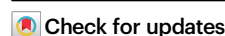


Fibroblastic aspartoacylase suppresses TGF β -mediated responses and cancer progression

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Metabolic reprogramming is a hallmark of cancer, and the field has predominantly focused on investigating metabolic alterations in tumour cells. However, the relevance, mechanism and consequences of metabolic adaptations in stromal cells remain understudied. Here, we identify aspartoacylase (ASP) as a metabolic enzyme consistently repressed in tumour stroma and cancer-associated fibroblasts (CAFs). Importantly, we report a reciprocal crosstalk between ASP and Transforming Growth Factor Beta (TGF β) signalling that influences fibroblast behaviour. TGF β suppresses ASP expression in fibroblasts, whereas ASP restrains TGF β -dependent myofibroblast conversion, extracellular cell matrix (ECM) remodelling, angiogenesis and pro-tumoral macrophage phenotypes. Analyses of human specimens revealed a strong negative prognostic value for ASP in different tumour types, associated with TGF β signalling levels and the generation of aggressive pro-tumoral responses. Our findings unveil ASP expression in fibroblasts as a gatekeeper of TGF β responses and activation in cancer progression.

Tumour cells exploit diverse metabolic strategies to support uncontrolled proliferation, resist cell death, and adapt to fluctuating nutrient and oxygen availability¹. Traditionally, studies have focused on cancer cell-autonomous metabolic alterations that support tumour

pathogenesis, progression and resistance to therapy^{1,2}. However, tumours are not composed of cancer cells in isolation. Instead, they function as complex ecosystems in which malignant, stromal, and immune cells engage in dynamic and reciprocal interactions that

influence tumour characteristics³. Within this heterogeneous micro-environment, nutrient availability, waste accumulation, and metabolic by-products shape not only the behaviour of malignant cells, but also impact tumour microenvironment (TME) components and the overall trajectory of the disease^{4,5}. However, how metabolic alterations shape the behaviour of stromal cells is not well understood.

Among the various stromal components of the TME, cancer-associated fibroblasts (CAFs) represent a predominant and functionally versatile population that plays a central role in supporting tumour growth, remodelling the extracellular matrix (ECM), regulating angiogenesis, and modulating immune responses⁶. CAFs do not represent a uniform cell type, but rather encompass a spectrum of phenotypic states that arise in response to contextual cues such as TGF β , interleukin-1 (IL-1), hypoxia, and mechanical stress⁷. These cues drive the activation and differentiation of CAFs into various subtypes, including myofibroblastic (myCAF), inflammatory (iCAF), and antigen-presenting (apCAF) populations, each contributing uniquely to tumour progression and therapy resistance. In addition to their structural and signalling roles, CAFs exert substantial influence over the metabolic landscape of the TME. Through reciprocal metabolic interactions with cancer cells and immune populations, CAFs can supply key nutrients, modulate oxidative stress, and recycle toxic metabolic wastes into energetic substances, allowing cancer cells to undergo biosynthesis⁸. Moreover, CAFs may compete with cancer and TME cells for specific nutrients to meet the bioenergetic demands of their pathologically activated status⁵. These metabolic exchanges are increasingly recognized as a hallmark of CAF function. However, the regulatory mechanisms and metabolic enzymes that govern this crosstalk remain incompletely characterized.

Aspartoacylase (ASPA) is a cytosolic enzyme primarily known for its role in the central nervous system (CNS), where it catalyses the hydrolysis of N-acetylaspartate (NAA) into aspartate and acetate—a process critical for myelin lipid synthesis and neuronal function⁹. Recent studies have begun to implicate ASPA in broader metabolic processes, and emerging evidence suggests that it may play an underappreciated role in non-neuronal tissues, including adipocytes^{10,11} and cancer cells¹². In cancer, high NAA levels have been associated with disease progression¹³. Impeding NAA production in ovarian cancer cells can affect glucose and glutamine metabolism, reduce tumour growth and influence pro-tumoral macrophage polarization^{13,14}.

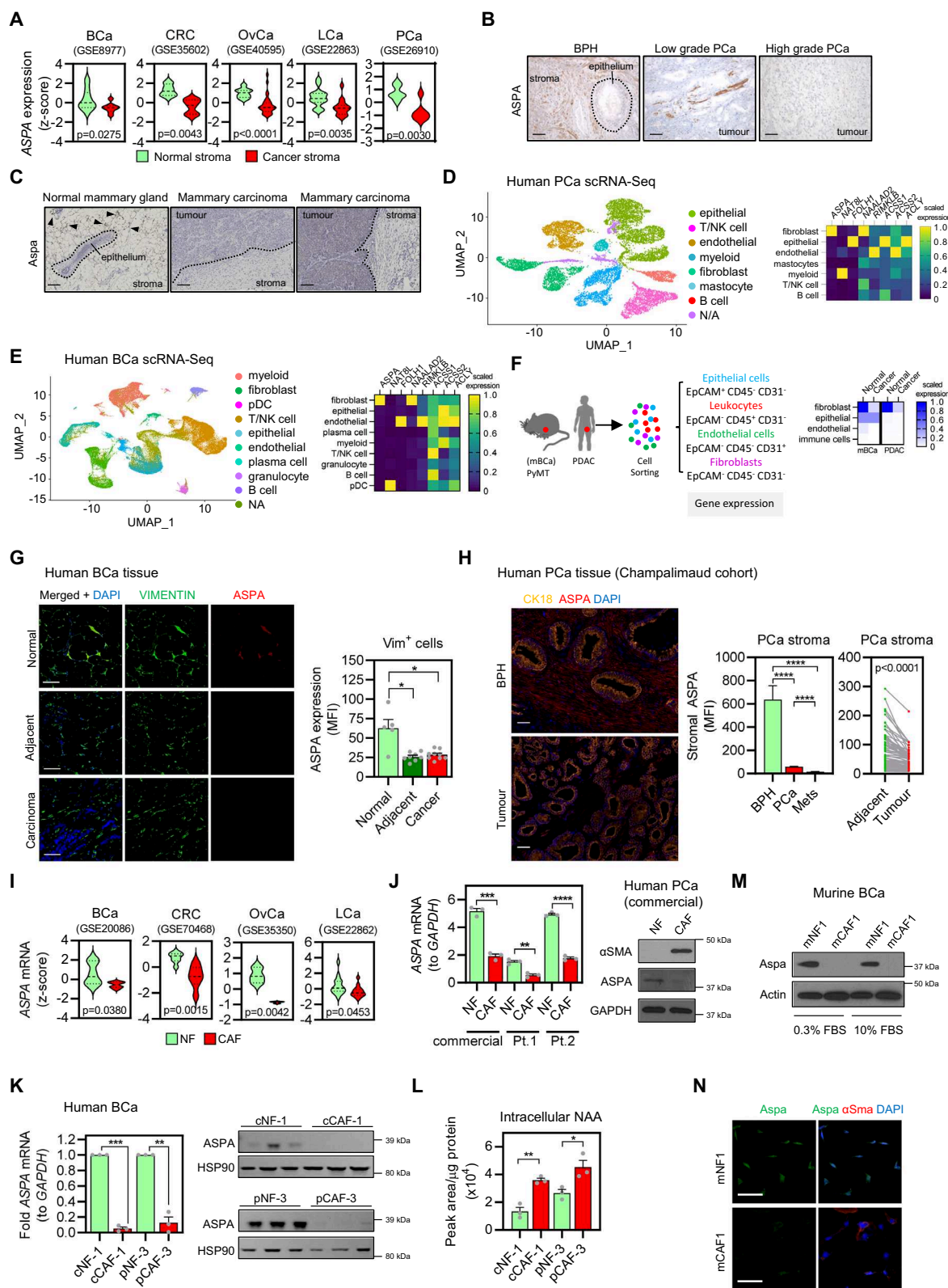
Here, we demonstrate that tumours consistently exhibit downregulation of ASPA in stromal fibroblasts, and that loss of ASPA regulates fibroblast activation and cancer progression, which is reflected in the strong negative prognostic value of the expression of this enzyme in human disease.

Results

Human tumour stroma and CAFs display reduced expression of ASPA

In search of new factors associated with aberrant TMEs, we interrogated stromal gene expression datasets from human breast (BCa), colorectal (CRC), ovarian (OvCa), lung (LCa) and prostate cancer (PCa) stroma against normal stroma (Supplementary Fig. 1A, B). This approach identified differentially expressed genes (DEGs) that were consistently altered in tumour stroma, including 13 upregulated and 19 downregulated genes (Supplementary Fig. 1C, D). Transcripts upregulated in tumour stroma included genes encoding for proteins with ECM organization (*COL3A1*, *COL11A1*, *COL12A1*, *MMPI1*, *CTHRC1*, *ITGB5*), underlying the well-characterized desmoplastic nature of most TMEs¹⁵. Downregulated transcripts were more heterogeneous, including genes encoding for transcriptional repressors (*SIN3A*, *HLF*, *EFCAB6*, *LRRFIP1*), for proteins with potential transcriptional-translational activity that are not well characterized (*EIF4EBP2*, *AFF4*, *EFCAB2*, *L3MBTL4*), or for proteins with metabolic functions (*SIN3A*, *PLD1*, *ASPA*,

