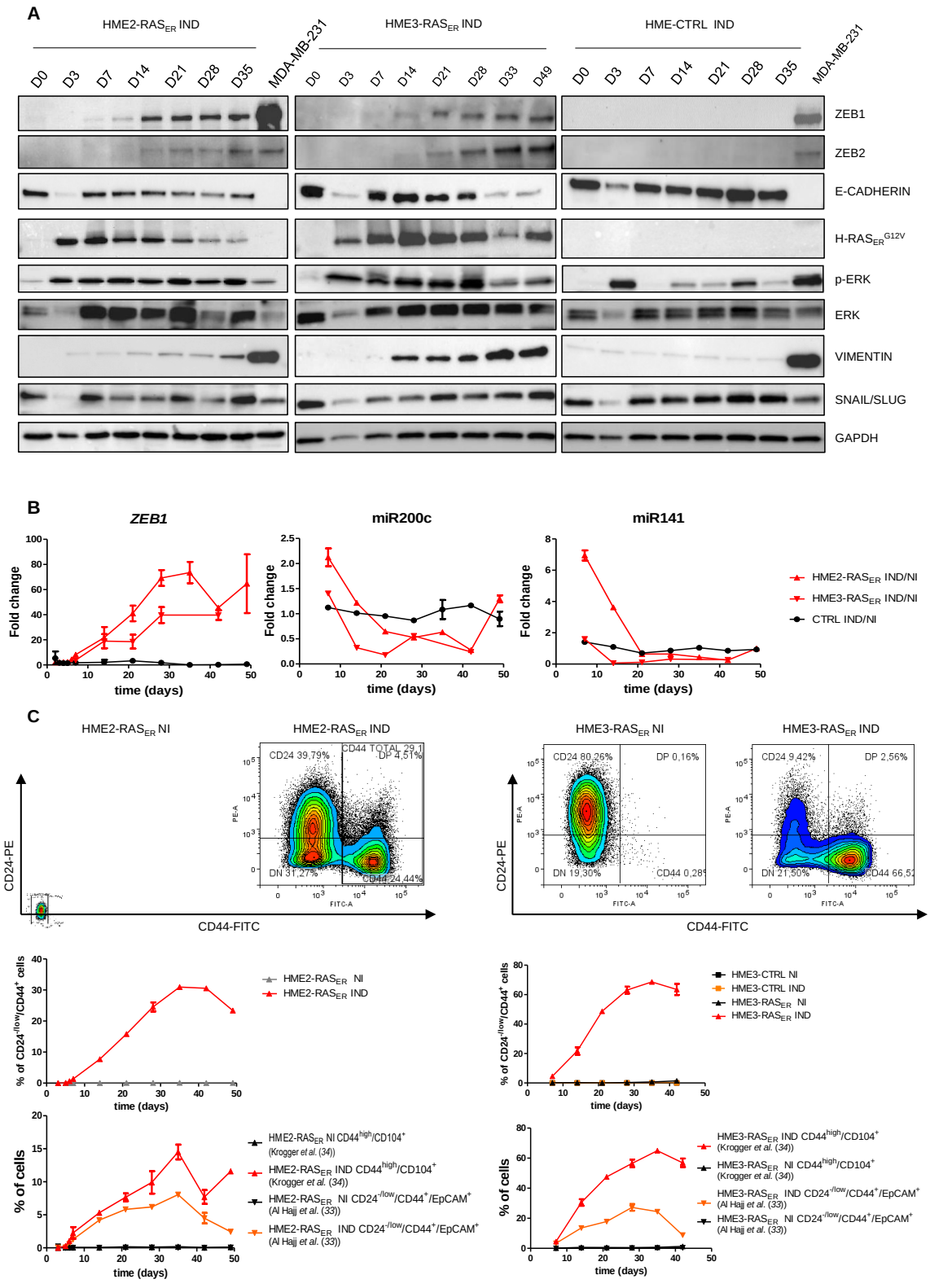


Fig. S3. RAS-activation in HME2-RAS$_{ER}$ and HME3-RAS$_{ER}$ cells induces expression of EMT-TFs and a shift in CD24/CD44/CD104/EpCAM phenotype. (**A**) Immunoblot showing the expression of HRAS$_{ER}^{G12V}$, pERK1/2, ERK, ZEB1, ZEB2, SNAIL/SLUG, E-CADHERIN, and VIMENTIN in HME2-RAS$_{ER}$ cells, HME3-RAS$_{ER}$ cells, and HME-CTRL cells at indicated time points after 4-OHT treatment. MDA-MB 231 cell line was used as a positive

