An Integrated Stress Response Pathway activates Perivascular Cancer-Associated Fibroblasts to Drive Angiogenesis and Tumor Progression

Ioannis Verginadis
University of Pennsylvania

Harris Avgousti
University of Pennsylvania Perelman School of Medicine

James Monslow
University of Pennsylvania, School of Veterinary Medicine

Giorgos Skoufos
University of Thessaly

Frank Chinga
University of Pennsylvania School of Medicine

Nektaria Maria Leli
University of Pennsylvania Perelman School of Medicine

Ilias Karagounis
University of Pennsylvania Perelman School of Medicine

Brett Bell
University of Pennsylvania Perelman School of Medicine

Carlo Salas Salinas
University of Pennsylvania School of Medicine

Yang Li
Stanford University

Jiangbin Ye
Stanford University https://orcid.org/0000-0003-1117-4869

David Scott
Sanford-Burnham Prebys Medical Discovery Institute

Andrei Osterman
Sanford-Burnham Prebys Medical Discovery Institute

Arjun Sengupta
University of Pennsylvania Perelman School of Medicine

Aalim Weljie
University of Pennsylvania Perelman School of Medicine

Enrico Radaelli
Article

**Keywords:** Tumor microenvironment, ISR pathway, ATF4, CAF, single cell transcriptomics, angiogenesis

**DOI:** [https://doi.org/10.21203/rs.3.rs-122088/v1](https://doi.org/10.21203/rs.3.rs-122088/v1)

**License:** [Creative Commons Attribution 4.0 International License.](https://creativecommons.org/licenses/by/4.0/) This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](https://creativecommons.org/licenses/by/4.0/)
Abstract

ATF4 is a major effector of the Integrated Stress Response (ISR), a homeostatic mechanism coupling cell growth and survival to bioenergetic demands. Although the pro-tumorigenic role of the ISR in a tumor cell-intrinsic manner has been established, its role in cell-extrinsic processes remains unexplored. Using novel conditional knockout ATF4 mouse models, we show that global, or fibroblast (FB)-specific loss of host ATF4 results in abnormal tumor vascularization and a pronounced tumor growth delay in syngeneic melanoma and pancreatic tumor models, a phenotype which is largely reversed by co-injection of ATF4<sup>wt/wt</sup> FBs. Single-cell tumor transcriptomics uncovered a reduction of markers associated with FB activation in a cluster of perivascular cancer-associated fibroblasts (CAF) in ATF4<sup>Δ/Δ</sup> mice. ATF4<sup>Δ/Δ</sup> FBs displayed significant defects in collagen biosynthesis and deposition and reduced ability to support angiogenesis in vitro. Mechanistically, ATF4 directly regulates the expression of the Col1α1 gene as well as the biosynthesis of glycine and proline, the major amino acids comprising collagen fibers. Analysis of human tumor samples revealed a strong correlation between ATF4 and collagen levels and between an ATF4 FB signature and expression of collagen genes. Our findings uncover a novel role of stromal ATF4 in shaping CAF functionality, a key driver of disease progression and therapy resistance.

Introduction

The tumor microenvironment (TME) is a diverse ecosystem comprised of multiple cell types, including malignant, as well as untransformed stromal and immune cells, with overlapping or sometimes opposing functions that impact tumor growth and progression to metastasis and shape therapeutic responses. Among stromal cells, cancer-associated fibroblasts (CAFs) constitute a distinct and heterogeneous population, characterized as one of the most active and functionally important components of the TME. These intratumoral fibroblasts are often co-opted to support multiple hallmarks of cancer. CAFs are the primary source of extracellular matrix (ECM) components, including collagens, fibronectin, matrix metalloproteinases (MMPs), which have been shown to modulate tumor stiffness and facilitate tumor progression. In addition to ECM production and organization, CAFs impact tumor metabolism and also secrete a plethora of cytokines, chemokines, growth factors, and exosomes to further support tumor progression and modulate responses to treatment.

Although diverse subtypes of CAFs express multiple markers such as α-smooth muscle actin (αSMA), platelet-derived growth factor receptor β (PDGFRβ), or fibroblast activation protein (FAP), none of these is considered to be sufficiently unique to distinguish all CAFs within the TME. However, recent advancements in single-cell RNA sequencing (scRNA-seq) and careful analysis of CAFs perspicuous spatial distribution and distinctive functional characteristics have identified distinct CAF subtypes in melanoma and pancreatic tumors. Interestingly, single-cell expression profiling on isolated CAFs from a genetically engineered mouse model of breast cancer identified a subset of vascular CAFs (termed vCAFs). These vCAFs are localized in the perivascular region and characterized by the expression of genes involved in vascular development. These findings further support previous reports establishing a
key role of CAFs in supporting tumor vasculature through pro-angiogenic functions of their secretome \cite{15,22} or by shaping mechanical forces within the TME \cite{23}.

Despite recent advances in prevention and treatment, including immune checkpoint inhibitors, malignant melanoma remains a particularly aggressive and deadly malignancy \cite{24}, which is partly attributed to its highly heterogeneous TME. Moreover, pancreatic ductal adenocarcinoma (PDAC) has one of the worst outcomes among all malignancies, with a median 5-year survival rate of 7\% \cite{25}. PDAC presents unique challenges for treatment primarily due to its diverse, highly desmoplastic microenvironment and its frequent diagnosis at the metastatic stage. In several solid malignancies, including melanoma and PDAC, this heterogeneous environment, coupled with disorganized tumor vasculature, conspire to limit the delivery of oxygen and nutrients to malignant cells, leading to the development of hypoxic and nutritional stress. Malignant cells exhibit altered signaling pathways, which enable them to adapt to both cell-intrinsic and extrinsic stressors within TME. The Integrated Stress Response (ISR) denotes an evolutionarily conserved mechanism that promotes cellular adaptation to stresses such as hypoxia, nutrient deprivation and oncogenic stress \cite{26,27,28}. The ISR kinases including PKR-like ER kinase (PERK), general control non-derepressible 2 (GCN2), double-stranded RNA-dependent protein kinase (PKR) and heme-regulated eIF2\(\alpha\) kinase (HRI), converge on phosphorylation of the \(\alpha\) subunit of the eukaryotic translation initiation factor eIF2 (eIF2\(\alpha\)), in response to such stresses. Phosphorylation of eIF2\(\alpha\) not only reduces energy-consuming global translation but also promotes efficient translation of stress-responsive genes including activating transcription factor 4 (ATF4). ATF4, a master transcriptional effector of the ISR, regulates the transcription of genes involved in the antioxidant response, autophagy, amino acid biosynthesis and transport \cite{29,30}. We and others have established a critical tumor cell-intrinsic role of ATF4 which culminates in the promotion of primary tumor growth and in the establishment of micro- and macro-metastases in xenograft, allograft and transgenic models \cite{31,32}. However, the potential roles of the ISR and particularly of ATF4-mediated responses in host-dependent, tumor-related processes, have not been yet extensively investigated.

Here, using a conditional global ATF4 knockout (KO) mouse model, we show that global host ATF4 deletion significantly delays both primary and metastatic tumor growth and prolongs survival in mice harboring melanoma and pancreatic syngeneic tumors. Immunofluorescence analysis revealed a severely impaired angiogenic phenotype in tumors grown in ATF4 KO mice which was accompanied by deficiencies in markers of CAF activation. Single-cell transcriptomic analysis of these tumors further localized this defect to a distinct CAF population, previously identified as vCAFs \cite{21}, and revealed a significant reduction in the expression of ECM components, primarily type I collagen, in tumors grown in ATF4 KO mice. Intriguingly, we have also identified a multifaceted impairment of the collagen biosynthetic pathway in ATF4-deficient lung and dermal fibroblasts along with profound deficiencies in fibroblast activation, proliferation, and secretion of critical angiogenic cytokines. Specific deletion of ATF4 in the fibroblast compartment produced a similar tumor growth delay as in the global ATF4 KO mice, and notably, co-injection of fibroblasts from ATF4-proficient mice led to significant recovery of tumor growth rates in ATF4-deficient mice. Finally, immunohistochemical staining of high-density
melanoma and pancreatic cancer tissue arrays revealed a significant positive correlation between collagen I and ATF4 levels and TCGA analysis showed a negative correlation between expression levels of collagen I with overall patient survival in multiple malignancies. These studies uncover a novel, pro-tumorigenic role of the ISR pathway via CAF-dependent mechanisms and suggest new modes for therapeutic intervention.

Results

Deletion of host ATF4 inhibits tumor growth and extends survival in syngeneic melanoma and pancreatic tumor models

To test the impact of host ATF4 on tumor growth, we generated a tamoxifen-inducible knockout mouse by crossing $Atf4^{fl/fl}$ mice with Rosa26::CreER$^{T2}$ (Fig. 1a and Extended Data Fig. 1a) as we previously described. Rosa26::CreER$^{T2}$:$Atf4^{wt/wt}$ and Rosa26::CreER$^{T2}$:$Atf4^{fl/fl}$ mice were treated with tamoxifen (Fig. 1a), resulting in almost complete (90–100%) excision of ATF4 as assayed by qRT-PCR analysis of ATF4 mRNA levels in whole liver, lung and spleen homogenates of $Atf4^{D/D}$ mice (Fig. 1b and Extended Data Fig. 1b). Ablation of ATF4 was well tolerated causing only a modest and transient decrease in hematocrit and body weight (Extended Data Fig. 1c and data not shown). We have followed $Atf4^{wt/wt}$ and $Atf4^{D/D}$ mice for over a year with no overt toxicities observed. Full necropsy analysis revealed no significant pathological aberrations in the $Atf4^{D/D}$ cohort apart from some mild/moderate toxicity on the small intestine, spleen and liver (data not shown).

Mouse melanoma B16F10 cells were injected orthotopically (subcutaneously) in the right flank of $Atf4^{wt/wt}$ and $Atf4^{D/D}$ mice (Extended Data Fig. 1d). A pronounced delay in tumor growth was observed in $Atf4^{D/D}$ mice accompanied by a significant increase in their survival, compared to $Atf4^{wt/wt}$ littermates (Fig. 1c,d and Extended Data Fig. 1e). These effects were not gender dependent (Extended Data Fig. 1f).

In addition, a significant inhibitory phenotype on tumor growth was observed when host ATF4 was excised following the establishment of palpable B16F10 tumors, indicating that host ATF4 expression contributes to both tumor initiation and progression (Fig. 1e). To test if these findings extend to other tumor types, we injected subcutaneously mice with syngeneic MH6419 pancreatic tumor cells, originated from the Kras$^{LSL-G12D/wt}$,Trp53$^{-/-}$;Pdx1-Cre (KPC) model of spontaneous pancreatic cancer. Similar to the melanoma growth results, global host ATF4 ablation resulted in a significant delay in tumor growth and extension of overall survival (Fig. 1f,g and Extended Data Fig. 1g). In total, these results suggest that host ATF4 significantly contributes to the establishment and growth of syngeneic tumors.

