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Running Title: CAFs promote gastric cancer via HSF1 and exosomes

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Abstract

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

Significance

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

Introduction

Gastric cancer is the 5th most common cancer and the 3rd 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-esophageal) and histological characteristics (diffuse *vs* intestinal; tubular *vs* papillary) have been used to classify gastric cancer subtypes (2). Recent advances in molecular understanding have enabled classification of gastric cancer into different subtypes based on chromosomal instability, microsatellite instability, genomic stability, presence of Epstein-Barr virus, and epithelial- mesenchymal transition (EMT), which were associated with different survival outcomes (3-6). Mutations in *CDH1* and *KRAS*, and overexpression of HER2, EGFR, FGFR2, VEGF, were shown to contribute to disease progression and correlate with poor outcome (7,8). Despite serving as valuable guides in deciphering the

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

Materials and Methods

Ethics statement

Clinical samples and patient data were collected following approval by the Rabin medical center Institutional Review Board (IRB, protocol #0297-11-RMC) with full exemption for consent form for anonymized samples. Human samples used for MxIF staining were obtained from the Israel National Biobank for Research (MIDGAM; https://www.midgam.org.il/) under IRB # 6141-19-SMC. These samples were collected from patients who provided written informed consent for collection, storage, 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).

Mice

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

Cell lines and primary cell cultures

N87 gastric cancer cells were kindly provided by Yosef Yarden (WIS; originally from ATCC). N87 cells were transduced with green fluorescent protein (GFP) using a 3rd-generation lentiviral system. MC38 colon cancer cells were kindly provided by Lea Eisenbach (WIS; originally from NCI). MC38 cells were transduced with mcherry-luciferase using a 2nd-generation lentiviral system. Primary MEFs were produced from WT and *Hsf1* null mice. HFF cells were purchased from ATCC. MEFs, MC38 cells and N87 cells were cultured in RPMI (#01-100-1A, Biological Industries) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and P/S (Biological Industries). HFF cells were cultured in DMEM (#01-052-1A, Biological Industries) supplemented with 15% FBS, 1.5% L-Glutamine and P/S. Cell lines were tested routinely for *Mycoplasma* using EZ-PCR Mycoplasma test kit (#20-700-20, Biological Industries). MEFs were used in passage 1. Other cell lines were maintained below passage 25.

Laser capture microdissection of human gastric cancer samples

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

(#73504, Qiagen) with one modification- proteinase K digestion at 56°C was carried out for 1 h.

Library preparation, RNA-sequencing and analysis of LCM samples

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

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

Gastric cancer was induced in *iLgr5;GLI2A* mice as described in (21). Gastric tumors were harvested post mortem, washed, minced and dissociated using a gentleMACS dissociator and enzymatic digestion with DMEM containing 3 mg ml^{-1} collagenase A (#11088793001, Sigma Aldrich,) and 0.1 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 CAFs were collected based on negative selection for ghost dye, CD45, EpCAM, and CD31 and positive selection for PDPN. RNA-seq was done by MARS-seq as described in (27). DEGs were filtered with the following parameters: baseMean > 5, padj < 0.01 and |logfoldchange| > 3. Pathway analysis was performed using Metascape, significant pathways were determined if $p < 0.05$ and FDR < 0.5.

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 cohorts (GSE62254) were downloaded, individual gene values were transformed to z-scores and the average of all known genes per sample was used to determine scores for the upregulated and downregulated signatures. For the *INHBA-THBS1-THBS2* gene signature individual gene values were transformed to z-scores and the average of genes per sample was determined. Gene symbols were matched through Affymetrix Human Genome U133 Plus 2.0 Array or Illumina HumanHT-12 V4.0 expression bead chip. For patient cohorts GSE15460 and GSE62254 we could match 109 DEGs from the CAF_up_sig and CAF_down_sig and for GSE26942 we could match 87 DEGs from the CAF_up_sig and CAF_down_sig (out of the total 129 DEGs). For the *iLgr5;GLI2A* mCAF_up_sig and mCAF_down_sig, 314 DEGs were matched in the GSE15460 and GSE62254 cohorts and 271 DEGs in the GSE26942 cohort (out of the total 361 DEGs). Median signature was calculated using patients with complete survival and signature information. Kaplan Meier (KM) analysis of overall survival with log rank p value was performed for each cancer type or patient cohort on patients stratified by median expression of each of these signatures.