control for ZEB1, ZEB2, and SNAIL/SLUG expression. GAPDH level was used as a loading control. (**B**) Fold change in expression across time of *ZEB1* mRNA, miR200C and miR141 in HME2-RAS_{ER} and HME3-RAS_{ER} cells or in HME-CTRL cells induced with 4-OHT (IND) or not (NI). Median± range (*n*=2 independent experiments). (**C**) Representative FACS analysis of CD24, CD44, CD104, and EpCAM markers in HME2-RAS_{ER} and HME3-RAS_{ER} cells at D28 of induction with 4-OHT (IND) or not (NI). Kinetics analysis and quantification across time of the percentage of $CD24^{-/low}/CD44^{+}$ cells, $CD24^{-/low}/CD44^{+}$ /$EpCAM^{+}$ cells, and $CD44^{+}/CD104^{+}$ cells after 4-OHT treatment. Median± range (*n*=2 independent experiments).

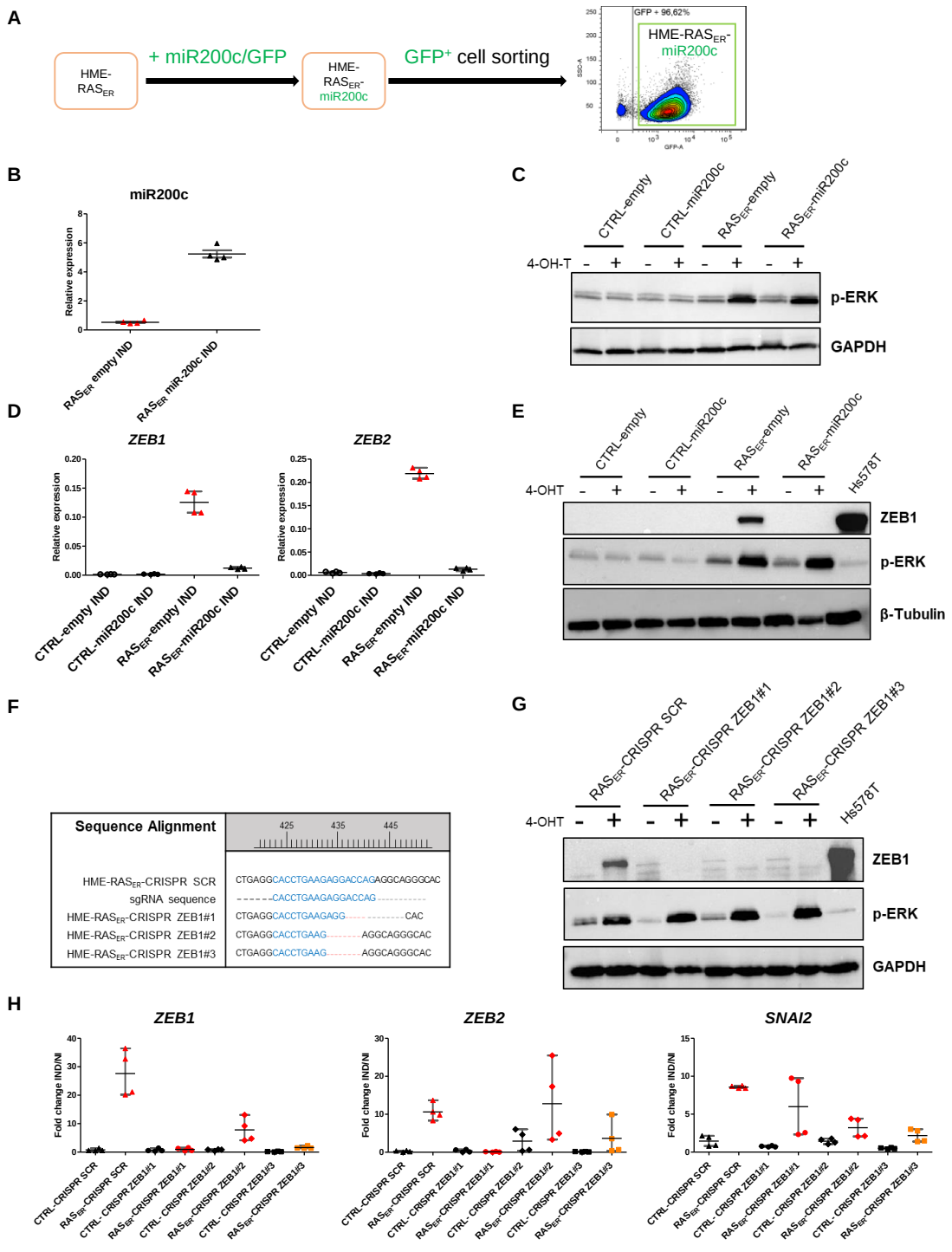


Fig. S4. Cellular plasticity and transformation capabilities after RAS activation are ZEB1-dependent. (**A**) Experimental outline. miR200c expression was associated with GFP expression. Cell sorting based on GFP^{high} expression in HME-RAS$_{ER}$ miR200c cells and HME-RAS$_{ER}$-empty cells. (**B**) miR200c expression in HME-RAS$_{ER}$-miR200c or HME-RAS$_{ER}$-empty cells induced with 4-OHT (IND). Median± range (*n*=4 independent experiments). (**C**) Immunoblotting of pERK in HME-RAS$_{ER}$-miR200c or in HME-RAS$_{ER}$-empty cells treated with 4-OHT (+) or not (-) and analyzed at D4. GAPDH level was used as a loading control. (**D**) *ZEB1* and *ZEB2* mRNA expression in HME-RAS$_{ER}$-miR200c or in HME-RAS$_{ER}$-empty cells

after 28 days of 4-OHT treatment (IND). Median±range (*n=4* independent experiments). **(E**) Immunoblotting of pERK and ZEB1 in HME-RAS_{ER}-miR200c or in HME-RAS_{ER}-empty cells treated with 4-OHT (+) or not (-) and analyzed at D28. β-Tubulin level was used as a loading control. Hs578T cell line was used as a positive control for ZEB1 expression. (**F**) Genomic DNA sequencing by Sanger method for assessing HME-RAS_{ER}-CRISPR ZEB1. Sequence alignment of a CRISPR SCR clone and three CRISPR ZEB1 KO clones (clones #1, #2 and #3). (**G**) Immunoblotting of pERK and ZEB1 in the three HME-RAS_{ER}-CRISPR ZEB1 clones (#1, #2 and #3) or in HME-RAS_{ER}-CRISPR SCR induced with 4-OHT (+) or not (-) and analyzed at D28. GAPDH level was used as a loading control. Hs578T cell line was used as a positive control for ZEB1 expression. (**H**) Fold change in expression (induced/not induced) of *ZEB1*, *ZEB2*, and *SNAI2* mRNA in the three HME-RAS_{ER}-CRISPR ZEB1 clones (#1, #2 and #3) or in HME-RAS_{ER}-CRISPR SCR induced with 4-OHT or not for 28 days. Relative expression was determined by the ΔΔCt method, normalized to *HPRT1* expression and divided by the expression of the untreated sample at the same time point. Median±range (*n=4* independent experiments).