ATF4 is essential for CAF activation in the tumor microenvironment

To delineate the role of host ATF4 in the sequence of events leading to tumor growth, we performed transcriptional profiling at the single-cell level (scRNA-seq) in smaller (150 mm$^3$) and larger (300 mm$^3$) size B16F10 tumors grown in $Atf4^{wt/wt}$ and $Atf4^{D/D}$ mice (Extended Data Fig. 2a). We acquired single-cell transcriptomes from a total of 7,414 cells from small and 28,166 cells from larger B16F10 tumors,
respectively, for downstream analysis. Graph-based clustering of cells following Uniform Manifold Approximation and Projection (UMAP), identified 7 distinct cell types in small B16F10 tumors (Fig. 2a), with CAFs accounting for 6.12% of the total cells. To identify the different cell types represented by the clusters, we cross-referenced the gene signature of each cluster with known markers of cell populations described in the literature (Fig. 2b) \(^\text{35,36}\). We then performed differential gene expression (DE) analysis for each cell type to identify potential transcriptome changes between the \(\text{Atf4}^{\text{wt/wt}}\) and \(\text{Atf4}^{\text{D/D}}\) cohorts. We confirmed host ATF4 deletion by the absence of \(\text{Atf4}\) mRNA expression in \(\text{Atf4}^{\text{D/D}}\) mice across all the “host” clusters (i.e. CAFs, dendritic cells, endothelial cells, etc.), while \(\text{Atf4}\) levels remained unchanged in melanoma clusters compared to the \(\text{Atf4}^{\text{wt/wt}}\) mice (Extended Data Fig. 2b). Interestingly, we observed a substantial decrease in the total number of endothelial cells in the \(\text{Atf4}^{\Delta/\Delta}\) grown tumors (Extended Data Fig. 2c), which implies a potential reduction in angiogenesis. Although there was a decrease in the total number of T cells/NK cells in \(\text{Atf4}^{\text{D/D}}\) grown tumors, anti-CD8 treatment caused a small increase in the rate of tumor growth in both \(\text{Atf4}^{\text{wt/wt}}\) and \(\text{Atf4}^{\text{D/D}}\) mice, suggesting that other mechanisms must account for the dramatic differences seen between these cohorts (data not shown). We did, however, observe striking differences in gene expression in a cluster that corresponds to CAFs. In this cluster, we identified 148 DE genes (Supplementary Table 1), including, a significant downregulation of \(\text{Col1a1}\) and \(\text{Col1a2}\) in the \(\text{Atf4}^{\text{D/D}}\) mice-grown tumors (Fig. 2c). These two genes encode the pro-alpha1(I) and pro-alpha2(I) chains, respectively, essential components of type I collagen, which is the most abundant collagen (~90%) in the body and in the ECM \(^\text{37}\). Furthermore, several additional collagen genes were downregulated in tumors grown in \(\text{Atf4}^{\text{D/D}}\) mice (data not shown). Notably, the expression levels of \(\text{Acta2}\) (encoding \(\alpha\text{SMA}\)) and \(\text{Pdgfr}\beta\) (platelet-derived growth factor receptor beta), which are broadly reported as markers of CAFs \(^\text{4,6}\), were nearly absent in the tumors grown in \(\text{Atf4}^{\text{D/D}}\) mice (Fig. 2c). Geneset enrichment analysis using Reactome pathways revealed that genes with higher levels of expression in \(\text{Atf4}^{\text{wt/wt}}\) compared to \(\text{Atf4}^{\text{D/D}}\) CAFs were mainly enriched for CAF-related functions, such as extracellular matrix organization, collagen formation and biosynthesis (Fig. 2d, left). In contrast, genes expressed at higher levels in \(\text{Atf4}^{\text{D/D}}\) CAFs were associated mainly with ISR activation, including response of GCN2 to amino acid deficiency and eukaryotic translation initiation (Fig. 2d, right), a finding consistent with loss of ATF4 in these cells. Several studies have defined distinct CAF subtypes with distinguishable functional programs based on specific gene expression signatures. Here, using the gene signatures described in Bartoschek et al. \(^\text{21}\) we identified 3 distinct cell subclusters, vascular CAFs (vCAFs), matrix CAFs (mCAFs) and cyclin/melanoma CAFs (cCAFs/melCAFs) (Fig. 2e). Interestingly, within the cCAFs we identified the melCAFs, a newly discovered sub-population which retains traits from CAFs but also melanoma cells. The vCAFs (Extended Data Fig. 2d) are considered essential for vascular development and angiogenesis and are also characterized by the highest levels of expression of \(\text{Acta2}\) and \(\text{Pdgfr}\beta\) among all CAF subclusters (Fig. 2f). Notably, the vCAFs were reduced substantially in the \(\text{Atf4}^{\text{D/D}}\) mice (Fig. 2g) and the \(\text{Col1a1}\) and \(\text{Col1a2}\) were significantly downregulated only in the vCAFs subcluster in the \(\text{Atf4}^{\text{D/D}}\) mice (Fig. 2h).
To track the regulation and fate of CAFs during B16F10 tumor progression and reorganization of the TME, we also performed scRNA-seq on larger sized tumors (Extended Data Fig. 2a). Graph-based clustering was used to identify 5 distinct cell clusters (Fig. 2i), which have been validated by a unique gene signature (Extended Data Fig. 2e). Atf4 levels remained extremely low in Atf4D/D mice across all the “host” clusters (Extended Data Fig. 2f). Similar to the smaller tumor data, the expression levels of Acta2 in CAFs cluster were significantly reduced in the tumors grown in Atf4D/D mice (Fig. 2j) and the number of endothelial cells was higher in the Atf4wt/wt mice indicating an important role of ATF4 in tumor vascularization (Extended Data Fig. 2g). In contrast to the smaller sized tumors, no difference was observed in the expression levels of Col1a1 and Col1a2 between the Atf4wt/wt and Atf4D/D cohorts (Fig. 2j), as observed in smaller sized tumors, suggesting the presence or activation of an alternative mechanism of Col1 gene expression regulation to compensate for the ATF4 loss during tumor progression. Geneset enrichment analysis revealed that genes upregulated in Atf4wt/wt CAFs were related mainly to ISR/UPR pathways as well as to smooth muscle contraction (Extended Data Fig. 2h). By following the aforementioned approach, we identified 4 distinct CAFs subclusters (Fig. 2k). The newly discovered sub-population melCAFs, was separated from the cCAFs sub-cluster and was found to form a distinct sub-cluster (Extended Data Fig. 2i,j). vCAFs cluster (Extended Data Fig. 2k), featured the highest expression of Acta2 and Pdgfrβ among all CAF subclusters again (Extended Data Fig. 2l) and critically, was diminished in tumors grown in Atf4D/D mice (Fig. 2l). Interestingly, the vCAFs remained as a distinct subcluster during the transition from smaller to larger sized melanoma tumors suggesting the importance of this subcluster in shaping the TME (Fig. 2m and Extended Data Fig. 2m,n). Collectively, these results suggest that host ATF4 deletion impairs CAF functionality at different stages of tumor development resulting in a tumor-inhibiting phenotype.

Host ATF4 loss results in abnormal tumor vascularization and reduced ECM component deposition

The results from the scRNA-seq data suggested specific defects in tumors grown in Atf4 Δ/Δ compared to Atf4wt/wt mice. To further explore these differences, we stained larger size B16F10 and MH6419 tumors of equal volume (approx. 300 mm³) from Atf4wt/wt and Atf4D/D mice for CD31 expression, a marker of tumor endothelium. Microvessel length (MVL) and microvascular density (MVD) were significantly reduced in the Atf4D/D mice indicating an abnormal vascularization in both tumor types (Fig. 3a,b and Extended Data Fig. 3a-c). Similar results were obtained in B16F10 tumors (approx. 1000 mm³) from endpoint euthanized mice, showing a persistent defect on vascularization post ATF4 loss (Extended Data Fig. 3d,e). Perfusion studies utilizing injected Texas Red-Dextran revealed significantly reduced vascular permeability in Atf4D/D mice further supporting an important role of ATF4 in tumor vascularization (Fig. 3c,d). Abnormal blood vessels provide reduced levels of nutrients and oxygen to tumor tissue resulting in intratumoral necrotic areas. Indeed, B16F10 tumors from Atf4 Δ/Δ mice presented a higher percentage of necrotic areas compared to the Atf4wt/wt tumors (Extended Data Fig. 3f-h). It is well-established that activated CAFs in the TME contribute to tumor neo-angiogenesis by providing structural support and local secretion of chemokines and angiogenic factors. Staining of B16F10 and MH6419
tumors for αSMA, showed dramatic reductions in tumors derived from Atf4Δ/Δ mice compared to levels observed in Atf4Δ/Δ mice, in which was primarily restricted in the blood vessels (Fig. 3e,f and Extended Data Fig. 3i-m). Levels of additional CAF markers such as fibroblast activation protein (FAP) in MH6419 tumors and PDGFRβ in B16F10 tumors, were also found to be significantly reduced in Atf4Δ/Δ mice (Fig. 3g,h and Extended Data Fig. 3n-p). These findings further corroborate the results from the scRNA-seq analysis, where the expression levels of Acta2 and Pdgfrβ in CAFs cluster were significantly reduced in the tumors grown in Atf4Δ/Δ mice. The expression of markers of other cell types which also contribute to blood vessel functionality such as neural/glial antigen 2 (NG2; pericytes) was not appreciably altered in the melanoma tumors from Atf4Δ/Δ mice (data not shown). A primary function of all fibroblasts is the synthesis and maintenance of ECM. Interestingly, collagen levels were significantly reduced in tumors grown in Atf4Δ/Δ mice (Fig. 3i,j and Extended Data Fig. 3q,r), further supporting a critically important role of ATF4 in CAF activation and stromagenesis in the solid tumors. Finally, immunofluorescence staining of human melanoma tissues revealed that ATF4 is highly expressed (apart from the tumor cells) on CAFs (aSMA) that localized on the perivascular area (CD34) compared to other CAFs reside away from the blood vessels (Fig. 3k).

We then sought to investigate if the absence of activated fibroblasts is related to the tumor-inhibitory effects observed in Atf4Δ/Δ mice. Since the syngeneic B16F10 tumors are grown subcutaneously, we isolated dermal fibroblasts from tumor-naïve Atf4Δ/Δ (DFBΔ/Δ) and Atf4Δ/Δ (DFBΔ/Δ) mice (Extended Data Fig. 4a). These were then co-injected with B16F10 cells (3:1 ratio) into the flanks of Atf4Δ/Δ or Atf4Δ/Δ mice (Fig. 3l). DFBΔ/Δ injected into Atf4Δ/Δ mice nearly completely reversed the tumor growth inhibition observed in Atf4Δ/Δ + DFBΔ/Δ group, while DFBΔ/Δ injected into Atf4Δ/Δ mice, caused a delay in tumor growth compared to the Atf4Δ/Δ + DFBΔ/Δ group (Fig. 3m). Although our results strongly implicated the fibroblast compartment in the tumor growth deficiency in Atf4Δ/Δ mice, we could not exclude a contribution of other cellular compartments. To further investigate the contribution of activated fibroblasts, we genetically excised ATF4 in a more tissue-specific manner by crossing Atf4Δ/Δ with Col1a1::CreERT2 mice (Extended Data Fig. 4b). The Col1a1 promoter has shown activity specifically in fibroblasts and osteoblast compartments. Deletion of ATF4 in these compartments following tamoxifen treatment did not result in any weight loss, reduction of hematocrit, or any other overt phenotypes (data not shown). Col1a1 driven-specific ATF4 deletion caused a significant B16F10 tumor growth delay, similar to that observed in Atf4Δ/Δ mice (Fig. 3n). This phenotype was accompanied by reduced MVD (Fig. 3o,p). Thus, these results lend strong support to the notion that ATF4 deficiency in fibroblasts creates an inhibitory TME through abnormal angiogenesis and reduced collagen deposition.