LDLR). Beyond antitumoral immune responses, the tumour suppressive features of stromal cells are not well delineated, so we decided to focus on downregulated genes. Among them, we selected *ASPA* (encoding for the metabolic enzyme aspartoacylase) (Fig. 1A) since (i) it exhibited robust and consistent downregulation in the stroma of all tumours analysed; and (ii) it was recently shown to have a tumour suppressive role in cancer cells¹², but less is known about its function in cancer stroma. *ASPA* catalyses the deacetylation of N-acetylaspartate (NAA), an amino acid derivative highly abundant in the central nervous system (CNS), to generate free acetate in the brain, showing a high level of activity localized primarily in oligodendrocytes⁹. Mutations in *ASPA* cause Canavan disease (OMIM ID#271900), a lethal autosomal recessive neurodegenerative disorder, which is associated with insufficient myelin synthesis resulting from a deficiency in the supply of NAA-derived acetate⁹. Employing public datasets, we confirmed that both mRNA and protein levels of *ASPA* are consistently downregulated in different tumour types (Supplementary Fig. 1E, F). Initial analyses also hinted at a preferential immunoreactivity of *ASPA* antibodies (validated in the *corpus callosum* of rat brain tissue, Supplementary Fig. 1G) in the stromal compartment in normal tissues that was reduced in tumour stroma (Supplementary Fig. 1H). Similar to gene expression levels, we observed a marked downregulation of *ASPA* protein expression in the stromal compartment of human PCa specimens and murine BCa models compared to normal stroma (Fig. 1B, C). Contrary to a previous report¹², no staining was observed in normal or cancerous epithelia. NAA is produced from acetyl-CoA and aspartate by *NAT8L*⁹ (Supplementary Fig. 1I). Alternatively, NAA can be interconverted to or from N-acetyl-aspartate-glutamate (NAAG) by *RIMKLB* or *FOLH1/NAALAD2*, respectively. When monitoring the complete metabolic pathway regulating NAA levels, no consistent expression changes were observed in the stroma of different tumour types beyond *ASPA* (Supplementary Fig. 1J), suggesting a distinct role for this enzyme. Next, we explored the cellular compartment contributing to *ASPA* expression using single-cell RNA-Seq (scRNA-Seq). First, we performed scRNA-Seq in a cancer adjacent region of a PCa patient, showing that *ASPA* expression was exclusively detected in fibroblasts (Fig. 1D). This result was validated using a public human BCa scRNA-Seq dataset, where *ASPA* expression was also restricted to fibroblast populations (Fig. 1E). *NAT8L*, *FOLH1*, *NAALAD2* and *RIMKLB* exhibited inconsistent expression patterns in both datasets, with immune, epithelial and endothelial compartments showing higher expression. Moreover, analyses of scRNA-Seq from normal bladder mucosa and bladder cancer (BLCa) specimens showed a higher expression of *ASPA* in normal fibroblasts (NFs) and a relevant reduction in CAFs (Supplementary Fig. 2A). Next, we used flow cytometry-based cell selection coupled with gene expression analyses as a complementary approach to study the expression of *ASPA* in a cell compartment-specific manner, employing human PDAC specimens and BCa murine models. These analyses confirmed that fibroblasts were the main cell type expressing *ASPA*, and that CAFs exhibited a remarkable downregulation (Fig. 1F). Co-immunostaining of *ASPA* with the fibroblast marker vimentin revealed a preferential expression in fibroblasts in normal human mammary glands, and a reduction in fibroblasts adjacent to or within BCa regions (Fig. 1G). Similar findings were obtained in murine models, which also showed no relevant expression in vimentin-negative cells (Supplementary Fig. 2B). Of note, we confirmed that *ASPA* expression in benign PCa was mainly stromal (i.e. undetected in CK18-positive epithelial cells) and significantly reduced after malignant progression (Fig. 1H). To validate these findings, we turned to tractable models of NFs/CAFs. Initial analyses showed a consistent downregulation of *ASPA* expression in CAFs from different tumour types when compared to NFs (Fig. 1I). Employing both human and murine BCa and PCa models, we confirmed that *ASPA* expression is indeed reduced at both protein and mRNA levels in established CAF populations (Fig. 1J, K and Supplementary Fig. 2C, D), in line with the predicted increase in the



accumulation of its substrate, NAA (Fig. 1L). ASPA protein levels in mNF1 and mCAF1 were independent of serum levels in culture (Fig. 1M) and similar results were obtained in murine mammary NF/CAFs freshly isolated from tumours (Supplementary Fig. 2E), ruling out any potential in vitro artefact. ASPA expression was low in cells positive for established markers of fibroblast activation such as α SMA, both in isolated cells and in murine normal mammary glands and carcinomas

(Fig. 1N and Supplementary Fig. 2F, G). These results demonstrate a loss of ASPA expression in fibroblasts associated with myofibroblast conversion, as opposed to the reported downregulation of ASPA in the epithelial compartment in cancer¹². To further compare the relative contribution of epithelial *vs* fibroblast ASPA expression in tumours, we measured its protein levels in multiple PCa and fibroblast cell lines, confirming predominant expression in fibroblasts (Supplementary

Fig. 1 | Identification of ASPA as a factor consistently downregulated in tumour stroma and CAFs. **A** Graphs show *ASPA* mRNA expression (z-score normalized) in normal and cancerous stroma from human BCa, CRC, OvCa, LCa and PCa tissues (BCa: normal, $n = 15$; cancer, $n = 8$; CRC: normal, $n = 4$; cancer = 13; OvCa: normal, $n = 8$; cancer, $n = 31$; LCa: normal, $n = 15$; cancer, $n = 15$; PCa: normal, $n = 6$; cancer, $n = 6$). Reference datasets are described. *P*-values are shown (unpaired heteroscedastic t-test). **B** Images show ASPA staining in human prostate tissues from benign prostatic hyperplasia (BPH), and low and high-grade PCa (representative images from a minimum of 40 independent samples). Epithelial/tumour and stromal areas are indicated. Bars, 100 μ m. **C** Images show representative ASPA staining in murine mammary glands and carcinomas. Representative results from 3 independent samples. Epithelial/tumour and stromal areas are indicated. Arrowheads indicate Aspa-positive stromal cells. Bars, 100 μ m. **D Left:** UMAP and annotation of cell types identified in human PCa samples by scRNA-Seq analysis ($n = 1$ patient). Epithelial cells ($n = 3788$); T/NK cells ($n = 1568$); Endothelial cells ($n = 1592$); myeloid ($n = 1543$); fibroblasts ($n = 1739$); mastocytes ($n = 305$); B cells ($n = 653$); NA ($n = 549$). **Right:** Heatmap showing scaled values of average gene expression for indicated genes associated with NAA metabolism in each cell type. **E** As in (**D**), but in human BCa samples. Myeloid ($n = 20,361$); fibroblasts ($n = 14,923$); pDC ($n = 707$); T/NK cells ($n = 32,828$); epithelial cells ($n = 17,111$); endothelial cells ($n = 9834$); plasma cells ($n = 2124$); granulocytes ($n = 2640$); B cells ($n = 4356$); NA ($n = 37,257$). **F** Diagram describing the strategy for isolation and mRNA analyses of epithelial, endothelial and immune cells, as well as fibroblasts from human PDAC and murine BCa and normal tissue counterparts. Heatmap shows scaled values of average *ASPA* mRNA expression in each cellular compartment. **G** Representative images showing DAPI (blue), Vimentin (green) and ASPA (red) staining in tissues from normal human mammary glands, adjacent to tumour, and from mammary carcinomas. Bars, 100 μ m. Graph shows ASPA expression (MFI) in Vimentin-positive cells from indicated tissues (Normal, $n = 5$; adjacent, $n = 8$; cancer, $n = 9$). Mann-Whitney test: *, $p < 0.05$. **H** Representative images showing DAPI (blue), CK18 (orange) and ASPA

(red) staining in tissues from prostatic BPH and PCa. Bars, 100 μ m. Graphs show ASPA expression (MFI) in stromal-rich areas from BPH, PCa and PCa metastases (**left**) as well as paired expression in pathologically normal adjacent tissues and from tumours (**right**) (Left: BPH, $n = 23$; PT, $n = 20$; Right: $n = 90$). *P*-values are shown or ****, $p < 0.0001$ (left, Mann-Whitney tests; right, Wilcoxon matched-pairs rank test). **I** Graphs show *ASPA* mRNA expression (z-score normalized) in NFs and CAFs from human BCa, CRC, OvCa and LCa. Reference datasets are described (BCa: NF, $n = 6$; CAF, $n = 6$; CRC: NF, $n = 7$; CAF, $n = 7$; OvCa: NF, $n = 3$; CAF, $n = 3$; LCa: NF, $n = 15$; CAF, $n = 15$). *P*-values are shown: unpaired t-test; except for LCa, Mann-Whitney test. **J Left:** Graph shows *ASPA* mRNA expression (relative to *GAPDH*) in primary NFs and CAFs from 2 independent human PCa patients (Pt) and from commercial origin (WPMY-1, NF; PF179T, CAF). Unpaired t-test ($n = 3$): ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$. **Right:** Western blots show expression levels of α SMA, ASPA and GAPDH in WPMY-1 and PF179T. **K** Graph shows fold *ASPA* mRNA expression (normalized to *GAPDH*) in human BCa NF and CAFs from 2 independent models (cNF/CAF and pNF/CAF) ($n = 3$). Paired t-test: ***, $p < 0.001$; **, $p < 0.01$. Western blots show expression levels of ASPA and HSP90, in triplicate. **L** Graph showing intracellular NAA levels in human BCa NF and CAFs from 2 independent models ($n = 3$). Unpaired t-test: **, $p < 0.01$; *, $p < 0.05$. **M** Western blot showing expression levels of Aspa and Actin in murine BCa NF and CAFs (mNF1/CAF1 model) in standard culture conditions (10% FBS) and in FBS-restricted conditions (0.3% FBS). Representative results from 3 independent repeats. **N** Images showing Aspa (green), α SMA (red) and DAPI (blue) staining in established murine BCa NF and CAFs (mNF1/CAF1 model). Bar, 100 μ m. Representative images from three independent repeats (quantification in Supplementary Fig. 2F). In all cases, bar graph data are presented as mean values $\pm$ SEM; violin plots display median (straight line), quartiles (dashed lines) and maximum/minimum values (limits). Unless stated otherwise, all statistical tests were two-tailed. Source data are provided as a Source Data file, which includes exact *p*-values and additional statistical tests.

Fig. 2H). Collectively, our data suggest that the metabolic enzyme ASPA is primarily expressed by fibroblasts outside the CNS and that it is consistently downregulated in tumour stroma and CAFs, suggesting a potential role in the generation of aberrant TMEs. Notably, ASPA protein and mRNA levels display a very low level of expression in normal and cancerous epithelia, suggesting a specific role in modulating fibroblast behaviour.

