Cancer cell – fibroblast crosstalk via HB-EGF/EGFR/MEK signalling promotes macrophage recruitment in squamous cell carcinoma

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Summary

Interactions between cells in the tumor microenvironment (TME) shape cancer progression and patient outcomes. To gain new insights into how the TME influences cancer outcomes, we derive gene expression signatures indicative of signalling between stromal fibroblasts and cancer cells, and demonstrate their prognostic significance in multiple and independent squamous cell carcinoma cohorts. We discover that the HB-EGF/EGFR axis represents a hub of tumor – stroma crosstalk, promoting the expression of CSF2 and LIF and favouring the recruitment of macrophages. Together these analyses demonstrate the utility of our approach for interrogating the extent and consequences of TME crosstalk. By focusing on the transcriptional consequences of cancer cell-fibroblast interactions we derive prognostic signatures and uncover molecular mechanisms promoting fibroblast to macrophage communication.

Keywords

Fibroblasts, Cancer-associated fibroblasts, CAFs, EGFR, HB-EGF, stroma, Macrophages, Immune Microenvironment, gene signature, RAS

48 ## Abbreviations

49 Activator protein 1 (AP-1)
50 Cancer associated fibroblasts (CAF)
51 Cervical squamous cell carcinoma (CESC)
52 Clear cell Renal Cell Carcinoma (ccRCC)
53 Chemokine (C-C motif) ligand (CCL)
54 Confidence Interval (CI)
55 Conditioned medium (CM)
56 False discovery rate (FDR)
57 Fibroblast growth factor (FGF)
58 Gene-set enrichment analysis (GSEA)
59 Hazard ratio (HR)
60 Head and neck squamous cell carcinoma (HNSCC)
61 Heparin-binding epidermal growth factor-like growth factor (HB-EGF)
62 Human papillomavirus (HPV)
63 Interleukin (IL)
64 Lung squamous cell carcinoma (LUSC)
65 Overall Survival (OS)
66 Mean fluorescent intensity (MFI)
67 Normalized enrichment score (NES)
68 Non-treated (NT)
69 Pancreatic ductal adenocarcinoma (PDAC)
70 Peripheral blood mononuclear cell (PBMC)
71 Platelet derived growth factor (PDGF)
72 Single-cell RNA sequencing (scRNAseq)
73 Standard deviation (SD)
74 Short tandem repeats (STR)
75 Squamous cell carcinoma (SCC)
76 The Cancer Genome Atlas (TCGA)
77 Transcription factor (TF)
78 Transforming Growth Factor (TGF)
79 Tumor microenvironment (TME)
80 Tumor necrosis factor (TNF)

Introduction

Cross-talk between cancer cells and non-malignant cells in the tumor microenvironment (TME) influences tumor growth, metastasis and therapy resistance through multiple signaling pathways and feedback mechanisms such as growth factors (TGFβ, PDGF, FGF), contact molecules (Notch, Ephrins), and inflammatory molecules (IL1, IL6, CCL12/CXCR4) [1]. Cancer-associated fibroblasts (CAFs) promote the invasion of cancer cells, reduce the efficacy of both targeted and cytotoxic therapies and modulate immune cell recruitment and functionality [2]. Crosstalk between cancer cells and CAFs have been demonstrated via in multiple tumors via, like oncogenic KRAS in colorectal cancer [3], EGFR in pancreatic ductal adenocarcinoma (PDAC) [4]. In addition, CAFs are correlated with a pro-tumorigenic immune landscape, including higher number of tumor-promoting myeloid cells [5], lower numbers of tumor-infiltrating lymphocytes [6] and worse prognosis [7] [8]. Of note, CAFs are linked to poor outcomes in squamous cell carcinoma (SCC) arising at multiple anatomical locations, including lungs (LUSC), cervix (CESC), and head and neck (HNSCC) [8–11]. Together, these different SCC account for over 800,000 deaths per year, highlighting the need for better understanding of the disease, new therapeutic strategies, and improved tools for clinical decision making[12].

Indeed, the development of advanced sequencing techniques allows multiple inferences about the type and abundance of different TME components, including CAFs, both from bulk transcriptome and genomic methylation data [9,13–16] . Both methods rely on the identification of cell type specific genes and the application of deconvolution strategies ultimately to infer the abundance of a particular population in a bulk dataset. However, these methods struggle to identify the functionally relevant interactions between cell types, such as signaling events [17,18] and the biological mechanisms associated with cell type crosstalk to be linked to patient outcomes remain incompletely understood.

Here, we propose an alternative approach to identify key players involved in tumor-stroma interaction. Instead of focusing on the abundance of CAFs or specific CAF subpopulations, we identify a signature indicative of signaling between cancer cells and CAFs. This signature is associated with worse overall survival in multiple types of SCC, pancreatic cancer, and kidney cancer. Moreover, we leverage information within the signature to identify a novel mechanism of interaction between cancer cells and CAFs. In co-culture, the RAS / MAPK pathway is strongly activated in both cell types, converging on the upregulation of Activator Protein 1 (AP-1) transcription factor (TF) components. We identify heparin-binding epidermal growth factor-like growth factor (HB-EGF) as a key mediator of cancer cell – CAF cross-talk, primarily expressed by cancer cells and able to upregulate the expression

114 cytokines through cross-talk with CAFs. In turn, we demonstrate that this upregulation can drive
115 attraction of macrophages, ultimately linked to worse overall survival in SCC patients (Figure S1A).

116 ## Results

117 ### Meta-analysis of transcriptomic data of cancer cell and cancer-associated fibroblast co-
118 cultures identifies gene signatures with prognostic value

119 To identify functionally and clinically relevant gene signatures based on cancer cell – CAF cross-talk,
120 we performed a meta-analysis of transcriptomic datasets that compare co-cultures and mono-cultures
121 of cancer cells and CAFs. The datasets were generated under similar direct co-culture conditions using
122 cells derived from different cancer types [19,20]. We applied two strategies to derive gene signatures
123 indicative of upregulated cancer cell – CAF signaling: i) selection of the most significantly enriched
124 pathways via gene set enrichment analysis (GSEA) in co-culture for each transcriptomic dataset,
125 followed by the selection of the up-regulated genes most frequently present in each enriched pathway
126 (Figure 1A); ii) selection of the most up-regulated genes in co-culture for each transcriptomic dataset
127 (Figure S1B). Using the first approach, we obtained a list of 5 genes upregulated in cancer cells and of
128 4 genes upregulated in CAFs upon co-culture, with one present in both. Therefore, this gene signature
129 comprised of 8 genes (named CoCu8) (Figure 1B). The second approach led to a list of 2 genes
130 upregulated in cancer cells and of 29 genes upregulated in CAFs upon co-culture, with one gene in
131 common. Therefore, this gene signature consisted of 30 genes (named CoCu30) (Figure S1C).
132
133 We tested CoCu8 and CoCu30 on publicly available dataset of breast cancer – fibroblast co-cultures[21]
134 confirming their relevance (Figure S1D). We also tested whether CoCu8 and CoCu30 are also
135 upregulated when cancer cells are co-cultured with other stromal cell types and for this reason, we
136 analyzed a dataset of co-culture between 1205Lu cancer cells and HUVEC endothelial cells[22]: CoCu8 is
137 neither enriched in cancer cells nor in endothelial cells when co-cultured, while CoCu30 shows only a
138 weak correlation with co-culture conditions both in 1205Lu and HUVEC cells (Figure S1E). Thus, we
139 establish new gene signatures specifically indicative of cancer cell - CAF communication.
140
141 We next sought to determine the clinical relevance of these signatures by testing the effect on patient
142 survival for the most frequent cancer types in The Cancer Genome Atlas (TCGA). We found that high
143 expression (Q4 vs Q1) of both gene signatures correlated with worse overall survival (OS) in most of
144 the tested datasets (Figure S2A). Among them, all tested SCC datasets presented the largest effect:
145 cervical squamous cell carcinoma (CESC, CoCu8 HR:2.79, CoCu30 HR: 2.08), HNSCC (CoCu8 Hazard
146 Ratio (HR): 1.95, CoCu30 HR: 1.57) and lung squamous cell carcinoma (LUSC, CoCu8 HR: 1.85, CoCu30
147 HR: 1.78) (Figure 1C; Figure S2B), with CoCu8 consistently showing a slightly higher hazard ratio (HR)
148 compared to CoCu30 in all three tumor types. A multivariate analysis including relevant clinical
149 variables such as age, sex and clinical stage to evaluate the co-culture signatures effect as a continuous

150 variable, confirmed the relevance of the signatures in these tumor types (Figure S3 for CoCu8, Figure
151 S4 for CoCu30,). Our signature was also associated with worse survival in pancreatic and clear cell
152 renal cell carcinoma (ccRCC) (Figure S2A). No significant link to outcome was observed in lung breast,
153 colorectal, bladder, or prostate cancer. Of note, given the clinical and biological differences between
154 HPV positive and negative tumors, which warrant a different staging classification and treatment
155 indications [23], we stratified HNSCC patients according to HPV status, observing that the strongest
156 prognostic effect was visible in HPV positive samples both for CoCu8 (Figure 1D) and CoCu30 (HR:
157 5.47) (Figure S2C).
158
159 We validated the association between CoCu8 / CoCu30 and outcome in a second independent cohort
160 of LUSC from the TRACERx study [24] (Figure 1E, Figure S2D). The multi-regional biopsies performed in
161 the study enabled us to ask if the expression of the CoCu8 signature was uniform across tumors. Of
162 the 117 tumors analyzed, 86 (74%) showed concordant expression of CoCu8 in all regions, which is
163 significantly greater than would be expected based on chance (Figure 1E). Similar results were
164 observed with CoCu30 (Figure S2D). This indicates that cancer cell-fibroblast crosstalk is typically
165 occurring across the whole tumor. Crucially, this analysis showed that the concordant up-regulation
166 of CoCu8 or CoCu30 across tumor regions is associated with worse prognosis. Overall, these data
167 indicate that CoCu8 and CoCu30 signatures are associated with worse overall survival in all SCC
168 datasets tested, therefore we decided to focus our attention on the effect of this crosstalk signature
169 in SCC.
170

171 ## The crosstalk gene signature has greater prognostic power than fibroblast abundance

172 Given that CoCu8 / CoCu30 reflects cancer cell-fibroblast crosstalk, the signature might be predicted
173 to correlate with fibroblast abundance. We performed a correlation analysis of CoCu8 / CoCu30 and
174 methyl CIBERSORT signatures in TCGA datasets, showing that there is a statistically significant
175 correlation between CAFs presence and CoCu8 signature (Figure S5A-B). Similar results were observed
176 with the CoCu30 signature (Figure S5C-D). Then, we sought to validate these results in the TRACERx
177 LUSC cohort and in a second independent UK_HPV positive cohort (Figure S5E-J). As methylome data
178 was not available for these cohorts, we used fibroblast subtype gene signatures defined in a pan-
179 cancer analysis by Galbo et al.[9]. We observed strong positive correlations between CoCu8 / CoCu30
180 signature and all of the fibroblast subtypes defined both in LUSC and UK_HPV positive HNSCC (Figure
181 S5E-J).
182

183 We speculated that our signatures of active cancer cell-fibroblast crosstalk might have better
184 prognostic power than simply CAF abundance. To test this, we analyzed the abundance of CAFs using
185 the methyl CIBERSORT deconvolution strategy in TCGA cohorts and probed links with overall survival[13].
186 This analysis indicated worse OS for HNSCC patients with higher CAF presence, but no significant
187 differences were observed in CESC or LUSC (Figure S6A-B). Of note, both CoCu8 and CoCu30 signatures
188 out-performed the methyl CIBERSORT method for HNSCC, CESC, and LUSC, which adds credence to
189 our method of deriving a signature based on the interaction with fibroblasts, not simply their
190 abundance. Overall, these data indicate that CoCu8 / CoCu30 signatures correlates with CAF
191 abundance but have greater prognostic value than gene signatures used to infer CAF abundance.
192

193 Pathway enrichment analysis of cancer-associated fibroblast and cancer cell co-culture
194 reveals a consistent upregulation of AP-1 transcription factor genes

195 To obtain insight in the molecular basis of the cancer cell-CAF interactions, we analyzed the pathways
196 that were enriched in all the transcriptomic datasets used to generate CoCu8 and CoCu30. This
197 revealed upregulation of multiple pathways linked to immune regulation, stress response, and
198 signaling (Figure 2A). Multiple genes belonging to the AP-1 transcription factor complex: *JUNB*, *FOS*,
199 *FOSB* were strongly enriched (Figure 2B). Moreover, *PLAUR* is regulated by AP-1 factors. As our meta-
200 analysis showed the strongest impact in the stratification of OS patients from HPV positive HNSCC, we
201 decided to explore *JUNB*, *FOS*, *FOSB* expression levels in 4 different co-culture combinations of human
202 HPV positive HNSCC cell lines, SCC154 and SCC47, and human oral CAFs, OCAF1 and OCAF2. Our results
203 confirmed that these three AP-1 TFs are upregulated when cancer cells and CAFs are in direct co-
204 culture, as compared to mono-culture (pooled RNA from both cell lines) (Figure 2C). Analysis of
205 indirect co-cultures [20] separated by a 0.4µm filter indicated that CoCu8 / CoCu30 are strongly enriched
206 with direct co-culture also when compared with indirect co-culture, implying that direct contact is
207 required for increased AP-1 TF expression (Figure S7A).
208

209 Interaction between cancer cells and cancer-associated fibroblasts is linked to increased RAS
210 activity

211 We next investigated possible mechanisms underlying the upregulation of the multifunctional AP-1
212 TFs both in cancer cells and CAFs[25]. RAS signaling via MAPK is known to be a major driver of AP-1 gene
213 expression[26,27]. Accordingly, we found that RAS signaling was strongly up-regulated upon direct co-
214 culture, as indicated by the enrichment of the KRAS_SIGNALLING_UP signature (Figure 2A) and of the
215 curated RAS84 gene signature [28], in all our cancer cell-CAF datasets (Figure 3A, Figure S7B).
216

217 To interrogate further the linkage between CAFs, the CoCu8 signature, and RAS signalling in HPV
218 positive patients from the TCGA cohort, we stratified them according to RAS activity. We split the
219 patient data into three groups (RAS84_0, RAS84_1 and RAS84max) according to the levels of RAS
220 activity as performed by East et al.[28]. This analysis shows that higher RAS activity (group RAS84_max)
221 correlates with worse OS, in a similar fashion to the effect observed with CoCu8 stratification (Figure
222 3B). Indeed, we observed a strong, positive correlation between RAS84 activity and CoCu8 expression
223 in both HPV positive HNSCC cohorts (TCGA - R = 0.79, Figure 3C and UK_HPV positive cohort - R = 0.84,
224 Figure S8A). We also observed a statistically significant enrichment in the extent of fibroblasts present
225 when RAS activity was higher in both cohorts (Figure 3D, Figure S8B).
226
227 Activation of the RAS pathway upon cancer cell-CAF co-culture was experimentally validated by co-
228 culturing SCC154 – OCAF1, which resulted in a strong increase of phosphorylated ERK1/2 (Figure 3E).
229 To test whether the RAS-MAPK pathway is responsible for the upregulation of *JUNB*, *FOS* and *FOSB*
230 genes, we used the MEK inhibitor, trametinib, in the SCC154-OCAF1 co-culture. We confirmed that
231 MEK inhibition downregulates ERK1/2 activation upon co-culture (Figure 3F) and observed a
232 significant downregulation of *JUNB*, *FOS* and *FOSB* genes (Figure 3G). Thus, multiple genes belonging
233 to the AP-1 TF complex are upregulated when cancer cells and CAFs are co-cultured and this is
234 mechanistically linked to the activation of RAS-MAPK kinase signaling.
235

236 ## HB-EGF activation is crucial to trigger RAS pathway signaling.