**ATF4-dependent, multilayered regulation of the collagen biosynthetic pathway contributes to protumorigenic fibroblast functionality**

The single-cell transcriptomic analysis revealed a profound impact of ATF4 on fibroblasts activation status and collagen mRNA levels. To cross-validate some of the scRNA-seq findings, we performed
genome-wide microarray analysis on isolated lung fibroblasts from Atf4<sup>wt/wt</sup> (LFB<sup>wt/wt</sup>) and Atf4<sup>D/D</sup> (LFB<sup>D/D</sup>) mice. We identified over 3000 genes to be differentially expressed, with a profound reduction in expression of collagen-associated (i.e. Col1a1, Co1a2, etc.) and fibroblast activation (i.e. Pdgfrβ) genes in LFB<sup>D/D</sup> (Fig. 4a), results which were confirmed by qRT-PCR analysis (Fig. 4b and Extended Data Fig. 4c). Similarly, Col1a1 levels were significantly reduced in DFB<sup>D/D</sup> (Extended Data Fig. 4d). Not surprisingly, ECM organization/degradation and collagen biosynthesis pathways were the most impaired in LFB<sup>D/D</sup>, validated by Geneset enrichment analysis on the 100 most downregulated genes in Atf4<sup>D/D</sup> mice (Fig. 4c). The biosynthesis of collagen is a highly coordinated process, involving mRNA synthesis and translation into pro-collagen, hydroxylation, glycosylation, and crosslink formation (Fig. 4d). Because both <em>in vitro</em> and <em>in vivo</em> RNA sequencing analysis showed downregulation of Col1a1 expression in the absence of ATF4, we reasoned that ATF4 directly regulates its expression. Analysis of mouse chromatin immunoprecipitation sequencing (ChIP-seq) data<sup>38</sup>, revealed potential binding sites of ATF4 inside intron 5 of Col1a1, as alternative transcription start sites (TSS) (Fig. 4e, left). ChIP-qRT-PCR validated the ChIP-seq results (Fig. 4e, right). We hypothesized that the severe phenotype of reduced collagen levels in tumors grown in ATF4 deficient mice could also involve additional steps in the pathway. Translation of Col1a1 mRNA requires adequate levels of glycine, proline and/or hydroxy-proline, which account for 70–100% of its polypeptide chain. Since ATF4 drives the transcription of genes involved in amino acid biosynthesis and transport<sup>29</sup>, we examined the levels of glycine and proline in ATF4 proficient and deficient LFB. Using NMR spectroscopy, we observed that intracellular levels of both amino acids were significantly reduced in ATF4 deficient cells (Fig. 4f). To further corroborate these findings, we measured the metabolic flux from serine to glycine and glutamine to proline by labeling the cells with serine-<sup>13</sup>C<sub>3</sub> (M + 3) and glutamine-<sup>13</sup>C<sub>5</sub><sup>15</sup>N<sub>2</sub> (M + 7), respectively. Notably, quantitative LC-ESI-MS/MS analysis revealed that although the labeling fractions of M + 3 serine and M + 7 glutamine were similar in LFB<sup>wt/wt</sup> and LFB<sup>D/D</sup> cells, there was a significant reduction of both labeled glycine (M + 2/<sup>13</sup>C<sub>2</sub>) and proline (M + 5/<sup>13</sup>C<sub>5</sub> and M + 6/<sup>13</sup>C<sub>5</sub><sup>15</sup>N) in LFB<sup>D/D</sup>, indicating that this reduction was not due to downregulation of precursor uptake, but due to reduction of ATF4-dependent metabolic flux from serine to glycine and glutamine to proline (Fig. 4g and Extended Data Fig. 4e,f). As expected, this defect in ATF4 deficient cell lines was accompanied by nearly undetectable intracellular procollagen levels (Fig. 4h and Extended Data Fig. 4g). The deficiencies in the synthesis of both mRNA as well as protein from low mRNA levels translated into a near-complete inability of ATF4 deficient fibroblasts to deposit collagen on gelatin-coated plates (Fig. 4i,j and Extended Data Fig. 4h,i). Consistent with an inability to synthesize or deposit collagen, ATF4 deficient fibroblasts also displayed significantly reduced proliferation capabilities (Fig. 4k,l and Extended Data Fig. 4j-m). Notably, re-expression of a mouse ATF4 homolog in LFB<sup>D/D</sup>, resulted in the detection of intracellular procollagen levels similar to the levels found in LFB<sup>wt/wt</sup> (Fig. 4m). Collectively, these results demonstrate that ATF4 is required to maintain a functional phenotype in fibroblasts, through the regulation of multiple steps of the collagen biosynthetic pathway.

**ATF4 deficient fibroblasts display significantly attenuated pro-angiogenic activity**
To further investigate the abnormal vascularization phenotype observed in both B16F10 and MH6419 tumors, primary lung endothelial cells were isolated from healthy \textit{Atf4}^{wt/wt} (EC\textit{wt/wt}) and \textit{Atf4}^{D/D} (EC\textit{Δ/Δ}) mice (Fig. 5a and Extended Data Fig. 5a), and tested for their ability to form endothelial tubes on Matrigel-coated plates. ATF4 was confirmed to be successfully excised in ECs (Fig. 5b,c and Extended Data Fig. 5b). After 4 and 8 hours of plating, EC\textit{Δ/Δ} did not show any differences on sprouting or tube formation parameters compared to the EC\textit{wt/wt}. In contrast, stimulation of endothelial cells with conditioned medium (CM) derived from LFB\textit{WT} and LFB\textit{D/D} revealed a significant defect in the response of EC\textit{Δ/Δ} (Fig. 5d,e and Extended Data Fig. 5c). Interestingly, the CM from LFB\textit{D/D} caused a significant reduction in both sprouting and tube formation of EC\textit{wt/wt} compared to those treated with CM from the LFB\textit{wt/wt}, indicating a possible deficiency in the LFB\textit{D/D} secretome (Fig. 5e). To further probe the reason for this deficiency, CM from ATF4 proficient and deficient LFB as well as from LFB\textit{D/D} expressing the mouse ATF4 homolog, were analyzed for multiple secreted angiogenesis-related proteins. Intriguingly, VEGF, SDF-1, IGFBP-2 and IGFBP-9 levels were significantly reduced in the CM from LFBs\textit{D/D}, and these levels were re-established in LFB\textit{D/D} with restored ATF4 levels (Fig. 5f,g). It is well established that TGF\textit{β}/Smad3 pathway is active in CAFs, which in turn secrete VEGF, among the other cytokines, to boost angiogenesis\textsuperscript{39}. Interestingly, here we show that expression of both p-SMAD3 and T-SMAD3 were downregulated in LFB\textit{D/D} post TGF\textit{b} treatment (Fig. 5h), in agreement with already published data\textsuperscript{40}. Taken together, these results suggest that ATF4 loss in fibroblasts impairs their pro-angiogenic activity through a defective secretome.

**Host ATF4 ablation inhibits lung metastasis of melanoma tumors**

Since acute deletion of host ATF4 caused a significant tumor-inhibitory phenotype, and since activated CAFs also play a critical role in the establishment of the metastatic niche\textsuperscript{41–43}, we speculated that ATF4 deficiency could also result in an inhibitory effect on lung metastasis. B16F10 melanoma tumors, similar to human melanoma metastasize to multiple sites, but primarily to the lung\textsuperscript{44}. We first examined the impact of host ATF4 deletion in the pre-metastatic niche by analyzing gene expression changes on lungs from \textit{Atf4}^{wt/wt} and \textit{Atf4}^{D/D} mice at 4 weeks post tamoxifen treatment. Genome-wide microarray analysis identified more than 170 genes as being differentially expressed, with 21 genes to be significantly downregulated in \textit{Atf4}^{D/D} lungs, including \textit{Col1a1}, validated by qRT-PCR analysis (Fig. 6a,b). Importantly, pathway analysis on the most dysregulated genes revealed defects on collagen formation, extracellular matrix organization and integrin cell surface interactions pathways (Fig. 6c), which is consistent with the gene expression data on LFBs. Mass spectrometry of lung tissue extracts revealed pronounced reductions in glycine and proline levels on \textit{Atf4}^{D/D} mice compared to their \textit{Atf4}^{wt/wt} littermates (Fig. 6d). These results indicate that loss of host ATF4 might cause an unfavorable metastatic niche, possibly through the regulation of fibroblast functionality. Tail vein injection of B16F10 cells resulted in efficient lung colonization in \textit{Atf4}^{wt/wt} mice at 3 weeks. In contrast, a significantly reduced number of lung metastases (evaluated macroscopically), as well as area covered by metastatic melanoma cells (determined by H&E stained serial lung sections), were observed in \textit{Atf4}^{D/D} compared to \textit{Atf4}^{wt/wt} mice.
(Fig. 6e-h). Critically, in a more physiologically relevant model of metastasis, where equal volume (appr. 300 mm$^3$) of B16F10 tumors in both genotypes were surgically excised and lungs were examined at 4 weeks post excision (Fig. 6i), the results were even more striking: 6 out of 9 lungs from Atf4$^{D/D}$ mice lacked any detectable metastases, while the other 3 presented with only a small single metastatic nodule. In contrast, a significantly higher number of metastases was observed in all the lungs of Atf4$^{N/wt/wt}$ littermates (Fig. 6j,k). Together, these results indicate that host ATF4 acts as a driving factor in the development of the pre-metastatic niche and efficient metastatic process in B16F10 melanoma tumors.

**Association between ATF4 levels, stromagenesis and poor prognosis in melanoma patients**

To investigate the relevance of our findings in human malignancies, we analyzed the expression of Col1a1, Acta2 and multiple other genes in relation to ATF4 activity in different cohorts of melanoma (SKCM) and pancreatic cancer patients (PAAD) from the TCGA database. Since ATF4 is primarily regulated at the translational level$^{29,33,38}$, we used ATF4 transcriptional targets as a surrogate for ATF4 activation. However, none of the well-characterized ATF4 targets in epithelial cells that we and others have previously reported$^{33}$ were expressed at significant levels in fibroblasts or showed any correlation with the collagen genes or Acta2 (data not shown). This is likely due to the tissue-specific and transformation-specific repertoire of genes regulated by ATF4. Therefore, we decided to use a “fibroblast ATF4 target signature” consisting of the 30 most downregulated genes in Atf4$^{D/D}$ compared to Atf4$^{N/wt/wt}$ fibroblasts as a surrogate for ATF4 activation in this cell type (Fig. 7a). Interestingly, we found that Col1a1, Acta2, Pdgfrβ and FAP displayed a significant positive correlation with this dataset primarily in melanoma (SKCM) and pancreatic tumors (PAAD) but also in several other tumor types, including lung adenocarcinoma (LUAD), kidney renal clear cell carcinoma (KIRC) etc. (Fig. 7b and Extended Data Fig. 6a). In contrast, no significant correlation was found using a list of 30 randomly chosen genes (Fig. 7c). To further probe this relationship, human malignant melanoma and high-density pancreatic cancer tissue arrays were stained for collagen (COL1) and ATF4 by immunohistochemistry (Extended Data Fig. 6b,c). Indeed, a significant positive correlation was found in melanoma tissue array (Fig. 7d,e and Extended Data Fig. 6d), which was stronger in the metastatic group compared to the primary tumor group (Extended Data Fig. 6e). These findings further corroborate the results from the mouse melanoma metastasis model. A significant positive correlation was observed in the pancreatic tissue array (Extended Data Fig. 7a,b). Interestingly, we noticed that this correlation was stronger in grade 2 compared to grade 3 pancreatic cancer patients, suggesting that ATF4 may exert a stronger regulatory role on collagen deposition at earlier disease stages (Extended Data Fig. 7c). These findings are consistent with the scRNA-seq results from B16F10 tumors, where we observed a gradual loss of Col1a1 regulation by ATF4 moving from smaller to larger size tumors. Notably, high expression of Col1a1 also correlated with poor prognosis in melanoma and many other tumor patients (Fig. 7f and Extended Data Fig. 7d). Together, these findings suggest that ATF4-dependent activation of CAFs dictates early ECM organization and CAFs-instructed angiogenesis to support the growth of primary tumors as well as the metastatic phenotype (Fig. 7g).
Discussion

The non-malignant ecosystem of the tumor microenvironment is increasingly being appreciated as a key driver of tumor progression, aggressiveness, and resistance to therapy. Given the cardinal features of CAFs in the tumor microenvironment, a better understanding of their transitory roles during tumor evolution and the mechanisms underlying these genotypic and phenotypic changes is crucial for developing novel therapeutic approaches. We have uncovered an essential, novel role for the master ISR effector ATF4 in shaping CAF functionality to dictate ECM organization and angiogenesis to support a tumor-promoting phenotype in experimental models of melanoma and pancreatic cancer (Fig. 7g).