ASPA repression is associated with myCAF phenotypes downstream of TGF β signalling

Next, we set out to explore the mechanisms regulating ASPA expression in fibroblasts. Recent studies suggest that CAFs are a collection of cells with diverse molecular features⁷. The current consensus groups them in populations enriched in ECM production with contractile properties that primarily emerge from TGF β signalling activation (myCAF); populations that produce high levels of chemokines/cytokines in response to inflammatory signals (iCAF); and emerging subtypes characterized by vascular phenotypes (vascular CAFs/vCAFs) or antigen-presentation features (antigen-presenting CAFs/apCAFs). Each subtype may emerge in response to specific signalling cues⁷ (Fig. 2A). Leveraging the BCa scRNA-Seq dataset, we observed that ASPA expression was heterogeneous, with myCAF subtypes presenting lower levels of expression (Fig. 2B and Supplementary Fig. 3A). ASPA mRNA levels negatively correlated with myCAF markers and processes in this analysis (Fig. 2B), as well as in datasets of NF/CAFs from different tumour types (Supplementary Fig. 3B). In addition, gene set enrichment analysis (GSEA) showed a negative association of ASPA mRNA levels with TGF β signatures in human stromal and NF/CAF datasets, whereas it had a positive association with NF phenotype gene signatures (Supplementary Fig. 3C). Therefore, we hypothesised that TGF β activation leads to the transcriptional repression of ASPA in NFs. To test this possibility, we first subjected murine mammary NFs to stimulation with a different set of modulating factors (Fig. 2A). We observed that only TGF β 1 stimulation downregulated ASPA mRNA and protein expression, which was concomitant with an activation of

TGF β /SMAD3 signalling and upregulation of α SMA (Fig. 2C, D and Supplementary Fig. 3D). Similar findings were obtained with human NFs from breast, colon and prostate tissues (Supplementary Fig. 3E–G). Interrogation of independent human datasets of human prostate NFs confirmed that ASPA expression was reduced upon TGF β 1 treatment and not by IL6 (Supplementary Fig. 3H). Consistently, incubation of murine and human NFs with SIS3, a SMAD3 inhibitor, blocked ASPA mRNA downregulation by TGF β 1 (Fig. 2E, F). These results demonstrate that ASPA is transcriptionally repressed by TGF β . To assess the potential mechanism of TGF β -induced ASPA repression in cancer, we analysed human BCa and PCa scRNA-Seq datasets. This showed that TGF β ligands can be expressed by different cells, with *TGFB1* primarily expressed by immune cells (including macrophages) and *TGFB2* and *TGFB3* by fibroblasts and epithelial/cancer cells (Fig. 2G). To independently validate this result, we employed: (i) established murine models of BCa cancer cells (TS1); (ii) macrophages (RAW 264.7) at basal state (M0) and subjected to stimulations to promote M2-like states (M2); and (iii) our mNF1/mCAF1 cells in normal culture conditions (See Methods for details). Analysis of *Tgfb1*, 2, 3 by RT-qPCR in these models confirmed the preferential expression of *Tgfb1* by macrophages, *Tgfb2* by cancer cells and *Tgfb3* by CAFs (Fig. 2H). Incubation of murine NFs with conditioned media (CM) from these models showed that M2-polarised macrophages had the greatest impact on Aspa repression in NFs, similar to levels induced by TGF β 1 (Fig. 2I), suggesting that ASPA downregulation is associated with inflammatory processes at early stages of tumour development (Fig. 2J). TGF β /SMAD3-dependent transcriptional repression may require the activity of histone deacetylases (HDACs)¹⁶, which participate in epigenetic rearrangements modulating gene expression. Epigenomic analysis of the 3' regulatory regions at the *Aspa* locus in murine CAFs, as compared to NFs, revealed a selective loss of H3K27Ac (Fig. 2K), an epigenetic mark of active enhancers. As expected, bona fide CAF marker genes such as *Acta2*, *Fap*, *Lrrc15* and others presented increased H3K27Ac signal at regulatory regions in mCAF1, whereas peaks at NF marker genes (*Cxcl1* and *Pil6*) were decreased

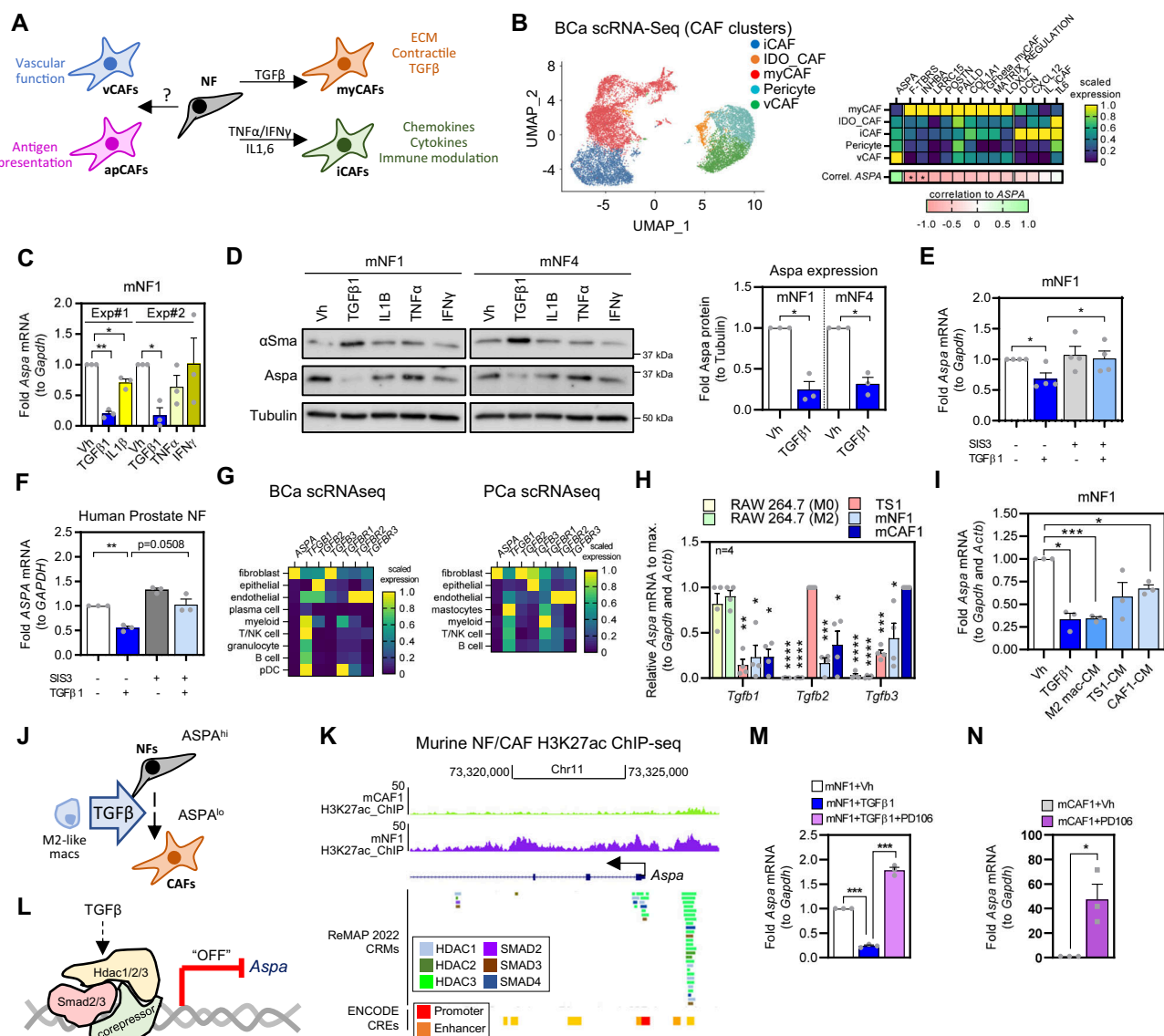


Fig. 2 | ASPA repression is associated with myCAF emergence downstream of TGFβ signalling. **A** Scheme illustrating CAF subtypes and main regulatory signals. **B** Left: UMAP and annotation of CAF subtypes identified in human BCa by scRNA-Seq analysis. *IDO_CAFs*: interferon-responsive CAFs. *iCAFs* ($n = 3492$); *IDO_CAFs* ($n = 518$); *myCAFs* ($n = 5851$); *pericytes* ($n = 22,524$); *vCAFs* ($n = 2534$); unclassified CAFs ($n = 4$). Right: Heatmap showing scaled values of average gene expression for ASPA and indicated genes/gene signatures associated with CAF subtypes, and Pearson correlation coefficient with ASPA (*, $p < 0.05$). **C** Graph shows fold *Aspa* mRNA expression (normalized to *Gapdh*) in mNF1 subjected to indicated treatments for 24 h ($n = 3$). Paired t-test: *, $p < 0.05$; **, $p < 0.01$. **D** Left: Western blot showing expression levels of αSma, Aspa and Tubulin in mNF1 and mNF4 subjected to the indicated treatments for 24 h. Right: Graph shows fold *Aspa* protein levels (normalized to Tubulin) in mNF1 and mNF4 subjected to vehicle (Vh) and TGFβ1 stimulation ($n = 3$). Paired t-test: *, $p < 0.05$. **E** Graph shows fold *Aspa* mRNA expression (normalized to *Gapdh*) in mNF1 treated with vehicle (Vh), TGFβ1 and/or SMAD2/3 inhibitor SIS3, as indicated ($n = 4$). Paired t-test: *, $p < 0.05$. **F** Graph shows fold *Aspa* mRNA expression (normalized to *Gapdh*) in human prostate NFs treated with vehicle (Vh), TGFβ1 and/or SMAD2/3 inhibitor SIS3, as indicated ($n = 3$). Paired t-test: **, $p < 0.01$. **G** Heatmaps showing scaled values of average gene expression for indicated genes associated with TGFβ signalling in cell types from BCa (left) and PCa (right) based on scRNA-Seq studies.