HSF1 scoring and analysis

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

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

MC38 ($2*10^5$) 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 Nude mice (Harlan laboratories). 48h later a second dose of 2.5 μg recombinant protein was injected. Tumors were measured by caliper for size and mice were sacrificed at day 15 due to high burden in the Activin A group.

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.

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.

Results

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

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

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

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

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

We next looked for potential associations between expression of our CAF signature and gastric cancer subtypes. In all 3 patient datasets, CAF_up_sig expression, but not CAF_down_sig expression, was significantly enriched in the diffuse gastric cancer subtype, which typically has a worse prognosis compared to the intestinal subtype (Fig. 2G-I and Supplementary Fig. S2A-C). In addition to the histological classification of gastric cancer to diffuse and intestinal subtypes, two independent molecular classification methods were recently described (4,5): A mesenchymal phenotype (MP) characterized by high genomic integrity and associated with poor survival, and an epithelial phenotype (EP) characterized by low genomic integrity and associated with favorable survival, were identified in the KUGH_KUCM cohort (5); and 4 molecular subtypes (MSS $TP53^-$, MSS $TP53^+$, MSI, EMT) were characterized in the ACRG cohort, of which the EMT subtype was associated with the worst outcome (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 ACRG cohort, the CAF_up_sig was significantly enriched in the EMT subtype while the CAF_down_sig was significantly enriched in MSS $TP53^{+/-}$ subtypes, associated with more favorable 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.

A transcriptional signature derived from mouse $PDPN^+$ 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

GLI2A induction, and CAFs were isolated by fluorescence activated cell sorting (FACS) based on negative selection for CD45 (immune), EpCAM (epithelial), and CD31 (endothelial cells), and positive selection for PDPN (fibroblasts) (Supplementary Fig. S3A and Supplementary Table 9) (16,28,29). 154 genes were differentially upregulated and 207 were differentially downregulated in CAFs compared to normal gastric fibroblasts (Supplementary Table 8). Pathway analysis highlighted similar pathways to those discovered in the stromal dissection of the human patient samples: ECM organization (*Adam12*, *Acan*, *Lox*), activation of matrix metalloproteinases (*Mmp3*, *Mmp9*, *Mmp10*, *Mmp13*), response to growth factors (*Inhba, Grem1, Runx3*) and regulation of hormone levels (*Inhba*, *Cnr1*, *Cpe*) were among the most differentially upregulated pathways in mouse CAFs, whereas digestion (*Apoa1*, *Tff1, Pgc*) and tissue homeostasis (*Atp4a*, *Car2*, *Cldn18*) were the most differentially downregulated pathways compared to normal gastric fibroblasts (Supplementary Table 10). We then checked whether a signature comprised of genes upregulated in mouse CAFs (mCAF_up_sig) or genes downregulated in mouse CAFs (mCAF_down_sig) would be associated with clinical characteristics in the Singapore, KUGH_KUCM, and ACRG cohorts (Supplementary Table 11). Similar to the CAF_up_sig from patient samples, high expression of the mCAF_up_sig significantly associated with poor overall survival in the Singapore cohort and in the KUGH_KUCM cohort, and the ACRG cohort showed a similar trend that was not statistically significant (Fig. 3A and Supplementary Fig. S3B-C). The mCAF_down_sig showed an opposite trend- it was significantly associated with favorable outcome in the Singapore cohort and a similar trend was seen in the KUGH_KUCM cohort (Fig. 3A and Supplementary Fig. S3D). The ACRG cohort showed no particular trend for this analysis (Supplementary Fig. S3E). The mCAF_up_sig also correlated with the more aggressive MP and EMT molecular subtypes similar to the CAF_up signature from patient samples (Fig. 3B and Supplementary Fig. S3F), whereas the mCAF_down_sig correlated with the less aggressive EP and MSS $TP53^{+/-}$ subtypes (Fig. 3B and Supplementary Fig. S3G). Collectively, the findings obtained from pure mouse CAFs support our findings from patient samples, indicate that CAFs support gastric cancer and provide potential targets and experimental systems for further characterization in mouse and human.