Employing unbiased single-cell transcriptomic analysis of small and large-sized melanoma tumors, we surprisingly detected impaired CAFs activation in \( \text{Atf4}^{D/D} \) mice, based on the expression levels of \( \text{Acta2} \) and \( \text{Pdgfr}\beta \), as one of the most commonly used CAF markers. Interestingly, the levels of aSMA, PDGFR\beta and FAP detected by immunofluorescent staining on melanoma and pancreatic tumors were substantially reduced to nearly undetectable in \( \text{Atf4}^{D/D} \) mice, indicating that ATF4 is essential for CAFs activation within the TME. A more constrained deletion of ATF4 in the fibroblast/osteoblast compartment in a similar tumor growth profile as in the global ATF4 KO mice. Notably, co-injection studies of fibroblasts from \( \text{Atf4}^{wt/wt} \) mice led to significant recovery of tumor growth rates in \( \text{Atf4}^{D/D} \) mice.

Our studies also provide for a putative mechanism underlying the effect of ATF4. These data suggest that transcriptional changes in CAFs elicited by ATF4 loss are associated with primary CAFs functions, such as extracellular matrix organization, collagen formation and biosynthesis. We have shown reduced levels of deposited collagen in melanoma and pancreatic tumors post-global ATF4 excision which has been validated by \( \text{ex vivo} \) fibroblast-derived matrix assays. Mechanistically, we identified and validated an ATF4 binding site at the intron 5 of the \( \text{Col1a1} \) gene. Intriguingly, in humans, this site has been identified as the second most common binding site region, with more than 20 transcription factors binding sites, deeming these loci regulatory hotspots. Therefore, our data suggest a new multifactorial role of ATF4 on the regulation of the collagen synthesis pathway which further contributes to the pro-tumorigenic fibroblast functionality.

There is overwhelming evidence related to the heterogeneity and plasticity of CAFs. The combination of scRNA sequencing with functional assays has uncovered unprecedented diverse and spatially distinct CAF subpopulations based on specific gene expression signatures. Among our key findings, we identified the vascular CAFs (vCAFs), a spatially distinct CAF subcluster featured by the highest levels of aSMA and PDGFR\beta, that were reduced in \( \text{Atf4}^{D/D} \) mice. CAFs have been ascribed well-established roles in supporting angiogenesis through the release of pro-angiogenic factors, like VEGFA, FGF2 and SDF-1/CXCL-12 (CAFs secretome) or via exertion of mechanical forces within the tumor milieu. Our data indicate that conditioned medium (CM) from LFB\(^{wt/wt}\) but not LFB\(^{D/D}\) could support vascular tube formation and sprouting in \( \text{in vitro} \) assays of angiogenic activity. Interestingly, CM from ATF4-replete LFBs exhibited significantly higher levels of VEGF and SDF-1/CXCL-12 which have
been shown to drive angiogenesis in malignancy\textsuperscript{15,53}. However, we cannot exclude a possible pro-angiogenic role of vCAFs through altering mechanical forces, as they appear to be predominantly localized in proximity to the vasculature, and future studies will be required to address this. Kojima and colleagues have demonstrated that TGFβ and SDF-1 autocrine signaling facilitates the differentiation of stromal fibroblasts into CAFs in invasive human breast carcinomas\textsuperscript{54}. Interestingly, we uncovered an impaired TGFβ/Smad3 signaling in TGFβ-treated LFBs lacking ATF4, suggesting a potential involvement of this pathway on the defective secretome of the LFB\textsuperscript{D/D}.

Collectively, our work highlights the vital importance of the ATF4 on regulating the differentiation of stromal fibroblasts to CAFs and the activation of the latter through the collagen I synthesis and TGFβ/Smad3 pathways, respectively. Overall, the lack of overt toxicities following transient ATF4 deletion in mice, coupled with the demonstrated pro-tumorigenic role of ATF4 in a tumor-intrinsic as well as tumor-extrinsic manner, further supports the notion that a clinically useful therapeutic window may exist for ISR and ATF4 inhibition as an attractive antitumor strategy.

**Methods**

**Plasmids and other reagents.** The list of reagents, assays and adenovirus used in this study is provided in the Supplementary Tables 2,3.

**Antibodies.** The list of antibodies is provided in the Supplementary Table 4.

**Cell culture.** All cell lines are listed in the Supplementary Table 5. B16F10 and MH6419 cells were cultured in RPMI-1640 supplemented with 10% FBS in the presence of 5% CO\textsubscript{2} at 37 °C. Isolated EC\textsuperscript{wt/wt} and EC\textsuperscript{Δ/Δ} cells were cultured in Endothelial Cell (EC) medium. Isolated LFB\textsuperscript{wt/wt}, LFB\textsuperscript{Δ/Δ}, DFB\textsuperscript{wt/wt} and DFB\textsuperscript{Δ/Δ} cells were cultured in phenol-free DMEM/F12. All cell lines were determined to be free of mycoplasma with repeated testing.

**Lung and dermal fibroblast isolation.** Lungs and skin were isolated from 8–10 week old Atf4\textsuperscript{wt/wt} and Atf4\textsuperscript{D/D} mice (males and females). Tissues from 4 mice were minced and digested in 20 ml of mixed collagenase lysis buffer (1 mg/ml of collagenase type I (Worthington, Cat#LS004214) and 1 mg/ml collagenase type II (Worthington, Cat#LS004176)) dissolved in phenol-free DMEM/F12 without FBS and Pen/Strep under continues rotation on a rocker at 37 °C for 40–50 min. An equal volume of phenol-free DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin were added to the lysed tissues to quench collagenase and then passed through 70 µµ and 40 µµ cell strainer (Falcon Cat#352350 and Cat#352340, respectively). Cells were spun at 300 g for 5 min and the pellet was resuspended in 10 ml of complete FB medium (phenol-free DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 µµ of 4-hydroxytamoxifen (4-HT) (Sigma-Aldrich, Cat#H7904), 1x non-essential amino acids (NEAA) (Gibco, Cat#11140-050) and 55 µM β-mercaptoethanol (β-ME) (Millipore, Cat#ES-007-E)). This is considered as passage zero (p0). All cell lines were also treated with 50 µg/ml of gentamycin (VWR, Cat#E737) until they reach p2.
**Lung endothelial cell isolation.** Lungs were isolated from 8–10 weeks old *Atf4*<sup>wt/wt</sup> and *Atf4*<sup>D/D</sup> mice (males and females). Tissues from 8 were minced and digested in 10 ml of collagenase lysis buffer (5 mg/ml of collagenase type II (Worthington, Cat#LS004176)) dissolved in phenol red-free EC medium (ScienCell, Cat#1001-prf) without FBS and Pen/Strep under continuous rotation on a rocker at 37 °C for 35–45 min. An equal volume of phenol-free EC medium supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin were added to the lysed tissues to quench collagenase and then passed through 70 µm and 40 µm cell strainer. Cells were spun at 300 g for 5 min, the pellet was resuspended in 10 ml of complete EC medium (phenol-free EC medium supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 µM of 4-hydroxytamoxifen (4-HT), 1x non-essential amino acids (NEAA) and 55 µM β-mercaptoethanol (β-ME)) and cells were plated in 10 cm plates and incubated at 37 °C for 1 h. Non-adherent cells were collected by 3-5x washes with HBSS, spun at 300 g for 5 min and washed again once with HBSS supplemented with 0.5% Fraction V BSA (Gibco, Cat#15260-037). Cells were incubated with beads and CD31<sup>+</sup> cells were isolated (positive selection) according to the manufacturer’s instructions (Miltenyi Biotec, Cat#130-097-418). Endothelial cells were plated on 0.1% gelatin-coated plates. This is considered as passage zero (p0).

**Western blot analysis**

Cells were harvested in ice-cold PBS and proteins were extracted using 1x RIPA buffer supplemented with protease (Sigma, #B8640) and phosphatase inhibitors (Sigma, #P5726 and #P0044). Proteins were separated by 4%-15% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% nonfat milk or bovine serum albumin in 1x PBS with Tween-20 (0.1%). Primary antibodies against ATF4 (Cell Signaling Technology, Cat#11815, RRID:AB_2616025), Collagen Type I (Millipore-Sigma, Cat#AB765P, RRID:AB_92259), p-SMAD3 (Cell Signaling Technology, Cat#9520, RRID:AB_2193207), SMAD2/3 (Cell Signaling Technology, Cat#8685, RRID:AB_10889933), VE cadherin (Cell Signaling Technology, Cat#2158, RRID:AB_2077970), b-actin (Cell Signaling Technology, Cat#3700, RRID:AB_2242334) and b-tubulin (Cell Signaling Technology, Cat#2146, RRID:AB_2210545) were added at 1:1000 and incubated overnight at 4 °C. Membranes were washed and secondary antibodies (ThermoFisher: Cat#31460, RRID:AB_228341 or Cat# 31430, RRID:AB_228307) were added at 1:2000. ECL (Thermo Scientific, Cat#32106 and GE Healthcare, Cat#RPN2232) was added and membranes were exposed to autoradiography films or ChemiDoc (Bio-Rad).

**Immunofluorescence.** Tumor tissues were cut in 8–10 µm (or 100 µm for confocal microscopy) thick sections, coded and stored at -80 °C. Slides thawed at room temperature and subsequently fixed with 2% paraformaldehyde for 20 min. After 3x washes with TBS, tissues were blocked with 8% BSA and 1% donkey serum in TBS-T (0.025% Triton X-100) at room temperature for 1 h. The primary antibodies against CD31 (1:50, BD Biosciences, Cat#550274 or Abcam, Cat#ab28364), Acta2 (1:100, Sigma, Cat#C6198), FAP (1:100, R&D systems, Cat#AF3715), PDGFRb (1:100, Abcam, Cat#ab69506), NG2 (1:20, ThermoFisher, Cat#MA5-24247) and collagen type I (1:400, Southern Biotech, Cat#1310-01) were applied for overnight incubation at 4 °C. After 3x washes with TBS-T, the secondary antibodies (ThermoFisher: Cat#A-11005; Cat#A-21206; Cat#A-11015; Cat#A-11006; Cat#A-11055) were added at 1:200 and
incubated for 1 hour at room temperature in a humidified chamber. After 3 × 5 min rinses with TBS, tissues were stained with 1 µg/ml Hoechst (Invitrogen, Cat#H3570) for 30 min at room temperature, washed with TBS and coverslips were mounted with Antifade Mounting Medium.

