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- 2 factor 1-mediated secretion of extracellular vesicles
- 3
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- 34 35

## 36 Abstract

37 Gastric cancer is the 3rd most lethal cancer worldwide, and evaluation of the genomic status of gastric 38 cancer cells has not translated into effective prognostic or therapeutic strategies. We therefore 39 hypothesize that outcomes may depend on the tumor microenvironment (TME), in particular, cancer-40 associated fibroblasts (CAF). However, very little is known about the role of CAFs in gastric cancer. 41 To address this, we mapped the transcriptional landscape of human gastric cancer stroma by 42 microdissection and RNA sequencing of CAFs from gastric cancer patients. A stromal gene signature 43 was associated with poor disease outcome, and the transcription factor heat shock factor 1 (HSF1) 44 regulated the signature. HSF1 upregulated inhibin subunit beta A (INHBA) and thrombospondin 2 45 (THBS2), which were secreted in CAF-derived extracellular vesicles (EV) to the TME to promote 46 cancer. Together, our work provides the first transcriptional map of human gastric cancer stroma and highlights HSF1 and its transcriptional targets as potential diagnostic and therapeutic targets in the 47 48 genomically stable tumor microenvironment.

49

## 50 Significance

51 We highlight a stromal transcriptional program associated with aggressive gastric cancer, describe a 52 role for HSF1 in regulating it, and propose that this program is communicated to cancer cells via 53 exosome-mediated signaling.

54

## 55 Introduction

Gastric cancer is the 5<sup>th</sup> most common cancer and the 3<sup>rd</sup> most lethal cancer, worldwide (1). Recent advances in treatment were made possible due to better classification of gastric cancer subtypes, but the prognosis of advanced gastric cancer remains poor and many patients get diagnosed at an advanced stage of the disease due to limited understanding of the underlying biology (2). There is an urgent need to better understand the molecular basis of this disease, and to identify biomarkers that may predict outcome and guide therapy.

Gastric cancer is a heterogeneous disease. Traditionally, anatomical location (true gastric vs gastro-62 63 esophageal) and histological characteristics (diffuse vs intestinal; tubular vs papillary) have been used 64 to classify gastric cancer subtypes (2). Recent advances in molecular understanding have enabled 65 classification of gastric cancer into different subtypes based on chromosomal instability, microsatellite 66 instability, genomic stability, presence of Epstein-Barr virus, and epithelial- mesenchymal transition 67 (EMT), which were associated with different survival outcomes (3-6). Mutations in CDH1 and KRAS, 68 and overexpression of HER2, EGFR, FGFR2, VEGF, were shown to contribute to disease progression 69 and correlate with poor outcome (7,8). Despite serving as valuable guides in deciphering the

70 complexity of gastric cancer, there has been little success in applying these molecular classifiers to 71 treatment stratification and development of targeted therapies (3). Prognosis in the clinic is still mostly 72 evaluated based on TNM staging (Tumor size, lymph Node involvement and Metastasis), and the 73 standard of care for localized gastric cancer is surgical intervention combined with chemotherapy (7). 74 Increasing evidence over the past decade highlighted the indispensable contribution of the tumor 75 microenvironment (TME) to disease progression and treatment resistance (9). The TME is comprised 76 of various cell types, including endothelial cells, fibroblasts, macrophages, and lymphocytes, as well 77 as extracellular matrix components (ECM) (10). The immune microenvironment of gastric cancer has 78 gained increasing attention over the last years, due to its potential effect on immunotherapy in patients 79 with high microsatellite instability (11). Yet little is known about the contribution of cancer-associated 80 fibroblasts (CAFs) to gastric cancer progression and metastasis. CAFs are the most abundant cell type in a variety of carcinomas (12). They support cancer cells by modifying the ECM, promoting 81 82 angiogenesis and maintaining a chronic inflammatory state (12-17). In gastric cancer, accumulation of 83 CAFs is correlated with increased tumor size, invasion and metastasis (18). Recently, the abundance 84 of natural killer cells, endothelial cells and CAFs was shown to predict chemotherapy benefit in gastric 85 cancer (19). However, the specific genes and molecular events contributing to these protumorigenic 86 effects are not well understood. To address this, we set out to map the transcriptional landscape of 87 gastric CAFs. Using laser-capture microdissection (LCM) and RNA-sequencing of CAFs from gastric 88 cancer patients we define a gene-signature associated with poor disease outcome. We characterize this 89 signature using mouse models and co-culture assays, and show that components of this signature are 90 regulated by the master transcriptional regulator heat shock factor 1 (HSF1) (20), and secreted from 91 CAFs via extracellular vesicles (EVs). These fibroblast-derived EVs contribute to tumor growth in an 92 HSF1-dependent manner. Together, our work provides a comprehensive map of gastric cancer stromal 93 transcription with potential implications on prognosis and treatment.

94

### 95 Materials and Methods

## 96 Ethics statement

97 Clinical samples and patient data were collected following approval by the Rabin medical center 98 Institutional Review Board (IRB, protocol #0297-11-RMC) with full exemption for consent form for 99 anonymized samples. Human samples used for MxIF staining were obtained from the Israel National 100 Biobank for Research (MIDGAM; https://www.midgam.org.il/) under IRB # 6141-19-SMC. These 101 samples were collected from patients who provided written informed consent for collection, storage, 102 distribution of samples and data for use in future research studies. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC protocol #15310619-2, #15140619-3,
#06690820-3).

105

## 106 **Mice**

107 Athymic Nude mice were purchased from Harlan biotech (Rehovot, Israel). These mice, the triple-108 transgenic *Lgr5-EGFP-IRES-CreERT2; R26-LSL-rtTA-IRES-EGFP; tetO-GLI2A* mice, (*iLgr5;GLI2A* 109 mice (21)), *Hsf1* null mice and their WT littermates (BALB/c  $\times$  129SvEV, by Ivor J. Benjamin (22)) 110 were maintained under specific-pathogen-free conditions at the Weizmann Institute's animal facility.

111

## 112 Cell lines and primary cell cultures

113 N87 gastric cancer cells were kindly provided by Yosef Yarden (WIS; originally from ATCC). N87 114 cells were transduced with green fluorescent protein (GFP) using a 3<sup>rd</sup>-generation lentiviral system. MC38 colon cancer cells were kindly provided by Lea Eisenbach (WIS; originally from NCI). MC38 115 cells were transduced with mcherry-luciferase using a 2<sup>nd</sup>-generation lentiviral system. Primary MEFs 116 117 were produced from WT and Hsfl null mice. HFF cells were purchased from ATCC. MEFs, MC38 118 cells and N87 cells were cultured in RPMI (#01-100-1A, Biological Industries) supplemented with 10% 119 fetal bovine serum (FBS; Invitrogen) and P/S (Biological Industries). HFF cells were cultured in 120 DMEM (#01-052-1A, Biological Industries) supplemented with 15% FBS, 1.5% L-Glutamine and P/S. 121 Cell lines were tested routinely for Mycoplasma using EZ-PCR Mycoplasma test kit (#20-700-20, 122 Biological Industries). MEFs were used in passage 1. Other cell lines were maintained below passage 123 25.