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

analysis as enriched in patients with poor outcome (Supplementary Fig. S2D and Supplementary Table 7). We added to this analysis thrombospondin 1 (THBS1), a close homologue of THBS2 that showed a similar trend of expression (Supplementary Table 3) and was also included in the enriched EMT gene set (Supplementary Table 7). *Inhba* was differentially upregulated also in mouse CAFs from *iLgr5;GLI2A* tumors, and *Thbs1/2* showed a similar trend (Fig. 3D-F). INHBA is a subunit of Activin and Inhibin, dimeric proteins belonging to the TGFß superfamily (31,32). Activin A is a homodimer of two INHBA subunits, whereas Inhibin A and Activin AB are heterodimers of INHBA with INHA and INHBB, respectively (32). INHBA is known to play a role in inflammation, tissue repair and activation of myofibroblasts, and increased levels of INHBA are associated with lymph node (LN) metastasis, gastric cancer cell proliferation and chemoresistance (33). THBS1/2 are adhesive glycoproteins involved in cell-cell and cell-matrix interactions. Increased levels of THBS2 are associated with LN metastasis and increased invasion in gastric cancer (34). The role of THBS1 is less clear since it was implicated both in pro- and antitumorigenic activities in gastric cancer (35-37). Both INHBA and THBS1/2 are known to play an important role in gastric cancer, however their role in the TME is not well studied (30). To validate our RNA-seq results, we extracted total RNA from *iLgr5;GLI2A* tumors and examined the levels of *Inhba*, *Thbs1* and *Thbs2* by qPCR. *Inhba* and *Thbs1* 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 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 and THBS1. INHBA and THBS1 were expressed at very low levels in normal gastric glands and muscle (Fig. 3J-K). Gastric tumors however exhibited high levels of INHBA and THBS1 both in stroma and in cancer cells (Fig. 3J-K). Together, these findings support our patient RNA-seq results and suggest that INHBA and THBS1/2 are upregulated in gastric cancer stroma.

Given their connectivity to other genes in the stromal network revealed by the STRING analysis (Fig. 3C), and the potential simplicity of a 3-gene signature (compared to a signature comprised of dozens of genes) we tested whether a minimal gene signature comprised of only *INHBA* and *THBS1/2* would correlate with disease outcome in our patient datasets. We found that the 3-gene signature (*INHBA/THBS1/THBS2*) correlated with poor disease outcome in the TCGA gastric cancer and colorectal cancer datasets, the Singapore cohort and the KUGH_KUCM cohort (Supplementary Fig. S4A-D and Supplementary Table 6). As with the other stromal signatures that we analyzed, the ACRG cohort showed a similar trend of disease outcome that was not statistically significant (Supplementary 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.

HSF1 activation in gastric CAFs is associated with poor disease outcome

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

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

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 exhibited low INHBA, THBS1 and HSF1 staining (Fig. 4D). To test whether HSF1 regulates INHBA and THBS1/2 stromal expression, and whether this regulation affects cancer cells, we measured the expression of INHBA and THBS1/2 in WT *vs Hsf1* null mouse embryonic fibroblasts (MEFs). THBS1/2 and INHBA protein levels were significantly higher in WT MEFs compared to *Hsf1* null MEFs (Fig. 5A-E). Next, we asked if INHBA and THBS1/2 expression in fibroblasts is affected by co-culture with cancer cells. 72h of co-culture with N87 human gastric cancer cells led to a significant increase in *Inhba*, *Thbs1* and *Thbs2* mRNA levels compared to cells grown in mono-culture (Fig. 5F-H). Some induction was also observed in *Hsf1* null MEFs upon co-culture, however the total levels 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 co-culture. 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).