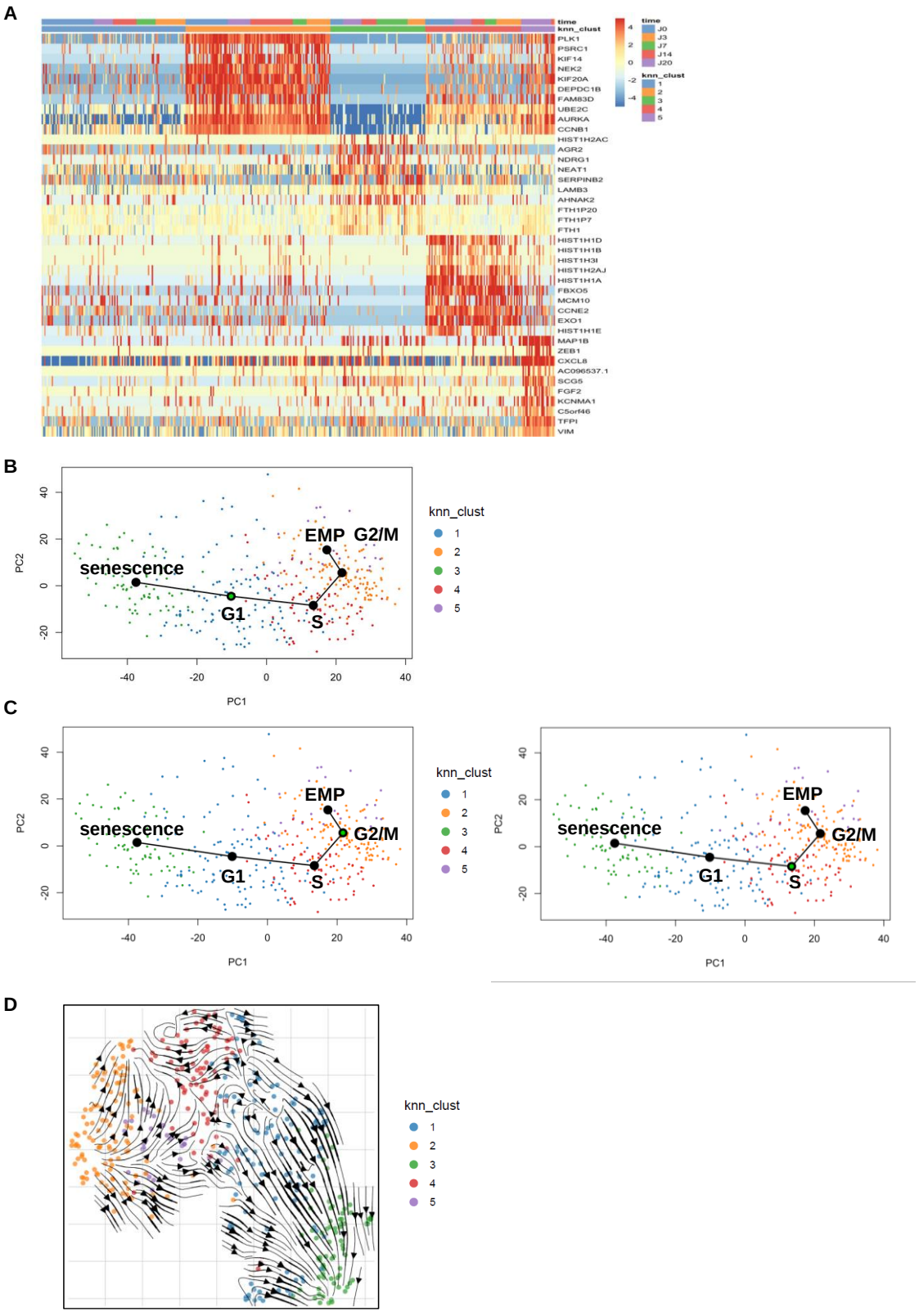