237 To explain why AP-1 TFs get upregulated in co-culture, we looked for possible activators of RAS-MAPK
238 signaling. We noted that *HB-EGF* was among the genes upregulated in all 6 transcriptomic datasets
239 together with *JUNB*, *FOS* and *FOSB* (Figure 2B). HB-EGF is an EGFR ligand and therefore can activate
240 MAPK pathway [29,30]. We evaluated the expression levels of all seven EGFR ligands. Importantly, *HB-*
241 *EGF* showed a strong and specific activation upon cancer cell - CAF co-culture (Figure 4A). Moreover,
242 *HB-EGF* expression strongly correlates with CoCu8 in HPV positive HNSCC patients' data from both
243 TCGA (R=0.6, p-value=2.2e-16) and UK_HPV positive (R=0.58, p-value=1e-8) datasets (Figure 4B).
244
245 HB-EGF can activate EGFR/MAPK as an un-cleaved pro-molecule at the plasma membrane [30,31] and, as
246 such, signaling by membrane-bound HB-EGF could explain the need of direct cell contact to trigger
247 the pathway. As membrane-bound HB-EGF should be expressed at about 20-25 kDa, we evaluated the
248 cellular levels of HB-EGF in OCAF1-SCC154 co-culture and found that it was strongly upregulated at
249 the protein level at a molecular weight previously reported as un-cleaved protein [32] (Figure 4C). We
250 also observed EGFR phosphorylation was increased upon direct co-culture (Figure 4C); importantly,

251 both these effects were abrogated by MEK inhibition (Figure 4D), suggesting the presence of a positive
252 feedback loop involving HB-EGF / EGFR / MAPK / AP-1 upon direct co-culture. Furthermore, the EGFR
253 inhibitor, afatinib, blocked AP-1 activation upon co-culture of SCC154-OCAF1 (Figure 4E).
254
255 These results suggest that HB-EGF might be the link that activates EGFR upon cancer cell – CAF co-
256 culture. Therefore, we evaluated the basal expression level of EGFR and HB-EGF in SCC154 and OCAF1
257 mono-cultures. Interestingly, SCC154 expressed HB-EGF at much higher levels than OCAF1, while both
258 cell types expressed similar levels of EGFR (Figure 4F). This suggests that both cell types can be reactive
259 to EGF ligands, but the activation of the positive feedback loop upon direct contact requires higher
260 levels of HB-EGF, expressed at the membrane of cancer cells. In that case, both cancer cells and CAFs
261 should be responsive to HB-EGF treatment, albeit with potentially different downstream effects. To
262 test this hypothesis, we incubated OCAF1 and SCC154 in mono-cultures with different concentrations
263 of HB-EGF. Firstly, HB-EGF caused an increase in proliferation in both cell types, as shown by a higher
264 proportion of EdU positive nuclei (Figure 4G). Moreover, SCC154 – but not OCAF1 – showed a scatter-
265 like phenotype when treated with high doses of HB-EGF, as visible from E-Cadherin staining (Figure
266 S8C). We then tested the effect of HB-EGF treatment on known CAF markers: after treatment of OCAF1
267 with HB-EGF for 48h we noticed a slight but significant downregulation of *ACTA2*, a CAF and
268 myofibroblast marker (Figure S8D). However, no effect was observed for other widely used CAF
269 markers (*FAP*, *LRRC15*, *FN1*) (Figure S8D).
270
271 Given these data, we asked whether HB-EGF expression in cancer cells is enough to induce
272 upregulation of AP-1 genes when cancer cells and CAFs are in co-culture: we therefore performed
273 knock down of HB-EGF in SCC154 (Figure S8E) and then co-cultured them with OCAF1. Importantly,
274 the downregulation of HB-EGF in SCC154 is enough to block the upregulation of *JUNB*, *FOS* and *FOSB*
275 when cancer cells and CAFs are co-cultured (Figure 4H).
276
277 These results point to an axis involving HB-EGF in cancer cells and EGFR in CAFs that activates MAPK /
278 AP-1 inducing a positive feedback loop when cancer cells and CAFs are co-cultured.
279

280 A paracrine HB-EGF/EGFR axis regulates cytokine expression and macrophage recruitment

281 To focus on the downstream effects of this crosstalk between cancer cells and CAFs and how HB-EGF
282 could affect CAFs functions and lead to unfavorable biology, we analyzed scRNAseq dataset of HNSCC
283 with both malignant and non-malignant samples published by Choi et al. [33]. Interestingly, by using
284 myofibroblast and inflammatory markers (Figure S8F), we found that EGFR is mainly expressed by

285 inflammatory fibroblasts (iFibroblasts), but not myofibroblastic CAFs (myoFibroblasts), while HB-EGF
286 is mainly expressed by endothelial and epithelial cells (Figure 5A).
287
288 Given that high expression of EGFR is linked to iCAFs, we performed a cytokine array of conditioned
289 medium from pooled mono-cultures and co-cultures (Figure 5B): interestingly, we found a strong
290 upregulation of macrophage attraction and differentiation markers (LIF and GM-CSF – gene name
291 *CSF2* –). We validated by qPCR that *LIF* and *CSF2* are transcriptionally upregulated in co-culture (Figure
292 5C) and that trametinib, MEK inhibitor, treatment is enough to downregulate their expression (Figure
293 5D). To further investigate the involvement of EGFR in this crosstalk pathway, we performed Afatinib
294 treatment in co-culture and observed that both *LIF* and *CSF2* are strongly downregulated by EGFR
295 inhibition (Figure 5E). Moreover, by reducing HB-EGF expression in cancer cells and then co-culturing
296 the cells with CAFs, we also observed strong downregulation of both *LIF* and *CSF2* expression (Figure
297 5F). Importantly, HB-EGF treatment in cancer cells and CAFs mono-cultures shows that: *LIF* is strongly
298 upregulated only by CAFs, indicating that these are the cells responsible for its production when in co-
299 culture (Figure S9A); *CSF2* instead is upregulated by HB-EGF treatment both in cancer cells and in CAFs
300 (Figure S9A). In line with these, transcriptomic data of HPV positive HNSCC patients from TCGA show
301 that there is a strong positive correlation between *LIF* / *CSF2* and *HBEGF* mRNA expression (Figure
302 S9B). These data establish that HB-EGF/EGFR signaling is required for the up-regulation of cytokines
303 and that EGFR is most highly expressed in human iCAFs.
304
305 Given the established literature behind GM-CSF and LIF involvement in macrophage biology [34,35] , we
306 isolated primary monocytes from peripheral blood mononuclear cells (PBMCs) from healthy donors,
307 differentiated them into macrophages and then performed a migration assay of macrophages using
308 conditioned medium (CM) to ask if cancer cell - CAF direct CM was sufficient to increase macrophage
309 attraction. Importantly, CM derived from the co-culture of OCAF1 and SCC154 increased the numbers
310 of migrating macrophages, compared with pooled CM derived from each cell in monoculture (Figure
311 5G). We next tested if the attraction of macrophages depended on the activation of EGFR upon cancer
312 cell-fibroblast interaction. We then tested whether MEK inhibition was enough to decrease the
313 number of macrophages attracted. Indeed, when cancer cells and CAFs are co-cultured in the presence
314 of trametinib, there is a stark decrease in the number of migrating macrophages attracted by the CM
315 (Figure 5H). Crucially, this was not the case when MEK inhibitor is freshly added to CM after it is
316 harvested from the cancer cell-CAF co-culture, indicating that any residual inhibitor in the CM is not
317 the cause of reduced macrophage attraction (Figure 5H). Moreover, blockade of EGFR using the

318 inhibitor afatinib during the co-culture phase significantly reduced the attraction of macrophages
319 (Figure 5H).
320
321 These data suggest that cancer cell – CAF crosstalk is consistently linked to macrophage recruitment,
322 therefore we asked whether CoCu8 high patients showed higher levels of macrophages. Of note, when
323 TCGA patients' data are separated according to CoCu8 expression, we observe a strong enrichment
324 for cells defined by a CD14-related methylation signature (monocytes/macrophages) (Figure 5I). We
325 found a similar pattern when patients were separated by fibroblast abundance and by RAS activity
326 (Figure S9C). Importantly, we also observed enrichment for monocyte/macrophage lineages in our
327 second cohort of 84 HPV positive HNSCC patients when separated for CoCu8 expression levels (Figure
328 5I).
329
330 In summary, we have demonstrated that cancer cell – CAF cross-talk uniquely increases expression of
331 different cytokines that, in turn, recruit higher numbers of macrophages. This loop is established by
332 HB-EGF expression in cancer cells that induces a paracrine cross-talk with CAFs via EGFR dependent
333 by RAS / MAPK activity. Activation of this pathway in both CAFs and cancer cells is needed to increase
334 the expression of both LIF and GM-CSF. MEK inhibitor and EGFR inhibitor are sufficient to reduce the
335 macrophage attraction.

336 ## Discussion

337 The presence of CAFs in tumors correlates with worse patient survival and an immune suppressive
338 TME in multiple tumor types [20,36–40], with recent studies attempting to link different CAF
339 subpopulations to prognosis [41]. However, analysis based on the presence or absence of CAFs does not
340 account for variability in the extent of crosstalk between cancer cells and CAFs. The approach we
341 develop here is based on the selection of genes that are commonly upregulated in both cancer cells
342 and CAFs upon direct cell-to-cell contact, thus focusing on the functional relevance of cancer cell-CAF
343 interaction, rather than just on the presence of CAFs in the tumor. We applied two different strategies
344 to select genes indicative of cancer cell-CAF interactions. The approach to define CoCu30 enriches for
345 genes that are strongly up-regulated, which has been employed previously [9]. To define the CoCu8
346 signature, we used a new approach based on the selection of a coherent set of genes linked by
347 function. Strikingly, this method generates a signature with prognostic power in all types of SCC
348 investigated and lung, pancreatic, and ccRCC, with particularly strong links to outcome in HPV positive
349 SCC. Our signature did not signify poor prognosis in breast, colorectal, or prostate cancer. We
350 speculate that the different relevance of the signature in cancer arising in different tissues might
351 reflect varying roles for fibroblasts in the tissue in coordinating wound healing responses, including
352 engagement with myeloid cells.
353
354 Comparative analysis of CoCu8 and CoCu30 with annotated gene sets (KRAS SIGNALLING UP and
355 RAS84 signature [28]) suggested a mechanism of cross-talk between cancer cells and CAFs based on the
356 activation of MAPK / AP-1 pathway (Figure S10). Consistent with this, the upregulation of CoCu30
357 genes – FOS, FOSB, JUNB, and HBEGF – required MEK activity. These data extend previous literature
358 showing that KRAS mutation is associated with higher stromal presence [42] and with higher cancer cell
359 – stromal interaction [43]. We hypothesize that our signatures are highly prognostic in HPV positive
360 HNSCC because it lacks oncogenic activation of EGFR or RAS, which frequently occurs in HPV negative
361 disease [44]. Thus, in HPV positive disease, RAS pathway activation and unfavorable downstream biology
362 are triggered by cancer cell – fibroblast interaction.
363
364 Our analyses indicate that HB-EGF is central to the activation of MAPK signaling upon cancer cell – CAF
365 contact. HB-EGF is the only EGF ligand to be consistently upregulated in co-culture across diverse
366 models. Accordingly, EGFR activation is upregulated in co-culture, suggesting the presence of a
367 positive feedback loop, including HB-EGF / EGFR / MAPK / AP-1. HB-EGF stimulation allowed us to
368 decipher the cell type-dependent consequences of the activation of the pathway. Indeed, while both
369 cancer cells and CAFs respond to HB-EGF by activating MAPK and inducing changes in AP-1 TF

370 expression, we observed different downstream activation mechanisms depending by the cell type.
371 These data are consistent with the finding that AP-1 activation leads to diverse molecular and
372 phenotypic consequences depending on the cell type studied [45]. The low level of HB-EGF expression
373 by cancer cells is not sufficient to initiate these events. We propose the presence of CAFs acts as a
374 mechanism to amplify the expression of HB-EGF, enabling a threshold for productive signaling to be
375 exceeded. CAFs also up-regulate inflammatory cytokines more strongly than cancer cells, meaning
376 that co-culture is required for HB-EGF to drive high levels of expression and subsequent macrophage
377 recruitment. The mechanism through which HB-EGF is upregulated could be associated with
378 proteolytic processing of HB-EGF at the interface between cancer cell and CAFs and will be interesting
379 to test in future studies.
380
381 Ultimately, we link increased MAPK and EGFR activity to the chemo-attraction of macrophages. Our
382 data provide insights into the molecular mechanism behind the correlation of CAFs and macrophages
383 in tumors and, more generally, for links between CAF and a pro-tumorigenic and immune-suppressive
384 milieu [37]. Indeed, GM-CSF is known to be associated with macrophage enrichment and chronic
385 inflammation and in cancer [34] and LIF can promote macrophage recruitment and induce a more pro-
386 tumorigenic polarization to alter immune response during anti PD-1 therapy [35]. Our data indicate that
387 different cytokines are regulated at different levels: GM-CSF is produced both by cancer cells and CAFs
388 when stimulated with HB-EGF, while LIF is specifically produced by CAFs. In contrast to our study,
389 Mucciolo and colleagues reported that stromal EGFR activated by AREG is involved in acquisition of
390 pro-tumorigenic properties that favor cancer cells via myofibroblast activation in pancreatic cancer [4].
391 This difference may reflect either difference between SCC, which is the experimental model in our
392 work, and pancreatic cancer, or that AREG and HB-EGF may trigger different patterns of gene
393 expression. Thus, EGFR is a critical determinant of CAF functions, with further studies required to
394 disentangle tissue- and ligand-specific biology.
395
396 Our findings have clinical implications for patient stratification and treatment. Although HPV positive
397 HNSCC patients typically have a better prognosis than HPV negative HNSCC patients, about 25% of
398 these patients still have poor overall survival [46,47].CoCu8 / CoCu30 signatures and CAF abundance could
399 help stratify those patients with worse prognosis within the HPV positive SCC. This improved patient
400 stratification would be especially relevant in the context of the recent unsatisfactory efforts to de-
401 escalate and de-intensify treatment for patients with HPV positive tumors [48] and could help reduce
402 toxicity without compromising outcomes. Moreover, our results suggest that this subset of patients
403 could benefit from a targeted approach, for example re-purposing the use of MEK or EGFR inhibitors.

404 Indeed, trametinib – MEK inhibitor – is already used in the treatment of melanoma [49] and non-small-
405 cell lung cancer [50] and it has been tested in phase I / II oral cavity SCC patients, showing some reduction
406 in RAS / MAPK activity as neoadjuvant treatment [51]. Our data argue that trametinib or EGFR inhibitors
407 may be beneficial for HPV positive HNSCC patients with high stromal content.
408
409 In conclusion, our results demonstrate a new approach to detect biologically meaningful stromal
410 signatures. We show that signatures based on signaling in the TME have the potential to both improve
411 patient stratification and to identify new mechanisms of cross-talk between cancer cells and CAFs.

412 # Materials and Methods

413 ## Cell lines and reagents

414 OCAF1 and OCAF2 human fibroblasts were isolated from patient tissues of oral cancer and
415 immortalized with lentiviral HTERT as described in [52]. These patient samples were collected under the
416 ethical approval REC reference 06/Q0403/125).
417 CRUK0764 were derived from patients with lung adenocarcinoma. These fibroblasts were established
418 from the tumor tissue. The primary CRUK0764 was immortalized by the following infection with
419 retroviruses expressing human telomerase reverse transcriptase.
420 PC9 was obtained from the Crick Institute Central Cell Services facility. PC9 were stably transfected
421 with Lipofectamine 2000 Reagent (Thermo Fisher Scientific) according to the manufacturer's
422 instructions. Briefly, PC9 cell line was seeded at 50-70 % confluence in a six-well plate and transfected
423 2 µg of Piggybac transposase (pPBase-piggyBac) and 2 µg of mEGFP (pPBbsr2-mEGFP) plasmid DNAs.
424 After 24h of incubation, the medium with Lipofectamine/plasmid DNA mix was replaced with a fresh
425 medium. Cells were selected using 2 µg ml -1 blasticidin.
426 SCC154 (UPCI-SCC154) and SCC47 (UM-SCC47) were purchased from ATCC.
427 OCAF1, OCAF2, SCC154, SCC47 and CRUK0764 cells were cultured in DMEM (ThermoFisher,
428 #41966052) containing 10% fetal bovine serum (Gibco, #10270-106), 1% penicillin/streptomycin
429 (Invitrogen, #15140122), 1% insulin–transferrin–selenium (Invitrogen, #41400045) and kept at 37°C
430 and 5% CO2.
431 PC9 were cultured in RPMI-1640 (Thermo Fisher Scientific, Rockford, IL) supplemented with 10% fetal
432 bovine serum (Gibco, #10270-106), 1% penicillin/streptomycin (Invitrogen, #15140122) and kept at
433 37 °C in 5% CO2.
434 Cells were not allowed to reach more than 90% confluency for routine cell culture cultivation. Cell
435 lines that are not commercially obtainable are available from the authors upon reasonable request.
436 Routine screening for *Mycoplasma* testing was performed for all cell lines with negative results. STR
437 profiles of human non-commercially available cell lines are included in Supplementary Table 1.
438

439 ## Cell cultures conditions and treatments

440 Co-cultures and mono-cultures were performed with a ratio of 1:2, typically plating 5.5 x 10^5 CAFs
441 and 2.75 x 10^5 cancer cells for a single well of a 6 well plate for the specified time point. When co-
442 cultures were compared to pooled mono-cultures, for the mono-culture condition, same number of
443 cells was plated but in two separated wells and then lysed together (pooled condition).
444 When cancer cells and CAFs mono-culture were compared among themselves, 1 x 10^6 OCAF1 and 1
445 x 10^6 SCC154 cells were plated in a 10cm dish.

446 For PC9 and CRUK0764 cells monocultures and co-cultures used for RNAseq, following 24 h co-
447 cultures, the culture media was replaced with fresh medium with DMSO, then harvested after an
448 additional 24 h. PC9 – CRUK0764 co-cultures were performed in a mixture of RPMI-1640 and DMEM
449 (1:1) containing 1% fetal bovine serum (Gibco, #10270-106).
450 For macrophage cultivation, please see "Macrophage migration assay" section.
451 For cell culture treatments: drugs / factors were added when cells were plated and then added fresh
452 after 24h. Drugs / factors used: trametinib (Selleckchem, #GSK1120212), afatinib (Selleckchem,
453 #BIBW2992), human recombinant HB-EGF (Peprotech, #100-47).
454 For trametinib treatment to collect conditioned medium (CM), in order to control the effect of the
455 drug presence regardless of its effect on secreted factors, we added fresh trametinib treatment to
456 DMSO co-culture CM at the same concentration used for the cell co-culture treatment.
457 All concentrations used are specified in the figures.
458 RNA interference was performed with Lipofectamine RNAimax reagent from Invitrogen, according to
459 the manufacturer's instructions. For transient knock down of HB-EGF, cells were subjected to reverse
460 transfection with 20 nM RNAi oligos plus forward transfection the day after, then analyzed 4 days after
461 reverse transfection. The following RNAi oligo (Dharmacon) was used: siHB-EGF A (Cat # D-019624-
462 02), siHB-EGF B (Cat # D-019624-03), as control the following non-targeting siRNA oligo (All Stars
463 Negative, Quiagen, Cat # 1027281).
464

465 ## Fluorescence-activated cell sorting

466 For PC9 – CRUK0764 RNAseq experiment, CRUK0764 were labelled with CellVue® Red Mini Kit for
467 Membrane Labeling (Polysciences, 25567-1) according to the manufacturer's instructions. Briefly, 1 ×
468 10^7 cells of CRUK0764 were resuspended in the Diluent C and mixed with CellVue® Red working dye
469 solutions (final concentration: 5 × 10^6 cells/mL, 2 × 10^6 M dye) and then incubated for 5 min at RT.
470 Cells were washed twice with DMEM, 10% FBS medium to ensure removal of unbound fluorescence
471 dye.
472 For fluorescence-activated cell sorting, cells were sorted using a flow cytometer–cell sorter BD
473 FACSAria™ II. PC9-GFP and CRUK0764 -CellVue Red were sorted by FACS 48 h after seeding them in
474 monoculture or direct co-culture. The cells were then trypsinised and resuspended in 3% FBS in PBS,
475 1 mM EDTA in preparation for sorting. Cells were separated into two populations: PC9-GFP and
476 CRUK0764 with CellVue Red using a 488 nm laser with collection filter 530 nm/30 nm for GFP and 561
477 nm laser with collection filter 582 nm/20 nm for CellVue Red. Gates were designed on the basis of
478 negative and single-color controls. All cell populations were tested for purity, and data were analyzed
479 using FlowJo software.