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, 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-cultured N87 cells (Fig. 5M and Supplementary Fig. S5G). A combined knockdown of *HSF1-INHBA-THBS2* had a similar effect on N87 growth (Fig. 5N), whereas the combination of *HSF1* and *INHBA* with *THBS1* had a milder effect (Supplementary Fig. S5H). Collectively these results support the hypothesis that HSF1, INHBA and THBS1/2 are part of a common stromal protumorigenic signaling network, in which HSF1 regulates the expression of *THBS1/2* and *INHBA*. While INHBA and THBS2 seem to play a protumorigenic role in fibroblasts, THBS1 may be antitumorigenic.

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

the tumorigenicity of these cells – larger and faster growing tumors formed in the presence of THBS2 or Activin A (Fig. 6A).

INHBA and THBS1/2 have been proposed to shuttle through EVs (43-49). Recently, THBS2 was shown to be a marker for exosomes secreted by tumors (50). We therefore hypothesized that the protumorigenic effects of stromal HSF1 may be mediated by secretion and delivery of these proteins to the TME, possibly via EVs. Small EVs are lipid bilayer-enclosed particles sized 30-150 nm, that mediate cell-cell communication via targeting, fusion and release of content from one cell to another (51). Their cargo includes bioactive molecules such as effector proteins, metabolites, large and small RNAs and even genomic DNA (50). Recently, EVs secreted from stromal cells were shown to contribute to disease progression and poor disease outcome by promoting vascularization and chemotherapy resistance (52). To test whether INHBA and THBS1/2 are secreted via EVs in an HSF1-dependent manner, we first confirmed the presence of INHBA and THBS1/2 in EVs by OptiPrep density gradient isolation of EVs secreted from WT MEFs (Fig. 6B-C and Supplementary Fig. S6A). ALIX and TSG101, two known exosome markers, were used as positive loading controls (53). HSF1 is not expected to be found in EVs and therefore served as a negative control. ALIX and TSG101 were found in fractions 3-8. Both proteins peaked in high density fractions (6-7), and TSG101 had an additional peak in low density fraction 4 (Fig. 6B-C). HSF1 was not detected in any of these fractions. INHBA and THBS1/2, however, were detected in fractions 2-7, and peaked in fractions 4-5 (Fig. 6B-C). To confirm that these fractions contain EVs we performed transmission electron microscope (TEM) analysis. We found that EVs are indeed observed in both low- and high-density fractions (Fig. 6D). These observations suggest that two populations of EVs are secreted by MEFs – a low density population, enriched in INHBA and THBS1/2 (Supplementary Fig. S6A) and a high-density population with lower levels of INHBA and THBS1/2. We also checked the presence of INHBA and THBS1/2 in EVs isolated from the serum of *iLgr5;GLI2A* mice. While we could not detect THBS1/2 in the serum (possibly due to low sensitivity of the assay), INHBA was detected, and its levels were significantly higher in EVs isolated from the serum of tumor-bearing *iLgr5;GLI2A* mice compared to 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 HSF1-dependent.

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

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

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

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.

HSF1 was previously shown by us and others to play protumorigenic roles in CAFs of breast, lung and colon carcinomas (17,20,38). The finding that HSF1 also acts in gastric CAFs implicates HSF1 as a 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^{-}, MSS TP53^{+}, 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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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).

Fig 2. High expression of the CAF signature is associated with aggressive disease and poor 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 based on expression of the upregulated (CAF_up_sig) stromal gene signature. **(D-F)** KM analysis showing overall survival of patients from the **(D)** Singapore cohort, **(E)** KUGH & KUCM cohort and **(F)** ACRG cohort stratified based on expression of the downregulated (CAF_down_sig) stromal gene signature. **(G-I)** Enrichment of the CAF_up_sig (mean of normalized counts) in patients with diffuse *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 patients with mesenchymal phenotype (MP) and epithelial phenotype (EP) subtypes in the KUGH & KUCM cohort (5). **(K)** Enrichment of the CAF_up_sig and CAF_down_sig (mean of normalized counts) in patients with molecular subtypes previously identified in the ACRG cohort (4). One-way ANOVA was used in **(G-K)**.