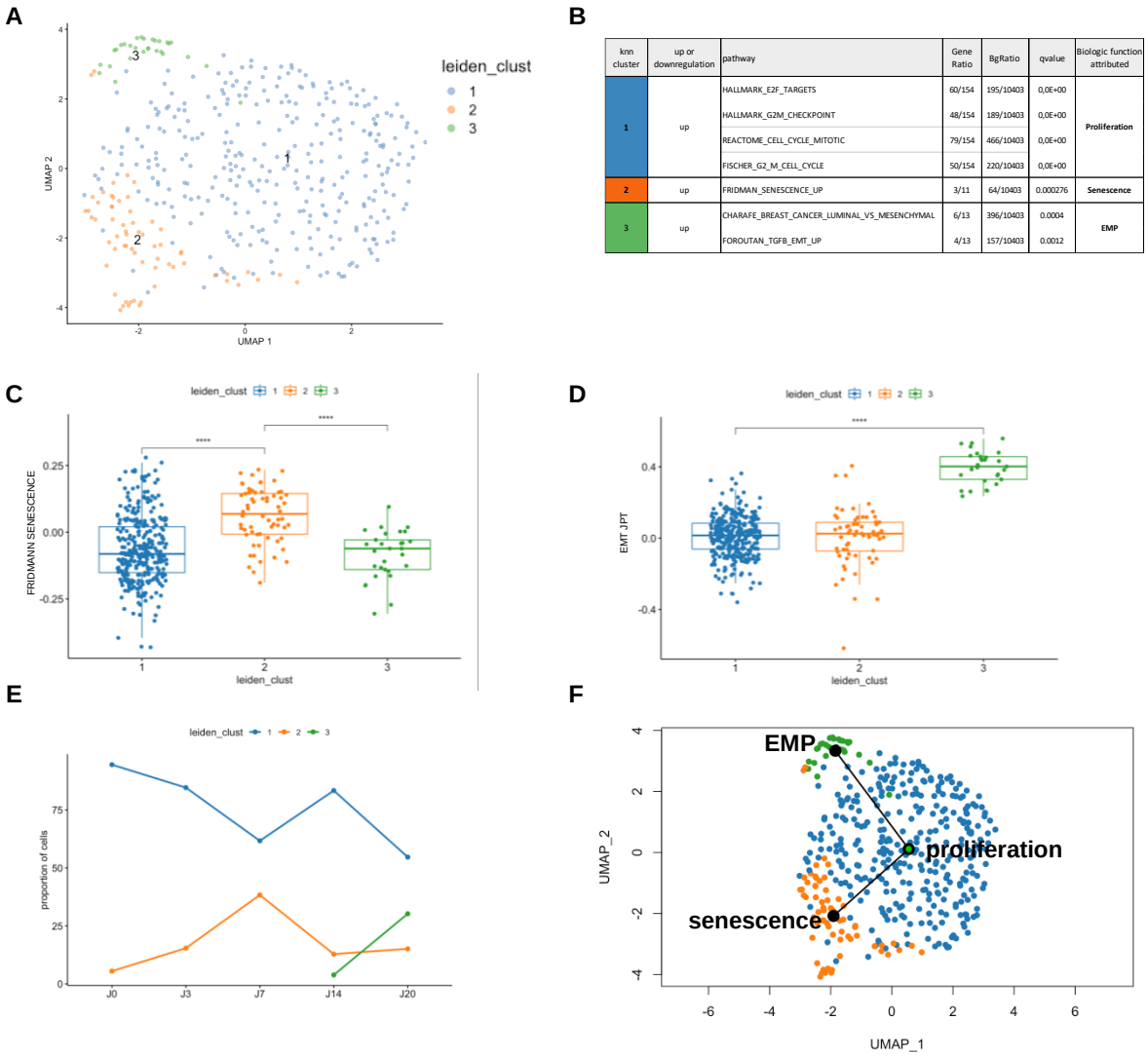