124

## 125 Laser capture microdissection of human gastric cancer samples

126 LCM cohort patients were selected based on patient outcome data (Supplementary Table 1). Stromal 127 and cancer regions were marked by a trained pathologist blinded to clinical and outcome data to include 128 >90% CAFs for stroma and >90% cancer cells for cancer. Gastric muscle, immune islands, and blood 129 vessels were excluded from microdissection. FFPE slides were deparaffinized and stained using 130 Arcturus Paradise Plus Staining kit (#KIT0312J, Thermo-Fischer) according to the instructions of the 131 manufacturer. Slides were left to dry for 5 min at RT followed by microdissection using the Arcturus 132 (XT) laser microdissection instrument (#010013097, Thermo-Fischer). Infrared capture was used to 133 minimize RNA damage. CapSure Macro LCM caps (#LCM0211, Thermo-Fischer) were used to 134 capture microdissected tissue. To obtain sufficient material from these highly degraded RNA samples 135 we performed microdissection from 6-10, 5 µm sections per sample. Microdissected tissue from each 136 sample was pooled together, and kept on dry ice until RNA isolation using the RNeasy FFPE kit 137 (#73504, Qiagen) with one modification- proteinase K digestion at 56°C was carried out for 1 h.

138

## 139 Library preparation, RNA-sequencing and analysis of LCM samples

140 Libraries were prepared using the SMARTer Stranded Total RNA-Seq v2-Pico Input Mammalian kit 141 (#634415, Takara Bio USA) according to the instructions of the manufacturer. Libraries were 142 sequenced on Illumina NextSeq 500, at 50M reads for stroma and 25M reads for cancer samples, to 143 provide sufficient reads to pass quality control filters of RNA-seq. Principal Component Analysis 144 (PCA) was performed on full RNA-seq datasets for each sample (for stroma and cancer samples, 145 separately). After calculating the first 3 main PCs (PCA1-3), we used the Robust Mahalanobis distance 146 function to exclude potential outlier samples (see GitHub https://github.com/privefl/bigutilsr, and (23-147 25)). These robust Mahalanobis distances are approximately Chi-square distributed, which enables 148 deriving p-values of outliers (Supplementary Table 2). Since we used 3 dimensions, we chose a p-value 149 threshold of 0.00111 (p-value < 0.01 with Bonferroni correction for multiple comparisons) which 150 concluded that patient 5 is an outlier in PCA2 & 3. This patient was removed from all downstream 151 analysis. Read counts of the 8 patients were normalized and tested for difference using DESeq2 (26). 152 Hierarchical clustering was carried out using Pearson correlation with complete linkage and on 153 differentially expressed genes (DEGs) which were filtered with the following parameters: baseMean > 154 5, padj < 0.1 and |logfoldchange| > 1. Pathway analysis was performed using Metascape, significant 155 pathways were determined if p < 0.05 and FDR < 0.5. STRING analysis was performed including all 156 DEGs.

157

## 158 CAF isolation and RNA sequencing from *iLgr5;GLI2A* mice

159 Gastric cancer was induced in *iLgr5;GLI2A* mice as described in (21). Gastric tumors were harvested 160 post mortem, washed, minced and dissociated using a gentleMACS dissociator and enzymatic 161 digestion with DMEM containing 3 mg ml<sup>-1</sup> collagenase A (#11088793001, Sigma Aldrich,) and 0.1 162 mg/ml Deoxyribonuclease I (#LS002007, Worthington) for 20 min at 37°C. The single cell suspension was washed, filtered using 100 µm cell strainer, and immunostained. Normal gastric fibroblasts or 163 164 CAFs were collected based on negative selection for ghost dye, CD45, EpCAM, and CD31 and positive 165 selection for PDPN. RNA-seq was done by MARS-seq as described in (27). DEGs were filtered with 166 the following parameters: baseMean > 5, padj < 0.01 and  $|\log foldchange| > 3$ . Pathway analysis was 167 performed using Metascape, significant pathways were determined if p < 0.05 and FDR < 0.5.

168

## Validation of the patient and *iLgr5;GLI2A* mouse stromal signatures in independent patient cohorts

Patient data from the TCGA, Singapore (GSE15460), KUGH\_KUCM (GSE26942), and ACRG 171 172 cohorts (GSE62254) were downloaded, individual gene values were transformed to z-scores and the 173 average of all known genes per sample was used to determine scores for the upregulated and 174 downregulated signatures. For the INHBA-THBS1-THBS2 gene signature individual gene values were 175 transformed to z-scores and the average of genes per sample was determined. Gene symbols were 176 matched through Affymetrix Human Genome U133 Plus 2.0 Array or Illumina HumanHT-12 V4.0 177 expression bead chip. For patient cohorts GSE15460 and GSE62254 we could match 109 DEGs from 178 the CAF up sig and CAF down sig and for GSE26942 we could match 87 DEGs from the 179 CAF up sig and CAF down sig (out of the total 129 DEGs). For the *iLgr5;GLI2A* mCAF up sig 180 and mCAF down sig, 314 DEGs were matched in the GSE15460 and GSE62254 cohorts and 271 181 DEGs in the GSE26942 cohort (out of the total 361 DEGs). Median signature was calculated using 182 patients with complete survival and signature information. Kaplan Meier (KM) analysis of overall 183 survival with log rank p value was performed for each cancer type or patient cohort on patients 184 stratified by median expression of each of these signatures.

185

## 186 HSF1 scoring and analysis

187 Nuclear HSF1 staining in stroma and cancer cells of 72 patients was analysed by a trained pathologist 188 who was blinded to both patient outcome and clinical data. A scale of 0-3 (0-3: low  $\leq$  1; 1.5 < 189 intermediate  $\leq 2$ ; high >2) was set by the pathologist and scores were given based on nuclear staining 190 of HSF1 in stroma and cancer cells (Supplementary Table 1). Tissue samples were obtained from 191 surgical specimens. Patients diagnosed as stage 1-3 did not present with metastases at diagnosis. 8 192 patients diagnosed as stage 4 gastric cancer with metastases were omitted from further analysis. Overall 193 survival was defined as the time from first diagnosis to death based on the clinical data outlined in 194 Supplementary Table 1. The scores in cancer cells and CAFs showed different distributions. Therefore 195 for survival analysis of HSF1 activation in cancer cells, patients with low and intermediate scores were 196 combined and compared to patients with high scores, whereas for survival analysis of HSF1 activation 197 in CAFs, patients with high and intermediate scores were combined and compared to patients with low 198 scores (Supplementary Table 1). One patient could not be scored for cancer and for CAF HSF1 due to 199 insufficient tumor tissue and was therefore excluded from all statistical analyses. Two patients could 200 not be scored for CAF HSF1 and were excluded from CAF HSF1 analysis. Stage 2/3 was scored as 201 stage 2 in the final clinical analysis.