**Immunohistochemistry.** For H&E staining, 5 µm thick paraffin sections were mounted on Superfrost Plus™ slides and stained using a Gemini AS Automated Slide Stainer. Slides were finally mounted with a resinous mounting medium (Thermo Scientific ClearVue™ coverslipper).

**Immunohistochemical staining of human tissue arrays for ATF4 and COL1.** Immunohistochemical staining was used to separately analyze ATF4 and COL1 content in human tissue arrays. FFPE-fixed tissue arrays were first deparaffinized and rehydrated following standard procedures. Antigen retrieval was then performed by submerging the slides in simmering (low-rolling boil) 0.05% Tween-20/10 mM sodium citrate (pH 6) for 20 min. After cooling for 5 min at RT, sections were consecutively treated to block endogenous peroxidase (3% H₂O₂ for 15 min) then with 10% normal serum blocking solution (dependent on host of the secondary antibody, in 1% BSA / PBS for 15 min). Sections were then incubated with primary antibodies against ATF4 (1:200, Abcam, Cat#ab31390) or COL1 (1:400, Southern Biotech, Cat#1310-01) or IgG isotype (Jackson ImmunoResearch, Cat#005-000-003 or Jackson ImmunoResearch, Cat#011-000-003) control in blocking solution overnight at 4 °C. Sections were then incubated with HRP-IgG secondary antibodies (Jackson ImmunoResearch, Cat#705-035-147 or Jackson ImmunoResearch, Cat#111-035-144) diluted in 1% BSA/PBS for 1 h at RT. Sections were then equilibrated in sterile H₂O for 5 min then developed with the DAB substrate kit (Dako, Agilent, Santa Clara, CA). Samples were counter-stained with hematoxylin, dehydrated, and mounted in Cytoseal-60.

**Multiplex immunostaining of human melanoma tissues.** Immunofluorescent multiplex staining was used to determine the colocalization of ATF4 with Acta2 (αSMA)- and/or CD34-positive cells. FFPE tissue sections were first rehydrated, submitted to antigen retrieval, peroxidase and serum blocking as described above. Sections were then incubated with primary antibodies against ATF4 (1:200, Abcam, Cat#ab31390) or CD34 (1:200, Biolegend, Cat#826401) or IgG isotype controls for ATF4 (Jackson ImmunoResearch, Cat#011-000-003) and CD34 (Southern Biotech, Cat#0102-01) in blocking solution overnight at 4 °C. Sections were then treated with an immunofluorescent-labeled secondary antibody for CD34 (ThermoFisher, Cat#A-11005), and a poly-HRP-secondary antibody then consecutive fluorescent-tyramide (ThermoFisher, Cat#B40926) for ATF4 (1 h at RT). Samples were then stained with an immunofluorescent-labeled primary antibody for Acta2 (1:500, Millipore-Sigma, Cat#F3777) or IgG isotype control (BD Biosciences, Cat#553456, RRID:AB_479604) for 1 h at RT. Finally, sections were counterstained for nuclei with DAPI then aqueous mounted in SlowFade Gold.

**EdU proliferation assay.** Fibroblast cell proliferation was detected using the Click-iT EdU Alexa Fluor 488 Imaging kit according to the manufacturer’s instructions.

**qRT-PCR.** Total RNA was isolated using the Macherey-Nagel kit and cDNA was synthesized using the High Capacity RNA-to-cDNA kit, according to manufacturer’s instructions. qRT-PCR was performed with Power
SYBR green PCR master mix. For data analysis, the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) was used. Relative gene expression levels were defined using the DDCt method and normalization was performed to 18S rRNA. All primers used in this study are described in the Supplementary Table 6.

**Chromatin immunoprecipitation (ChIP).** The ChIP assay was performed as previously described \(^ {55} \). IgG was used for pulldown as an experimental control. The primer sequences are listed in Supplementary Table 6.

**Conditioned medium.** Fibroblasts were plated and cultured until 80% confluence. After 3x washes with PBS, phenol-free DMEM/F12, without any supplement, was added and kept for 24 h. The medium was spun at 300 g for 5 min, filtered through 0.22 µm filter and stored at -80 °C until further use.

**Fibroblast-derived matrices (FDMs).** Decellularized FDMs were generated as described previously \(^ {56} \), using lung and dermal fibroblasts cultured at confluence in the presence of 75 µg/ml ascorbic acid for 8 days.

**Tube formation assay.** Each well of the 8-well slide chamber was coated with matrigel. \(10^5\) cells endothelial cells in 300 µl of complete EC medium were plated on matrigel on each well. Cells were allowed to attach for 4 h, and phase-contrast images were taken at 8, 12 and 16 h post-plating, using the Zeiss Observer.Z1 (Zeiss).

**Protein angiogenesis array.** Conditioned medium was added to the membranes of the proteome profiler mouse angiogenesis array and processed according to the manufacturer's instructions.

**Flow cytometry.** Endothelial and fibroblast cells were stained with Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen, Cat#L34957) for live/dead cell discrimination. Cell proliferation was detected using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, Cat#C10632) according to the manufacturer's instructions. For endothelial cells, cell surface staining against CD31 (Biolegend, Cat#102508) was performed for 30 min at 4 °C. All data acquisition was done using a FACSCanto II (BD Biosciences).

**Gas chromatography–mass spectrometry.** Samples (up to 50 mg – cut and reweighed as needed) were added to 0.5 ml 10 mM pH 7.4 Hepes/ 1 mM EDTA/ 0.1% Triton X100/ 2 mM L-norvaline (internal standard) in Omni Bead Ruptor tubes, and lysed with setting 5.5 for 30 s. The equivalent of 2.5 mg tissue (25–114 ul or 50–228 nmol norvaline) was transferred to 0.5 ml Eppendorf tubes (duplicates for 2 lung samples L7, L10 and 1 skin sample S3), and dried for 1 h in Speedvac. Added 100 ul 6 N HCl, incubated at 105C for 15.5 h. Dried 5 ul (lung), 7.5 ul (skin – this was a guess – too much!), or 10 ul (melanoma) by Speedvac (25 min, 2.5–22.8 nmol norvaline). Sets of standards to run in parallel were also dried. Derivatized with 60 ul 1:1 mix pyridine: MTBSTFA, 60 min 80C. After derivatization, samples were transferred to GC-MS vials and left at room temp for 4 days before running GC-MS (this was to stabilize the norvaline signal).
**NMR spectrometry.** Cell or tissue samples were extracted using a biphasic extraction protocol. LC-MS grade Methanol and chloroform were purchased from Thermo Fisher. 0.22 micron filtered milli-Q water was used for extraction purpose. Fifty mg of tissue and/or 106 cells were extracted using 500 µl 2:2:1 methanol/chloroform/water. The cell samples were sonicated using a sonicator bath and the tissue samples were homogenized using steel bids in a Tissuelyzer II system (Eppendorf). The samples were further centrifuged (13300 rpm, 4°C). The upper fraction containing the polar metabolites were carefully collected and dried using a vacuum centrifuge (Eppendorf).

The dried samples were dissolved in 200 µl phosphate buffer (pH ~ 7.1) containing Sodium-2,2-Dimethyl-2-Silapentane-5-Sulfonate (DSS, Cambridge Isotope Limited, Andover, MA) and 10% D2O for field frequency lock purpose (Cambridge Isotope Limited). The samples were transferred to NMR tubes (3 mm I.D, Bruker Biospin, Billerica, MA).

NMR spectra were acquired using Avance III HD 700 MHz NMR spectrometer (Bruker Biospin, Billerica, MA) fitted with a 3 mm NMR triple resonance inverse probe and SampleJet system for automated high throughput spectral acquisition. All spectra were acquired at 298K. The pulse program of the acquired NMR spectra took the shape of the first transient of a 2-dimensional NOESY and generally of the form RD-90-t-tm-90-ACQ. Where RD = relaxation delay, t = small time delay between pulses, tm = mixing time and ACQ = acquisition. Continuous irradiation of water during RD and tm was used to suppress the water signal. The spectra were acquired using 1-second interscan delay, 0.1 second mixing time, 76K data points and 14 ppm spectral width with a variable number of scans depending on starting sample mass. The FIDs were zero-filled to 128K; 0.1 Hz of linear broadening was applied followed by Fourier transformation.

NMR spectra were imported into Chenomx v 8.0. (Edmonton, Canada) for quantitative targeted profiling. The processor module was used to phase and baseline correct the spectra followed by internal standard calibration and deletion of the water region. The processed spectra were then imported to the profiler module for the targeted profiling of selected metabolites. Quantified data from this process were exported for further analysis.

**Metabolic tracing study.** For serine-13C3 labeling experiments, cells were cultured in RPMI medium lacking glucose, serine, and glycine (TEKnova) supplemented with 2 g/L glucose and 0.03 g/L serine-13C3 (Sigma Aldrich) for 1 and 3 hours before harvesting. Cells were washed twice with ice-cold PBS prior to extraction with 600 µL of 80:20 acetonitrile:water over ice for 15 min. Cells were scraped off plates to be collected with supernatants, sonicated for 30 sec, then spun down at 15,000 RPM for 15 min. 200 µL of supernatant was taken out for LC-MS/MS analysis immediately. Quantitative LC-ESI-MS/MS analysis was performed using an Agilent 1290 UHPLC system equipped with an Agilent 6545 Q-TOF mass spectrometer (Santa Clara, CA, US). A hydrophilic interaction chromatography method (HILIC) with a ZIC-pHILIC column (150 x 2.1 mm i.d., 5 µm; Merck) was used for compound separation at 35 °C with a flow rate of 0.3 ml/min. The mobile phase A consisted of 20 mM ammonium bicarbonate in water and mobile phase B was acetonitrile. The gradient elution was 0–1.5 min, 80%B; 1.5–7 min, 80%B → 40%B;
7–8.5 min, 40%B; 8.5–8.7 min, 40% → 80%B; 8.7–10 min, 80%B. The overall runtime was 10 min and the injection volume was 6 µL. Agilent Q-TOF was operated in negative mode and the relevant parameters were as listed: ion spray voltage, 3500 V; nozzle voltage, 1000 V; fragmentor voltage, 125 V; drying gas flow, 11 L/min; capillary temperature, 300 °C, drying gas temperature, 320 °C; and nebulizer pressure, 40 psi. A full scan range was set at 50 to 1200 (m/z). The reference masses were 119.0363 and 980.0164. The acquisition rate was 2 spectra/s. Data processing was performed with Agilent Profinder B.08.00 (Agilent technologies). The mass tolerance was set to +/- 15 ppm and RT tolerance was +/- 0.2 min.