Fig. S5. Identification of distinct senescence and EMP clusters in induced HME-RAS$_{ER}$. Single-cell RNAseq analysis related to Fig.3 (**A**) Heatmap representing the expression of the ten most up-regulated genes of each cluster compared to all other clusters. (**B** and **C**) Projection of the trajectory analysis performed by the Slingshot algorithm along the first two axes of an

unsupervised Principal Component Analysis (PCA) of all cells. Cells are colored by clusters. (**B)** Starting point of the trajectory analysis is cluster 1 (green circle), (**C**) Left panel: Starting point of the trajectory analysis is cluster 2 (green circle); Right panel: Starting point of the trajectory analysis is cluster 4 (green circle). (**D**) Cell fate deciphered by RNA velocity analysis projected onto the computed UMAP. Cells are colored by cluster. Arrows represent the probable next state of each cell based as resulted by scVelo.



knn cluster	up or downregulation	pathway	Gene Ratio	BgRatio	qvalue	Biologic function attributed
1	up	HALLMARK_E2F_TARGETS	60/154	195/10403	0,0E+00	Proliferation
		HALLMARK_G2M_CHECKPOINT	48/154	189/10403	0,0E+00	
		REACTOME_CELL_CYCLE_MITOTIC	79/154	466/10403	0,0E+00	
		FISCHER_G2_M_CELL_CYCLE	50/154	220/10403	0,0E+00	
2	up	FRIDMAN_SENESCENCE_UP	3/11	64/10403	0.000276	Senescence
3	up	CHARAFE_BREAST_CANCER_LUMINAL_VS_MESENCHYMAL	6/13	396/10403	0.0004	EMP
		FOROUTAN_TGFB_EMT_UP	4/13	157/10403	0.0012	

Fig. S6. Identification of distinct senescence and EMP clusters in induced HME-RAS$_{ER}$. Single-cell RNAseq analysis with partial cell-cycle regression. (**A**) Unsupervised UMAP of the transcriptome of all cells at all time points (D0, D3, D7, D14 and D20). Cells are colored by their attributed cluster. (**B**) Main altered pathways by marker genes for each cluster. Gene ratio presented as k/n, where k is the size of the overlap of our input with the specific gene set and n is the size of the overlap of our input with all members of the collection of gene sets. qvalue refers to false discovery rate. (**C** and **D**) Scores per cell for two transcriptomic pathways: FRIDMAN SENESCENCE (ssgsea score) and EMT JPT (EMT cell line score from (*29*)). Cells are grouped by clusters, each box representing the median and interquartile ranges. Individual Wilcoxon tests, p-value is represented by stars (**p<=0.01 and ****p<=0.0001). (**E**) Proportion of cells by cluster at each time point. Emerging clusters across time were colored (cluster 1 in blue, cluster 2 in orange, and cluster 3 in green). (**F**) Projection of the trajectory analysis performed by the Slingshot algorithm along the first two axes of an unsupervised PCA of all cells. Cells are colored by clusters. Starting point of the trajectory analysis is cluster 1 (green circle).