480

481 ## RNA sequencing analysis for co-cultures

482 PC9 and CRUK0764 cells were immediately centrifuged at 300 × g for 4 min to remove supernatant
483 and add 350 µl RLT buffer (Qiagen, 79216) containing 1% β-mercaptoethanol (Sigma, M6250) and total
484 RNA was extracted using the RNAeasy Mini kit (Qiagen, 74104; n = 3 independent experiments). Prior
485 to library construction, the quality of total RNA was assessed by Bioanalyzer 2100 (Agilent
486 Technologies Inc).
487 For RNAseq analysis: biological replicates libraries were prepared using the polyA KAPA mRNA
488 HyperPrep Kit and sequenced on the Illumina HiSeq 4000 platform generating ~28 million 75bp single
489 end reads per sample. FASTQ_files were quality trimmed and adaptor removed using Trimmomatic
490 (version 0.36) [53]. The RSEM package (version 1.3.30) [54] in conjunction with the STAR alignment
491 software (version 2.5.2a) [55] was used for the mapping and subsequent gene level counting of the
492 mapped reads with respect to the Ensembl human GRCh38 (release 89) transcriptome. Normalization
493 of raw count data was performed with the DESeq2 package (version 1. 18.1) [56]. All the analysis was
494 done (version 1. 18.1) [56] within the R programming environment (version 3. 4. 3).
495 To check the purity of the samples, we analyzed the resulting transcriptomic data for the expression
496 of 'lineage markers'. CDH1, EPCAM, CD24, and KRT genes were used as markers of carcinoma cells and
497 for fibroblasts we used COL1A1, COL1A2, DCN, CD248, and PDGFR genes. This revealed high sample
498 purity for all transcriptomic data, except in the PC9 – CRUK0764 experiment that had variable purity
499 between samples. Therefore, we estimated the impurity in each sample based on the expression of
500 the lineage marker genes and calculated the expected level of transcript if the two mono-cultures
501 (cancer cells alone and CAFs alone) were mixed in proportion with the impurity estimate. The
502 observed transcript in the co-culture condition was then normalized to account for the effect of
503 contamination.

504

505 ## EdU proliferation assay

506 The Click-iT Plus EdU Imaging Kit (Invitrogen #c10640) was used to perform the assay. Briefly, 48h after
507 mono-cultures of OCAF1 and SCC154 were seeding, a solution with Edu 20µM was prepared and then
508 diluted 1:1 with cell media to add EdU 10µM final concentration. After 90 minutes incubation, cells
509 were washes twice in PBS, then fixed for 15 minutes with paraformaldehyde 3.7% and then washed
510 twice in BSA 3%. Following this step, cells were incubated for 20 minutes with 0.5% Triton X-100 in
511 PBS. After two BSA 3% washes, the Click-iT reaction buffer was added for 30 minutes, followed by one
512 wash in BSA 3% and one wash in PBS. Subsequently, nuclei were stained with Hoechst 33342 at 5
513 µg/mL in PBS incubation for 30 minutes, followed by two PBS washes.

514 Samples were imaged with Zeiss 980 microscope.

515

516 Immunofluorescence assay

517 The samples used to perform EdU proliferation assay have been then stained for E-Cadherin. Briefly,
518 samples were washes twice in PBS, followed by incubation for 30 minutes in BSA 3%. Then samples
519 were incubated over night at 4°C. After two washes in BSA 3% of 5 minutes each, samples were
520 incubated with secondary antibody Alexa Fluor 555 in BSA 3% for 45 min. Following this step, samples
521 were washed with PBS twice. Subsequently, nuclei were stained with Hoechst 33342 at 5 µg/mL in
522 PBS incubation for 30 minutes, followed by two PBS washes. Samples were imaged with Zeiss 980
523 microscope.

524

525 Peripheral Blood Mononuclear Cell extraction and monocytes selection

526 Donations of healthy blood donors were received from the Francis Crick Institute. PBMCs were
527 isolated from whole blood using Lymphoprep (Stemcell Technologies #7811) with SepMate™ density
528 centrifugation tubes in line with manufacturer's instructions (Stemcell Technologies #85450). Freshly
529 isolated PBMCs were then counted before isolation of monocytes (Miltenyi Biotec #130-096-537).
530 Monocytes were then counted for plated in normal plastic dishes.

531

532 Cytokine array

533 Cytokine array used is "Proteome Profiler Human XL Cytokine Array Kit" (R&D Systems, # ARY022B)
534 following manufacturer's instruction. Briefly, conditioned media was isolated and filtered through a
535 0.4µm low protein binding PVDF Miltex syringe-driven filter (Millipore #SLHV033RS) to remove cellular
536 debris. Media was then concentrated to 4X using Amicon® Ultra-15 and used for subsequent
537 incubation with array.

538

539 Macrophage migration assay

540 Monocytes were plated into 12-well plates ($1x10^5$ cells / well) in RPMI 1640 media (ThermoFisher
541 #12633-012) containing 10% FBS, 1% streptomycin/penicillin and 50ng/mL of M-CSF (Peprotech #300-
542 25) and kept at 37°C and 5% CO2 for 5 days to allow macrophage differentiation. During incubation
543 period, OCAF1 – SCC154 mono- and co-cultures were set-up for 48h. Conditioned media was isolated
544 and filtered through a 0.4µm low protein binding PVDF Miltex syringe-driven filter (Millipore
545 #SLHV033RS) to remove cellular debris. Media was then concentrated to 4X using Amicon® Ultra-15
546 centrifugal filter units (Millipore #UFC901024) and frozen into aliquots until needed. Conditioned

547 media was added to 24-well plates, 8µm hanging cell culture inserts (Millipore #MCEP12H48) were
548 placed on top of each well. The now differentiated macrophages were seeded inside the hanging cell
549 culture insert and left to settle for 10 minutes before topping up media. Plates were left in the
550 incubator for 5 hours to allow macrophages to migrate through membrane pores. After this time, the
551 inserts were removed and the macrophages sat on top of the membrane were wiped off with a cotton
552 bud, leaving behind the migrated macrophages at the bottom. Inserts were stained with 0.05% crystal
553 violet for 30 minutes before washing and then imaged. Inserts were imaged using Zeiss Observer Z1
554 mounted with a QImaging Color camera. Quantification of crystal violet staining was carried out using
555 ImageJ through 'Cell Counter' function.
556

557 ## Gene Set Enrichment Analysis

558 Gene set enrichment analysis was performed with GSEA software v4.1.0. The dataset used to perform
559 the comparative analysis are: RAS84 derived from [28], CoCu8 derived from our own analysis, Hallmarks
560 (h.all.v7.5.symbols.gmt) for all the other analysis. All the parameters have been used as defaults
561 except: permutation type (gene set) and metric for ranking genes (Student's t-test). Gene signatures
562 with a false discovery rate < 0.05 were considered as statistically significant.
563

564 ## Transcriptomic data

565 The transcriptomic data used are: microarray data of A431 / VCAF2b under conditions of mono-
566 cultures, co-cultures in direct contact and indirect contact are available at the Gene Expression
567 Omnibus under record GSE121058. The microarray data of MAF2 under conditions of mono-culture
568 and co-culture in direct contact is available at the Gene Expression Omnibus under record GSE63160.
569 The microarray data of 5555 under conditions of mono-culture and co-culture in direct contact will be
570 submitted at the Gene Expression Omnibus.
571 The RNAseq data of PC9 / CRUK0764 under conditions of mono-cultures and co-cultures in direct
572 contact will be submitted to the European Genome-Phenome Archive before publication.
573 The microarray data used for HUVEC – 1205Lu analysis is available at Gene Expression Omnibus under
574 record GSE8699.
575 The microarray data used for breast cancer cell lines co-culture with fibroblasts analysis is available at
576 Gene Expression Omnibus under record GSE41678.
577

578 ## Co-culture gene signature generation

579 CoCu8 gene signature generation: the A431/VCAF2b, 5555/MAF2, PC9/CRUK0764 co-cultures vs
580 mono-cultures transcriptional datasets have been analysed with GSEA (see Gene Set Enrichment
581 Analysis method) to obtain a list of enriched pathways in co-culture with FDR < 0.05 for each condition.
582 For each cell type, all the genes statistically upregulated have been pulled together. From this list,
583 genes that were present in 20% or more of the enriched pathways have been selected. The results
584 obtained for each sample have been merged according to the cell type: the three cancer cells in co-
585 culture have been pulled together, same for the three CAFs. To select the final list, only genes present
586 in the three different cancer cells or in the three different CAFs have been selected to generate CoCu8.
587

588 CoCu30 gene signature generation: the genes with a fold change upregulation of 1.5 or higher have
589 been selected for each cell type upon co-culture. The results of the three cancer cells in co-culture
590 have been pulled together, same for the three CAFs in co-culture. To select the final list, only genes
591 present in the three different cancer cells or in the three different CAFs have been selected to generate
592 CoCu30.
593

594 ## TCGA analysis

595 Clinical data, RSEM (RNA-Seq by Expectation-Maximization) normalized expression data (Illumina
596 RNASEQ platform) and Methylation data (Illumina Human Methylation 450 platform) for TCGA cohorts
597 were downloaded from the Firebrowse website hosed by Broad Institute of MIT and Harvard.
598 [http://firebrowse.org/]. Data downloads were all version 2016012800.0.0.
599 De-convolution strategies:
600 MethylCIBERSORT: signature matrix and mixture files were obtained using MethylCIBERSORT R
601 package, hosted on Zenodo. The detailed origin of the signatures and the procedure to create the
602 deconvolution strategy is explained in Chakravarthy et al. [13].
603 Absolute-CIBERSORT: To calculate the immune infiltrate per sample, the library 'CIBERSORT' (version
604 1.04 [57]) was run within R version 3.4.3 on the RSEM normalized data and the LM22 signature using the
605 parameters absolute=TRUE and abs_method ="no.sumto1".
606

607 ## RNA extraction and RT-qPCR

608 Cells were collected and lysed with RLT buffer and total RNA was extracted using the RNeasy Mini kit
609 (Qiagen, #74104), according to the manufacturer's protocol.

610 The cDNA was prepared using M-MLV reverse transcriptase (Promega, #M3682), and quantitative PCR
611 was performed using PowerUp™ SYBR™ Green Master Mix (ThermoFisher, #A25778), using the
612 QuantStudio 3 and 7 Real-Time PCR systems (Applied Biosystems).
613 Custom primers were acquired from Sigma; sequences are available in Supplementary Table 2. RNA
614 levels were normalized using three house-keeping genes using the ΔΔC method and reported as
615 relative fold change compared with Ctr/not treated cells/mono-culture. For each sample, technical
616 triplicates were obtained performed and, if one of the three technical replicates was an outlier, it has
617 been excluded. Samples with expression levels below 37 or undetected have been considered as not
618 expressed and – in order to perform statistics – a Ct value of 40 has been assigned.
619

620 ## scRNAseq analysis

621 scRNAseq data from Choi et al. [33] was downloaded from GEO (GSE181919) and analyzed using Seurat
622 package (version 4) [58].
623

624 ## Protein extraction, quantification and Western Blot analysis

625 Cells were lysed in RIPA buffer (50 mM TrisHCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium
626 deoxycholate, 0.1% SDS), supplemented with a protease and phosphatase inhibitors (PhosSTOP tablet
627 Roche #04906837001, cOmplete EDTA-free Roche #11873580001, 50 mM NaF). Lysis was performed
628 directly in the cell culture plates using a cell scraper, lysates were kept for 10min on ice and then
629 clarified by centrifugation at 16,000 g for 30 min at 4 °C.
630 Total protein was quantified using the bicinchoninic acid method in accordance with manufacturer's
631 instructions (ThermoFisher, 23225). Following protein quantification, 20 µg of sample was loaded on
632 a 4–15% gradient Mini-PROTEAN TGX Gels (Biorad, #4561084) and transferred to a Trans-Blot Turbo
633 Mini 0.2 µm PVDF membrane (Biorad, 1704156) for blotting. The membrane was blocked for 1 h in
634 5% BSA or 5% milk in TBST and then incubated overnight at 4°C or 1 h at room temperature with
635 antibodies. The membrane was then washed before adding the horseradish peroxidase-conjugated
636 secondary antibody (ThermoFisher), and incubating for 1 h at room temperature. The membrane was
637 washed again before developing with Luminata Classico Western HRP substrate (Millipore,
638 #WBLUR0100) Luminata Classico Western HRP substrate (Millipore, # WBLUF0100) and imaging.
639 Antibody information are listed in Supplementary Table 3. All original blots are provided as source
640 data.
641

642 ## Software and visualization

643 Graphs were generated with Prism software (Graphpad Software v9.4.0) and R (version 4.2.1) using
644 package 'ggplot' except for correlation plot in Figure S10B that was generated with cBioportal [59].
645 scRNAseq data were analyzed with Seurat package (version 4).
646

647 ## Statistics

648 Statistical analysis was performed using Prism software (Graphpad Software v9.4.0), Excel software
649 (Microsoft Corporation v16.0) and R (version 4.2.1).
650 All Student's t-tests have been performed with two tailed strategy.
651 P-value information: * is p-value<0.05; ** is p-value<0.01, *** is p-value<0.001, **** is p-
652 value<0.0001.
653 For GSEA, we used FDR with a threshold below 0.05 to definite the significance.
654 Kaplan-Meier, Log-Rank and Cox regression on survival data was calculated using the R package
655 'survminer' using univariable analysis. Correlations were calculated using the Spearman method in R
656 and the package 'corplot' was used to generate the graphs.
657

658 ## UK_HPV positive cohort

659 FFPE tumor samples from 2 studies formed this cohort:
660 - INOVATE (MR/R015589/1 ISRCTN32335415), a prospective sample collection study in patients
661 with T1-T2/N1-3 or T3-T4/N0-3 oropharyngeal cancer (AJCC TNM classification 7.0) receiving
662 treatment with radical radiotherapy with or without additional platin-based chemotherapy.
663 - INSIGHT-2 (C7224/A23275 NCT04242459), a prospective study of optimizing radiation therapy
664 in head and neck cancers using functional image-guided radiotherapy and novel biomarkers.
665 INOVATE was approved by the London -Bloomsbury Research Ethics Committee (19/LO/1558) and
666 INSIGHT-2 was approved by London – Queen Square Research Ethics Committee (19/LO/0638).
667 Written informed consent was obtained from all participants prior to any study procedure.
668

669 ## UK_HPV positive cohort RNAseq and data analysis

670 Baseline diagnostic biopsies embedded in paraffin blocks were obtained from the above-mentioned
671 cohort. Relevant tumor sections were selected and RNA was extracted from 3-4 slides using the
672 Qiagen AllPrep® DNA/RNA FFPE kit (#80234). Ribosomal RNA was depleted using QIAGEN FastSelect
673 rRNA H/M/R kit (#334375). RNA sequencing libraries were prepared using the NEBNext Ultra II RNA
674 Library Prep Kit (#E770) for Illumina following manufacturer's instructions. The sequencing libraries

675 were multiplexed and loaded on the flow cell on the Illumina NovaSeq 6000 instrument according to
676 manufacturer's instructions. The samples were sequenced using a 2x150 Pair-End (PE) configuration
677 v1.5 for an estimated output of ~50M paired-end reads per sample. Image analysis and base calling
678 were conducted by the NovaSeq Control Software v1.7 on the NovaSeq instrument. Raw sequence
679 data (.bcl files) generated from Illumina NovaSeq was converted into fastq files and de-multiplexed
680 using Illumina bcl2fastq program version 2.20. One mismatch was allowed for index sequence
681 identification. Sample adequacy was confirmed using FASTQC, low quality bases and reads were
682 trimmed using Trimmomatic, we run Hisat2-Stringtie for alignment.
683 RNAseq was performed on 103 patient samples, of which 7 were from the INSIGHT2 and 96 from
684 INOVATE. The data from RNAseq was analyzed to identify samples with presence of HPV (by aligning
685 the unmapped sequences to the whole HPV16 genome sequence obtained from GEO using HISAT2
686 and StingTie) and these samples were classed as HPV positive. 84 samples (77 INOVATE and 7
687 INSIGHT2) were classified at HPV positive and RNAseq data from these was used for analysis in this
688 study.
689

690 ### TRACERx cohort

691 Tumor samples used in this study were collected from LUSC patients enrolled as a part of TRACERx
692 study (accession code: NCT01888601) which is sponsored by University College London (UCL/12/0279)
693 and has been approved by an independent research ethics committee (13/LO/1546). Multiple regions
694 were sampled per tumor and processed as described by Frankell et al. [24] yielding whole-RNA
695 sequencing data for 295 regions from 117 LUSC patients. Expression count and transcript per million
696 (TPM) were quantified by the RSEM package [54]. Genes with expression level of at least 1 TPM in at
697 least 20% of the samples were included. A variance stabilizing transformation (VST) was then applied
698 to filtered count using the DESeq2 package [56].
699

700 ## Supplementary tables

701 Supplementary Tables 1, 2 and 3 are provided with this article.
702

703 ## Data accessibility

704 The transcriptomic data for UK_HPV positive cohort is part of ongoing clinical trials, therefore the data
705 cannot be deposited in a public repository until the trial is finalised. Data can be shared upon
706 reasonable request following corresponding Ethical Research Committee approval following the ICR-
707 Clinical Trials and Statistics Unit policy.