H Graph shows fold expression (relative to maximum expression) of *Tgfb1*, *Tgfb2* and *Tgfb3* (normalized to average of *Gapdh* and *Actb*) in murine M0 and M2 macrophage models (RAW 264.7), murine BCa models (TS1), mNF1 and mCAF1 ($n = 4$). Paired t-test to maximum value: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. **I** Graph shows fold *Aspa* mRNA expression (normalized to average of *Gapdh* and *Actb*) in mNF1 subjected to vehicle (Vh), TGFβ1 and conditioned media (CM) from murine M2 macrophage models (M2 mac), TS1 and mCAF1 ($n = 3$). Paired t-test: *, $p < 0.05$; ***, $p < 0.001$. **J** Diagram illustrating the mechanism modulating ASPA expression in stromal fibroblasts in tumours. **K** Genome browser H3K27ac ChIP-seq tracks at the *Aspa* locus in mNF1 and mCAF1. ReMap cis regulatory motifs (CRMs) for HDAC1-3 and SMAD2-4, and cis regulatory elements (CREs) for putative enhancers and promoters are shown. Ranges for read counts and gene structure are indicated. Scale bar, 5 kb. **L** Model for the TGFβ-driven repression mechanism of *Aspa* in stromal fibroblasts. **M** Graph shows fold *Aspa* expression (normalized to *Gapdh*) in mNF1 subjected to vehicle (Vh), TGFβ1 or TGFβ1 + PD106 for 24 h ($n = 3$). Paired t-test: ***, $p < 0.001$. **N** Graph shows fold *Aspa* expression (normalized to *Gapdh*) in mCAF1 subjected to vehicle (Vh) or PD106 for 24 h ($n = 3$). Paired t-test: *, $p < 0.05$. In all cases, bar graph data are presented as mean values ± SEM. Unless stated otherwise, all statistical tests were two-tailed. Source data are provided as a Source Data file, which includes exact p -values and additional statistical tests.

(Supplementary Fig. 3I). ReMap analysis¹⁷ of the H3K27Ac peaks in *Aspa* promoter and enhancers identified SMAD2/3 motifs overlapping HDAC1-3 *cis* regulatory modules (CRMs) that constitute potential TGF β inhibitory elements. Moreover, GSEA using a signature of genes repressed by HDAC1 and 2 in MEFs demonstrated the inverse association of *ASPA* levels and HDAC1/2 activities in NF/CAFs from different tumour types (Fig. S3J). These results suggest a model whereby a TGF β -mediated repression mechanism promotes the loss of *ASPA* in fibroblasts (Fig. 2L). In line with this idea, incubation of murine NFs with pan-HDAC inhibitors such as trichostatin A (TSA), or class I-specific HDAC inhibitors (PD106 and Romidepsin), abolished *ASPA* protein repression by TGF β 1 (Supplementary Fig. 3K). This finding was validated at the level of mRNA expression with PD106 in murine NFs (Fig. 2M). Importantly, incubation of unstimulated murine CAFs with both TSA and PD106 induced *ASPA* protein and mRNA expression, respectively (Fig. 2N and Supplementary Fig. 3L). Altogether, these results demonstrate the critical role of HDAC-mediated repression in the modulation of *ASPA* during CAF emergence, both downstream of TGF β /SMAD3 and through epigenetic rearrangements.

ASPA loss in stromal fibroblasts induces myofibroblast conversion

To explore the role of *ASPA* in the function and regulation of fibroblasts, we first characterised the murine CAF system after *ASPA* ectopic expression. Analyses of cell growth in vitro showed no differences between control and *ASPA*-expressing murine CAFs in normal culture conditions (Supplementary Fig. 4A). Furthermore, expression of *ASPA* in different mCAF1 clones did not affect the expression of myofibroblast marker α SMA, suggesting no observable effect on CAF activation (Supplementary Fig. 4B). Functional analyses of ECM remodelling activities employing the collagen: matrigel contraction assay, which measures the ability of a cell to remodel collagen by analysing changes in collagen plug area (Supplementary Fig. 4C) showed no relevant differences after *ASPA* expression in CAFs, or observable consequences in any system after addition of NAA (Supplementary Fig. 4D). Similar findings were obtained when the ability of CAFs to support murine BCa cell growth in 2D co-culture assays (Supplementary Fig. 4E) was analysed (Supplementary Fig. 4F, G). Together, these results indicate that recovery of *ASPA* expression in pathologically activated CAFs has no relevant phenotypic or functional consequences.

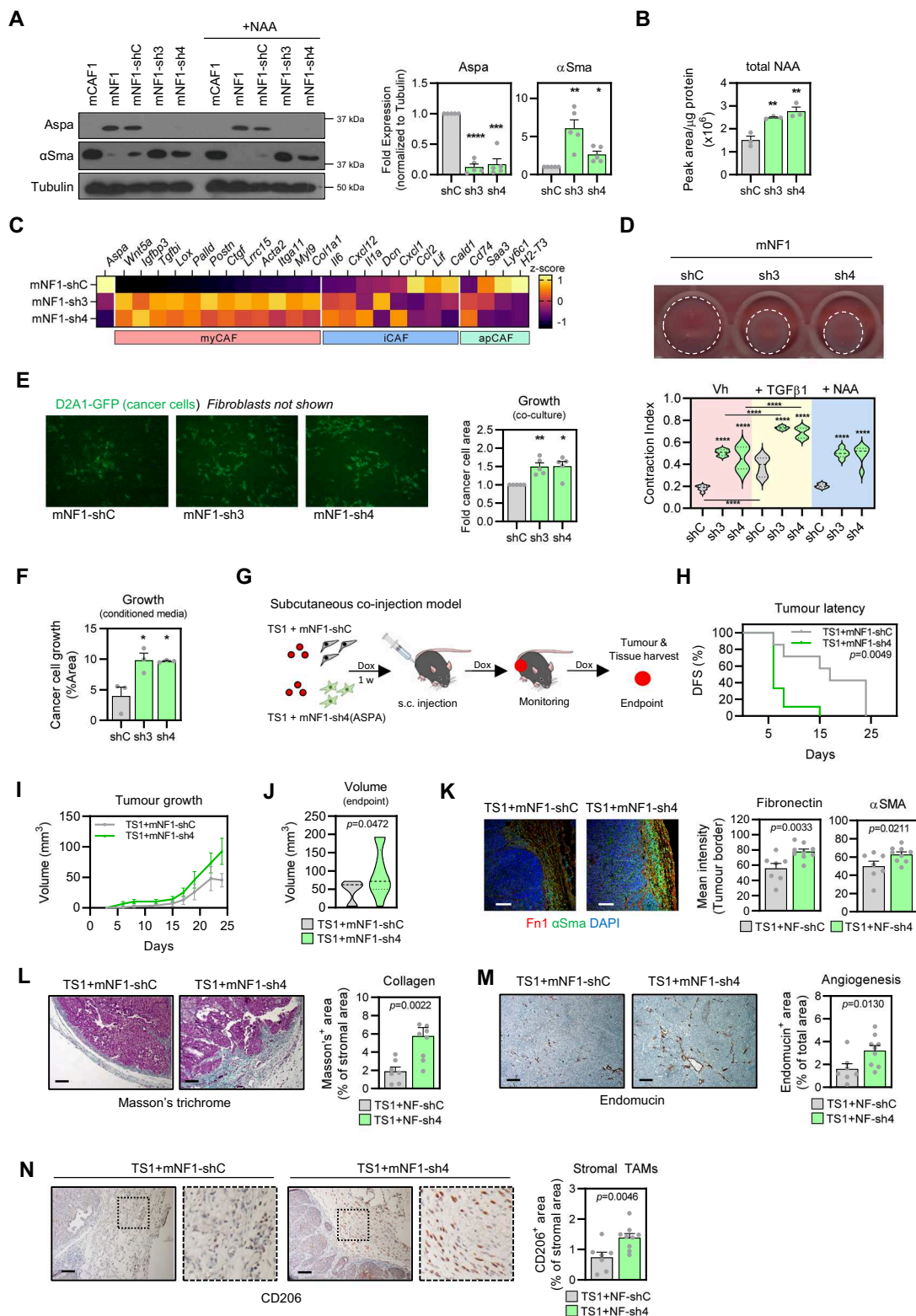
Next, we assessed whether *ASPA* downregulation played any role in early stages of fibroblast activation by knocking down *Aspa* expression in murine normal mammary gland fibroblasts (mNF) with two independent shRNAs. This approach reduced *ASPA* levels to those of CAFs (Fig. 3A) and was associated with a significant increase in NAA levels, indicative of defects in NAA metabolism (Fig. 3B). Interestingly, depletion of *ASPA* in mNF1 was associated with an increase in α SMA expression, and NAA addition in control or *ASPA*-silenced mNF1 had no effect in α SMA levels (Fig. 3A). Moreover, gene expression analyses confirmed that mNF1 present a general upregulation in the expression of myofibroblastic/myCAF genes after *Aspa* knock-down (Fig. 3C). These data suggest that *ASPA* may be suppressing fibroblast activation towards myofibroblast phenotypes, possibly through NAA-independent mechanisms. To test this notion, we investigated the ECM remodelling and promotion of cancer cell growth abilities, which are generally attributed to myofibroblast/myCAF phenotypes^{6,18} (Supplementary Fig. 4C–E). These analyses demonstrated that silencing *Aspa* expression in murine NFs increased these activities (Fig. 3D–F). Again, NAA supplementation had no overall effect on the evaluated parameters (Fig. 3D). Interestingly, we noticed that *Aspa* depletion potentiated the effects of TGF β 1 stimulation in gel contraction assays. Knocking down *Aspa* in mNF1 elicited a tumour-promoting phenotype as informed by in vivo co-injection assays (Fig. 3G), reducing tumour latency and accelerating tumour growth

(Fig. 3H–J). Mammary tumours generated by co-injection of *Aspa*-silenced mNF1 were more desmoplastic when compared to control-mNF1 counterparts, as shown by the significant increase in fibronectin content and collagen deposition (Fig. 3K, L), α SMA expression (Fig. 3K), angiogenesis (Fig. 3M), and the presence of CD206⁺ macrophages (Fig. 3N). Altogether, these results indicate that *ASPA* expression restrains fibroblast activation and the generation of pro-tumorigenic fibroinflammatory phenotypes.