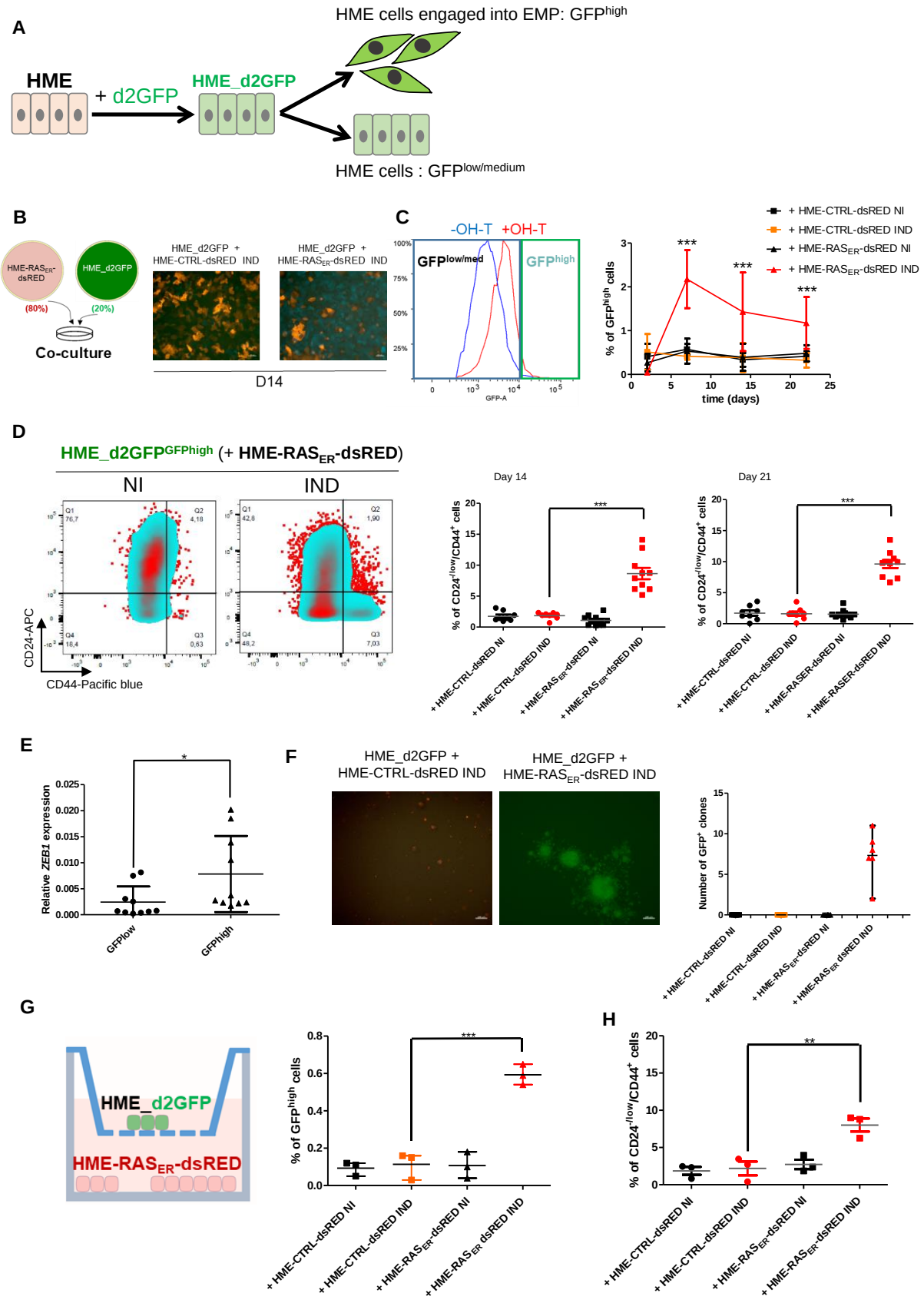


Fig. S7. ZEB1-dependent cellular plasticity is driven by a paracrine mechanism. (**A**) Experimental outline of HME_d2GFP generation and expected read out; (**B-E**) Co-culture