Authors contributions

G.G. and A.R. conceptualized and designed the research, with guidance from E.S. GG performed the experimental analysis and identified the cancer cell – CAF crosstalk gene signature strategy. A.R. performed the clinical data analysis on TCGA and UK_HPV positive cohorts and performed the deconvolution strategies. G.G., A.R., S.H., E.H., Y.N., P.N. and P.C. acquired and analyzed the data, except for TRACERx data that were analyzed by D.B. and UH.L.. C.S., A.M., K.H., S.B., M.C. and E.S. provided funding and access to samples and reagents. G.G., A.R. and E.S. wrote the manuscript. All authors reviewed and edited the manuscript. All authors authorized the final version.

Declaration of Interests and acknowledgments

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756 options in Apogen Biotechnologies and GRAIL until June 2021, and currently has stock options in Epic
757 Bioscience, Bicycle Therapeutics, and has stock options and is co-founder of Achilles Therapeutics.
758 C.S. holds patents relating to assay technology to detect tumor recurrence (PCT/GB2017/053289); to
759 targeting neoantigens (PCT/EP2016/059401), identifying patent response to immune checkpoint
760 blockade (PCT/EP2016/071471), determining HLA LOH (PCT/GB2018/052004), predicting survival
761 rates of patients with cancer (PCT/GB2020/050221), identifying patients who respond to cancer
762 treatment (PCT/GB2018/051912), US patent relating to detecting tumor mutations
763 (PCT/US2017/28013), methods for lung cancer detection (US20190106751A1) and both a European
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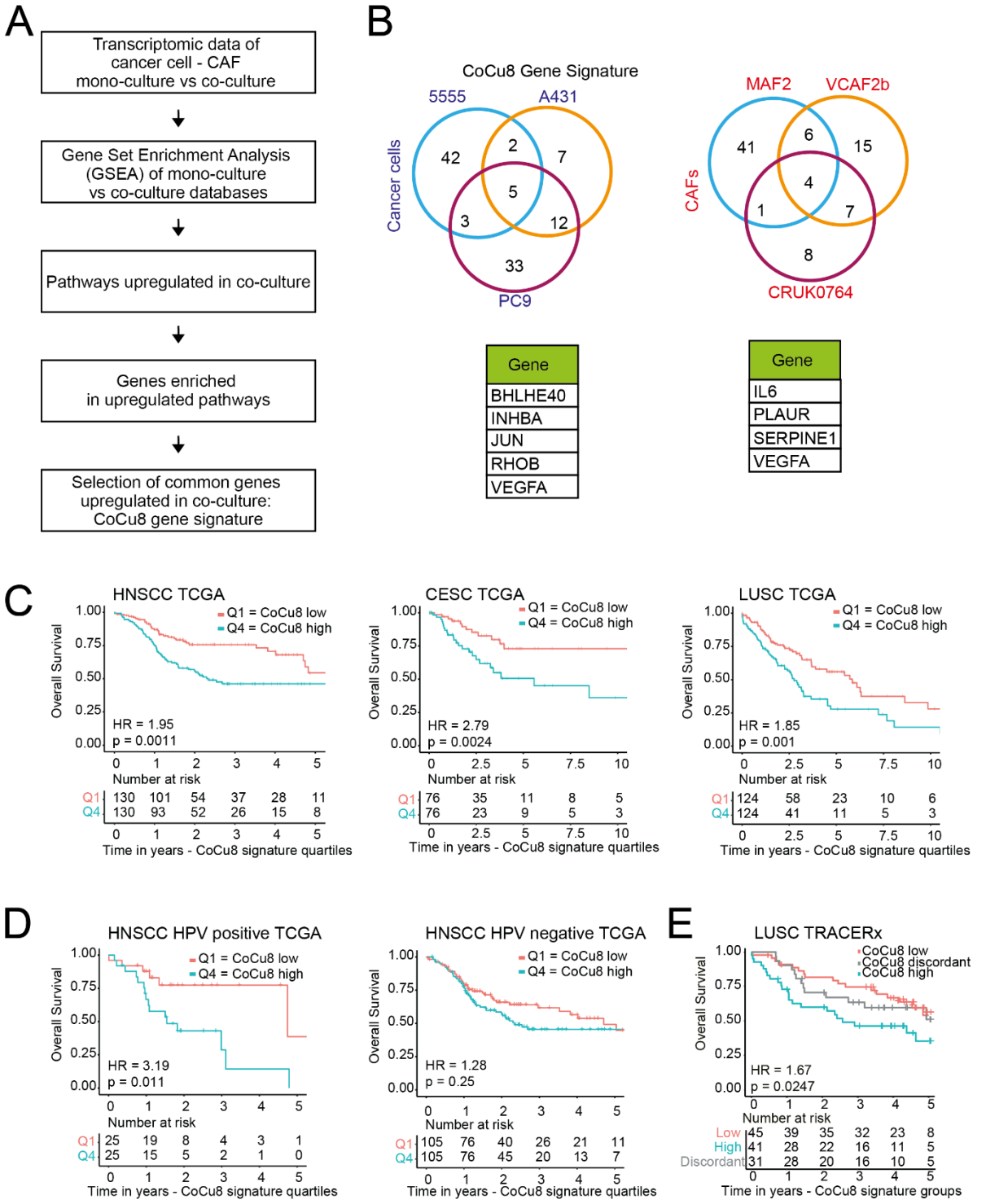
979

980 # Figures



981 Figure 1

982 **Figure 1:** Cancer cell / CAF co-culture gene signature CoCu8 is associated with worse overall survival
983 in multiple squamous cell carcinoma datasets. **A)** Strategy used to obtain CoCu8 gene signature. **B)**
984 Venn diagrams of the genes upregulated in the different datasets (top) and summary table of the
985 genes upregulated in all the datasets (bottom) for cancer cells (right) and CAFs (left). **C)** Kaplan-Meier
986 overall survival analysis of HNSCC (right), CESC (centre), LUSC (left) TCGA datasets stratified for CoCu8
987 first vs last quartile. Numbers at risk shown in tables below graphs. HNSCC HR=1.95 (95% Confidence
988 Interval (CI) 1.29-2.93), p-value=0.0011. CESC HR=2.79 (95%CI 1.40-5.56), p-value=0.0024, LUSC
989 HR=1.85 (95%CI 1.28-2.70), p-value=0.001. HR and CI were calculated using Cox regression. p-value
990 was calculated using logRank test. **D)** Kaplan-Meier overall survival analysis of HNSCC HPV positive
991 (right) and negative (left) TCGA datasets stratified for CoCu8 first vs last quartile. Numbers at risk
992 shown in tables below graphs. HNSCC HPV positive HR=3.19 (95%CI 1.24-8.18), p-value=0.011. HPV
993 negative HR=1.28 (95%CI 0.84-1.93), p-value=0.25. HR and CI were calculated using Cox regression. p-
994 value was calculated using logRank test. **E)** Kaplan-Meier overall survival analysis of LUSC TRACERx
995 dataset. Individual tumors stratified as high-, discordant or low-risk according to expression profile of
996 CoCu8 signature across multiple regions, as previously described and stratified according to Biswas et
997 al. [60]. Briefly, patients were classified as discordant when different tumour regions from the same
998 patient presented not unique signature levels. Below are shown the numbers at risk in years. HR=1.67
999 (95%CI 1.13-2.47), p-value=0.0247. HR and CI calculated using Cox regression and are referred to
1000 CoCu8 low vs CoCu8 high. p-value was calculated using logRank test.

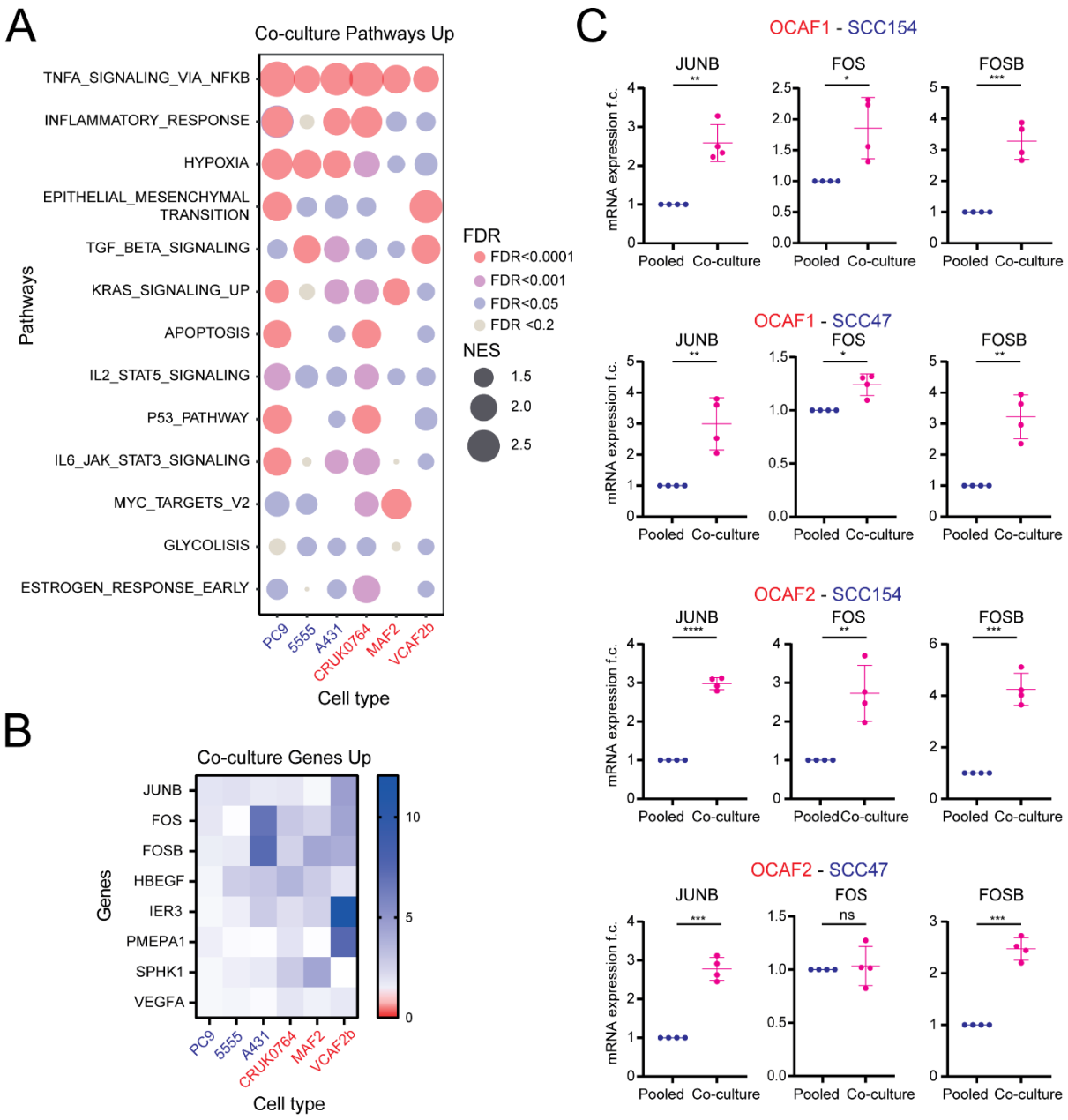

A
Co-culture Pathways Up
TNFA_SIGNALING_VIA_NFKB
INFLAMMATORY_RESPONSE
HYPOXIA
EPITHELIAL_MESENCHYMAL TRANSITION
TGF_BETA_SIGNALING
KRAS_SIGNALING_UP
APOPTOSIS
IL2_STAT5_SIGNALING
P53_PATHWAY
IL6_JAK_STAT3_SIGNALING
MYC_TARGETS_V2
GLYCOLISIS
ESTROGEN_RESPONSE_EARLY
Pathways
PC9
5555
A431
CRUK0764
MAF2
VCAF2b
Cell type
FDR
FDR<0.0001
FDR<0.001
FDR<0.05
FDR <0.2
NES
1.5
2.0
2.5
B
Co-culture Genes Up
JUNB
FOS
FOSB
HBEGF
IER3
PMEPA1
SPHK1
VEGFA
Genes
10
5
0
PC9
5555
A431
CRUK0764
MAF2
VCAF2b
Cell type
C
OCAF1 - SCC154
OCAF1 - SCC47
OCAF2 - SCC154
OCAF2 - SCC47
JUNB
FOS
FOSB
mRNA expression f.c.
Pooled
Co-culture
ns

Figure 2

1003 **Figure 2:** Cancer cell / CAFs co-culture upregulates AP-1 TF genes. **A)** Bubble plot of the Hallmarks
1004 pathways upregulated by cancer cells / CAFs culture. Normalised enrichment score (NES) is depicted
1005 as bubble size; False discovery rate (FDR) is depicted as colour intensity. **B)** Heatmap of expression of
1006 the genes commonly upregulated upon co-culture in all the tested conditions from the
1007 TNFA_SIGNALLING_VIA_NKFB pathway. **C)** qPCR analysis of *JUNB*, *FOS* and *FOSB* genes in
1008 OCAF1/OCAF2 with SCC154/SCC47 pooled mono-cultures and co-culture after 24h. mRNA expression
1009 is reported as mean ± standard deviation (SD) fold change difference over pooled mono-culture.
1010 Genes have been normalized over the average of *GAPDH*, *ACTB* and *RPLP0* housekeeping genes. n = 4
1011 independent experiments. Two tailed paired Student's t-test.

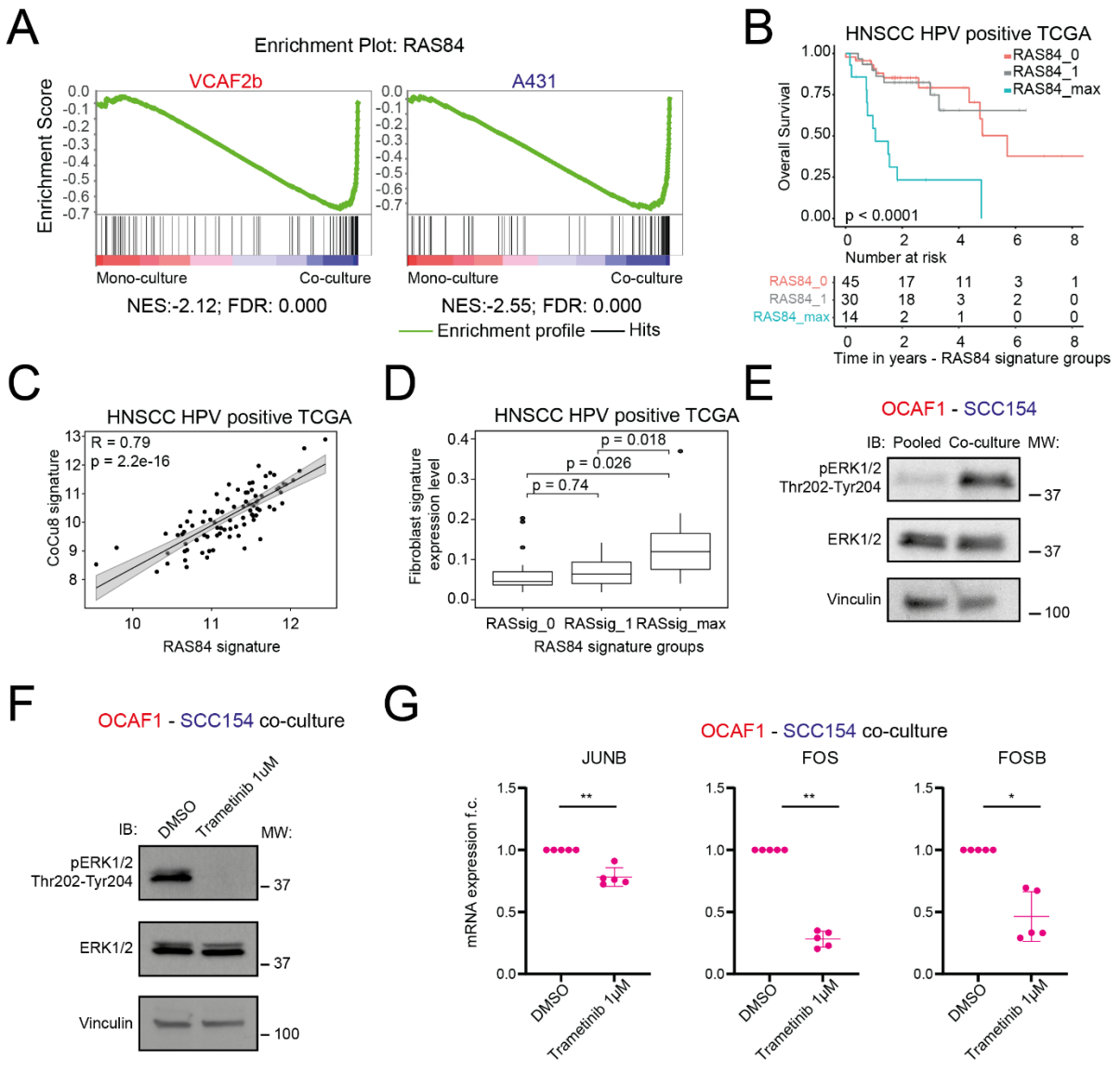

A
Enrichment Plot: RAS84
VCAF2b
A431
Enrichment Score
Mono-culture
Co-culture
NES:-2.12; FDR: 0.000
NES:-2.55; FDR: 0.000
Enrichment profile
Hits
B
HNSCC HPV positive TCGA
RAS84_0
RAS84_1
RAS84_max
Overall Survival
p < 0.0001
Number at risk
Time in years - RAS84 signature groups
C
HNSCC HPV positive TCGA
R = 0.79
p = 2.2e-16
CoCu8 signature
RAS84 signature
D
HNSCC HPV positive TCGA
p = 0.018
p = 0.026
p = 0.74
Fibroblast signature expression level
RASsig_0
RASsig_1
RASsig_max
RAS84 signature groups
E
OCAF1 - SCC154
IB: Pooled Co-culture MW:
pERK1/2 Thr202-Tyr204
ERK1/2
Vinculin
F
OCAF1 - SCC154 co-culture
IB:
DMSO
Trametinib 1uM
MW:
pERK1/2 Thr202-Tyr204
ERK1/2
Vinculin
G
OCAF1 - SCC154 co-culture
JUNB
FOS
FOSB
mRNA expression f.c.
DMSO
Trametinib 1μM