ASPA represses TGF β signalling in stromal fibroblasts

To shed light on the mechanisms whereby *ASPA* affected fibroblast behaviour, we performed transcriptomic analyses of mNF1 after knockingdown *Aspa* with two independent shRNAs. Principal Component analysis (PCA) of RNA-Seq data showed that *Aspa*-depleted NFs presented a distinct transcriptional landscape apart from control NFs (Supplementary Fig. 5A), in line with our functional observations. This was also evidenced by the number of up- or downregulated DEGs after *Aspa* silencing (Supplementary Fig. 5B and Supplementary Data 1). Amongst the genes consistently upregulated after *Aspa* depletion, we observed genes associated with ECM remodelling, TGF β and Wnt targets, as well as genes upregulated in BCa stroma and myCAFs (Fig. 4A). These data prompted us to perform GSEA against public databases (MSigDB ‘Hallmarks’ and ‘CTD’) and gene sets associated with processes related to fibroblast activation/CAFs (‘Curated’). This analysis showed that genes downregulated after *Aspa* knockdown were significantly associated with metabolic processes, including fatty acid metabolism, oxidative phosphorylation and synthesis of amino acids (Supplementary Fig. 5C). Conversely, genes upregulated in *Aspa*-depleted mNF1 were markedly enriched in signalling pathways and biological processes associated with fibroblast activation, which included signatures of collagen production and modification, EMT, myCAF phenotypes and BCa stromal signatures (Supplementary Fig. 5C, D). Of note, we observed a relevant upregulation of TGF β and Wnt signalling signatures, as well as gene sets associated with ECM regulation after *Aspa* depletion in mNF1 (Fig. 4B). Together, these results suggest that *Aspa* represses signalling cascades associated with fibroblast activation, such as TGF β and Wnt/ β -catenin^{19,20} to modulate fibroblast behaviour. Using orthogonal approaches, we confirmed that *Aspa* knockdown in mNF1 increased activated levels of β -catenin and the transcriptional activity of TGF β /SMAD3 in basal conditions (Fig. 4C, D). In line with functional results, *Aspa*-depleted mNF1 also presented increased SMAD2/3 transcriptional activity in response to TGF β stimulation (Fig. 4D). In addition, stratification of fibroblasts from BCa and PCa scRNA-Seq datasets based on *ASPA* expression showed a negative association between *ASPA* levels and activation of TGF β and Wnt signalling (Fig. 4E), or EMT and desmoplastic CAF signatures (Supplementary Fig. 5E). Of note, signatures for NF phenotypes were significantly upregulated in *ASPA*-high CAFs (Supplementary Fig. 5E). There were no relevant associations with other signatures related to CAFs such as NOTCH and NF κ B (Supplementary Fig. 5E). Hierarchical analyses indicated that Wnt/ β -catenin activation was downstream TGF β signalling in this system, as the TGFBR inhibitor SB431542 inhibited both β -catenin and SMAD2/3 activation after *Aspa* silencing (Fig. 4F, G). In agreement, we observed that blocking TGF β signalling reversed the functional activation elicited by *Aspa* knockdown in mNF1 at the level of α SMA expression (Fig. 4F) and ECM remodeling/gel contraction (Fig. 4H). Wnt/ β -catenin inhibition with XAV939 had no functional consequences (Fig. 4H).

These data indicate that *ASPA* may be tuning the responsiveness of stromal fibroblasts to TGF β stimulation. Together with previous data showing how TGF β 1 reduced *ASPA* expression, our results suggest that *ASPA* may be functioning as a critical gatekeeper of TGF β responses in fibroblasts, and that *ASPA* loss is a critical step to elicit full myofibroblast activation. To test this hypothesis, we investigated the effects of constitutive *ASPA* expression in TGF β 1-induced



myofibroblast activation of mNF1 (Fig. 4I). Gene expression analyses revealed that constitutive ectopic expression of ASPA reduced the upregulation of myofibroblast gene expression induced by TGFβ1 (Fig. 4J). GSEA analyses showed that gene sets associated with Wnt and TGFβ activation, EMT and desmoplastic CAF function were also significantly affected by constitutive expression of ASPA in TGFβ1-stimulated mNF1 (Supplementary Fig. 5F, G). In agreement, orthogonal analyses showed that ectopic expression of ASPA negatively impacted

the transcriptional activity of TGFβ/SMAD3 in TGFβ1-stimulated mNF1 (Fig. 4J, K). These effects were accompanied by a substantial reduction in myofibroblastic functions such as gel remodelling activity (Fig. 4L). Moreover, to examine whether these effects relied on the catabolic activity of ASPA, we generated a loss-of-function E178D mutation of ASPA (Glu178Asp, ASPA^{MUT}), which has been associated with Canavan disease and that substantially diminishes its enzymatic activity towards NAA²¹ (Supplementary Fig. 5H). Notably, constitutive ectopic

Fig. 3 | ASPA loss in stromal fibroblasts induces myofibroblast conversion and pro-tumoral features. **A** Western blots showing expression of *Aspa*, α Sma and Tubulin in mCAF1, mNF1, and mNF1 stably transfected with control (shC) and 2 individual Doxycycline-induced *Aspa*-targeting shRNAs (sh3, sh4) after 5 days of induction with Doxycycline. Where indicated, NAA was added for 24 h. Graphs on the right show fold α Sma and *Aspa* levels (normalized to tubulin) in unstimulated mNF1 ($n = 5$). Paired t-test: ****, $p < 0.0001$; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$. **B** Graph shows total NAA levels (intracellular and extracellular) in control mNF1 (mNF1-shC) and *Aspa*-depleted mNF1 (mNF1-sh3 and sh4) after addition of NAA ($n = 3$ biological replicates). Unpaired t-test: **, $p < 0.01$. **C** Heatmap showing average z-score levels ($n = 3$) of expression of indicated genes in unstimulated mNF1-shC, mNF1-sh3 and mNF1-sh4. Genes are grouped based on their association with myCAF, iCAF and apCAF phenotypes. **D** Representative images of gel contraction assays of unstimulated mNF1-shC, mNF1-sh3 and mNF1-sh4. Bottom graph shows contraction index of mNF1-shC, mNF1-sh3 and mNF1-sh4 in unstimulated conditions (Vh) or stimulated with TGF β 1 or NAA ($n = 6$). Ordinary one-way ANOVA: ****, $p < 0.0001$. **E** Images show D2A1-GFP cancer cells (green) after 2 days of co-culture with unlabelled mNF1-shC, mNF1-sh3 and mNF1-sh4 (fibroblasts not shown). The graph shows fold cancer cell area (D2A1) from co-culture experiments ($n = 5$). Paired t-test: *, $p < 0.05$; **, $p < 0.01$. **F** Graph shows cancer cell area (D2A1-GFP) after 48 h of culture in 2% FBS conditioned media from mNF1-shC, mNF1-sh3 and mNF1-sh4 ($n = 3$). Unpaired t-test: *, $p < 0.05$. **G** Experimental design for the in vivo subcutaneous BCa model generated by co-injection of TS1 murine carcinoma cells with

mNF1-shC and mNF1-sh4 (*Aspa* knock-down). **H** Graph shows disease-free survival (%) of mice from experiments in (G) (mNF1-shC, $n = 7$; mNF1-sh4, $n = 9$). *P*-value is shown (Mantel-Cox test). **I** Graph shows tumour volume at different time points of experiment in (G) (mNF1-shC, $n = 7$; mNF1-sh4, $n = 9$). **J** Graph shows tumour volume at endpoint of experiment in (G) (mNF1-shC, $n = 7$; mNF1-sh4, $n = 9$). *P*-value is shown (one-tailed unpaired t-test). **K** Images show Fn1 (red), α Sma (green) and DAPI (blue) of tumour border regions in endpoint tumours from (G). Bars, 100 μ m. Graphs on the right show MFI of Fibronectin/Fn1 (left) and α Sma staining (right) at tumour borders (mNF1-shC, $n = 7$; mNF1-sh4, $n = 9$). *P*-values are shown (one-tailed unpaired t-test). **L** Images of Masson's trichrome staining (collagen) of tumours from (G). Bars, 100 μ m. Graph shows quantification of percentage of Masson's positive area in tumour border (mNF1-shC, $n = 7$; mNF1-sh4, $n = 9$). *P*-value is shown (one-tailed unpaired t-test). **M** Images of Endomucin staining (blood vessels) of tumours from (G). Bars, 100 μ m. Graph shows quantification of percentage of Endomucin positive area in tumours (mNF1-shC, $n = 7$; mNF1-sh4, $n = 9$). *P*-value is shown (one-tailed unpaired t-test). **N** Images of CD206 staining (TAMs) of tumours from (G). Bars, 100 μ m. Squares indicate zoomed-up areas shown in additional panels. Graph shows quantification of percentage of CD206 positive area in stroma (mNF1-shC, $n = 7$; mNF1-sh4, $n = 9$). *P*-values are shown (one-tailed unpaired t-test). In all cases, bar graph data are presented as mean values $\pm$ SEM. Unless stated otherwise, all statistical tests were two-tailed. Source data are provided as a Source Data file, which includes exact *p*-values and additional statistical tests.

expression of ASPA^{MUT} behaved similarly to wild-type ASPA (ASPA^{WT}) in all evaluated features (Fig. 4K, L), underlying a role of ASPA independent of NAA metabolism in restraining fibroblast activation.

Association between stromal ASPA levels and prognosis and fibroinflammation in cancer patients

To investigate the relevance of our findings in human cancer, we first evaluated the prognostic value of *ASPA* bulk mRNA expression in BCa and PCa patients. In both cases, low *ASPA* expression was associated with poorer disease-free survival in independent public datasets and with different stratification strategies (Fig. 5A, B and Supplementary Fig. 6A, B). Importantly, *ASPA* repression was significantly associated with disease progression in various PCa datasets that contained information on metastatic specimens^{22–24}, thus reinforcing its relevance in cancer aggressiveness (Supplementary Fig. 6C). We validated the bioinformatics strategy in an independent cohort composed of 140 cases with benign hyperplasia (BPH) or PCa (Supplementary Fig. 6D). Using nCounter from Nanostring, we corroborated that *ASPA* expression was reduced in PCa specimens, and that its repression was associated with shorter time to biochemical recurrence (Supplementary Fig. 6E, F). At the protein level, we observed that non-cancerous tissue consistently exhibited the highest protein immunoreactivity (Fig. 1B and Supplementary Fig. 6G) and that *ASPA* expression stratified patients with better clinical outcomes after surgery (Fig. 5C). We previously showed that *ASPA* expression is restricted to stromal regions in normal tissues and repressed in cancer stroma (Fig. 1). Accordingly, stromal *ASPA* expression also presented prognostic value in both PCa and BCa specimens at the level of protein or mRNA expression, respectively (Fig. 5D, E). Together, our results reveal that the tumour suppressive activity of *ASPA* is translated into prognostic potential in bulk molecular or histological analyses.