For glutamine-13C5,15N2 labeling experiments, cells were cultured in DMEM medium lacking glutamine (Gibco) supplemented with 2 mM glutamine-13C5,15N2 (Cambridge Isotopes) for 2 and 6 hours before harvesting. Metabolites extraction was performed as described above. Quantitative LC-ESI-MS/MS analysis was performed using an Agilent 1290 UHPLC system equipped with an Agilent 6545 Q-TOF mass spectrometer (Santa Clara, CA, US). A hydrophilic interaction chromatography method (HILIC) with an Atlantis Silica HILIC Column (100 x 2.1 mm i.d., 3 µm; Waters) was used for amino acids separation at 35 °C with a flow rate of 0.3 ml/min. The mobile phase A consisted of 10 mM ammonium formate and 0.1% formic acid in water and mobile phase B was acetonitrile. The gradient elution was 0–1 min, 85%B; 1–4 min, 85%B → 65%B; 4–4.5 min, 65%B → 50%B; 4.5–5.5 min, 50% → 40%B; 5.5–6 min, 40%B → 25%B; 6–7 min, 25%B; 7–7.5 min, 25%B → 85%B. After the gradient, the column was re-equilibrated at 85%B for 2.5 min. The overall runtime was 10 min and the injection volume was 3 µL. Agilent Q-TOF was operated in positive mode and the relevant parameters were as listed: ion spray voltage, 3000 V; nozzle voltage, 500 V; fragmentor voltage, 125 V; drying gas flow, 11 L/min; capillary temperature, 300 °C, drying gas temperature, 320 °C; and nebulizer pressure, 40 psi. A full scan range was set at 50 to 1200 (m/z). The reference masses were 121.0509 and 922.0098. The acquisition rate was 2 spectra/s. Data processing was performed with MAVEN (http://genomics-pubs.princeton.edu/mzroll/index.php).

Genome-wide gene expression microarray analysis. Microarray services were provided by the UPENN Molecular Profiling Facility, including quality control tests of the total RNA samples by Agilent Bioanalyzer and Nanodrop spectrophotometry. All protocols were conducted as described in the Affymetrix WT Plus Reagent Kit Manual and the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, 250 ng of total RNA was converted to the first-strand cDNA using reverse transcriptase primed by poly(T) and random oligomers that incorporated the T7 promoter sequence. Second-strand cDNA synthesis was followed by in vitro transcription with T7 RNA polymerase for linear amplification of each transcript, and the resulting cRNA was converted to cDNA, fragmented, assessed by Bioanalyzer, and biotinylated by terminal transferase end labeling. Five and a half micrograms of labeled cDNA were added to Affymetrix hybridization cocktails, heated at 99°C for 5 min and hybridized for 16 h at 45°C to Clariom D Mouse Arrays using the GeneChip Hybridization oven 645. The microarrays were then washed at low (6X SSPE) and high (100 mM MES, 0.1M NaCl) stringency and stained with streptavidin-phycoerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycoerythrin stain. A GeneChip 3000 7G scanner was used to collect fluorescence signal. Affymetrix Command Console and Expression Console were used to quantitate expression levels
for targeted genes; default values provided by Affymetrix were applied to all analysis parameters. The GEO accession number is GSE159020.

**Single-cell RNA-sequencing.** Cells were loaded into a 10X Genomics Chromium Single-Cell controller following manufacturer’s instructions using the 10 × 3’ RNA-Seq V2 kit. Illumina sequencing libraries were prepared then sequenced either on three lanes of a HiSeq 4000 (28 bp x 98 bp) or a NovaSeq 6000 (28 bp x 91 bp). Samples were sequenced to a median depth of 14,188 +/- 932.6 reads per cell with a median 2,291 +/- 334 median gene count detected per cell. The fraction of reads mapping confidently to the transcriptome was 60% +/- 4.6%. The percent of reads from mitochondrial genes had a median of 6 +/- 0.41%. Initial data processing was performed with Cell Ranger V3.0.1. The GEO accession number is GSE159996.

**Mouse necropsy.** Mouse necropsy was performed according to the Comparative Pathology Core’s standardized approach for rodent studies.

**Correlation and survival analysis.** Gene expression profiles (RSEM-normalized gene expression values) were obtained from TCGA for the following cancer types: Skin Cutaneous Melanoma (SKCM), Pancreatic adenocarcinoma (PAAD), Bladder Urothelial Carcinoma (BLCA), Kidney renal clear cell carcinoma (KIRC), Kidney renal papillary cell carcinoma (KIRP) and Brain Lower Grade Glioma (LGG). Expression profiles were downloaded from Broad GDAC Firehose. For each dataset, Pearson correlation coefficients between Log_{2}-transformed expression values of COL1A1, COL1A2, ACTA2, PDGFRb, and FAP and Log_{2}-transformed mean expression values of 30 ATF4-target genes were estimated. As a baseline, Pearson correlation coefficients were also estimated between COL1A1, ACTA2, PDGFRb, and 30 genes that were randomly chosen using RSAT.

Subsequently, patients in each cancer type were divided into two groups according to COL1A1 expression: low COL1A1 expression (below 1st quartile) and high COL1A1 expression (above 3rd quartile). Survival analysis using Kaplan–Meier and the log-rank test between COL1A1-low and -high groups, was performed using R package survival and OncoLnc. Correlation analysis and Kaplan-Meier plots were produced using R package ggplot2.

**In vivo mouse studies.** All animal experiments have been approved by the University Laboratory Animal Resources and Institutional Animal Care and Use Committee of the University of Pennsylvania regulations. Both males and females were used in in vivo experimental procedures. Mice were housed in pathogen-free conditions. ATF4 excision was achieved by oral gavage of tamoxifen (200 mg/kg BW) for 5 consecutive days. For tumor growth studies, 5 × 10^5 B16F10 or MH6419 cells were injected into the flanks of 9-10-week-old Atf4wt/wt and Atf4Δ/Δ mice. For co-injection studies, 5 × 10^4 B16F10 mixed with 1.5 × 10^5 fibroblast (Atf4wt/wt or Atf4Δ/Δ) cells were injected into the flanks of 9-10-week-old Atf4wt/wt and Atf4Δ/Δ mice. For sc-RNA-seq studies, 5 × 10^5 B16F10 or MH6419 cells were injected into the flanks of 9-10-week-old Atf4wt/wt and Atf4Δ/Δ mice. For lung colonization studies, 1.5 × 10^5 B16F10 cells were injected in the tail vein of Atf4wt/wt and Atf4Δ/Δ mice. For lung metastasis studies, 5 × 10^5 B16F10 were
injected into the flanks of 9-10-week-old \textit{Atf4}\textsuperscript{wt/wt} and \textit{Atf4}\textsuperscript{Δ/Δ} mice. Tumors reached appr. 300 mm\(^3\) were surgically removed and 4 weeks later mice were euthanized the lungs were harvested and stored in 10% formalin. The primers for genotyping used in this study are described in Supplementary Table 6. Also, the mouse models used in this study are described in Supplementary Table 7.

**Patient samples.** Malignant melanoma with normal skin tissue array and pancreas cancer tissue array with adjacent normal pancreas tissue were purchased from US Biomax, Inc. Formalin-fixed paraffin-embedded human melanoma tumors were obtained from patients resected at the Hospital of the University of Pennsylvania upon signing the informed consent in accordance with the IRB protocol No. 703001 (Supplementary Table 8).

**Statistical analysis.** Parameters such as sample size, the number of independent experiments (mean ± SEM), and statistical significance are reported in Figures and Figure Legends. Results were considered statistically significant when \(p < 0.05\) by the appropriate test (ANOVA, Log-rank, t-test, Pearson correlation). The Student’s t-test and hypergeometric test were utilized for comparisons in experiments with two sample groups. Tukey’s multiple comparisons test was utilized for comparison in the tube formation experiments. Survival data were summarized with Kaplan-Meier methods and tested using the log-rank test. Statistical analyses were performed with GraphPad Prism version 8.

**Declarations**

**Acknowledgments**

This work was supported by NIH grants P01 CA165997 and P01 CA128814 to C.K. and P01 CA217805 to E.P. A.G.H. was supported by the Hellenic Foundation for Research and Innovation (H.F.R.I.) under the “First Call for H.F.R.I. Research Projects to support Faculty members and Researchers and the procurement of high-cost research equipment grant” (Project Number: 2563). G.S. is supported by the Operational Programme ‘Human Resources Development, Education and Lifelong Learning’ in the context of the project ‘Strengthening Human Resources Research Potential via Doctorate Research’ [MIS-5000432], implemented by the State Scholarships Foundation (IKY), in the form of a PhD Scholarship. We thank the members of the Koumenis lab for helpful suggestions and for critically reading the manuscript. We would like to thank Dr. Ben Stanger for providing the pancreatic cancer cell line. Authors would like to thank the University of Pennsylvania Veterinary Comparative Pathology Core, Molecular Profiling Facility, Next-Generation Sequencing Core and Microscopy Core for their valuable assistance with this project.

**Author contributions**

I.I.V. designed and conducted the experiments and acquired, analyzed, and interpreted the data. H.A., N.M.L., I.V.K., B.I.B., F.C., C.S.S., Y.L., J.Y., D.A.S., A.L.O., A.S. and A.W. conducted the experiments and acquired the data. J.M. and E.P. performed immunofluorescence and immunohistochemistry and analyzed and interpreted the results. F.R., P.K., and J-C.M., aided in the conceptual design of scRNA-seq experiments and interpreted the results. G.S. and A.G.H. performed the scRNA-seq analysis, analyzed the
TCGA data and interpreted the results. E.R. performed the mouse necropsy. J.W.T. analyzed the data from the genome-wide microarray analysis. X.X. provided the human melanoma tissues and contributed to the interpretation of the resultant data. S.R. provided guidance and support on the tube formation assays. A.J.D. and S.Y.F. aided in the conceptual design of metastasis experiments and provided key reagents. All authors read and edited the manuscript. I.I.V. and C.K. conceptualized and designed the research study and wrote the manuscript.

Data availability

Genome-wide gene expression microarray and single-cell RNA-sequencing data that support the findings of this study have been deposited into the Gene Expression Omnibus (GEO) repository under accession codes GSE159020 and GSE159996.

References

1. Ho, W. J., Jaffee, E. M. & Zheng, L. The tumour microenvironment in pancreatic cancer - clinical challenges and opportunities. *Nature reviews. Clinical oncology*, doi:10.1038/s41571-020-0363-5 (2020).

2. McGranahan, N. & Swanton, C. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. *Cell* **168**, 613-628, doi:10.1016/j.cell.2017.01.018 (2017).

3. Augsten, M. Cancer-associated fibroblasts as another polarized cell type of the tumor microenvironment. *Frontiers in oncology* **4**, 62, doi:10.3389/fonc.2014.00062 (2014).

4. Kalluri, R. The biology and function of fibroblasts in cancer. *Nat Rev Cancer* **16**, 582-598, doi:10.1038/nrc.2016.73 (2016).

5. Hanahan, D. & Coussens, L. M. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* **21**, 309-322, doi:10.1016/j.ccr.2012.02.022 (2012).

6. Sahai, E. *et al.* A framework for advancing our understanding of cancer-associated fibroblasts. *Nat Rev Cancer* **20**, 174-186, doi:10.1038/s41568-019-0238-1 (2020).

7. Tian, C. *et al.* Proteomic analyses of ECM during pancreatic ductal adenocarcinoma progression reveal different contributions by tumor and stromal cells. *Proceedings of the National Academy of Sciences of the United States of America* **116**, 19609-19618, doi:10.1073/pnas.1908626116 (2019).

8. Laklai, H. *et al.* Genotype tunes pancreatic ductal adenocarcinoma tissue tension to induce matricellular fibrosis and tumor progression. *Nat Med* **22**, 497-505, doi:10.1038/nm.4082 (2016).

9. Olivares, O. *et al.* Collagen-derived proline promotes pancreatic ductal adenocarcinoma cell survival under nutrient limited conditions. *Nature communications* **8**, 16031, doi:10.1038/ncomms16031 (2017).
10 Mohammadi, H. & Sahai, E. Mechanisms and impact of altered tumour mechanics. *Nature cell biology* **20**, 766-774, doi:10.1038/s41556-018-0131-2 (2018).

11 Sanford-Crane, H., Abrego, J. & Sherman, M. H. Fibroblasts as Modulators of Local and Systemic Cancer Metabolism. *Cancers (Basel)* **11**, doi:10.3390/cancers11050619 (2019).