202

#### 203 Co-injection of recombinant Activin A and THBS2 with MC38 cancer cells into Nude mice

- 204 MC38 (2\*10<sup>5</sup>) were incubated with either PBS, 2.5 μg of Activin A (#CYT -146, ProSpec), or 2.5 μg
- of THBS2 (#1635-T2, R&D Systems) and co-injected in a total volume of 100 µl subcutaneously into
- 206 Nude mice (Harlan laboratories). 48h later a second dose of 2.5 µg recombinant protein was injected.
- 207 Tumors were measured by caliper for size and mice were sacrificed at day 15 due to high burden in the
- 208 Activin A group.
- 209

## 210 Co-injection of EVs with MC38 cancer cells into Nude mice

- MC38 cells  $(2*10^5)$  were co-injected with either PBS or  $1*1^{10}$  WT or *Hsf1* null EVs subcutaneously into Nude mice (Harlan laboratories). 48h later a second dose  $(5*10^9)$  of EVs was injected. Tumors were measured by caliper for size and the mice were sacrificed at day 17 due to high tumor burden.
- 214

## 215 Data availability statement

RNA sequencing data of *iLgr5;GLI2A* mice and patient samples were deposited in Gene Expression
Omnibus (GEO) and can be accessed via GSE162301 and GSE165211, respectively. All other data
supporting the findings of this study are available from the corresponding author on reasonable request.

## 219 Results

# CAFs express a transcriptional program that promotes malignancy and correlates with poor disease outcome in gastric cancer

222 Gastric CAFs have been attributed protumorigenic effects, however the genes contributing to these 223 effects are largely unknown. Therefore, we mapped the transcriptome of gastric CAFs in the 224 intratumoral stroma by laser capture microdissection (LCM) followed by RNA-sequencing 225 (Supplementary Fig. S1A). We isolated and sequenced CAF-rich stromal regions from formalin-fixed 226 paraffin-embedded (FFPE) tumor sections of 9 gastric cancer patients (Supplementary Fig. S1B-C and 227 Supplementary Table 1), representing favorable (survival) and poor prognostic (lethality) outcomes 228 (Supplementary Table 3). Principal component analysis (PCA) showed that stromal samples from these 229 patients clustered based on disease outcome (Fig. 1A and Supplementary Fig. S1D), while cancer 230 samples from the same patients did not (Fig. 1B and Supplementary Fig. S1E). Differential expression 231 analysis of stromal samples (see Materials and Methods, Supplementary Table 2 and Supplementary 232 Fig. S1F-G) revealed 129 differentially expressed genes (DEGs) between favorable and poor outcome 233 groups (Fig. 1C; Supplementary Table 3). ECM organization (involving genes such as AEBP1, 234 COL10A1, COL11A1, SPOCK1, THBS2, EMILIN1, and TPM2), response to growth factors (INHBA,

235 FGFR1, HSPB1) and mesenchymal cell proliferation (LMNA, UACA) were the most differentially 236 upregulated pathways in the stroma of patients with poor outcome (compared to patients with favorable 237 outcome; Fig. 1C and Supplementary Table 4). The humoral immune response (involving genes such 238 as LCN2, PGC, REG1A, ITLN1, BPIFB1, and BIRC3), digestive tract development (GATA6, ITGA6, 239 CLDN18), and tissue homeostasis (LYZ, MUC6) were most significantly downregulated in these 240 patients' stroma, compared to patients with favorable outcome (Fig. 1C and Supplementary Table 4). 241 Analysis of cancer samples from the same patients highlighted only 13 DEGs, and no significant 242 differentially regulated pathways (Supplementary Table 3 and Supplementary Fig. S1H).

243 The observed changes in stromal gene expression could be driven by differences in stromal abundance 244 between the patient groups. To test this, we performed image analysis to quantify stroma, cancer, and 245 immune regions in Hematoxylin Eosin (H&E) stained FFPE sections from the patients. We found no 246 significant difference in the percentage of stroma, cancer and immune cells between the favorable and 247 poor outcome patients, suggesting that it is not the abundance, but the transcriptional program that is 248 different between the two groups (Supplementary Fig. S1I-L). These findings suggest that as tumors 249 progress, stromal pathways involved in maintaining normal stomach functions are replaced by 250 pathways resulting from tumor-stroma interactions that support tumor growth.

251 We next set out to test the correlation between our stromal signature and clinical characteristics in 252 independent datasets. Since no pure gastric CAF datasets with reported disease outcome are available, 253 to the best of our knowledge, we turned to published datasets from bulk tumors and asked whether a 254 stromal signature comprised of genes upregulated in poor outcome patients in our dataset 255 (CAF up sig) could be detected in bulk tumors (including both stroma and cancer cells). First, we 256 analyzed The Cancer Genome Atlas (TCGA) datasets for gastro-intestinal (GI) tract cancers (gastric, 257 colorectal, pancreatic, hepatocellular, esophageal; Fig. 1D-E and Supplementary Table 5), and found 258 that the CAF up sig is significantly associated with poor outcome in gastric cancer and in colorectal 259 cancer (Fig. 1D-E). Genes downregulated in the stroma (CAF down sig) did not show any significant 260 association with survival (Supplementary Fig. S1M-N).

We then analyzed datasets from three other large patient cohorts: The Singapore cohort, the KUGH\_KUCM cohort, and the ACRG cohort (Supplementary Table 6). CAF\_up\_sig expression significantly associated with poor overall survival in the Singapore cohort and in the KUGH\_KUCM cohort, and a similar trend was found with the ACRG cohort (Fig. 2A-C). Our CAF\_down\_sig showed an opposite trend – high expression of CAF\_down\_sig significantly correlated with favorable outcome in the Singapore and KUGH\_KUCM cohorts, and a similar mild trend was observed with the ACRG cohort (Fig. 2D-F). Univariate analysis showed that CAF\_up\_sig expression, cancer stage and presence

- of metastasis were associated with poor overall survival in the Singapore and the KUGH\_KUCM
  cohorts and the ACRG cohort showed a similar trend (Supplementary Table 6).
- 270 We next looked for potential associations between expression of our CAF signature and gastric cancer 271 subtypes. In all 3 patient datasets, CAF up sig expression, but not CAF down sig expression, was 272 significantly enriched in the diffuse gastric cancer subtype, which typically has a worse prognosis 273 compared to the intestinal subtype (Fig. 2G-I and Supplementary Fig. S2A-C). In addition to the 274 histological classification of gastric cancer to diffuse and intestinal subtypes, two independent 275 molecular classification methods were recently described (4,5): A mesenchymal phenotype (MP) 276 characterized by high genomic integrity and associated with poor survival, and an epithelial phenotype 277 (EP) characterized by low genomic integrity and associated with favorable survival, were identified in 278 the KUGH KUCM cohort (5); and 4 molecular subtypes (MSS TP53<sup>-</sup>, MSS TP53<sup>+</sup>, MSI, EMT) were 279 characterized in the ACRG cohort, of which the EMT subtype was associated with the worst outcome 280 (4). Analyzing the KUGH KUCM cohort, we found that the CAF up sig was significantly enriched in the MP class, and the CAF-down sig was significantly enriched in the EP class (Fig. 2J). In the 281 282 ACRG cohort, the CAF up sig was significantly enriched in the EMT subtype while the CAF down sig was significantly enriched in MSS TP53<sup>+/-</sup> subtypes, associated with more favorable 283 284 outcomes (Fig. 2K).
- Supporting this classification, gene set enrichment analysis (GSEA) using MSigDB (Hallmark gene sets, see Supplementary Materials and Methods) on the full stromal RNA-seq dataset highlighted EMT as the most significantly enriched pathway in patients with poor outcome compared to patients with favorable outcome (Supplementary Fig. S2D and Supplementary Table 7). These analyses collectively indicate that the stromal signature correlates with diffuse, mesenchymal and aggressive gastric cancer subtypes, further reinforcing the clinical relevance of our stromal classification and pointing to specific genes for dissection and targeting.
- 292