Figure 3

1014 **Figure 3:** RAS activity is upregulated in cancer cell and CAFs upon co-culture. **A)** Gene set enrichment
1015 analysis (GSEA) plot of RAS84 gene signature in mono-culture and co-culture. NES and FDR are
1016 specified below each plot. **B)** Kaplan-Meier overall survival analysis of HNSCC HPV positive TCGA
1017 dataset stratified for Ras84 activity according to [28]. Below are shown the numbers at risk in years.
1018 RAS84_0 vs RAS84_1 HR=1.01 (95%CI 0.387-2.62) p-value=0.98. RAS84_0 vs RAS84_max HR=5.85
1019 (95%CI 2.46-13.9) p-value<0.001. HR and CI calculated using Cox regression. **C)** Correlation plot of
1020 RAS84 expression level and CoCu8 expression level in HNSCC HPV positive TCGA dataset. R is
1021 Spearman correlation coefficient. n=97. **D)** Box plot analysis of fibroblast abundance via Methyl
1022 CIBERSORT deconvolution strategy in HNSCC HPV positive TCGA dataset according to RAS84 activity.
1023 Independent Student's t-test. **E)** Western blot analysis of OCAF1 – SCC154 pooled mono-culture vs co-
1024 culture for 48h showing the indicated antibodies. Vinculin is used as loading control. **F)** Western blot
1025 analysis of OCAF1 – SCC154 co-cultures for 48h at the indicated conditions showing the indicated
1026 antibodies. Vinculin is used as loading control. **G)** qPCR analysis of *JUNB*, *FOS* and *FOSB* genes in OCAF1
1027 - SCC154 co-cultures for the indicated treatments after 48h. mRNA expression is reported as
1028 mean ± standard deviation (SD) fold change difference over co-culture DMSO. Genes have been
1029 normalized over the average of *GAPDH*, *ACTB* and *RPLP0* housekeeping genes. n = 4 independent
1030 experiments. Two tailed paired Student's t-test.

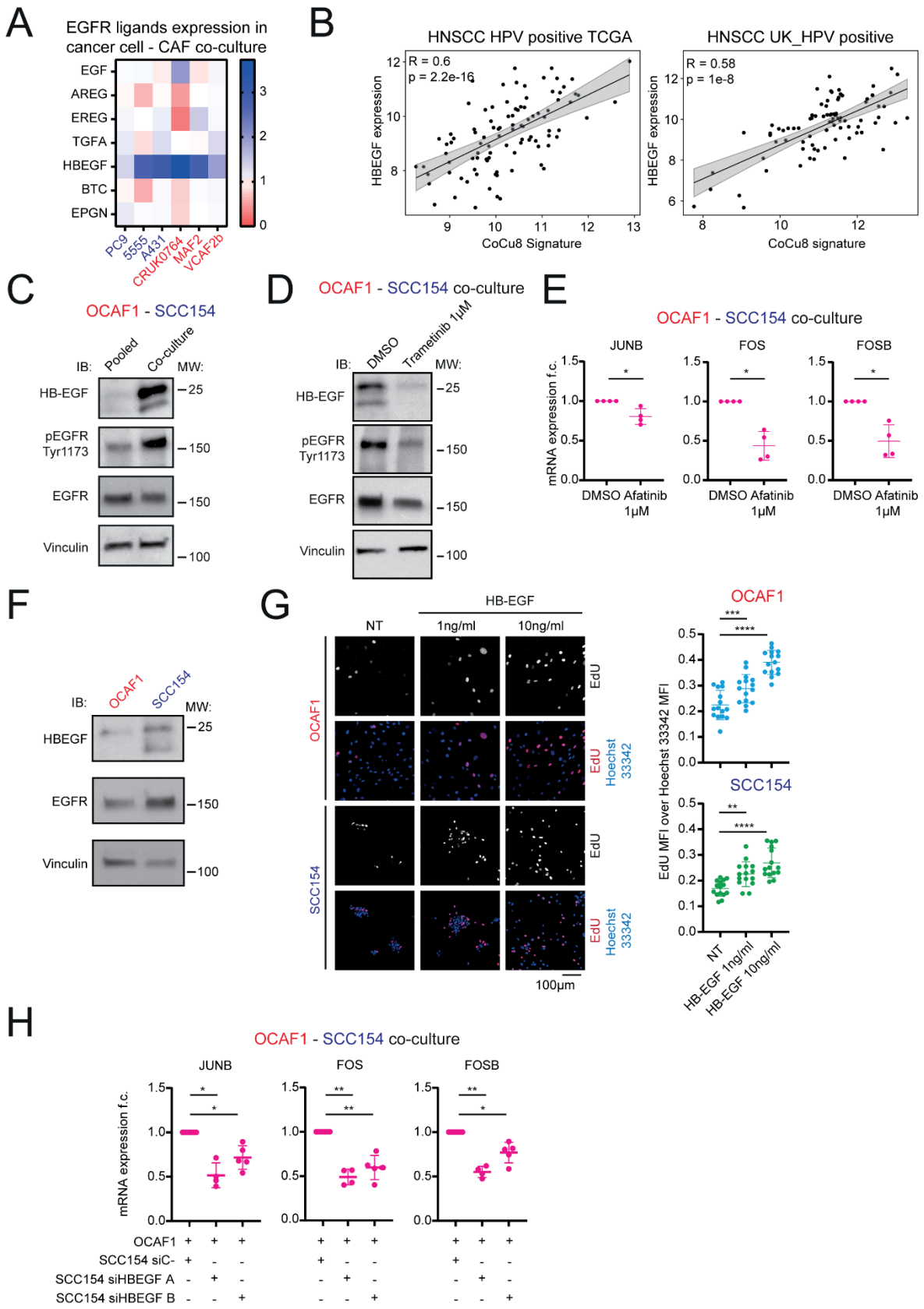

A
EGFR ligands expression in cancer cell - CAF co-culture
EGF
AREG
EREG
TGFA
HBEGF
BTC
EPGN
PC9
5555
A431
CRUK0764
MAF2
VCAF2b
B
HNSCC HPV positive TCGA
R = 0.6
p = 2.2e-16
HBEGF expression
CoCu8 Signature
HNSCC UK_HPV positive
R = 0.58
p = 1e-8
HBEGF expression
CoCu8 signature
C
OCAF1 - SCC154
IB:
Pooled
Co-culture
MW:
HB-EGF
pEGFR Tyr1173
EGFR
Vinculin
D
OCAF1 - SCC154 co-culture
IB:
DMSO
Trametinib 1µM
MW:
HB-EGF
pEGFR Tyr1173
EGFR
Vinculin
E
OCAF1 - SCC154 co-culture
JUNB
FOS
FOSB
mRNA expression f.c.
DMSO
Afatinib 1µM
F
IB:
OCAF1
SCC154
MW:
HBEGF
EGFR
Vinculin
G
HB-EGF
NT
1ng/ml
10ng/ml
OCAF1
SCC154
EdU
Hoechst 33342
100µm
OCAF1
SCC154
EdU MFI over Hoechst 33342 MFI
NT
HB-EGF 1ng/ml
HB-EGF 10ng/ml
H
OCAF1 - SCC154 co-culture
JUNB
FOS
FOSB
mRNA expression f.c.
OCAF1
SCC154 siC-
SCC154 siHBEGF A
SCC154 siHBEGF B

Figure 4

1033 **Figure 4:** HB-EGF / EGFR axis activates AP-1 TF genes in cancer cells and CAFs upon co-culture via RAS
1034 pathway. **A)** Pattern of expression of the 7 EGFR ligands in all the tested transcriptomic datasets. **B)**
1035 Correlation plot of *HB-EGF* expression level and CoCu8 expression level in HNSCC HPV positive TCGA
1036 dataset (left, n=97) and UK_HPV positive cohort (right, n=84). R is Spearman correlation coefficient.
1037 **C)** Western blot analysis of OCAF1 – SCC154 pooled mono-culture vs co-culture for 48h showing the
1038 indicated antibodies. Vinculin is used as loading control. **D)** Western blot analysis of OCAF1 – SCC154
1039 co-cultures for 48h at the indicated conditions showing the indicated antibodies. Vinculin is used as
1040 loading control. **E)** qPCR analysis of *JUNB*, *FOS* and *FOSB* genes in OCAF1 - SCC154 co-cultures for the
1041 indicated treatments after 48h. mRNA expression is reported as mean ± standard deviation (SD) fold
1042 change difference over co-culture DMSO. Genes have been normalized over the average of *GAPDH*,
1043 *ACTB* and *RPLP0* housekeeping genes. n = 4 independent experiments. The DMSO treated sample is
1044 the same used for Figure 3G. Two tailed paired Student's t-test. **F)** Western blot analysis of OCAF1 and
1045 SCC154 mono-cultures after 48h showing the indicated antibodies. Vinculin is used as loading control.
1046 **G)** Proliferation assay of OCAF1 and SCC154 mono-cultures for the indicated treatments after 48h and
1047 stained with EdU and Hoechst 33342. On the left, a representative image is shown with bar graph. On
1048 the right, dot plot of mean fluorescent intensity (MFI) of EdU over Hoechst 33342 with
1049 mean ± standard deviation (SD) highlighted. Each dot is a field of view. n = 3 independent experiments.
1050 Two tailed Student's t-test. **H)** qPCR analysis of *JUNB*, *FOS* and *FOSB* genes in OCAF1 - SCC154 co-
1051 cultures after 48h with SCC154 pre-treated with the indicated conditions. mRNA expression is
1052 reported as mean ± standard deviation (SD) fold change difference over siC- condition. Genes has been
1053 normalized over the average of *GAPDH*, *ACTB* and *RPLP0* housekeeping genes. n ≥ 4 independent
1054 experiments. Two tailed paired Student's t-test.