assays. (**B**) Experimental outline and representative fluorescence images of co-culture assay. (**C**) Representative flow cytometry analysis of GFP expression in HME_d2GFP cells co-cultured with HME-RAS_{ER}-dsRED (+HME-RAS_{ER}-dsRED) cells after 14 days of induction with 4-OHT (IND) or not (NI) and quantification of GFP^{high} population across time. Median±range (*n=8* independent experiments) (Scale bars, 50 µm). (**D**) Representative flow cytometry analysis of CD24 and CD44 markers in HME_d2$GFP^{GFPhigh}$ cells co-cultured with HME-RAS_{ER}-dsRED or HME-CTRL-dsRED cells, induced with 4-OHT (IND) or not (NI) for 21 days and quantification of $CD24^{-/low}/CD44^{+}$ population from HME_d2$GFP^{GFPhigh}$ cells at D14 and D21 (*n=8* independent experiments). (**E**) *ZEB1* expression in HME-d2GFP cells co-cultured with HME-RAS_{ER}-dsRED cells after induction with 4-OHT and sorted at D14 based on their GFP expression. Median±range (*n*=5 independent experiments in duplicate). (**F**) Transformation potential analysis as assessed by a soft agar colony formation assay of HME_d2GFP cells co-cultured with HME-RAS_{ER}-dsRED (+HME-RAS_{ER}-dsRED) or HME-CTRL-dsRED (+HME-CTRL-dsRED) cells, induced with 4-OHT (IND) or not (NI) for 21 days. Representative images and quantification of GFP-positive colonies (as defined by > 20 cells). Median±range (*n*=6 independent experiments) (Scale bars, 200 µm). (**G** and **H**) Transwell culture assays. (**G**) Quantification of GFP^{high} population from HME_d2GFP cells co-cultured with HME-RAS_{ER}-dsRED (+HME-RAS_{ER}-dsRED) or HME-CTRL-dsRED (+HME-CTRL-dsRED) cells, induced with 4-OHT (IND) or not (NI) for 21 days. Median±range (*n*=3 independent experiments). (**H**) Quantification of $CD24^{-/low}/CD44^{+}$ population from HME_d2$GFP^{GFPhigh}$ cells co-cultured with HME-RAS_{ER}-dsRED (+HME-RAS_{ER}-dsRED) or HME-CTRL-dsRED (+HME-CTRL-dsRED) cells, induced with 4-OHT (IND) or not (NI) for 21 days. Median±range (*n*=3 independent experiments). P-values are calculated by one-way ANOVA, Tukey multiple comparison test (****p<=0.001), or Student's t-test (*<=0.1) as appropriate.