Next, we investigated the relationship between *ASPA* expression and processes associated with its function in CAFs, including TGF β signalling activation, CAF phenotypes and pro-tumoural macrophage content. These analyses showed that *ASPA*-high PCa and BCa tumours displayed significantly lower levels of TGF β activity, expression of desmoplastic myCAF gene sets, and gene signatures associated with tumour aggressiveness (Fig. 5F and Supplementary Fig. 6H). Conversely, *ASPA* loss was associated with a decrease in signatures associated with NF phenotypes (Fig. 5F and Supplementary Fig. 6H) and a concomitant increase in M2 pro-tumoural macrophage content (Fig. 5G and Supplementary Fig. 6I). As we have shown, *ASPA* downregulation

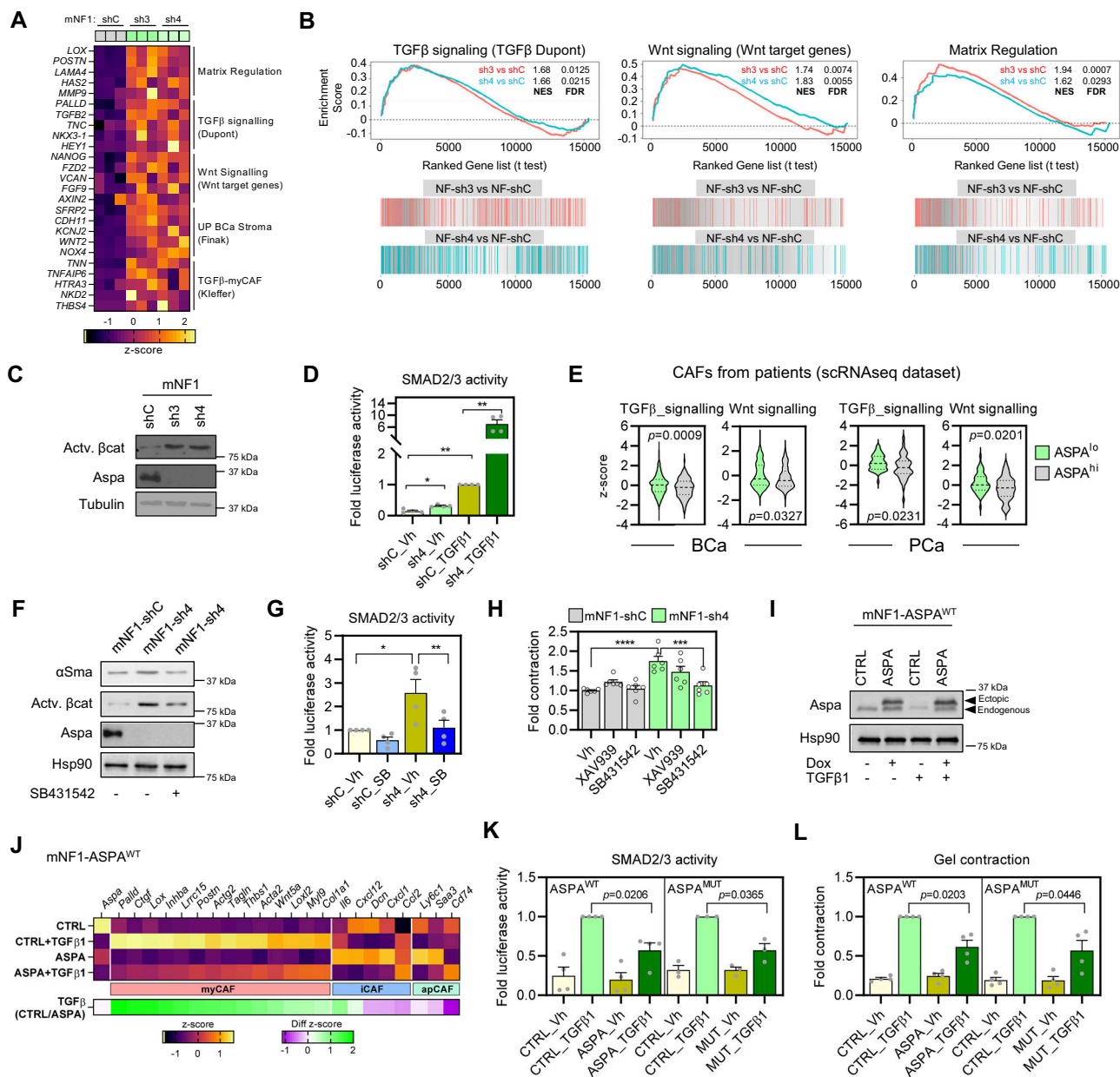
can be a consequence of increased TGF β 1 levels in the TME, which may also impact some of the processes under investigation. This raises the possibility that our observations are not directly linked to *ASPA* activity in CAFs but rather through TGF β -dependent responses. To address this issue, we generated a gene signature containing the top downregulated genes after *ASPA* depletion in murine NFs as a surrogate for *ASPA* activity (Supplementary Fig. 7A), obtaining similar results to *ASPA* expression in PCa and BCa (Supplementary Fig. 7B–D). Finally, we leveraged a BCa dataset containing RNA and metabolite measurements in cancerous and pathologically normal adjacent tissues (Fig. 5H)²⁵. These analyses showed that *ASPA* downregulation in cancer is associated with an accumulation of NAA (Fig. 5I, J), showing a negative correlation between both parameters (Fig. 5K). Importantly, stratification based on NAA levels showed a significant increase in M2 macrophage content in NAA-high cancerous tissues (in line with the role of the metabolite in macrophage polarization¹⁴, Fig. 5L), which were also associated with poorer patient prognosis (Fig. 5M).

Altogether, our observations establish stromal *ASPA* as a multifaceted tumour suppressor gene affecting fibroblast behaviour. Conceptually, these findings inform a framework whereby *ASPA* operates as a critical TME regulator by repressing fibroblast activation downstream of TGF β signals through a mechanism independent from its metabolic activity.

Discussion

Given the crucial role of the TME in tumour progression, understanding the mechanisms governing their tumour-regulatory properties is essential for the refinement of prognostic and therapeutic strategies, as exemplified by immunotherapies and associated biomarkers²⁶. Reciprocal metabolic interactions between tumour and the TME are emerging as key mechanisms influencing tumour characteristics^{4,5}, both fuelling cancer cell fitness as well as altering the behaviour of cancer and stromal cells^{27–30}. In this study, we provide evidence that repression of *ASPA* in stromal fibroblasts unleashes cell-autonomous TGF β -SMAD2/3 signalling leading to pro-tumoural myCAF phenotypes. These findings represent an unappreciated role for *ASPA* in the TME that underlie the complex and intertwined nature of tumour-stroma interactions in human cancer.

Through a comprehensive analysis of altered genes in stroma, we uncovered that *ASPA* is consistently repressed in cancer stroma and CAFs across tumour types. A recent study proposed that *ASPA* repression was a key process in PCa cells promoting proliferation,



migration, and invasion¹². No assessment of ASPA expression in stroma or cellular components of the TME was reported. Despite our efforts, we were unable to corroborate a dominant role for ASPA or NAA in PCa cells in normal or nutrient-restricted conditions (Supplementary Fig. 8), suggesting that its contribution within tumour cells is minor compared to its role in the stroma. To further expand our findings, we have employed data mining analysis (including scRNA-Seq datasets), tissues from human specimens and GEMMs, and tractable cellular systems from different cancer types. Our findings illustrate that, both at mRNA and protein levels, ASPA is primarily expressed by stromal fibroblasts outside the CNS, where it plays a relevant tumour suppressive role.

ASPA is an enzyme responsible for catalysing the conversion of NAA to aspartate and acetate. Mutations in this gene result in the accumulation of NAA, leading to Canavan disease^{9,13}. Increased NAA levels in cancer have been reported as a result of NAT8L overexpression in cancer cells or alterations in NAA-associated enzymes e.g. FOLH1/NAALAD2^{13,14,31–33}. Interestingly, NAA can promote protumoral macrophage polarization¹⁴, and our correlative analyses suggested an association between ASPA expression, NAA levels, pro-

tumoral macrophage content and cancer patient prognosis (Fig. 5H–M). Further analyses confirmed that PCa cells produce and secrete elevated levels of NAA (Supplementary Fig. 9A). Moreover, our analyses showed that NAA supplementation in vivo augmented the number of tumour-infiltrating pro-tumoral macrophages in xenografts and in a genetic mouse model of PCa without overt systemic effects in the immune compartment (Supplementary Fig. 9B–K). However, the extracellular NAA-clearing capacity of ASPA-overexpressing CAFs (which did not show changes in activation status, Supplementary Fig. 4) from DU145 cell conditioned media was limited (Supplementary Fig. 9L–N), thus suggesting that other experimental conditions or additional factors may be considered.

Our study reveals that ASPA depletion in fibroblasts influences their activation status. Silencing ASPA in unstimulated NFs induced myofibroblast conversion, increasing αSMA expression, ECM remodelling, fibroblast-dependent cancer cell proliferation, angiogenesis and tumour associated macrophage modulation. Mechanistically, we provide evidence that ASPA deficiency induces TGFβ-SMAD2/3 signalling in NFs, whereas ectopic expression of ASPA in NFs prevents their transition to a myofibroblast phenotype. These observations were

Fig. 4 | Aspa loss in stromal fibroblasts promotes TGF β -SMAD2/3 signalling.