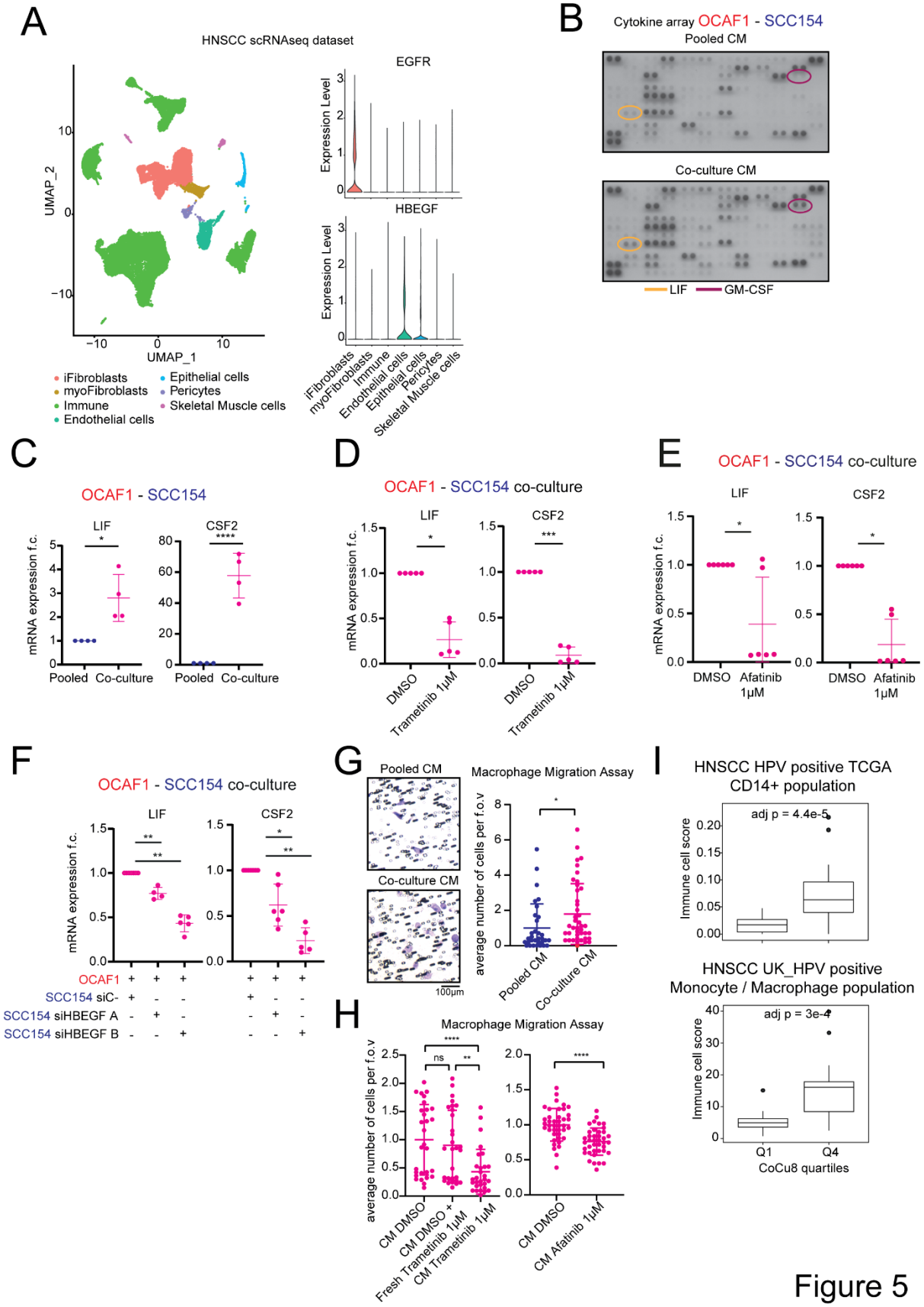


Figure 5

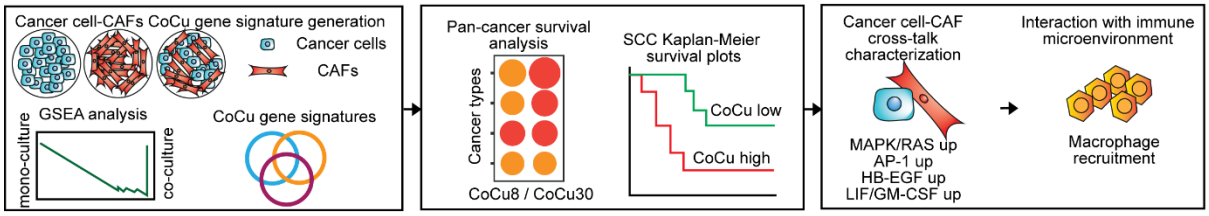
1055

1056

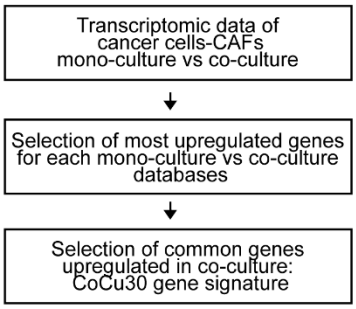
1057 **Figure 5:** Cancer cells – CAFs co-culture induces production of specific cytokines to attract
1058 macrophages. **A)** On the right is shown scRNAseq UMAP analysis from Choi et al. [33]. On the left is
1059 shown violin plot of EGFR and HBEGF mRNA expression levels in the indicated clusters. **B)** Cytokine
1060 array of conditioned medium from pooled mono-culture and co-culture of OCAF1 and SCC154.
1061 Highlighted relevant cytokines. n = 2 independent experiments. **C)** qPCR analysis of *LIF* and *CSF2* genes
1062 in OCAF1 – SCC154 pooled mono-culture vs co-culture after 24h. mRNA expression is reported as
1063 mean ± standard deviation (SD) fold change difference over pooled mono-culture. Genes have been
1064 normalized over the average of *GAPDH*, *ACTB* and *RPLP0* housekeeping genes. n = 4 independent
1065 experiments. Two tailed paired Student's t-test. **D)** qPCR analysis of *LIF* and *CSF2* genes in OCAF1 -
1066 SCC154 co-cultures for the indicated treatments after 48h. mRNA expression is reported as
1067 mean ± standard deviation (SD) fold change difference over co-culture DMSO. Genes have been
1068 normalized over the average of *GAPDH*, *ACTB* and *RPLP0* housekeeping genes. n = 5 independent
1069 experiments. Paired t-test. **E)** qPCR analysis of *LIF* and *CSF2* genes in OCAF1 - SCC154 co-cultures for
1070 the indicated treatments after 48h. mRNA expression is reported as mean ± standard deviation (SD)
1071 fold change difference over co-culture DMSO. Genes have been normalized over the average of
1072 *GAPDH*, *ACTB* and *RPLP0* housekeeping genes. n = 6 independent experiments. The DMSO treated
1073 sample is the same used for Figure 5D. Two tailed paired Student's t-test. **F)** qPCR analysis of *LIF* and
1074 *CSF2* genes in OCAF1 - SCC154 co-cultures after 48h with SCC154 pre-treated with the indicated
1075 conditions. mRNA expression is reported as mean ± standard deviation (SD) fold change difference
1076 over siC- condition. Gene has been normalized over the average of *GAPDH*, *ACTB* and *RPLP0*
1077 housekeeping genes. n ≥ 4 independent experiments. Two tailed paired Student's t-test. **G)** Migration
1078 assay of macrophages plated in transwells with conditioned medium (CM) from OCAF1-SCC154 pooled
1079 mono-culture or co-culture. CM have been obtained after 48h culture. On the left, a representative
1080 field of view is shown with bar graph. On the right, dot plot of number of cells per field of view as
1081 mean ± standard deviation (SD). Each dot is a field of view normalized by the average of the pooled
1082 mono-culture CM sample. n = 4 different donors. Two tailed Student's t-test. **H)** Migration assay of
1083 macrophages plated in transwells with CM from OCAF1-SCC154 co-culture with the indicated
1084 treatments. CM have been obtained after 48h culture and, for the fresh trametinib sample the drug
1085 has been added after the CM was collected. Dot plot of number of cells per field of view as
1086 mean ± standard deviation (SD) is shown. Each dot is a field of view normalized by the average of the
1087 co-culture DMSO CM sample. n = 3 different donors for trametinib effect and 4 donors for afatinib
1088 effect. Two tailed Student's t-test. **I)** At the top, box plot analysis of CD14+ monocytic/macrophage
1089 lineage immune cell absolute score via Methyl CIBERSORT deconvolution strategy in HNSCC HPV
1090 positive TCGA dataset separate by first and last quartile of CoCu8 expression. Independent Student's

1091 t-test, Bonferroni correction for multiple comparisons. At the bottom, box plot analysis of monocyte
1092 and macrophage immune cell score using Absolute CIBERSORT deconvolution strategy in HNSCC HPV
1093 positive UK_HPV positive dataset separate by first and last quartile of CoCu8 expression. Independent
1094 Student's t-test.
1095

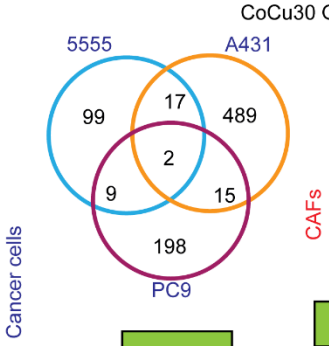
A



B



C



Gene
JUNB
FOSB

Gene			
ADAM19	FOSB	HHIP	MTHFD2
ASNS	GLIS3	IER3	MYO10
BAALC	GRB10	IFIT2	MTHFD2
CCL2	HBEGF	IL6	OAS2
F2RL1	HECW2	ITGA5	SERPINE1
FLNB	HES1	KCNN4	SPHK1
FOS	HES4	MLPH	TMEM51
			TRIB1

D

Cancer cell / CAF cell co-culture

NES	CoCu8	CoCu30
Wi38 with MDA-MB-231	-1.99	-2.30
CDD1112Sk with MDA-MB-231	-2.00	-2.12
HFF1 with MDA-MB-231	-2.05	-2.41
HFF2 with MDA-MB-231	-1.88	-2.15
MDA-MB-231 with Wi38	-1.91	-2.39
MDA-MB-231 with CDD1112Sk	-1.26	-1.48
MDA-MB-231 with HFF1	-1.89	-2.22
MDA-MB-231 with HFF2	-1.85	-2.07
Cal51 with Wi38	-1.92	-2.52
Cal51 with CDD1112Sk	-1.36	-1.76
Cal51 with HFF1	-1.97	-2.28
Cal51 with HFF2	-1.79	-1.82
Wi38 with Cal51	-1.33	-1.16
CDD1112Sk with Cal51	-1.56	-1.61
HFF1 with Cal51	-1.68	-1.97
HFF2 with Cal51	-1.69	-1.96

FDR<0.01
FDR<0.05
FDR n.s.

E

Cancer cell / endothelial cell co-culture

NES	CoCu8	CoCu30
HUVEC with 1205Lu	+0.73	-1.46
1205Lu with HUVEC	-1.12	-1.64

FDR<0.01
FDR<0.05
FDR n.s.

Figure S1

1098 **Figure S1:** Generation and validation of CoCu8 / CoCu30 gene signatures. **A)** Flow chart description of
1099 the manuscript is provided. **B)** Strategy used to obtain CoCu30 gene signature. **C)** Venn diagram of the
1100 genes upregulated in the different datasets (top) and a table to summarize the genes constantly
1101 upregulated in the datasets (bottom) for cancer cells (left) and CAFs (right). **D)** Table with Normalized
1102 Enrichment Score (NES) values of different combinations of cancer cells and CAFs breast cancer cell
1103 lines from Rajaram et al. [21]. Negative values represent enrichment towards co-culture condition.
1104 Colour legend is shown. **E)** Table with NES values of CoCu8 and CoCu30 gene signatures for cancer
1105 cells and endothelial cells mono-culture vs co-culture available from Stine et al. [22]. Negative values
1106 represent enrichment towards co-culture condition. Colour legend is shown.
1107
1108

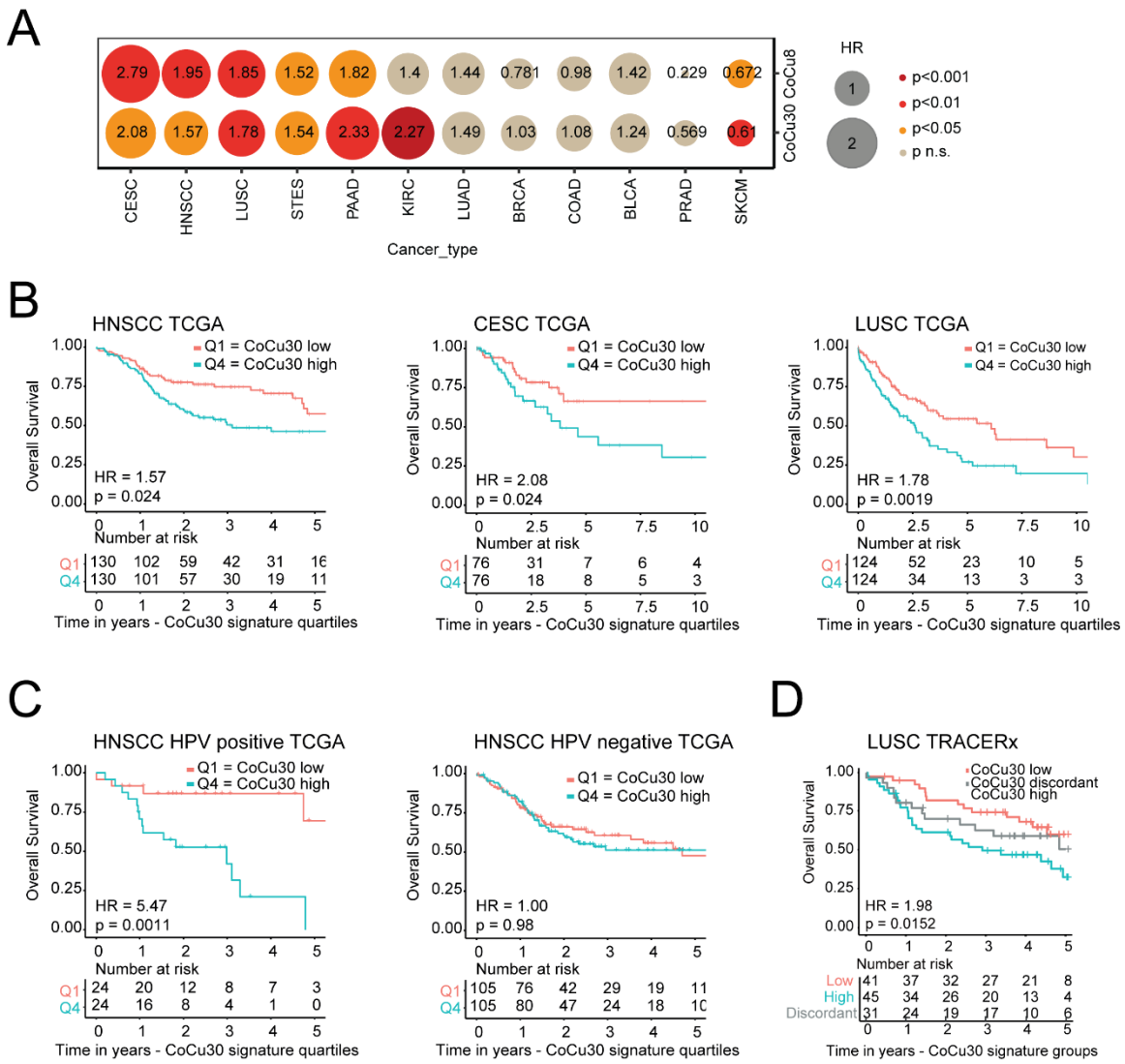

A
2.79 1.95 1.85 1.52 1.82 1.4 1.44 0.781 0.98 1.42 0.229 0.672
2.08 1.57 1.78 1.54 2.33 2.27 1.49 1.03 1.08 1.24 0.569 0.61
CoCu30 CoCu8
CESC HNSCC LUSC STES PAAD KIRC LUAD BRCA COAD BLCA PRAD SKCM
Cancer_type
HR
1
2
p<0.001
p<0.01
p<0.05
p n.s.
B
HNSCC TCGA
Q1 = CoCu30 low
Q4 = CoCu30 high
Overall Survival
HR = 1.57
p = 0.024
Number at risk
Q1 130 102 59 42 31 16
Q4 130 101 57 30 19 11
Time in years - CoCu30 signature quartiles
CESC TCGA
Q1 = CoCu30 low
Q4 = CoCu30 high
HR = 2.08
p = 0.024
Number at risk
Q1 76 31 7 6 4
Q4 76 18 8 5 3
Time in years - CoCu30 signature quartiles
LUSC TCGA
Q1 = CoCu30 low
Q4 = CoCu30 high
HR = 1.78
p = 0.0019
Number at risk
Q1 124 52 23 10 5
Q4 124 34 13 3 3
Time in years - CoCu30 signature quartiles
C
HNSCC HPV positive TCGA
Q1 = CoCu30 low
Q4 = CoCu30 high
HR = 5.47
p = 0.0011
Number at risk
Q1 24 20 12 8 7 3
Q4 24 16 8 4 1 0
Time in years - CoCu30 signature quartiles
HNSCC HPV negative TCGA
Q1 = CoCu30 low
Q4 = CoCu30 high
HR = 1.00
p = 0.98
Number at risk
Q1 105 76 42 29 19 11
Q4 105 80 47 24 18 10
Time in years - CoCu30 signature quartiles
D
LUSC TRACERx
CoCu30 low
CoCu30 discordant
CoCu30 high
HR = 1.98
p = 0.0152
Number at risk
Low 41 37 32 27 21 8
High 45 34 26 20 13 4
Discordant 31 24 19 17 10 6
Time in years - CoCu30 signature groups

Figure S2

1111 **Figure S2:** CoCu8 / CoCu30 gene signatures are associated with worse overall survival in multiple
1112 squamous cell carcinoma datasets. **A)** Bubble plot hazard ratios and p- values for overall survival for
1113 multiple TCGA cancer types using both CoCu8 and CoCu30 signatures. CESC - cervical squamous cell
1114 carcinoma; HNSCC - head and neck squamous cell carcinoma; LUSC – lung squamous cell carcinoma;
1115 PRAD - prostate adenocarcinoma; PAAD - pancreatic adenocarcinoma; LUAD - lung adenocarcinoma;
1116 KIRC - kidney clear cell carcinoma; COAD - colorectal adenocarcinoma; BLCA - bladder urothelial
1117 carcinoma; STES - esophagogastric carcinoma; SKCM – melanoma; BRCA - breast cancer. **B)** Kaplan-
1118 Meier overall survival analysis of HNSCC (left), CESC (centre), LUSC (right) TCGA datasets stratified for
1119 CoCu30 first vs last quartile. Below each analysis are shown the corresponding numbers at risk, time
1120 in years. HNSCC HR=1.57 (95%CI 1.06-2.35), p-value=0.024. CESC HR=2.08 (95%CI 1.09-4.01), p-
1121 value=0.024. LUSC HR=1.78 (95%CI 1.23-2.59), p-value=0.0019. HR and CI were calculated using Cox
1122 regression. p-value was calculated using logRank test. **C)** Kaplan-Meier overall survival analysis of
1123 HNSCC HPV positive (left) and HNSCC HPV positive (right) TCGA datasets stratified for CoCu30 first vs
1124 last quartile. Below each analysis are shown the corresponding numbers at risk, time in years. HPV
1125 positive HR=5.47 (95%CI 1.76-17.0), p-value=0.0011. HPV negative HR=1.00 (95%CI 0.67-1.51), p-
1126 value=0.98. HR and CI were calculated using Cox regression. p-value was calculated using logRank test.
1127 **D)** Kaplan-Meier overall survival analysis of LUSC TRACERx dataset. Individual tumors stratified as high-
1128 , discordant or low-risk according to expression profile of CoCu30 signature across multiple regions,
1129 as previously described and stratified according to Biswas et al. [60]. Below are shown the numbers at
1130 risk in years. HR=1.98 (95% CI 1.09-3.6), p-value=0.0152. HR and CI calculated using Cox regression
1131 and are referred to CoCu30 low vs CoCu30 high. p-value was calculated using logRank test.

CoCu8 Overall Survival

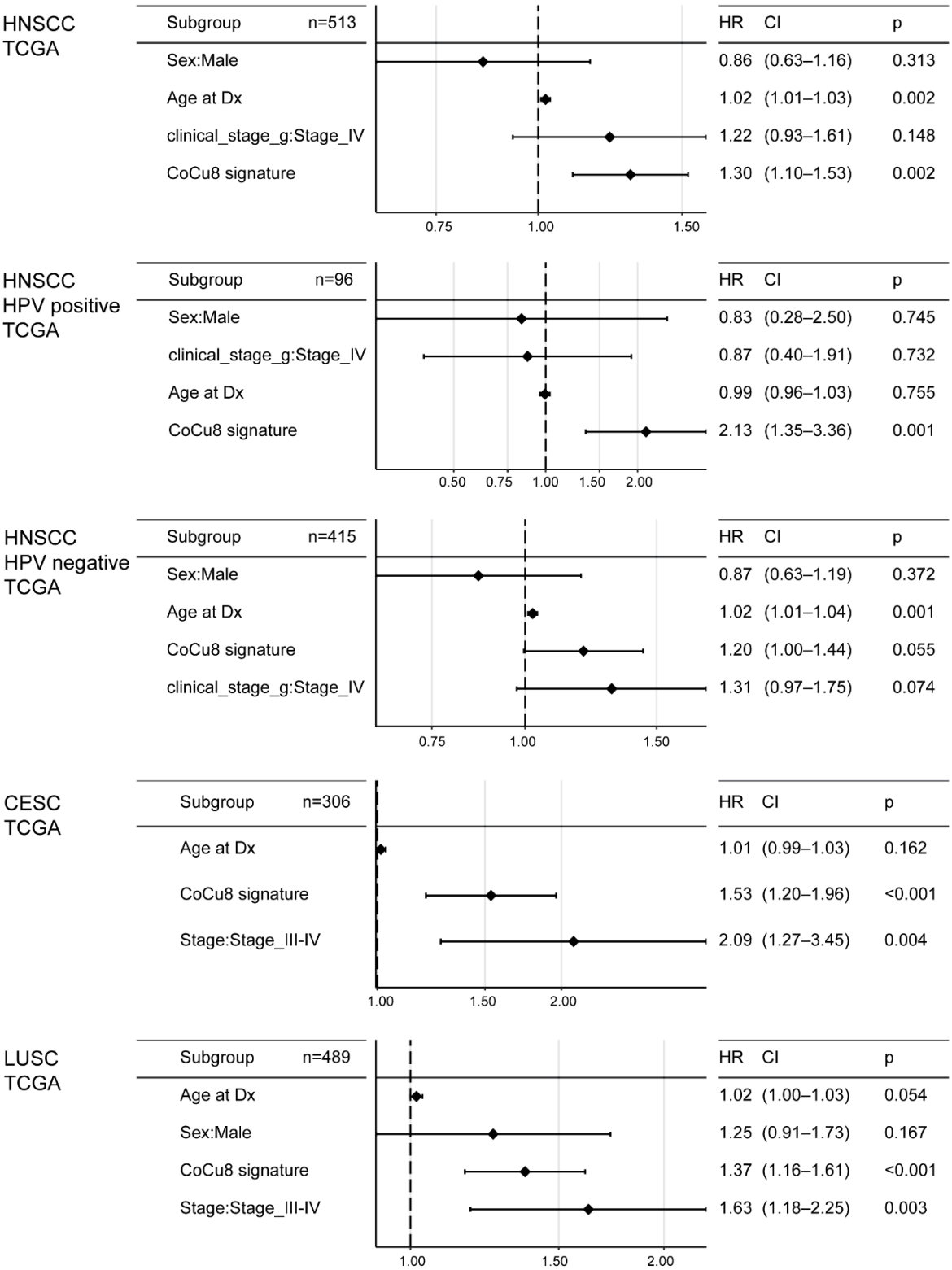


Figure S3

1133 **Figure S3:** Multivariate analysis of CoCu8 overall survival. Forest plot showing Hazard Ratios, 95%
1134 confidence interval and p value calculated using multivariate Cox regression from patients with HNSCC
1135 (HPV positive and negative), LUSC and CESC from the TCGA cohort. Variables include: age (continuous,
1136 years), sex (male vs female, except for CESC as all patients were female), clinical stage (categorical)
1137 and the CoCu8 signature (continuous variable).