12 Schworer, S., Vardhana, S. A. & Thompson, C. B. Cancer Metabolism Drives a Stromal Regenerative Response. *Cell Metab* **29**, 576-591, doi:10.1016/j.cmet.2019.01.015 (2019).

13 LeBleu, V. S. & Kalluri, R. A peek into cancer-associated fibroblasts: origins, functions and translational impact. *Dis Model Mech* **11**, doi:10.1242/dmm.029447 (2018).

14 Luga, V. *et al.* Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell* **151**, 1542-1556, doi:10.1016/j.cell.2012.11.024 (2012).

15 Orimo, A. *et al.* Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* **121**, 335-348, doi:10.1016/j.cell.2005.02.034 (2005).

16 Bhowmick, N. A., Neilson, E. G. & Moses, H. L. Stromal fibroblasts in cancer initiation and progression. *Nature* **432**, 332-337, doi:10.1038/nature03096 (2004).

17 Santos, A. M., Jung, J., Aziz, N., Kissil, J. L. & Pure, E. Targeting fibroblast activation protein inhibits tumor stromagenesis and growth in mice. *The Journal of clinical investigation* **119**, 3613-3625, doi:10.1172/JCI38988 (2009).

18 Davidson, S. *et al.* Single-Cell RNA Sequencing Reveals a Dynamic Stromal Niche That Supports Tumor Growth. *Cell Rep* **31**, 107628, doi:10.1016/j.celrep.2020.107628 (2020).

19 Elyada, E. *et al.* Cross-Species Single-Cell Analysis of Pancreatic Ductal Adenocarcinoma Reveals Antigen-Presenting Cancer-Associated Fibroblasts. *Cancer Discov* **9**, 1102-1123, doi:10.1158/2159-8290.CD-19-0094 (2019).

20 Ohlund, D. *et al.* Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. *J Exp Med* **214**, 579-596, doi:10.1084/jem.20162024 (2017).

21 Bartoschek, M. *et al.* Spatially and functionally distinct subclasses of breast cancer-associated fibroblasts revealed by single cell RNA sequencing. *Nature communications* **9**, 5150, doi:10.1038/s41467-018-07582-3 (2018).

22 De Palma, M., Biziato, D. & Petrova, T. V. Microenvironmental regulation of tumour angiogenesis. *Nat Rev Cancer* **17**, 457-474, doi:10.1038/nrc.2017.51 (2017).
23 Sewell-Loftin, M. K. et al. Cancer-associated fibroblasts support vascular growth through mechanical force. *Sci Rep* **7**, 12574, doi:10.1038/s41598-017-13006-x (2017).

24 Geller, A. C. et al. Melanoma epidemic: an analysis of six decades of data from the Connecticut Tumor Registry. *J Clin Oncol* **31**, 4172-4178, doi:10.1200/JCO.2012.47.3728 (2013).

25 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2018. *CA Cancer J Clin* **68**, 7-30, doi:10.3322/caac.21442 (2018).

26 Pakos-Zebrucka, K. et al. The integrated stress response. *EMBO Rep* **17**, 1374-1395, doi:10.15252/embr.201642195 (2016).

27 Costa-Mattioli, M. & Walter, P. The integrated stress response: From mechanism to disease. *Science* **368**, doi:10.1126/science.aat5314 (2020).

28 Tameire, F., Verginadis, II & Koumenis, C. Cell intrinsic and extrinsic activators of the unfolded protein response in cancer: Mechanisms and targets for therapy. *Semin Cancer Biol*, doi:10.1016/j.semcancer.2015.04.002 (2015).

29 Harding, H. P. et al. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* **11**, 619-633, doi:10.1016/s1097-2765(03)00105-9 (2003).

30 Hart, L. S. et al. ER stress-mediated autophagy promotes Myc-dependent transformation and tumor growth. *The Journal of clinical investigation* **122**, 4621-4634, doi:10.1172/JCI62973 62973 [pii] (2012).

31 Dey, S. et al. ATF4-dependent induction of heme oxygenase 1 prevents anoikis and promotes metastasis. *The Journal of clinical investigation* **125**, 2592-2608, doi:10.1172/jci78031 (2015).

32 Nguyen, H. G. et al. Development of a stress response therapy targeting aggressive prostate cancer. *Sci Transl Med* **10**, doi:10.1126/scitranslmed.aar2036 (2018).

33 Tameire, F. et al. ATF4 couples MYC-dependent translational activity to bioenergetic demands during tumour progression. *Nature cell biology* **21**, 889-899, doi:10.1038/s41556-019-0347-9 (2019).

34 Li, J. et al. Tumor Cell-Intrinsic Factors Underlie Heterogeneity of Immune Cell Infiltration and Response to Immunotherapy. *Immunity* **49**, 178-193.e177, doi:10.1016/j.immuni.2018.06.006 (2018).

35 Tirosh, I. et al. Dissecting the multicellular ecosystem of metastatic melanoma by single-cell RNA-seq. *Science* **352**, 189-196, doi:10.1126/science.aad0501 (2016).

36 Guerrero-Juarez, C. F. et al. Single-cell analysis reveals fibroblast heterogeneity and myeloid-derived adipocyte progenitors in murine skin wounds. *Nature communications* **10**, 650, doi:10.1038/s41467-018-08247-x (2019).
37 Gelse, K., Poschl, E. & Aigner, T. Collagens—structure, function, and biosynthesis. *Advanced drug delivery reviews* **55**, 1531-1546, doi:10.1016/j.addr.2003.08.002 (2003).

38 Han, J. *et al.* ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death. *Nature cell biology* **15**, 481-490, doi:10.1038/ncb2738 (2013).

39 Shimoda, M., Mellody, K. T. & Orimo, A. Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression. *Semin Cell Dev Biol* **21**, 19-25, doi:10.1016/j.semcdb.2009.10.002 (2010).

40 Gonzalez-Gonzalez, A. *et al.* Activating Transcription Factor 4 Modulates TGFbeta-Induced Aggressiveness in Triple-Negative Breast Cancer via SMAD2/3/4 and mTORC2 Signaling. *Clin Cancer Res* **24**, 5697-5709, doi:10.1158/1078-0432.CCR-17-3125 (2018).

41 Chen, X. & Song, E. Turning foes to friends: targeting cancer-associated fibroblasts. *Nat Rev Drug Discov* **18**, 99-115, doi:10.1038/s41573-018-0004-1 (2019).

42 Zhang, X. H. *et al.* Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma. *Cell* **154**, 1060-1073, doi:10.1016/j.cell.2013.07.036 (2013).

43 Gui, J. *et al.* Activation of p38α stress-activated protein kinase drives the formation of the pre-metastatic niche in the lungs. *Nat Cancer* in press (2020).

44 Overwijk, W. W. & Restifo, N. P. B16 as a mouse model for human melanoma. *Curr Protoc Immunol* Chapter **20**, Unit 20 21, doi:10.1002/0471142735.im2001s39 (2001).

45 Ohlund, D., Elyada, E. & Tuveson, D. Fibroblast heterogeneity in the cancer wound. *J Exp Med* **211**, 1503-1523, doi:10.1084/jem.20140692 (2014).

46 Pereira, B. A. *et al.* CAF Subpopulations: A New Reservoir of Stromal Targets in Pancreatic Cancer. *Trends Cancer* **5**, 724-741, doi:10.1016/j.trecan.2019.09.010 (2019).

47 Gerstein, M. B. *et al.* Architecture of the human regulatory network derived from ENCODE data. *Nature* **489**, 91-100, doi:10.1038/nature11245 (2012).

48 Wang, J. *et al.* Sequence features and chromatin structure around the genomic regions bound by 119 human transcription factors. *Genome Res* **22**, 1798-1812, doi:10.1101/gr.139105.112 (2012).

49 Puram, S. V. *et al.* Single-Cell Transcriptomic Analysis of Primary and Metastatic Tumor Ecosystems in Head and Neck Cancer. *Cell* **171**, 1611-1624 e1624, doi:10.1016/j.cell.2017.10.044 (2017).

50 Lambrechts, D. *et al.* Phenotype molding of stromal cells in the lung tumor microenvironment. *Nat Med* **24**, 1277-1289, doi:10.1038/s41591-018-0096-5 (2018).
51 Fukumura, D. et al. Tumor induction of VEGF promoter activity in stromal cells. Cell 94, 715-725, doi:10.1016/s0092-8674(00)81731-6 (1998).

52 Anderberg, C. et al. Paracrine signaling by platelet-derived growth factor-CC promotes tumor growth by recruitment of cancer-associated fibroblasts. Cancer research 69, 369-378, doi:10.1158/0008-5472.CAN-08-2724 (2009).

53 Compagni, A., Wilgenbus, P., Impagnatiello, M. A., Cotten, M. & Christofori, G. Fibroblast growth factors are required for efficient tumor angiogenesis. Cancer research 60, 7163-7169 (2000).

54 Kojima, Y. et al. Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. Proceedings of the National Academy of Sciences of the United States of America 107, 2009-20014, doi:10.1073/pnas.1013805107 (2010).

55 Soufi, A., Donahue, G. & Zaret, K. S. Facilitators and impediments of the pluripotency reprogramming factors' initial engagement with the genome. Cell 151, 994-1004, doi:10.1016/j.cell.2012.09.045 (2012).

56 Beacham, D. A., Amatangelo, M. D. & Cukierman, E. Preparation of extracellular matrices produced by cultured and primary fibroblasts. Curr Protoc Cell Biol Chapter 10, Unit 10 19, doi:10.1002/0471143030.cb1009s33 (2007).

57 Beckonert, O. et al. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. Nat Protoc 2, 2692-2703, doi:10.1038/nprot.2007.376 (2007).

58 Weljie, A. M., Newton, J., Mercier, P., Carlson, E. & Slupsky, C. M. Targeted profiling: quantitative analysis of 1H NMR metabolomics data. Anal Chem 78, 4430-4442, doi:10.1021/ac060209g (2006).

59 Cancer Genome Atlas Research, N. et al. The Cancer Genome Atlas Pan-Cancer analysis project. Nat Genet 45, 1113-1120, doi:10.1038/ng.2764 (2013).

60 Nguyen, Nga Thi T. et al. RSAT 2018: regulatory sequence analysis tools 20th anniversary. Nucleic Acids Research 46, W209-W214, doi:10.1093/nar/gky317 (2018).

61 Anaya, J. OncoLnc: linking TCGA survival data to mRNAs, miRNAs, and IncRNAs. PeerJ Comput Sci, doi:ARTN e67 10.7717/peerj-cs.67 (2016).