## A transcriptional signature derived from mouse PDPN<sup>+</sup> gastric CAFs is associated with aggressive gastric cancer phenotypes and poor disease outcome in patients

To further dissect the contribution of CAFs to gastric cancer we induced gastric cancer in mice using a triple-transgenic gastric cancer mouse model- *Lgr5-EGFP-IRES-CreERT2; R26-LSL-rtTA-IRES-EGFP; tetO-GLI2A* mice, (*iLgr5;GLI2A* mice) (21). This model is based on deregulated activation of the Hedgehog pathway by expression of GLI2A, an activated form of GLI2, in *Lgr5* expressing stem cells in the stomach (21). We isolated CAFs and normal fibroblasts from the stomachs of gastric cancer-induced and naïve *iLgr5;GLI2A* mice, and performed RNA-sequencing to obtain a pure mouse CAF transcriptional signature (Supplementary Table 8). To that end tumors were excised 3 weeks after 302 GLI2A induction, and CAFs were isolated by fluorescence activated cell sorting (FACS) based on 303 negative selection for CD45 (immune), EpCAM (epithelial), and CD31 (endothelial cells), and positive 304 selection for PDPN (fibroblasts) (Supplementary Fig. S3A and Supplementary Table 9) (16,28,29). 305 154 genes were differentially upregulated and 207 were differentially downregulated in CAFs 306 compared to normal gastric fibroblasts (Supplementary Table 8). Pathway analysis highlighted similar 307 pathways to those discovered in the stromal dissection of the human patient samples: ECM 308 organization (Adam12, Acan, Lox), activation of matrix metalloproteinases (Mmp3, Mmp9, Mmp10, 309 Mmp13), response to growth factors (Inhba, Grem1, Runx3) and regulation of hormone levels (Inhba, 310 Cnr1, Cpe) were among the most differentially upregulated pathways in mouse CAFs, whereas 311 digestion (Apoa1, Tff1, Pgc) and tissue homeostasis (Atp4a, Car2, Cldn18) were the most differentially 312 downregulated pathways compared to normal gastric fibroblasts (Supplementary Table 10). We then 313 checked whether a signature comprised of genes upregulated in mouse CAFs (mCAF up sig) or genes 314 downregulated in mouse CAFs (mCAF down sig) would be associated with clinical characteristics in 315 the Singapore, KUGH KUCM, and ACRG cohorts (Supplementary Table 11). Similar to the 316 CAF up sig from patient samples, high expression of the mCAF up sig significantly associated with 317 poor overall survival in the Singapore cohort and in the KUGH KUCM cohort, and the ACRG cohort 318 showed a similar trend that was not statistically significant (Fig. 3A and Supplementary Fig. S3B-C). 319 The mCAF down sig showed an opposite trend- it was significantly associated with favorable 320 outcome in the Singapore cohort and a similar trend was seen in the KUGH KUCM cohort (Fig. 3A 321 and Supplementary Fig. S3D). The ACRG cohort showed no particular trend for this analysis 322 (Supplementary Fig. S3E). The mCAF up sig also correlated with the more aggressive MP and EMT 323 molecular subtypes similar to the CAF up signature from patient samples (Fig. 3B and Supplementary 324 Fig. S3F), whereas the mCAF down sig correlated with the less aggressive EP and MSS TP53<sup>+/-</sup> 325 subtypes (Fig. 3B and Supplementary Fig. S3G). Collectively, the findings obtained from pure mouse 326 CAFs support our findings from patient samples, indicate that CAFs support gastric cancer and provide 327 potential targets and experimental systems for further characterization in mouse and human.

328

## 329 INHBA and THBS1/2 are upregulated in gastric cancer stroma

To characterize stromal pathways highlighted by our transcriptional profiling, we queried our patient gene list for potential interactions of translated proteins using STRING (Fig. 3C). Based on this analysis we chose to focus on two targets upregulated in poor outcome patients: inhibin Subunit Beta A (INHBA) and thrombospondin 2 (THBS2), suggested to be part of a common signaling network (30). Both targets were recently found by us to be highly expressed in a subset of wound-healing CAFs in breast cancer (16). Moreover, they were both part of the EMT gene set highlighted by the GSEA 336 analysis as enriched in patients with poor outcome (Supplementary Fig. S2D and Supplementary Table 337 7). We added to this analysis thrombospondin 1 (THBS1), a close homologue of THBS2 that showed 338 a similar trend of expression (Supplementary Table 3) and was also included in the enriched EMT gene 339 set (Supplementary Table 7). Inhba was differentially upregulated also in mouse CAFs from 340 *iLgr5;GLI2A* tumors, and *Thbs1/2* showed a similar trend (Fig. 3D-F). INHBA is a subunit of Activin 341 and Inhibin, dimeric proteins belonging to the TGFB superfamily (31,32). Activin A is a homodimer 342 of two INHBA subunits, whereas Inhibin A and Activin AB are heterodimers of INHBA with INHA 343 and INHBB, respectively (32). INHBA is known to play a role in inflammation, tissue repair and 344 activation of myofibroblasts, and increased levels of INHBA are associated with lymph node (LN) 345 metastasis, gastric cancer cell proliferation and chemoresistance (33). THBS1/2 are adhesive 346 glycoproteins involved in cell-cell and cell-matrix interactions. Increased levels of THBS2 are 347 associated with LN metastasis and increased invasion in gastric cancer (34). The role of THBS1 is less 348 clear since it was implicated both in pro- and antitumorigenic activities in gastric cancer (35-37). Both 349 INHBA and THBS1/2 are known to play an important role in gastric cancer, however their role in the 350 TME is not well studied (30). To validate our RNA-seq results, we extracted total RNA from 351 *iLgr5;GL12A* tumors and examined the levels of *Inhba*, *Thbs1* and *Thbs2* by qPCR. *Inhba* and *Thbs1* 352 levels were significantly upregulated in gastric tumors compared to normal gastric tissue and Thbs2 showed a similar trend (Fig. 3G-I). To define the tissue localization of INHBA and THBS1/2, and 353 354 confirm their expression at the protein level we performed immunohistochemistry (IHC) staining of sections from *iLgr5;GLI2A* tumors and from normal stomach controls using antibodies against INHBA 355 356 and THBS1. INHBA and THBS1 were expressed at very low levels in normal gastric glands and 357 muscle (Fig. 3J-K). Gastric tumors however exhibited high levels of INHBA and THBS1 both in 358 stroma and in cancer cells (Fig. 3J-K). Together, these findings support our patient RNA-seq results 359 and suggest that INHBA and THBS1/2 are upregulated in gastric cancer stroma.