CoCu30 Overall Survival

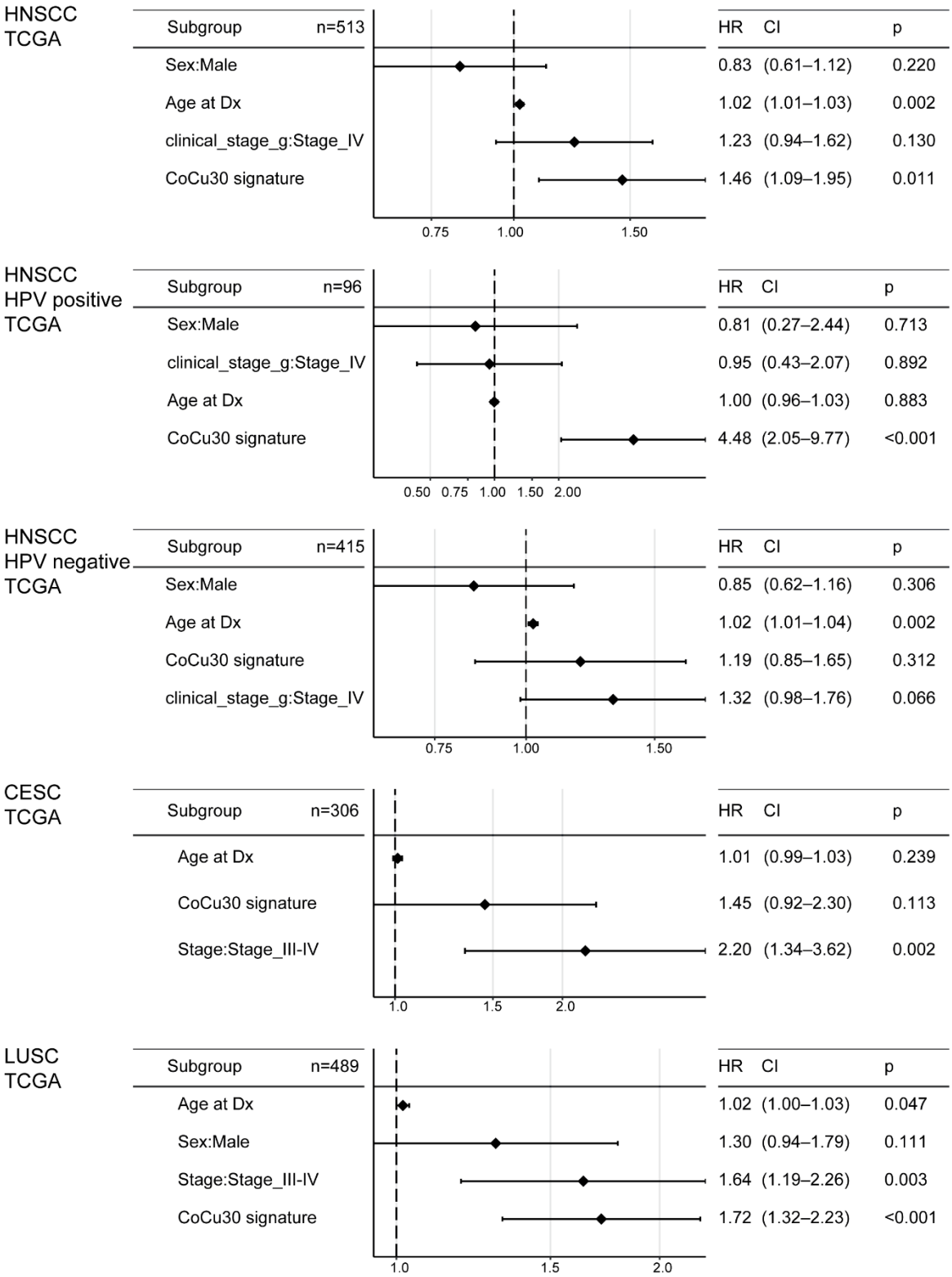


Figure S4

1139 **Figure S4:** Multivariate analysis of CoCu30 overall survival. Forest plot showing Hazard Ratios, 95%
1140 confidence interval and p value calculated using multivariate Cox regression from patients with HNSCC
1141 (HPV positive and negative), LUSC and CESC from the TCGA cohort. Variables include: age (continuous,
1142 years), sex (male vs female, except for CESC as all patients were female), clinical stage (categorical)
1143 and the CoCu30 signature (continuous variable).

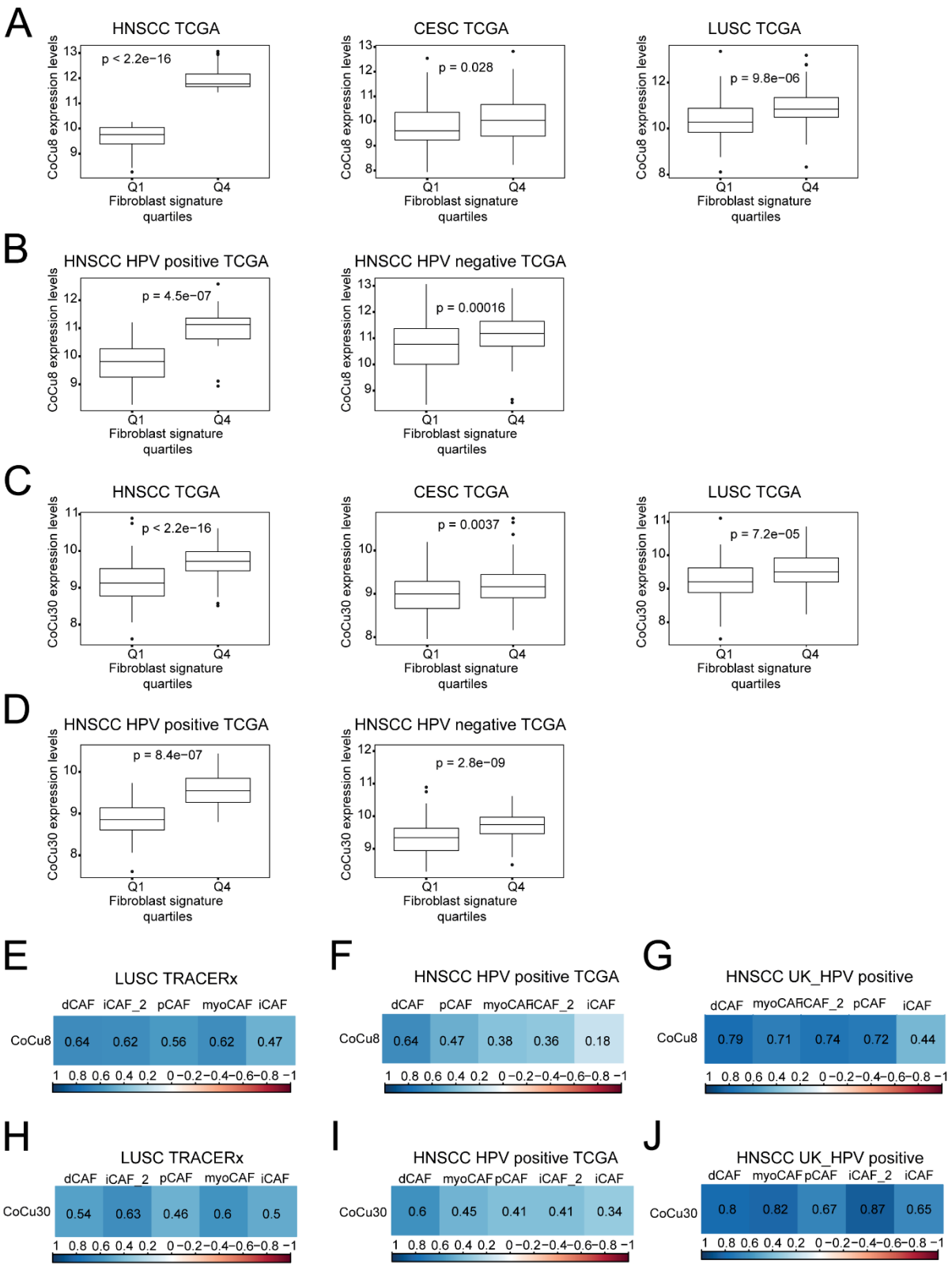

A
HNSCC TCGA
p < 2.2e−16
CESC TCGA
p = 0.028
LUSC TCGA
p = 9.8e−06
CoCu8 expression levels
Q1
Q4
Fibroblast signature quartiles
B
HNSCC HPV positive TCGA
p = 4.5e−07
HNSCC HPV negative TCGA
p = 0.00016
C
HNSCC TCGA
p < 2.2e−16
CESC TCGA
p = 0.0037
LUSC TCGA
p = 7.2e−05
CoCu30 expression levels
D
HNSCC HPV positive TCGA
p = 8.4e−07
HNSCC HPV negative TCGA
p = 2.8e−09
E
LUSC TRACERx
dCAF iCAF_2 pCAF myoCAF iCAF
CoCu8 0.64 0.62 0.56 0.62 0.47
F
HNSCC HPV positive TCGA
dCAF pCAF myoCAF iCAF_2 iCAF
CoCu8 0.64 0.47 0.38 0.36 0.18
G
HNSCC UK_HPV positive
dCAF myoCAF iCAF_2 pCAF iCAF
CoCu8 0.79 0.71 0.74 0.72 0.44
H
LUSC TRACERx
dCAF iCAF_2 pCAF myoCAF iCAF
CoCu30 0.54 0.63 0.46 0.6 0.5
I
HNSCC HPV positive TCGA
dCAF myoCAF pCAF iCAF_2 iCAF
CoCu30 0.6 0.45 0.41 0.41 0.34
J
HNSCC UK_HPV positive
dCAF myoCAF pCAF iCAF_2 iCAF
CoCu30 0.8 0.82 0.67 0.87 0.65
1 0.8 0.6 0.4 0.2 0 −0.2 −0.4 −0.6 −0.8 −1

Figure S5

1146 **Figure S5:** Fibroblast abundance correlates with CoCu8 / CoCu30 gene signature in different squamous
1147 cell carcinoma datasets. **A)** Box plot analysis of CoCu8 expression in HNSCC, CESC and LUSC TCGA
1148 separated by first and last quartile of fibroblast abundance via Methyl CIBERSORT deconvolution
1149 strategy. Independent Student's t-test. **B)** Box plot analysis of CoCu8 expression in HPV positive and
1150 negative TCGA separated by first and last quartile of fibroblast abundance via Methyl CIBERSORT
1151 deconvolution strategy. Independent Student's t-test. **C)** Box plot analysis of CoCu30 expression in
1152 HNSCC, CESC and LUSC TCGA separated by first and last quartile of fibroblast abundance via Methyl
1153 CIBERSORT deconvolution strategy. Independent Student's t-test. **D)** Box plot analysis of CoCu30
1154 expression in HPV positive and negative TCGA separated by first and last quartile of fibroblast
1155 abundance via Methyl CIBERSORT deconvolution strategy. Independent Student's t-test. **E-J)**
1156 Correlation plot of different fibroblast subpopulations derived from Galbo et al. [9] with CoCu8 gene
1157 signature in LUSC TRACERx **(E)**, HPV positive HNSCC TCGA **(F)**, UK_HPV positive HNSCC **(G)** patients
1158 and with CoCu30 gene signature in LUSC TRACERx **(H)**, HPV positive HNSCC TCGA **(I)**, UK_HPV positive
1159 HNSCC **(J)** patients. The number inside the square represents the R, Spearman correlation coefficient.
1160 The colour legend is shown at the bottom. All correlations are significant p-value<0.05.

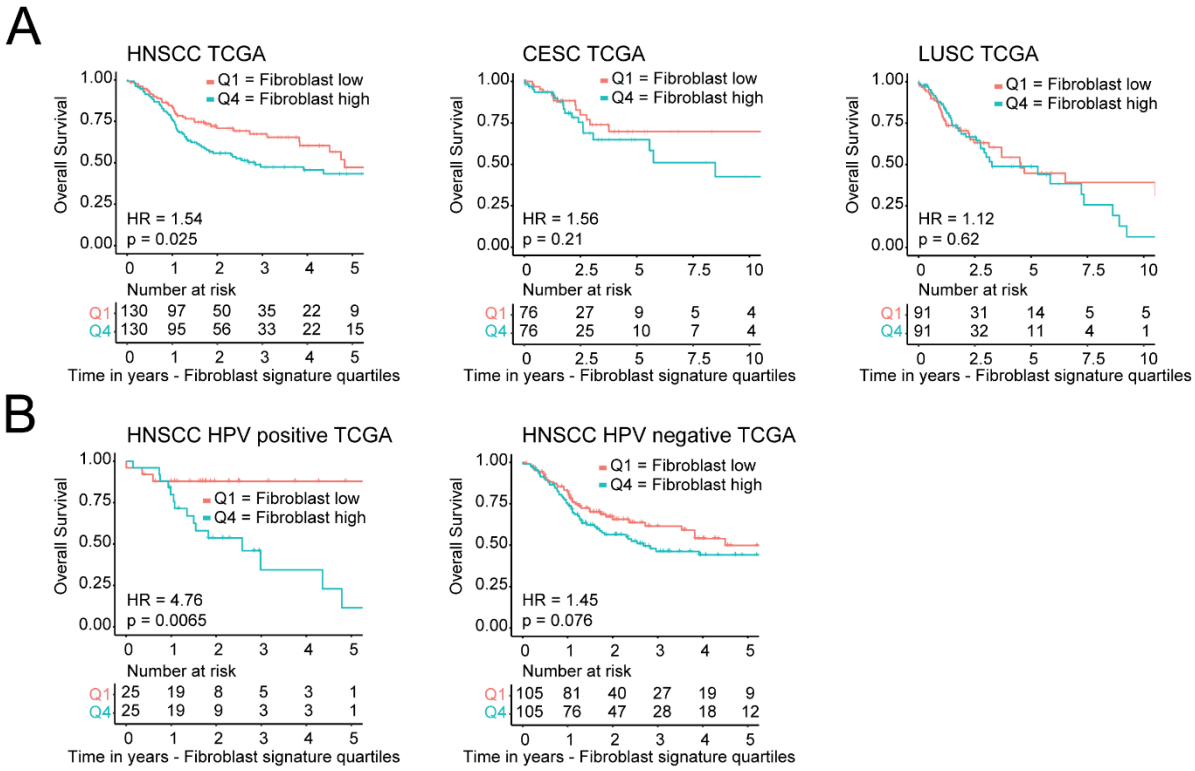
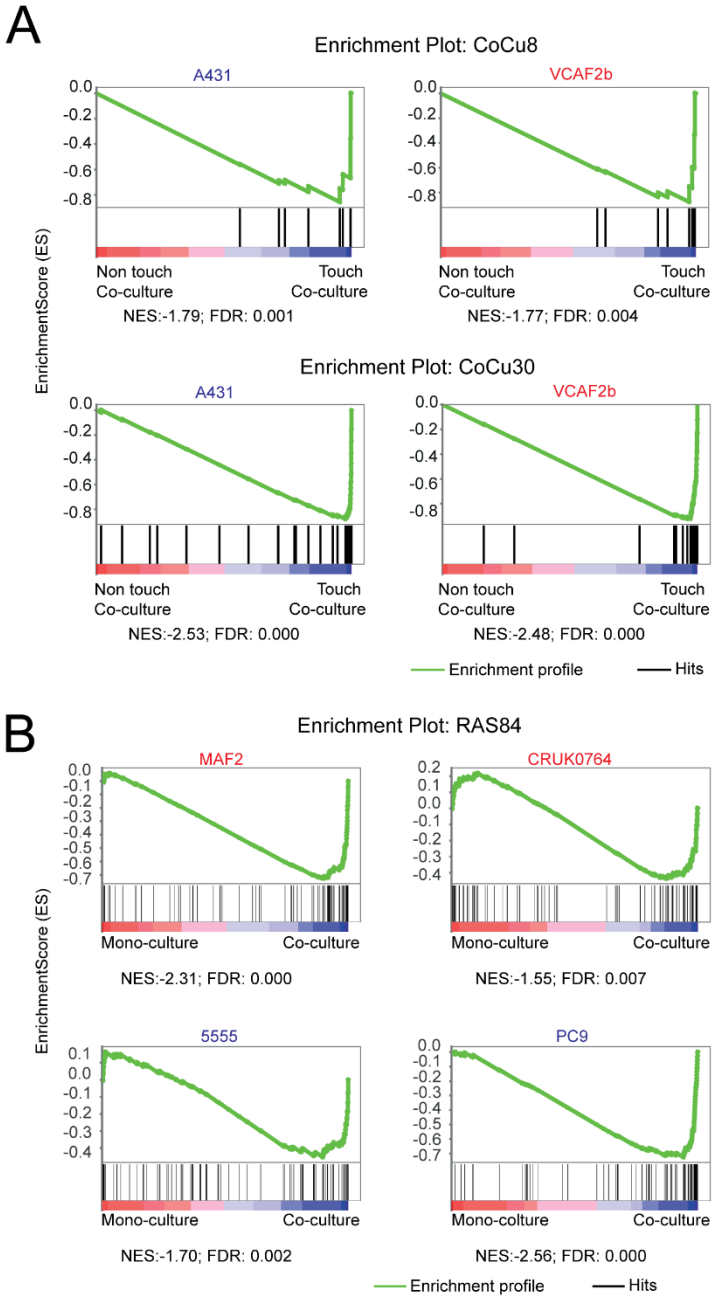