A Heatmap showing the expression levels of indicated genes (z-score normalized) in mNF1 stably transfected with control (shC) and 2 individual Doxycyclin-induced Aspa-targeting shRNAs (sh3, sh4) after 5 days of culture with Doxycyclin. **B** GSEA plots showing the enrichment of indicated gene signatures in Aspa-depleted mNF1 (sh3/4) when compared to control mNF1 (shC). Normalized Enriched Score (NES) and False Discovery Rate (FDR) coefficients are also shown. **C** Western blots showing non-phospho (active) β -catenin (Ser33/37/Thr41), Aspa and Tubulin levels in mNF1 transducing control (shC) and Aspa targeting shRNAs (sh3/4). Representative blots of 3 independent experiments. **D** Graph shows fold SMAD2/3 reporter activity in control (shC) and Aspa-silenced (sh4) mNF1 stimulated with vehicle (Vh) or TGF β 1 for 24 h ($n = 4$). P -values are shown (ratio paired t-test). **E** Graphs show expression of indicated gene signatures (z-score normalized) in individual fibroblasts from human BCa (left) or human PCa (right) stratified based on the levels of ASPA expression (BCa: ASPA^{lo}, $n = 949$; ASPA^{hi}, $n = 128$. PCa: ASPA^{lo}, $n = 46$; ASPA^{hi}, $n = 46$). P -values are shown (unpaired t-test). **F** Western blots showing α Sma, non-phospho (active) β -catenin (Ser33/37/Thr41), Aspa and Hsp90 levels in control (shC) and Aspa-silenced (sh4) mNF1 cultured with vehicle (-) or TGF β i (SB431542) for 24 h, as indicated. Representative blots of 3 independent experiments. **G** Graph shows fold SMAD2/3 reporter activity in control (shC) and Aspa-silenced (sh4) mNF1 cultured with vehicle (Vh) or TGF β i SB431542 (SB) for 24 h ($n = 4$). Ratio paired t-test: *, $p < 0.05$; **, $p < 0.01$. **H** Graph shows fold gel contraction activities of control (shC) and Aspa-silenced (sh4) mNF1 cultured with

Wnt inhibitor (XAV939) or TGF β i (SB431542) for 24 h ($n = 6$). Ordinary one-way ANOVA: ***, $p < 0.001$; ****, $p < 0.0001$. **I** Western blots showing Aspa and Hsp90 levels in mNF1 transfected with a doxycyclin-inducible vector expressing wild-type (ASPA^{WT}) after induction with vehicle (CTRL) or with Doxycyclin (Dox) for 5 days (ASPA), and TGF β 1 stimulation for 24 h, as indicated. Endogenous and ectopic forms of ASPA are indicated. Representative blots of 3 independent experiments. **J** Heatmap showing average z-scores of mRNA expression of genes associated with myCAF, iCAF and apCAF phenotypes in control mNF1 (CTRL) or mNF1 stably expressing wild-type ASPA (ASPA) and subjected to vehicle or TGF β 1 stimulation for 24 h. Bottom heatmap shows the difference in average z-score values between mNF1-CTRL and mNF1-ASPA stimulated with TGF β 1. **K** Graph shows fold SMAD2/3 reporter activity in control mNF1 (CTRL) or mNF1 stably expressing wild-type ASPA (ASPA) or E178D mutant (MUT), stimulated with vehicle (Vh) or TGF β 1 for 24 h (ASPA, $n = 4$; MUT, $n = 3$). P -values are shown (paired t-test). ASPA^{WT}/ASPA^{MUT} expression was induced by 5-day doxycycline treatment. **L** Graph shows fold gel contraction activities in control mNF1 (CTRL) or mNF1 stably expressing wild-type ASPA (ASPA) or E178D mutant (MUT), stimulated with vehicle (Vh) or TGF β 1 (ASPA, $n = 4$; MUT, $n = 3$). P -values are shown (paired t-test). In all cases, bar graph data are presented as mean values $\pm$ SEM; violin plots display median (straight line), quartiles (dashed lines) and maximum/minimum values (limits). Unless stated otherwise, all statistical tests were two-tailed. Source data are provided as a Source Data file, which includes exact p -values and additional statistical tests.

further supported with scRNA-Seq data from clinical specimens, where ASPA levels were significantly associated with TGF β -SMAD2/3 activation and desmoplastic CAF features. Our data suggests that this phenotype does not require the catalytic activity of the enzyme, since (i) NAA supplementation has no observable effects on these parameters; and (ii) an enzymatic dead mutant of ASPA²¹ had similar effects as the wild-type counterpart. This NAA-independent regulation may rely on protein-protein interactions with specific factors that influence cell signalling cascades³⁴, as previously described for the tyrosine kinase LYN¹². Further research is required to identify the specific effector and mechanism of action. Interestingly, our analyses also show that ASPA downregulation is primarily associated with the emergence of myofibroblast CAF phenotypes (myCAFs) downstream TGF β -SMAD2/3 signalling. The impact of TGF β signalling in the activation of pro-tumour transcriptional programs in fibroblasts is well documented, but little is known about TGF β -mediated gene repression³⁵. Our results indicate that this mechanism depends on class I HDAC activity, which likely removes acetyl groups at ASPA promoters and enhancers, driving ASPA downregulation. Similar findings have been recently reported for TGF β -mediated repression of *PPARGC1A* in lung fibrosis via HDAC³⁶, or the identification of TGF β inhibitory elements (containing HDACs) promoting *Pkcz* loss in stromal fibroblasts in colon cancer models¹⁶. Given that other inflammatory stimuli altering fibroblast behaviour, such as interleukins or TNF α , do not appear to influence ASPA repression, these results suggest an unappreciated role of gene repression programs in modulating phenotypic conversion in fibroblasts that requires further investigation. Together, these observations demonstrate the existence of a reciprocal regulation between TGF β and ASPA critical for the establishment of fibrodesmoplastic responses. This axis may be particularly relevant during early tumour development, when inflammation increases TGF β levels leading to ASPA repression in fibroblasts and, as a consequence, to the potentiation of the cytokine signalling resulting in the acquisition of myofibroblast phenotypes.

The biological and mechanistic analyses of stromal ASPA in cancer provide a feasible explanation for the negative prognostic potential of this enzyme in several tumour types. Tumours from different tissues with low ASPA expression (mRNA or protein) exhibit poorer prognosis in the form of shorter recurrence-free survival. In line with our molecular findings, BCa and PCa patient data analyses corroborated that ASPA expression (or ASPA-dependent transcriptional signatures in fibroblasts) inversely correlated with TGF β -SMAD2/3 activity, NAA

levels and pro-tumour macrophage content. Whether stromal ASPA influences the efficacy of current lines of treatment and has any predictive value remains to be explored. It is plausible that ASPA repression in fibroblasts can lead to myCAF phenotypes⁷ that elicit a tumour-promoting immune reprogramming^{14,37}, which could influence the efficacy of immunotherapies.

In summary, our observations establish ASPA as a stromal tumour suppressive gene regulating fibroblast activation within the TME. In turn, developing selective strategies to regulate the activity and function of ASPA could support the establishment of innovative therapeutic strategies.

Methods

Patient samples

All prostate specimens from Basurto cohort were obtained upon informed consent and with evaluation and approval from the corresponding ethics committee (Comité de Ética en Investigación Clínica de Euskadi, codes OE Hun11-12, OE Hun14-14 and OE Hun19-20). Clinic-pathological information is included in Supplementary Data 2, 3. Prostate tissue sample from Champalimaud cohort was approved by the Champalimaud Foundation's Ethics Committee. These specimens were retrospectively identified from the archives of the Service of Anatomic Pathology of the Champalimaud Clinical Centre and were classified by a pathologist. Clinic-pathological data are resumed in Supplementary Data 4. All human samples and the corresponding clinical data were received in a de-identified manner through the Basque and Champalimaud Foundation Biobanks. Analyses of human breast tissues were performed on material obtained from US Biomax, Inc. (BR1503H). Normal and cancerous pancreatic tissue was obtained from patients receiving partial pancreatoduodenectomy at the Department of General, Visceral and Transplantation Surgery, University of Heidelberg (Germany). Patients were part of the HIPO-project. The study was approved by the ethical committee of the University of Heidelberg (case number S-206/2011 and EPZ-Biobank Ethic Vote #301/2001); written informed consent was obtained from all patients.

Mouse strains

All the experimental procedures were carried out according to the ethical guidelines defined by Biosafety and Animal Welfare Committees at CIC bioGUNE (codes P-CBBA-0715 and 0121) and Universidad de Cantabria (codes PI-05-19 and PI-02-23), in concordance with the