360 Given their connectivity to other genes in the stromal network revealed by the STRING analysis (Fig. 361 3C), and the potential simplicity of a 3-gene signature (compared to a signature comprised of dozens 362 of genes) we tested whether a minimal gene signature comprised of only INHBA and THBS1/2 would 363 correlate with disease outcome in our patient datasets. We found that the 3-gene signature 364 (INHBA/THBS1/THBS2) correlated with poor disease outcome in the TCGA gastric cancer and 365 colorectal cancer datasets, the Singapore cohort and the KUGH KUCM cohort (Supplementary Fig. 366 S4A-D and Supplementary Table 6). As with the other stromal signatures that we analyzed, the ACRG 367 cohort showed a similar trend of disease outcome that was not statistically significant (Supplementary 368 Fig. S4E), possibly due to differences in patient follow up time or cohort characteristics

(Supplementary Table 6). These results imply that stromal INHBA and THBS1/2 are associated with
 aggressive disease phenotypes in gastric cancer, and serve as attractive targets for characterization.

371

## 372 HSF1 activation in gastric CAFs is associated with poor disease outcome

373 In search for potential transcriptional regulators of the stromal signature in general, and INHBA and 374 THBS1/2 in particular, we examined heat-shock factor 1 (HSF1). Previously we and others have shown 375 that HSF1, the master transcriptional regulator of the heat shock response, plays an important role in 376 the conversion of fibroblasts into CAFs in the TME (20,38). Moreover, INHBA and THBS1 were 377 shown to be transcriptional targets of HSF1 (39,40). In gastric cancer, activation of HSF1 in cancer 378 cells was shown to correlate with poor disease outcome (41), yet the contribution of stromal HSF1 to 379 disease outcome has not been assessed. HSF1 translocates from the cytoplasm to the nucleus and binds 380 to heat shock elements in the DNA upon activation (39). Therefore, its nuclear localization is 381 commonly used as a proxy for HSF1 activation (39). Indeed, IHC staining of FFPE sections from 382 gastric cancer patients revealed nuclear HSF1 staining both in cancer cells and in CAFs, while normal 383 stomach glands and muscle exhibited low or no HSF1 staining (Fig. 4A).

384 To systematically test whether stromal activation of HSF1 is associated with disease outcome in gastric 385 cancer, we performed IHC staining for HSF1 and scored its nuclear localization in cancer cells and 386 CAFs, in sections from 64 gastric cancer patients (including the sub-cohort of LCM-RNA-seq patients) 387 with documented clinical characteristics and patient outcome data (Supplementary Table 1). High 388 HSF1 activation in cancer cells correlated with shorter overall survival time and stromal HSF1 showed 389 a similar trend (Fig. 4B-C and Supplementary Table 12). In the cohort of patients analyzed by LCM 390 and RNA-seq, all patients with poor outcomes also exhibited intermediate or high HSF1 activation (i.e. 391 nuclear localization) in cancer and stromal cells, while patients with favorable outcomes differed in 392 their HSF1 activation status (Supplementary Table 1). Interestingly, stromal HSF1 activation also 393 significantly correlated with HER2 status – HER2<sup>-</sup> patients exhibited high HSF1 levels whereas HER2<sup>+</sup> 394 patients had low stromal HSF1 activation levels (Supplementary Table 1). These results imply that in 395 addition to its previously described roles in gastric cancer cells, HSF1 activates complementary 396 pathways in gastric stroma that promote aggressive disease phenotypes. This conclusion was further 397 supported by a multivariate Cox proportional-hazards regression analysis (Supplementary Table 12). 398 In an additive multivariate model considering tumor stage and HSF1 score, stromal HSF1 score and 399 tumor stage were significantly associated with overall survival (p=0.006), and this association was 400 more significant than that of cancer HSF1 and tumor stage with survival (p=0.016).

401

## 402 Stromal INHBA and THBS1/2 are targets of HSF1, *in vitro*

- Multiplexed immunofluorescent staining (MxIF) of gastric cancer patient samples showed that HSF1
   is co-expressed with INHBA and THBS1, in cancer cells and in CAFs, while normal stomach tissue
- 405 exhibited low INHBA, THBS1 and HSF1 staining (Fig. 4D). To test whether HSF1 regulates INHBA
- 406 and THBS1/2 stromal expression, and whether this regulation affects cancer cells, we measured the
- 407 expression of INHBA and THBS1/2 in WT vs Hsfl null mouse embryonic fibroblasts (MEFs).
- 408 THBS1/2 and INHBA protein levels were significantly higher in WT MEFs compared to *Hsf1* null
- 409 MEFs (Fig. 5A-E). Next, we asked if INHBA and THBS1/2 expression in fibroblasts is affected by
- 410 co-culture with cancer cells. 72h of co-culture with N87 human gastric cancer cells led to a significant
- 411 increase in Inhba, Thbs1 and Thbs2 mRNA levels compared to cells grown in mono-culture (Fig. 5F-
- 412 H). Some induction was also observed in *Hsf1* null MEFs upon co-culture, however the total levels
- 413 were lower in *Hsf1* null MEFs compared to WT MEFs (Fig. 5F-H).
- To determine how this stromal network affects cancer cells, we monitored cancer cell growth in coculture. N87 cells showed a significant growth reduction when co-cultured with *Hsf1* null MEFs compared to WT MEFs (Fig. 5I-K), and similar results were observed upon co-culture of N87 cells with human foreskin fibroblasts (HFFs) in which HSF1 was knocked down by siRNA (Fig. 5L and Supplementary Fig. S5A-C).
- 419 Next we knocked down INHBA, THBS1 and THBS2 in fibroblasts and monitored gastric cancer cell growth in co-culture. Knock down of THBS2 in HFFs led to a minor decrease in N87 cell proliferation, 420 421 and knockdown of THBS1 led to a minor increase in N87 proliferation (Supplementary Fig. S5D-F). Knock down of INHBA however led to a substantial and significant decrease in the growth of co-422 423 cultured N87 cells (Fig. 5M and Supplementary Fig. S5G). A combined knockdown of HSF1-INHBA-424 THBS2 had a similar effect on N87 growth (Fig. 5N), whereas the combination of HSF1 and INHBA 425 with THBS1 had a milder effect (Supplementary Fig. S5H). Collectively these results support the 426 hypothesis that HSF1, INHBA and THBS1/2 are part of a common stromal protumorigenic signaling 427 network, in which HSF1 regulates the expression of THBS1/2 and INHBA. While INHBA and THBS2 428 seem to play a protumorigenic role in fibroblasts, THBS1 may be antitumorigenic.
- 429