Figures
Host ATF4 deletion inhibits tumor growth and extends survival. a, LoxP sites flank exons 2 and 3 of the ATF4 gene. Tamoxifen treatment scheme of 200mg/kg body weight (BW) for 5 consecutive days by oral gavage. b, qRT-PCR of ATF4 in whole lung and liver (n = 4 per group). Values represent mean + SEM, unpaired t-test. c, Tumor growth curves of Atf4wt/wt (n=14) (blue line) and Atf4D/D (n=10) (red line) mice following 5x10^5 B16F10 cell injection. Values represent mean + SEM. Two-way ANOVA analysis (until
day 18). d, Kaplan-Meier survival analysis of the mice from C. Log-rank (Mantel-Cox) test. e, Growth curves post-B16F10 cell injection of Atf4wt/wt and Atf4D/D mice with the tamoxifen administered after the tumors reached around 100 mm³ and continued for 5 days (dark green line on x-axis). Values represent mean ± SEM. Two-way ANOVA analysis (until day 18). f, Tumor growth curves of Atf4wt/wt (n=11) and Atf4D/D (n=10) mice following 5x10⁵ MH6419 cell injection. g, Kaplan-Meier survival analysis of the mice from F. Values represent mean ± SEM. Two-way ANOVA analysis for F (until day 24). Log-rank (Mantel-Cox) test for G.
**Figure 2**

Single-cell transcriptomic analysis reveals ATF4-dependent changes in CAFs in B16F10 tumors. a, Uniform manifold approximation and projection (UMAP) plot of cells from 2 pooled small B16F10 tumors (150 mm³) from each genotype. Different cell type clusters are color coded. b, Dot plot displaying selected gene markers across all clusters. The color intensity represents the average expression and size of dots indicates the percentage of cells expressing each gene. c, Violin plots showing the expression of the Acta2, Pdgfrb, Col1a1 and Col1a2 at the CAFs cluster identified in B16F10 tumors. The y axis shows the mean expression level. Red (KO) and blue (WT) colors represent the Atf4D/D and Atf4wt/wt, respectively. d, Bar plot displaying the negative log10 FDR of the ten most significantly upregulated gene ontology terms enriched in WT (left) or KO (right) CAFs. fc: fold change. e, UMAP plot after re-clustering of the CAF cell type in the data set from A. f, Violin plots showing the expression of the indicated CAF markers in the CAFs subclusters. g, Bar plot displaying the normalized Log2 fold change (WT/KO) of CAFs subclusters in tumors grown in each genotype. h, Violin plots showing the expression of Col1a1 and Col1a2 in the vCAFs subcluster. fc: fold change. i, UMAP plot of cells from 4 pooled large B16F10 tumors (300 mm³) from each genotype. j, Violin plots showing the expression of the indicative markers at the CAF clusters. k, UMAP plot after re-clustering of the CAF cell type in the data set from I. l, Bar plot displaying the normalized Log2 fold change (WT/KO) of CAFs subclusters in each genotype. m, Slingshot-based pseudo-time ordering suggests that mCAFs move along a differentiation trajectory to become vCAFs, cCAFs and melCAFs.
Figure 3

ATF4 loss is associated with abnormal tumor vascularization and reduced extracellular matrix component deposition culminating in a tumor-inhibiting phenotype. a, Representative IF images from B16F10 tumors (appr. 300 mm3) stained for CD31 (green). Original magnification, 10x, 28x (insets). b, Quantification of the microvessel length from A (in mm) (n=4 per group). Values represent mean + SEM, unpaired t-test. c, Representative IF images from mice i.v.-injected with Texas Red-Dextran post-ATF4
deletion. Magnification, 20x. d, Quantification of % Dextran positive area from C (n=3-5 per group). Values represent mean + SEM, unpaired t-test. e, Representative IF images from B16F10 tumors stained for aSMA (red) and CD31 (green). Magnification, 20x. f, Quantification of the % aSMA positive area from E (n=3-4 per group). g, Representative IF images from MH6419 tumors stained for FAP (green). Magnification, 10x. h, Quantification of the % FAP positive area from G (n=4-6 per group). Values represent mean + SEM, unpaired t-test. i, Representative IF images from B16F10 tumor sections stained for collagen (green). j, Quantification of the % positive collagen area from I (n=7 per group). Values represent mean + SEM, unpaired t-test. k, Representative IF image from human melanoma tissues stained for CD34 (red), aSMA (green) and ATF4 (white). Arrows denote the aSMA+ cells with high ATF4 expression located in the perivascular area. Asterisks denote high ATF4 expression in tumor cells. Cropped images from 10x original magnification. l, Schematic of co-engraftment strategy to examine the ATF4-dependent tumor promoting role of fibroblasts in the TME. m, Tumor growth curves of mice of the indicated genotype (Atf4wt/wt or Atf4D/D) co-engrafted with DFB from the indicated ATF4 genotypes in ratios as described in L (n = 5-6 per group). Values represent mean + SEM. Two-way ANOVA analysis (until day 17 for Atf4wt/wt groups and day 24 for Atf4D/D groups). n, Tumor growth curves of mice with fibroblast/osteoblast specific ATF4 excision (Col1a1Cre;Atf4wt/wt (n = 11) and Col1a1Cre;Atf4D/D (n = 7)) following 5x10^5 B16F10 cell sub-cutaneous injection. Values represent mean + SEM. Two-way ANOVA analysis (until day 14). o, Representative IF images from B16F10 tumors from N stained for CD31 (green). p, Quantification of the microvascular density from O (n=5 per group). Values represent mean + SEM, unpaired t-test. Scale bars, 100mm (A, C, E, G, and O), 50mm (K) and 1 mm (I).
Figure 4

ATF4-dependent Col1a1 gene expression and multistep regulation of the collagen biosynthetic pathway contribute to fibroblast functionality. a, Volcano plot from genome-wide gene expression microarray on LFBs. b, qRT-PCR analysis was performed to validate the findings from A (n=4-7 per group). Values represent mean + SEM, unpaired t-test. c, Bar plot displaying the negative log10 FDR of the fifteen most significantly upregulated gene ontology terms enriched in LFBwt/wt compared to LFBD/D cells. d,
Schematic of the collagen synthesis pathway. e, Predicted binding site of ATF4 on intron 5 of Col1a1 gene (left). ATF4 ChIP followed by qRT-PCR at the Col1a1 locus as well as Eif4ebp1 (positive control) (representative from two biological replicates; n=3-4 technical replicates) (right). NEG = PCR amplification of a site with no predicted ATF4 binding sites, located at intron 6 of Col1a1 gene. f, NMR spectrometry analysis of intracellular glycine and proline levels (mM/cell) in LFBwt/wt and LFBD/D cells (n=4 per group). Values represent mean ± SEM, unpaired t-test. g, LC-ESI-MS/MS analysis to measure the metabolic flux from serine to glycine and glutamine to proline in LFBwt/wt and LFBD/D cells (n=3 per group). Values represent mean ± SEM. # and & indicate a statistically significant change from the LFBwt/wt at each isotopologue (&=p<0.01, #=p<0.001). h, Proteins were detected by immunoblotting in untreated LFBs. B-actin was used as a loading control. i, Representative images of collagen deposition from LFBwt/wt and LFBD/D using second harmonic generation (SHG) microscopy. j, quantification of the fluorescent signal from i. Values represent mean ± SEM, unpaired t-test. k, Representative IF images of proliferating LFBwt/wt and LFBD/D cells post EdU pulsing. I, Quantification of % EdU+ cells from K. Values represent mean ± SEM, unpaired t-test. m, Re-expression of a mouse ATF4 homolog from an Adenoviral vector in LFBD/D cells restores collagen I levels. Proteins were detected by immunoblotting. B-actin was used as a loading control.
Figure 5

ATF4 deficient fibroblasts fail to support endothelial tube formation and secrete reduced levels of specific angiogenic cytokines. a, Schematic of in vitro analysis of angiogenic activity of fibroblast-derived conditioned medium (CM). b, qRT-PCR of ATF4 in endothelial cells (ECs) isolated from Atf4wt/wt and Atf4D/D mice (n=2 per group). Values represent mean + SEM, unpaired t-test. c, Proteins were detected by immunoblotting in untreated ECs. B-actin was used as a loading control. d, ECwt/wt and ECA/Δ were treated with CM collected from LFBwt/wt or LFBD/D and plated for tube formation assay and analyzed 4h after plating. Magnification, 10x. e, The average number of sprouts (left) and tubes (right) per field was quantified. Values represent mean + SEM, unpaired t-test. Scale bars, 100mm. f, CM collected from LFBwt/wt, LFBD/D and LFBD/D+AdmATF4 cells was used for analysis of pro-angiogenic cytokines using antibody arrays. g, Membranes were subjected to immunoblotting and protein levels were quantified. h,
Proteins were detected by immunoblotting in untreated or TGFβ treated LFBwt/wt or LFBD/D. B-tubulin was used as a loading control.

Figure 6

Host ATF4 ablation severely impairs lung colonization and metastasis of melanoma cells. a, Volcano plot from genome-wide gene expression microarray on lungs from Atf4wt/wt and Atf4Δ/Δ mice at 4 weeks post tamoxifen treatment (Atf4D/D vs Atf4wt/wt). b, qRT-PCR of indicative markers in Atf4wt/wt
and Atf4D/D lungs (n=4 per group). Values represent mean + SEM, unpaired t-test. c, Bar plot displaying the fifteen most significantly enriched gene ontology terms in lungs from Atf4wt/wt compared to Atf4D/D mice from A. d, Quantitative mass spectrometry analysis in Atf4wt/wt and Atf4D/D lungs for glycine and proline (n=4 per group). Values represent mean + SEM, unpaired t-test. e, Representative images from Atf4wt/wt and Atf4Δ/Δ lungs at 3 weeks post tail vein injection with 1.5x10^5 B16F10 cells. f, Quantification of the number of macroscopic lung metastases (n=7-8 per group). Values represent mean + SEM, unpaired t-test. g, Representative images of serial sections of lungs from E stained for H&E. h, Quantification of the percentage of lung tumor area from G (n=5-6 per group). Values represent mean + SEM, unpaired t-test. i, Schematic of the process to analyze metastatic activity. Mice were injected subcutaneously with 5x10^5 B16F10 cells and the primary tumors were surgically excised when they reached appr. 300 mm^3. The mice were sutured and followed for a period of 4 weeks. j, Representative images from lungs, harvested at 4 weeks post tumor excision. k, The number of macroscopic lung metastases was quantified (n=7-9 per group). Values represent mean + SEM, unpaired t-test.
Figure 7

High ATF4 levels or ATF4-dependent gene expression correlate with increased COL1 expression or deposition in human tumors. a, “Fibroblast ATF4 target signature” comprised of the 30 most downregulated genes in LFBD/D compared to LFBwt/wt cells. b, Pearson correlation between the fibroblast ATF4 target signature and COL1A1, ACTA2, PDGFRb and FAP in Skin Cutaneous Melanoma (SKCM) and Pancreatic adenocarcinoma (PAAD). The linear regression lines along with 95% confidence
intervals (shaded regions) are shown. c, Pearson correlation between 30 randomly chosen genes and COL1A1 and PDGFRb in SKCM. The linear regression lines along with 95% confidence intervals (shaded regions) are shown. d, Representative images from human melanoma tissue arrays stained for COL1 and ATF4 proteins (Up: high expression; Down: low expression). Scale bars, 100mm. e, Pearson correlation between the % ATF4 area and % COL1 area. f, Kaplan-Meier plot of survival time of SKCM patients with high (n=151) or low (n=151) COL1A1 expression. Log-rank (Mantel-Cox) test. g, Working model for host ATF4's role in tumor progression and metastasis. ATF4 is essential for the CAF activation via direct regulation of Col1a1 expression and by impacting multiple additional steps in the collagen synthesis pathway, including Glycine (Gly) and Proline (Pro) pools. The resulting abrogation of Collagen I (and potentially additional collagen isoforms) in ATF4-deficient FBs leads in dramatic reduction in secreted extracellular matrix collagen, which in turn results in defective CAF activation and reduced levels of angiogenic cytokine signaling to endothelial cells. The resulting defective angiogenesis leads to reduced support for primary and metastatic tumor growth.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- [ExtendedDataLegends.docx](#)
- [ExtendedDataFigures17.pdf](#)
- [SupplementaryTables1232020.docx](#)