A
HNSCC TCGA
Q1 = Fibroblast low
Q4 = Fibroblast high
Overall Survival
HR = 1.54
p = 0.025
Number at risk
Q1 130 97 50 35 22 9
Q4 130 95 56 33 22 15
Time in years - Fibroblast signature quartiles
CESC TCGA
HR = 1.56
p = 0.21
Q1 76 27 9 5 4
Q4 76 25 10 7 4
LUSC TCGA
HR = 1.12
p = 0.62
Q1 91 31 14 5 5
Q4 91 32 11 4 1
B
HNSCC HPV positive TCGA
HR = 4.76
p = 0.0065
Q1 25 19 8 5 3 1
Q4 25 19 9 3 3 1
HNSCC HPV negative TCGA
HR = 1.45
p = 0.076
Q1 105 81 40 27 19 9
Q4 105 76 47 28 18 12

Figure S6

1162 **Figure S6:** Fibroblast abundance meta-analysis on different squamous cell carcinoma datasets. **A)**
1163 Kaplan-Meier overall survival analysis of HNSCC (left), CESC (centre), LUSC (right) TCGA datasets
1164 stratified for fibroblast abundance first vs last quartile. Below each analysis are shown the
1165 corresponding numbers at risk, time in years. HNSCC HR=1.54 (95%CI 1.05-2.25), p-value=0.025. CESC
1166 HR=1.56 (95%CI 0.77-3.17), p-value=0.21. LUSC HR=1.12 (95%CI 0.71-1.77), p-value=0.62. HR and CI
1167 were calculated using Cox regression. p-value was calculated using logRank test. **B)** Kaplan-Meier
1168 overall survival analysis of HNSCC HPV positive (left) and HNSCC HPV positive (right) TCGA datasets
1169 stratified for fibroblast abundance first vs last quartile. Below each analysis are shown the
1170 corresponding numbers at risk, time in years. HPV positive HR=4.76 (95%CI 1.36-16.5), p-
1171 value=0.0065. HPV negative HR=1.45 (95%CI 0.96-2.18), p-value=0.076. HR and CI were calculated
1172 using Cox regression. p-value was calculated using logRank test.

1173



1174

Figure S7

1175 **Figure S7:** Enrichment of CoCu8, CoCu30 and RAS84 gene signatures in different co-culture conditions.
1176 **A)** Gene set enrichment analysis (GSEA) plot of CoCu8 gene signature (top) and CoCu30 (bottom) in
1177 co-culture indirect vs direct condition in A431 / VCAF2b transcriptomic dataset. NES and FDR are
1178 specified below each plot. **B)** Gene set enrichment analysis (GSEA) plot of RAS84 gene signature [28] in
1179 mono-culture and co-culture. NES and FDR are specified below each plot.

1180

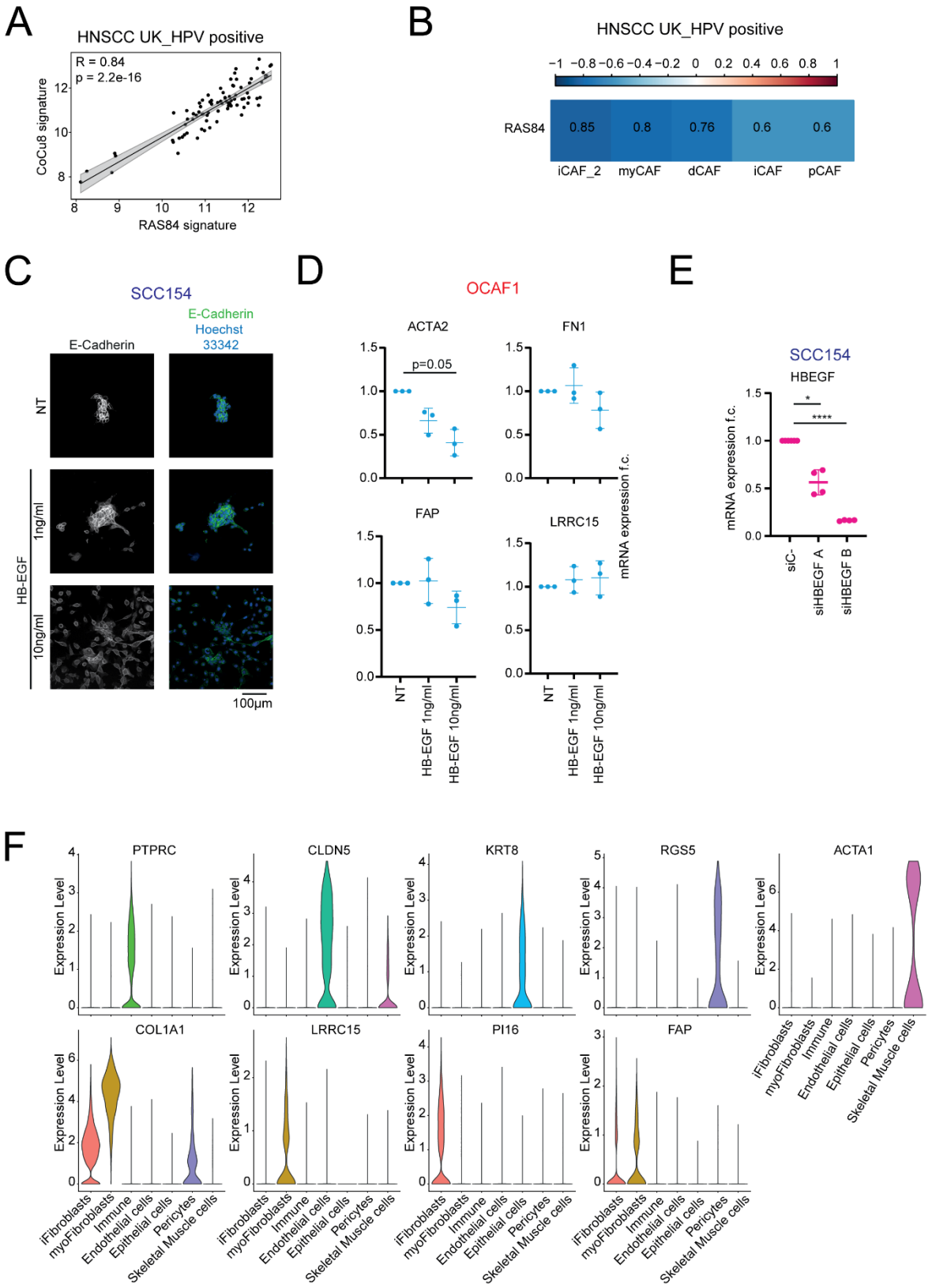


Figure S8

1181

1182 **Figure S8:** HB-EGF/RAS/MAPK activity in cancer cells and CAFs mono-culture vs co-culture. **A)**
1183 Correlation plot of CoCu8 and RAS84 expression levels in HNSCC HPV positive UK_HPV positive cohort.
1184 R is Spearman correlation coefficient. **B)** Correlation table of different fibroblast subpopulations
1185 derived from Galbo et al. [9] with RAS84 gene signature in the independent cohort UK_HPV positive of
1186 HNSCC HPV positive patients. The number inside the square represents the Spearman R, correlation
1187 coefficient. The colour legend is shown on the top. All correlations are significant at p-value<0.001.
1188 n=97. **C)** Immunofluorescence staining of E-Cadherin and Hoechst 33342 for SCC154 mono-culture for
1189 the indicated treatments after 48h. **D)** qPCR analysis of *ACTA2*, *FAP*, *FN1* and *LRRC15* genes in OCAF1
1190 mono-culture for the indicated treatments after 48h. mRNA expression is reported as
1191 mean ± standard deviation (SD) fold change difference over non-treated (NT) condition. Genes have
1192 been normalized over the average of *GAPDH*, *ACTB* and *RPLP0* housekeeping genes. n = 3 independent
1193 experiments. **E)** qPCR analysis of *HBEGF* gene in SCC154 after 96h of treatment with the indicated
1194 conditions. mRNA expression is reported as mean ± standard deviation (SD) fold change difference
1195 over siC- condition. Gene has been normalized over the average of *GAPDH*, *ACTB* and *RPLP0*
1196 housekeeping genes. n ≥ 4 independent experiments. Two tailed paired Student's t-test. **F)** Violin plot
1197 of mRNA expression levels of the indicated genes for each cluster from Choi et al. [33].

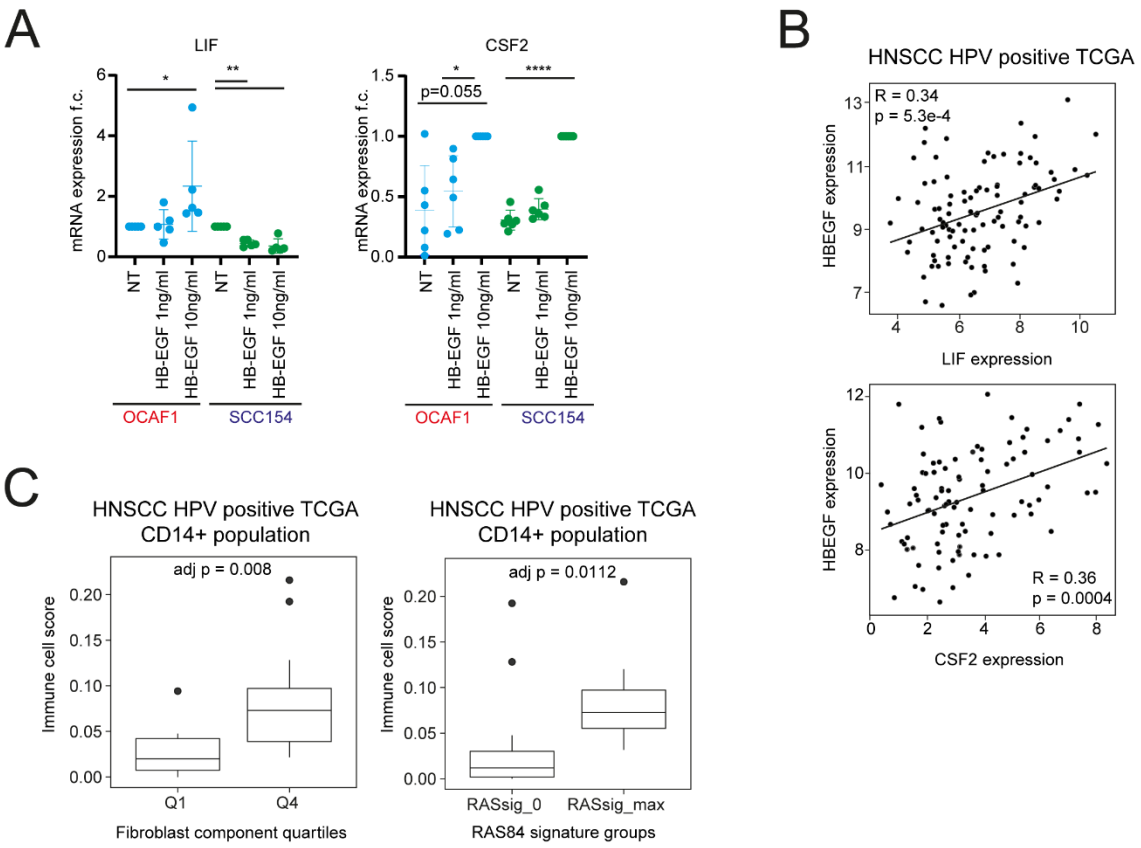

A
LIF
CSF2
mRNA expression f.c.
p=0.055
NT
HB-EGF 1ng/ml
HB-EGF 10ng/ml
OCAF1
SCC154
B
HNSCC HPV positive TCGA
R = 0.34
p = 5.3e-4
HBEGF expression
LIF expression
R = 0.36
p = 0.0004
CSF2 expression
C
HNSCC HPV positive TCGA
CD14+ population
adj p = 0.008
adj p = 0.0112
Immune cell score
Q1
Q4
Fibroblast component quartiles
RASsig_0
RASsig_max
RAS84 signature groups

Figure S9

1199 **Figure S9:** HB-EGF effect on cytokine production and monocyte/macrophage enrichment. **A)** qPCR
1200 analysis of *LIF* and *CSF2* genes in OCAF1 and SCC154 mono-cultures for the indicated treatments after
1201 48h. mRNA expression is reported as mean ± standard deviation (SD) fold change difference over non-
1202 treated (NT) condition for each cell type with *LIF*, while for *CSF2* fold change difference is reported
1203 over Hb-EGF 10ng/ml treated sample. Genes have been normalized over the average of *GAPDH*, *ACTB*
1204 and *RPLP0* housekeeping genes. n ≥ 5 independent experiments. Two tailed paired Student's t-test. **B)**
1205 Correlation plot of *HBEGF* with *LIF* (left) and *HBEGF* with *CSF2* (right) expression levels in HNSCC HPV
1206 positive TCGA dataset. R is Spearman correlation coefficient. **C)** (Right) Box plot analysis of immune
1207 cell absolute score via Methyl CIBERSORT deconvolution strategy in HNSCC HPV positive separated by
1208 first and last quartile of fibroblast abundance. Independent Student's t-test. Bonferroni correction for
1209 multiple comparisons. (Left) Box plot analysis of immune cell absolute score via Methyl CIBERSORT
1210 deconvolution strategy in HNSCC HPV positive separated by RAS84_0 and RAS84_max. Student's t-
1211 test Bonferroni correction for multiple comparisons.

1212

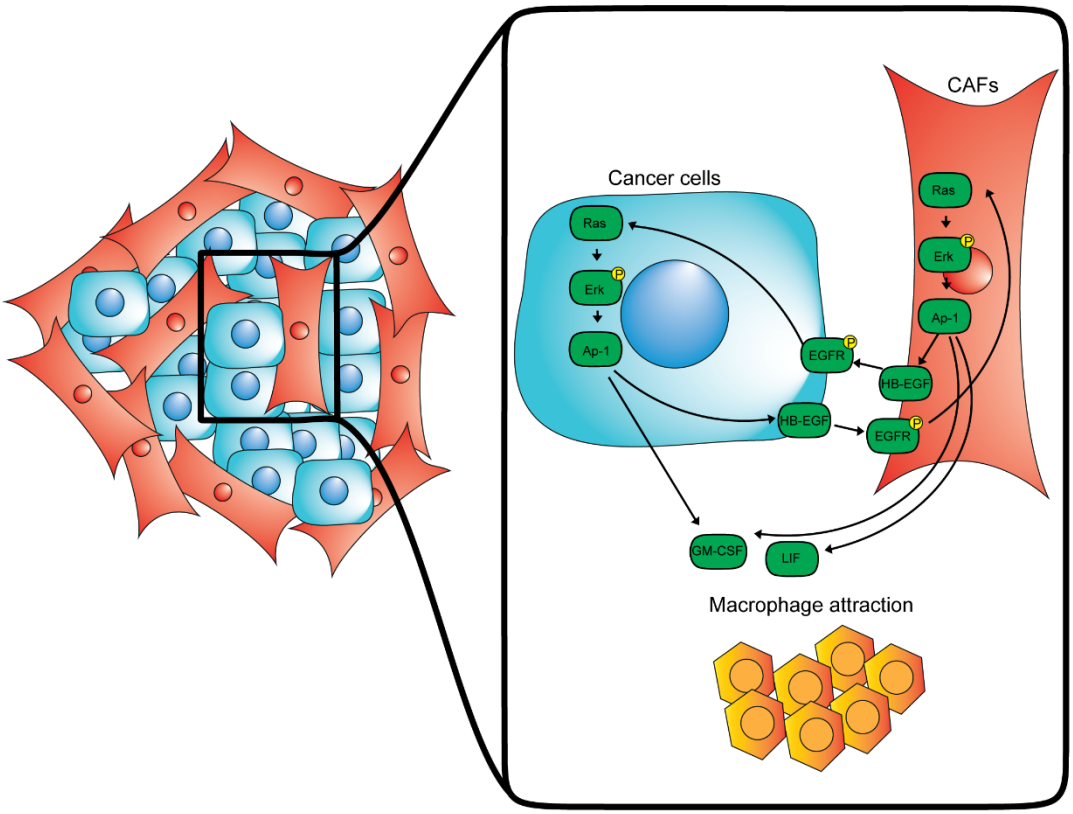


Figure S10

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1214

1215 **Figure S10:** Schematic representation of the current cross-talk model of cancer cells – CAFs co-culture.