## 430 THBS2 and INHBA are secreted from fibroblasts via EVs, in an HSF1-dependent manner

INHBA and THBS1/2 are secreted proteins (42). We therefore hypothesized that INHBA and THBS2 are secreted from CAFs to the TME where they act on cancer cells, and that this process could be mimicked by exogenous treatment with recombinant proteins. To test this, we co-injected MC38 colon cancer cells with recombinant proteins into mice, subcutaneously, followed by another injection of recombinant protein two days later, and monitored tumor growth. Co-injection of either THBS2 or Activin A (a homodimer of two INHBA subunits (31)) with MC38 cancer cells significantly increased

- 437 the tumorigenicity of these cells larger and faster growing tumors formed in the presence of THBS2
- 438 or Activin A (Fig. 6A).
- 439 INHBA and THBS1/2 have been proposed to shuttle through EVs (43-49). Recently, THBS2 was 440 shown to be a marker for exosomes secreted by tumors (50). We therefore hypothesized that the 441 protumorigenic effects of stromal HSF1 may be mediated by secretion and delivery of these proteins 442 to the TME, possibly via EVs. Small EVs are lipid bilayer-enclosed particles sized 30-150 nm, that 443 mediate cell-cell communication via targeting, fusion and release of content from one cell to another 444 (51). Their cargo includes bioactive molecules such as effector proteins, metabolites, large and small 445 RNAs and even genomic DNA (50). Recently, EVs secreted from stromal cells were shown to 446 contribute to disease progression and poor disease outcome by promoting vascularization and 447 chemotherapy resistance (52). To test whether INHBA and THBS1/2 are secreted via EVs in an HSF1-448 dependent manner, we first confirmed the presence of INHBA and THBS1/2 in EVs by OptiPrep 449 density gradient isolation of EVs secreted from WT MEFs (Fig. 6B-C and Supplementary Fig. S6A). 450 ALIX and TSG101, two known exosome markers, were used as positive loading controls (53). HSF1 451 is not expected to be found in EVs and therefore served as a negative control. ALIX and TSG101 were 452 found in fractions 3-8. Both proteins peaked in high density fractions (6-7), and TSG101 had an 453 additional peak in low density fraction 4 (Fig. 6B-C). HSF1 was not detected in any of these fractions. 454 INHBA and THBS1/2, however, were detected in fractions 2-7, and peaked in fractions 4-5 (Fig. 6B-455 C). To confirm that these fractions contain EVs we performed transmission electron microscope 456 (TEM) analysis. We found that EVs are indeed observed in both low- and high-density fractions (Fig. 457 6D). These observations suggest that two populations of EVs are secreted by MEFs – a low density 458 population, enriched in INHBA and THBS1/2 (Supplementary Fig. S6A) and a high-density 459 population with lower levels of INHBA and THBS1/2. We also checked the presence of INHBA and 460 THBS1/2 in EVs isolated from the serum of *iLgr5;GLI2A* mice. While we could not detect THBS1/2 461 in the serum (possibly due to low sensitivity of the assay), INHBA was detected, and its levels were 462 significantly higher in EVs isolated from the serum of tumor-bearing *iLgr5;GLI2A* mice compared to 463 EVs isolated from the serum of naïve *iLgr5;GLI2A* mice (Fig. 6E-F).
- We then compared the expression levels of INHBA and THBS1/2 in EVs isolated from WT *vs Hsf1* null fibroblasts. While THBS1 levels were similar between WT and *Hsf1* null-derived EVs, THBS2 and INHBA levels were significantly higher in EVs derived from WT MEFs compared to EVs from *Hsf1* null MEFs (Fig. 6G-J). These results suggest that INHBA and THBS2 expression in EVs is HSF1dependent.
- To examine whether the differential expression of INHBA and THBS2 was due to impaired EV biogenesis in *Hsf1* null MEFs, we compared the number and size of EVs produced by each genotype

471 using nanoparticle tracking analysis (NTA). We could not detect differences in size or in quantity 472 between EVs secreted from WT and Hsfl null fibroblasts (Supplementary Fig. S6B-E). We extended 473 our analysis to field-flow fractionation (FFF), to better separate EV populations and assess smaller EV 474 populations shown to be biologically active (54). Similar to our NTA analysis, FFF did not detect 475 consistent differences between EVs derived from Hsf1 null MEFs compared to WT MEFs 476 (Supplementary Fig. S6F). We next tested whether the differences in protein content could be due to 477 impaired uptake of EVs derived from Hsfl null compared to WT MEFs. We incubated N87 gastric 478 cancer cells and MC38 colon cancer cells with CFSE stained EVs, and analyzed uptake 12-16h later 479 by imaging the cells in an ImageStream imaging flow cytometer. We could not detect differences in 480 the percentage of CFSE<sup>+</sup> N87 and MC38 cells incubated in the presence of EVs from Hsfl null 481 compared to WT MEFs (Supplementary Fig. S6G-O), indicating that HSF1 does not affect EV 482 biogenesis or uptake, yet it plays an important role in the protein content of EVs.

483 To assess the biological relevance of these findings we co-injected EVs derived from WT vs Hsfl null MEFs together with MC38 cancer cells into nude mice, and monitored tumor growth. Co-injection 484 485 with EVs derived from WT MEFs caused a significant increase in the growth of MC38-injected tumors 486 (Fig. 6K). This effect was completely abolished when EVs from *Hsf1* null MEFs were co-injected with 487 MC38 cells. Taken together these experiments show that EVs derived from WT and Hsfl null MEFs 488 are similar in size, quantity, biogenesis and uptake into cancer cells. However, there is a significant 489 difference in their content and, consequently, their effect on tumor growth. These findings imply that 490 HSF1 regulates the expression of INHBA and THBS1/2 in stromal cells. INHBA and THBS2 are then 491 packaged into EVs in an HSF1-dependent manner and secreted to the TME, where they are taken up 492 by cancer cells and promote a more aggressive disease phenotype (Fig. 6L).

493

## 494 **Discussion**

Despite recent advances in molecular subtyping, the backbone of gastric cancer treatment remains chemotherapeutic combinations. Molecular classifications, based largely on mutations and genomic alterations in the cancer cells, do not translate to guide treatment modality. Here we chose a complementary approach - searching for transcriptional changes in the gastric TME. We defined a stromal gene signature associated with poor disease outcome in patients, and found a role for the stromal master transcriptional regulator HSF1 in driving it, through exosome-mediated secretion of protumorigenic proteins that are taken up by cancer cells to promote aggressive disease phenotypes.

502 HSF1 was previously shown by us and others to play protumorigenic roles in CAFs of breast, lung and 503 colon carcinomas (17,20,38). The finding that HSF1 also acts in gastric CAFs implicates HSF1 as a 504 master regulator of CAF activities in carcinomas across different tissues, and suggests that its 505 protumorigenic effects - in gastric cancer and other carcinomas - may be mediated via delivery of 506 targets to the TME in EVs.