Fig 3. **INHBA and THBS1/2 are upregulated in gastric cancer. (A-B)** Gastric cancer was induced in *iLgr5;GLI2A* mice, $PDPN^+$ 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 genes upregulated (mCAF_up_sig;) or downregulated (mCAF_down_sig) in $PDPN^+$ CAFs vs $PDPN^+$ normal fibroblasts were derived. **(A)** KM analysis of overall survival in patients from the Singapore 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 the MP and EP subtypes in the KUGH & KUCM cohort. One-way ANOVA was used for statistical analysis. **(C)** STRING analysis of potential interactions between protein products of genes differentially expressed in gastric cancer patients with favorable *vs* poor outcome. Proteins with no connections were omitted from the image. THBS2 and INHBA are highlighted in red. **(D-F)** Log normalized counts and p-adjusted values of the indicated genes taken from DESeq analysis of the *iLgr5;GLI2A* $PDPN^+$ CAF RNA-seq data (Supplementary Table 8). **(G-I)** Total RNA levels of the indicated genes normalized to HPRT in normal stomachs and tumors (cancer) from *iLgr5;GLI2A* mice. N=3 mice per group, means ± 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 indicated proteins in gastric tumors and control stomachs (naïve) from *iLgr5;GLI2A* mice. N=5 mice for cancer and N=3 mice for normal control. C- cancer, S- stroma. Scale bar- 100 μm. Arrows indicate INHBA and THBS1 positive CAFs.

Fig 4. HSF1 is co-expressed with INHBA and THBS1 in human gastric CAFs. (A) Formalin-fixed paraffin-embedded (FFPE) sections from 64 gastric cancer patients and 4 normal controls were stained by H&E and immunohistochemical staining for HSF1. **(Upper panel)** Images representing high (pt 6) *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 as described above were scored for high/intermediate (int)/low nuclear HSF1 staining in cancer cells/CAFs, and KM analysis of overall survival in these patients was performed. **(B)** Patients were 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 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.

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

Fig 6. Fibroblast-derived EVs promote tumor growth in an HSF1-dependent manner. (A) Nude mice were injected subcutaneously with MC38 cancer cells alone, or co-injected with either recombinant THBS2 or Activin A followed by another injection of recombinant protein two days later. Tumor size measured by caliper is presented as mean ± SEM for N=8 mice per group (across 2 experiments). Repeated measures Two-way ANOVA using least-squares means to adjust for group 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 exosomal markers ALIX and TSG101, as well as THBS1/2, INHBA and HSF1. EVs from 3 WT MEFs were pooled together for the isolation. The experiment was repeated twice (with different biological 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 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-bearing and naïve *iLgr5;GLI2A* mice. ALIX was used as loading control. Arrow indicates expected size of ALIX. **(F)** INHBA levels from EVs isolated from the serum of tumor-bearing and naïve *iLgr5;GLI2A* mice were analyzed using western blot. INHBA levels were normalized to ALIX. Average expression of INHBA normalized to ALIX in 5 biological replicates (across 2 experiments) is presented in as mean ± SEM. Two-tailed Student's t-test was used for statistical analysis. **(G-J)** INHBA, THBS1 and THBS2 levels in EVs derived from WT and *Hsf1* null primary MEFs were analyzed using western blot. ALIX and TSG101 were used as loading controls. Representative blots are shown in **(G)**. **(H)** Average expression of INHBA normalized to TSG101 in 8 biological replicates (across 3 experiments for INHBA) is presented as mean ± SEM. **(I)** Average expression of THBS1 normalized to TSG101 in 5-7 biological replicates (across 3 experiments) is presented as mean ± SEM.

(J) Average expression THBS2 normalized to TSG101 in 10-11 biological replicates (across 4 experiments) is presented as mean ± SEM. Two-tailed Student's t-test was used for statistical analysis 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 ± SEM for N=14-15 mice per group (across 4 experiments). Repeated measures Two-way ANOVA 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 THBS1/2. INHBA and THBS2 from CAFs are packaged into EVs and secreted to the TME, where they are taken up by cancer cells.