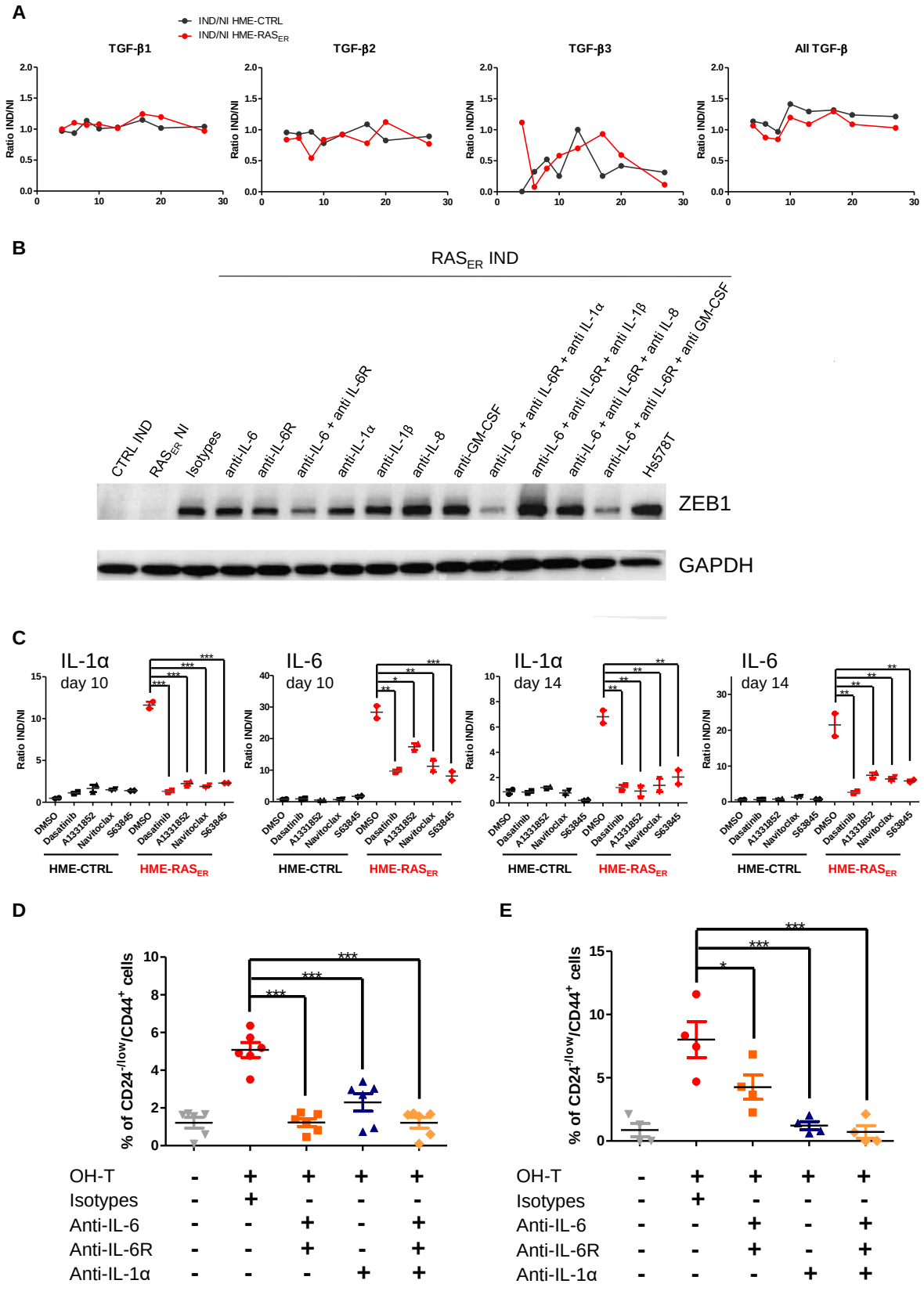


Fig. S8. ZEB1-dependent plasticity is driven by cytokines IL-6 and IL-1α secreted by senescent cells. (**A**) Quantification of TGF-β ligands levels in HME-RAS$_{ER}$ supernatants following 4-OHT treatment compared to controls at D4, D6, D8, D10, D13, D17, D20, and

D27. Data are presented as fold change of secreted TGF-β ligands concentrations in HME-RAS_{ER} or HME-CTRL cells induced by 4-OHT (IND) or not (NI). (**B**) Immunoblotting of ZEB1 expression in HME-CTRL (CTRL) or HME-RAS_{ER} (RAS_{ER}) cells induced by 4-OHT (IND) or not (NI), treated with neutralizing antibodies or isotype controls and analyzed at D28. Hs578T cell line was used as a positive control for ZEB expression. Data is representative of three independent experiments (*n*=3 independent experiments). (**C**) Quantification of IL-1α and IL-6 levels in HME-RAS_{ER} or HME-CTRL supernatants following 4-OHT treatment and treatment with senolytic drugs or the equivalent concentration of DMSO at D10 and D14. Data are presented as fold change of secreted cytokine concentrations in HME-RAS_{ER} or HME-CTRL cells induced by 4-OHT (IND) or not (NI) in each treatment condition. Median±range (*n*=2 independent experiments). (**D**) Quantification of $CD24^{-/low}/CD44^{+}$ population from HME-$d2GFP^{GFPhigh}$ cells co-cultured with HME-RAS_{ER}-dsRED cells induced with 4-OHT (+) or not (-), treated with indicated neutralizing antibodies or isotype controls and analyzed at D20. Median±range (*n*=6 independent experiments). (**E**) Quantification of $CD24^{-/low}$/CD44+ population from HME-$d2GFP^{GFPhigh}$ cells cultured in transwell plate with HME-RAS_{ER}-dsRED cells induced with 4-OHT (+) or not (-), treated with indicated neutralizing antibodies or isotype controls and analyzed at D20. Median±range (*n*=4 independent experiments). P-values are calculated by one-way ANOVA, Tukey multiple comparison test (*p <= 0.05, **p <= 0.01 and ***p <= 0.001).

SUPPLEMENTARY TABLES

Table S1: Cluster 1, downregulated genes (refers to Fig. 3)

Table S2: Cluster 2, up regulated genes (refers to Fig. 3)

Table S3: Cluster 3, down- and upregulated genes (refers to Fig. 3)

Table S4: Cluster 4, down- and upregulated genes (refers to Fig. 3)

Table S5: Cluster 5, down- and upregulated genes (refers to Fig. 3)

Table S6: Cluster 1, upregulated genes (refers to Fig. S6)

Table S7: Cluster 2, upregulated genes (refers to Fig. S6)

Table S8: Cluster 3, upregulated genes (refers to Fig. S6)