- 507 INHBA and THBS1/2 are involved in tumor progression and were shown to be co-regulated (30,55,56) 508 possibly sharing common signaling pathways. While INHBA and THBS2 are protumorigenic, THBS1 509 was proposed to exert both pro and antitumorigenic effects, depending on the system examined 510 (44,55,57). Our findings suggest that all 3 proteins are upregulated in CAFs in an HSF1-dependent 511 manner. Our in vitro experiments and mouse co-injections with recombinant proteins show a clear 512 protumorigenic role of Activin A and THBS2, while the effect of stromal THBS1 on cancer cells (in 513 vitro) is less clear. Taken together with the finding that INHBA and THBS2 are delivered into 514 exosomes in an HSF1-dependent manner, while THBS1 exosomal expression is not affected by HSF1 515 status, it is possible that selective delivery of INHBA and THBS2 to exosomes leads to the 516 protumorigenic effect observed, while THBS1 is antitumorigenic.
- 517 EV cargo includes proteins, metabolites, RNA and genomic DNA (50), which could serve as bioactive 518 molecules in the TME. In GI-tract cancers, EVs from CAFs were shown to promote cancer through 519 delivery of miRNAs to gastric cancer cells to suppress ferroptosis (58), and Wnt glycoproteins to 520 colorectal cancer cells to induce cancer stemness and chemoresistance (59). In our study, differential 521 protein expression in EVs affects their activity. Though biogenesis and uptake of EVs was not 522 impaired, loss of HSF1 abolished the protumorigenic effect of EVs derived from WT MEFs. Our 523 findings indicate that EV cargo is selective and the content is affected by HSF1.
- 524 Over the last years, efforts were made to identify gastric cancer drivers and gene signatures that may 525 serve as biomarkers for diagnosis and treatment (3). Trastuzumab revolutionized the treatment of 526 HER2-positive gastric cancers (60), and immunotherapy has proven to be an effective therapy for 527 patients with microsatellite instability (MSI) (61). Other signatures, such as those associated with 528 *Helicobacter pylori* and *EBV* infections (62,63), germline mutations of *CDH1*, mismatch repair genes 529 (64,65), epithelial vs mesenchymal cell types (5), and MSS TP53<sup>-</sup>, MSS TP53<sup>+</sup>, MSI, EMT subtypes 530 (4) enabled associations between molecular landscape and gastric cancer subtyping (3,60). However, 531 the TME of gastric cancer in general, and the molecular composition of gastric CAFs in particular, 532 have been scarcely studied. Our profiling of CAFs from patient tumors highlights stromal compositions 533 associated with the aggressive diffuse and EMT-like gastric cancer subtypes. These targets should be 534 further explored, certainly as prognostic targets and hopefully as robust therapeutic targets in gastric
- 535 cancer.
- 536

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- (CDH1) mutations predispose to familial gastric cancer and colorectal cancer. Hum Mol Genet 1999;8:607-10
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## 694 **Figure Legends**

Fig 1. The transcriptional landscape of gastric cancer stroma changes with disease aggressiveness. CAF-rich or cancer-rich regions of tumor sections from 9 gastric cancer patients were laser-capture microdissected and analyzed by RNA-sequencing. Principal component analysis (PCA) was performed for (A) CAFs and (B) cancer cells. Purple/orange dots - survival/lethality, as indicated. (C) Heatmap showing hierarchical clustering of 129 genes differentially expressed in CAF-rich samples with favorable *vs* poor outcome. Pathway analysis was performed using Metascape. Selected significant pathways (p < 0.05, FDR < 0.5) are shown (see Supplementary Table 4). Purple/orange bars - survival/lethality, as indicated. (D-E) Kaplan-Meier (KM) analysis showing overall survival of
 (D) gastric or (E) colorectal cancer patients from the TCGA stratified based on median expression of
 the stromal gene signature (CAF up sig).

705 Fig 2. High expression of the CAF signature is associated with aggressive disease and poor 706 disease outcome in gastric cancer patients. (A-C) KM analysis showing overall survival of patients from (A) the Singapore cohort; (B) the KUGH & KUCM cohort; and (C) the ACRG cohort stratified 707 based on expression of the upregulated (CAF up sig) stromal gene signature. (D-F) KM analysis 708 709 showing overall survival of patients from the (D) Singapore cohort, (E) KUGH & KUCM cohort and 710 (F) ACRG cohort stratified based on expression of the downregulated (CAF down sig) stromal gene 711 signature. (G-I) Enrichment of the CAF up sig (mean of normalized counts) in patients with diffuse 712 vs intestinal gastric cancer in the (G) Singapore cohort; (H) KUGH & KUCM cohort; and (I) ACRG cohort. (J) Enrichment of the CAF up sig and CAF down sig (mean of the normalized counts) in 713 714 patients with mesenchymal phenotype (MP) and epithelial phenotype (EP) subtypes in the KUGH & 715 KUCM cohort (5). (K) Enrichment of the CAF up sig and CAF down sig (mean of normalized 716 counts) in patients with molecular subtypes previously identified in the ACRG cohort (4). One-way 717 ANOVA was used in (G-K). 718

719 Fig 3. INHBA and THBS1/2 are upregulated in gastric cancer. (A-B) Gastric cancer was induced 720 in *iLgr5;GLI2A* mice, PDPN<sup>+</sup> fibroblasts were isolated from the resulting tumors and RNA-seq was performed using fibroblasts isolated from stomachs of naïve mice as control. Signatures comprised of 721 722 genes upregulated (mCAF up sig;) or downregulated (mCAF down sig) in PDPN<sup>+</sup> CAFs vs PDPN<sup>+</sup> 723 normal fibroblasts were derived. (A) KM analysis of overall survival in patients from the Singapore 724 cohort stratified based on expression of the mCAF up sig (left) or mCAF down sig (right). (B) Enrichment of the mCAF up sig and mCAF down sig (mean of normalized counts) in patients with 725 the MP and EP subtypes in the KUGH & KUCM cohort. One-way ANOVA was used for statistical 726 727 analysis. (C) STRING analysis of potential interactions between protein products of genes 728 differentially expressed in gastric cancer patients with favorable vs poor outcome. Proteins with no 729 connections were omitted from the image. THBS2 and INHBA are highlighted in red. (D-F) Log 730 normalized counts and p-adjusted values of the indicated genes taken from DESeq analysis of the 731 *iLgr5;GLI2A* PDPN<sup>+</sup> CAF RNA-seq data (Supplementary Table 8). (G-I) Total RNA levels of the 732 indicated genes normalized to HPRT in normal stomachs and tumors (cancer) from *iLgr5;GLI2A* mice. 733 N=3 mice per group, means  $\pm$  SEM are presented. Two-tailed Student's t-test was used for statistical analysis. (J-K) Representative images showing H&E and immunohistochemical staining of the 734 indicated proteins in gastric tumors and control stomachs (naïve) from *iLgr5;GLI2A* mice. N=5 mice 735 736 for cancer and N=3 mice for normal control. C- cancer, S- stroma. Scale bar- 100 µm. Arrows indicate 737 INHBA and THBS1 positive CAFs.

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739 Fig 4. HSF1 is co-expressed with INHBA and THBS1 in human gastric CAFs. (A) Formalin-fixed 740 paraffin-embedded (FFPE) sections from 64 gastric cancer patients and 4 normal controls were stained 741 by H&E and immunohistochemical staining for HSF1. (Upper panel) Images representing high (pt 6) 742 vs low (pt 18) nuclear HSF1 staining in CAFs. (Lower panel) Representative images of normal gastric glands and muscle. C- cancer, S- stroma. Scale bar- 100 µm. (B-C) 64 gastric cancer samples stained 743 744 as described above were scored for high/intermediate (int)/low nuclear HSF1 staining in cancer 745 cells/CAFs, and KM analysis of overall survival in these patients was performed. (B) Patients were 746 stratified by high vs int/low HSF1 scores in cancer cells. (C) Patients were stratified by high/int vs low HSF1 scores in CAFs (see Supplementary Table 1). (D) FFPE sections from 4 gastric cancer patients 747 748 and 2 normal stomach controls were stained by multiplexed immunofluorescence for HSF1, INHBA,

THBS1 and DAPI (nuclear marker). Representative images from 3 different patients and one control
 are shown. Scale bar- 50 μm.

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752 Fig 5. Stromal INHBA and THBS1/2 expression is HSF1-dependent. (A-E) INHBA, THBS1 and 753 THBS2 protein expression levels in WT and *Hsf1* null primary MEFs were analyzed by western blot. 754 Representative blots are shown in (A-B). An arrow indicates the expected size of INHBA bands. (C) 755 INHBA western blot results of 5-10 biological replicates (across 2 experiments) were quantified, 756 normalized to actin and are presented as mean ± SEM. (D-E) THBS1 western blot results of 5 biological replicates (across 2 experiments) and THBS2 western blot results of 5-10 biological 757 758 replicates (across 3 experiments) were quantified, normalized to actin and are presented as mean  $\pm$ 759 SEM. Two-tailed Student's t-test was used for statistical analysis in (C-E). (F-K) WT and Hsfl null MEFs were co-cultured with N87-GFP cells for 72h, and each cell type was grown in mono-culture as 760 control. Co-cultures were sorted by flow cytometry using GFP. (F-H) The levels of the indicated genes 761 762 in (GFP-negative) MEFs were determined by qPCR. Average expression in 6-8 biological replicates 763 (across 3 experiments for *INHBA* and *THBS1* and 2 experiments for *THBS2*), normalized to *HPRT*,  $\pm$ 764 SEM are presented. Two-way ANOVA was used for statistical analysis. (I) Representative GFP 765 (upper panel), and brightfield (lower panel) images of mono and co-cultures are shown. N=3 766 biological replicates. Scale bar- 50 µm. (J) Representative FACS plots showing the percentage of N87-767 GFP cells co-cultured with WT (left) and *Hsf1* null MEFs (right). N=3 biological replicates. (K) The 768 average percentage (± SEM) of N87-GFP cells co-cultured with WT and Hsfl null MEFs in 3 769 biological replicates is shown. Two-tailed Student's t-test was used for statistical analysis. (L-N) HFF 770 cells treated with siHSF1, siINHBA, siHSF1-INHBA-THBS2 (siCombined) or siControl as indicated 771 were co-cultured with N87-GFP cells for 72h. The percentage of N87-GFP in the co-cultures averaged 772 across 5-9 biological replicates (± SEM) (across 3 experiments for siINHBA and siHSF1-INHBA-773 THBS2 and 2 experiments for siHSF1) is shown. Two-tailed Student's t-test was used for statistical 774 analysis.

775 Fig 6. Fibroblast-derived EVs promote tumor growth in an HSF1-dependent manner. (A) Nude 776 mice were injected subcutaneously with MC38 cancer cells alone, or co-injected with either 777 recombinant THBS2 or Activin A followed by another injection of recombinant protein two days later. 778 Tumor size measured by caliper is presented as mean  $\pm$  SEM for N=8 mice per group (across 2 779 experiments). Repeated measures Two-way ANOVA using least-squares means to adjust for group 780 pairwise comparisons was used for statistical analysis. (B-C) Western blot analysis of fractions obtained from Optiprep density gradient isolation of EVs secreted by WT MEFs blotted against 781 782 exosomal markers ALIX and TSG101, as well as THBS1/2, INHBA and HSF1. EVs from 3 WT MEFs 783 were pooled together for the isolation. The experiment was repeated twice (with different biological 784 replicates), representative results are shown. (D) Representative transmission electron microscope (TEM) images of low (i-ii) and high (iii) density EV fractions (repeated 2 times, from 2 biological 785 786 replicates). (i) -1.03% sucrose; (ii) -1.04% sucrose; (iii) -1.07% sucrose. Scale bars- 100 nm. (E) Representative western blot showing INHBA levels from EVs isolated from the serum of tumor-787 bearing and naïve iLgr5; GLI2A mice. ALIX was used as loading control. Arrow indicates expected 788 789 size of ALIX. (F) INHBA levels from EVs isolated from the serum of tumor-bearing and naïve 790 iLgr5;GLI2A mice were analyzed using western blot. INHBA levels were normalized to ALIX. 791 Average expression of INHBA normalized to ALIX in 5 biological replicates (across 2 experiments) 792 is presented in as mean  $\pm$  SEM. Two-tailed Student's t-test was used for statistical analysis. (G-J) 793 INHBA, THBS1 and THBS2 levels in EVs derived from WT and Hsf1 null primary MEFs were 794 analyzed using western blot. ALIX and TSG101 were used as loading controls. Representative blots 795 are shown in (G). (H) Average expression of INHBA normalized to TSG101 in 8 biological replicates 796 (across 3 experiments for INHBA) is presented as mean  $\pm$  SEM. (I) Average expression of THBS1 797 normalized to TSG101 in 5-7 biological replicates (across 3 experiments) is presented as mean  $\pm$  SEM.

798 (J) Average expression THBS2 normalized to TSG101 in 10-11 biological replicates (across 4 799 experiments) is presented as mean  $\pm$  SEM. Two-tailed Student's t-test was used for statistical analysis 800 in (H-I). (K) Nude mice were injected subcutaneously with MC38 cancer cells alone, or co-injected with EVs derived from WT or *Hsf1* null MEFs. Tumor size measured by caliper is presented as mean 801 802 ± SEM for N=14-15 mice per group (across 4 experiments). Repeated measures Two-way ANOVA 803 using least-squares means to adjust for group pairwise comparisons was used for statistical analysis. (L) Graphic summary of the proposed model. HSF1 in CAFs regulates expression of INHBA and 804 THBS1/2. INHBA and THBS2 from CAFs are packaged into EVs and secreted to the TME, where 805 806 they are taken up by cancer cells.

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