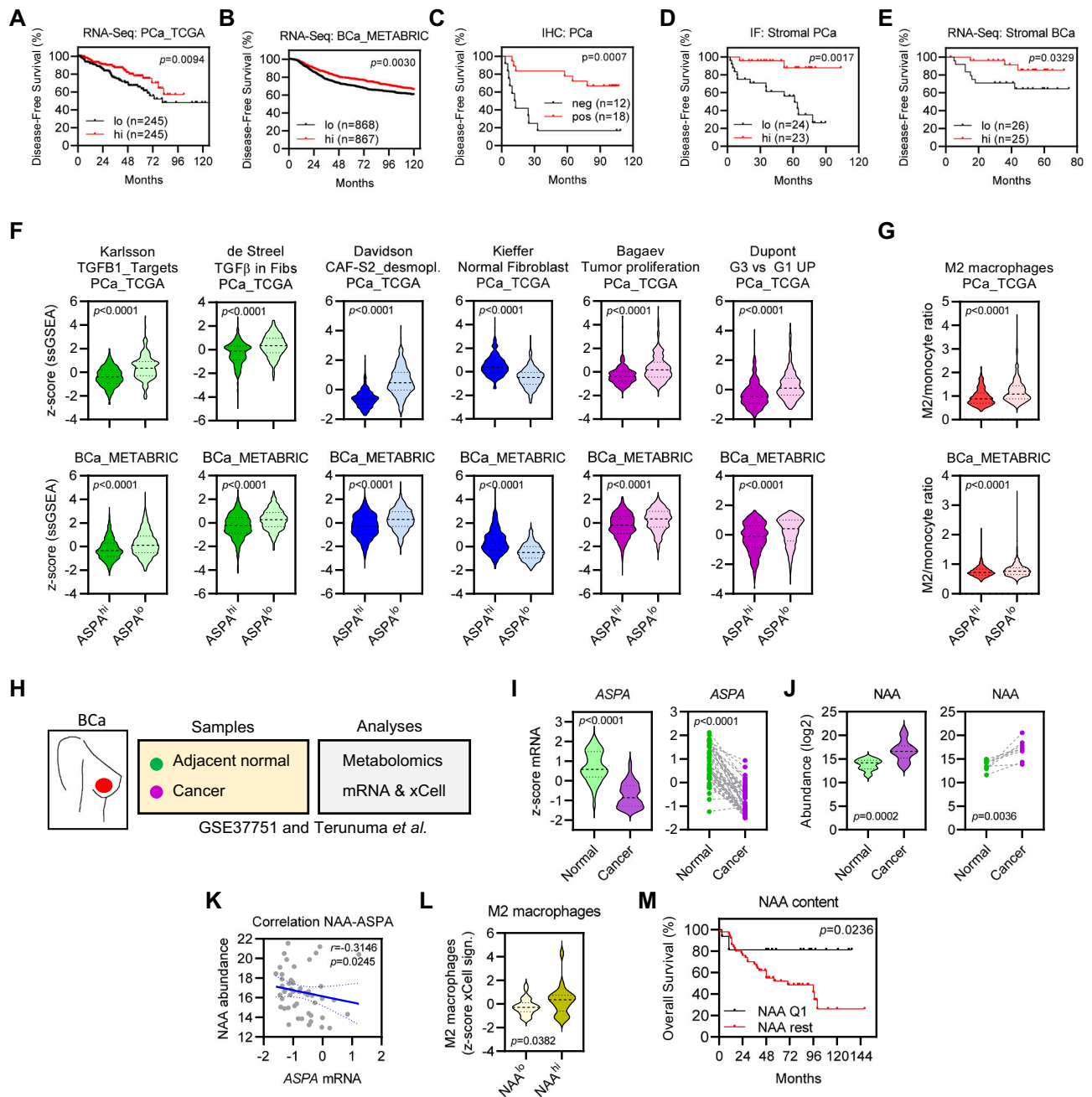


Fig. 5 | Association between stromal ASPA levels and prognosis and fibroinflammation in cancer patients. **A, B** Kaplan-Meier curves showing the percentage of disease-free survival over time of PCa patients from TCGA (**A**) and BCa patients from METABRIC (**B**) stratified by median ASPA expression. *P*-values are shown (Mantel-Cox test). **C** Kaplan-Meier curve showing the percentage of disease-free survival of PCa patients from the Basurto cohort stratified by ASPA immunoreactivity (positive/negative). *P*-value is shown (Mantel-Cox test). **D, E** Kaplan-Meier curves showing the percentage of disease-free survival of PCa patients from Champalimaud cohort stratified by stromal ASPA immunoreactivity (**D**) and of BCa patients from GSE9014 stratified by stromal ASPA mRNA levels (**E**). *P*-values are shown (D, Mantel-Cox test; E, Gehan-Breslow-Wilcoxon test). **F** Graphs show expression levels of indicated gene signatures (ssGSEA z-score normalized) in samples from PCa-TCGA (*top*) and BCa-METABRIC (*bottom*) patients stratified by median ASPA mRNA levels (PCa: ASPA^{hi}, *n* = 245; ASPA^{lo}, *n* = 245. BCa: ASPA^{hi}, *n* = 867; ASPA^{lo}, *n* = 868). *P*-values are shown (Mann-Whitney test). **G** Graphs show M2-macrophage to monocyte ratios (extracted by xCell algorithm) in samples from PCa-TCGA (*top*) and BCa-METABRIC (*bottom*) patients stratified by median ASPA mRNA levels (PCa: ASPA^{hi}, *n* = 245; ASPA^{lo}, *n* = 245. BCa: ASPA^{hi}, *n* = 867; ASPA^{lo}, *n* = 868). *P*-values are shown (Mann-Whitney test). **H** Scheme representing the

comprehensive analysis of metabolomics and gene expression in normal and BCa samples from GSE37751 and Terunuma et al. **I** Graph shows ASPA mRNA levels (z-score normalized) in normal and cancer samples from (**H**). Analyses are represented unpaired (*left*) or paired (*right*) (*n* = 47). *P*-values are shown (left, unpaired t-test; right, paired t-test). **J** Graph shows NAA levels in normal and cancer samples from (**H**). Analyses are represented unpaired (*left*) or paired (*right*) (Left: normal, *n* = 9; cancer, *n* = 53. Right: *n* = 8). *P*-values are shown (left, unpaired t-test; right, paired t-test). **K** Graph shows correlation between ASPA mRNA and NAA levels in patients from (**H**) with matched metabolomics and gene expression data. Each dot represents an individual sample (*n* = 51). Spearman correlation coefficient and *p*-value are shown. **L** Graph shows M2-macrophage content (z-score normalized from xCell algorithm) in cancer samples from (**H**) stratified by average NAA levels (NAA^{lo}, *n* = 27; NAA^{hi}, *n* = 21). *P*-value is shown (one-tailed unpaired t-test). **M** Kaplan-Meier curve showing the overall survival of BCa patients from (**H**) stratified by quartile NAA levels (Q1 vs rest). *P*-value is shown (Mantel-Cox test). In all cases, violin plots display median (straight line), quartiles (dashed lines) and maximum/minimum values (limits). Unless stated otherwise, all statistical tests were two-tailed. Source data are provided as a Source Data file, which includes exact *p*-values and additional statistical tests.

recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care. Mice were maintained in a controlled environmental condition, with time-controlled lighting on standard 12:12 light: dark cycles, 30–50% of humidity and controlled temperature at $22 \pm 2^\circ\text{C}$. Diet (Teklad Global Diets, 2914) and water were provided ad libitum. For analyses with PCa models, mice were fasted for 6 h prior to tissue harvest to prevent metabolic alterations due to immediate food intake. At the experimental endpoint, all mice were sacrificed by CO_2 inhalation followed by cervical dislocation. Transgenic FVB/n mice expressing the Polyoma Middle T antigen oncogene under the Mouse Mammary Tumour Virus promoter (MMTV-PyMT)³⁸ were used for cell isolation and FACS analysis. Wild-type FVB/n 6–8 week-old females (Charles River) were used as recipients for tumours (TS1 mammary carcinoma model from MMTV-PyMT). Athymic Nude-Foxn1nu mice 6-week-old males (Hsd: Athymic Nude-Foxn1nu from Envigo) were used as recipients of PCa xenographs (subcutaneous injection of prostate model PC3). Eight-week-old male mice of the conditional tissue-specific *Pten* knockout (C57/BL6/129sv; Pb-Cre4; *Pten* lox/lox) were used for NAA supplementation studies. Paraffined rat brain tissues were kindly provided by Fuescila Pilar-Cuellar (IBB-TEC, Spain).

Cell lines

Established murine fibroblasts from FVB/n normal mammary glands (NFs) and MMTV-PyMT mammary carcinoma (CAFs) have been previously described^{18,39}. Patient-derived NF and CAF from BCa and CRC were provided by Clare Isacke (Institute of Cancer Research, UK) and Danijela Vignjevic (Institut Curie, France), respectively. Fibroblasts from human prostate tissue (WPMY-1, ATCC® CRL-2854TM) and from PCa (PF179T, ATCC® CRL-3290TM), human prostate carcinoma 22RV1 and human benign prostate RWPE-1 were purchased from American Type Culture Collection, which provided an authentication certificate. Primary cell lines of patient-derived prostate CAFs (CAF 85-AD and 81-BI) and fibroblasts from adjacent pathologically normal tissue (NF 85-BI and 81-AI) from two independent patients (patient #1 and #2) were kindly provided by Noemi Eiro and Francisco J. Vizoso (Fundación Hospital de Jove, Spain). Human primary fibroblasts were expanded and immortalized using hTERT virus (pCSII vector backbone), followed by selection with hygromycin. Human prostate cell lines BPH-1, LnCaP, DU145 and PC3 were obtained from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH with the corresponding certificate of authenticity. 293FT cells were generously provided by the laboratory of Rosa Barrio (CIC bioGUNE, Spain). MMTV-PyMT TS1 and D2A1 murine BC cells were a kind gift from Erik Sahai (Francis Crick Institute, UK). RAW 264.7 murine macrophages were provided by Juan Rodríguez-Vita (Príncipe Felipe Research Center Foundation, Spain).

Fibroblasts, DU145, PC3, 293FT, TS1, D2A1 and RAW 264.7 were cultured in DMEM high glucose (Sigma) supplemented with GlutaMax (Gibco). LnCaP and 22RV1 were maintained in RPMI 1640 supplemented with GlutaMax. The RWPE-1 cell line was cultured in a specific Keratinocyte-Serum Free Medium (Gibco) supplemented with recombinant Epidermal Growth Factor and Bovine Pituitary Extract (Gibco). All culture media, except for RWPE-1 culture medium, were supplemented with 10% FBS and 1% penicillin-streptomycin. All cell lines were grown in a standard humidified 5% CO_2 incubator at 37°C and tested negative for mycoplasma infection.

Reagents

D2A1 murine BCa cells were fluorescently labelled using the lentiviral vector (pCSII-IRES2-blasti-eGFP). For ASPA ectopic expression, DU145 cells were transduced with a modified TRIPZ (Dharmacon) doxycycline inducible lentiviral construct in which the RFP and miR30 region was substituted by HA-tagged ASPA (HA-ASPA). For ectopic ASPA expression in mNF1 and mCAF1 cell lines, the same plasmid was employed,

replacing the puromycin resistance cassette with a hygromycin cassette. Loss-of-function E178D mutant of ASPA (Glu178Asp, ASPA^{MUT}) was generated by site-directed mutagenesis kit (Thermo, F541) on the aforementioned plasmid using primers described in Supplementary Data 7. To silence *Aspa* in mNF1, a pLKO-TET-on doxycycline-inducible plasmid was used. Scramble shRNA and two individual shRNA targeting *Aspa* (Sh3-ASPA and Sh4-ASPA) were transfected into mNF1, and selection was performed using Blasticidin at $10\text{ }\mu\text{g/mL}$. shRNA sequences can be found in Supplementary Data 5. For luciferase-based SMAD2/3 reporter assays, CAGA-luc (pGL3 basic backbone) and basic pEGFP plasmids were employed.

Collagenase type I (C0130), doxycycline (D9891), N-acetylaspartate (NAA) (00920), SB431542 (S4317), XAV939 (X3004), trichostatin A (TCA, T8552), AGK2 (A8231), tubastatin A (Sml0044), puromycin (P8833) and LPS (L2654) were purchased from Sigma; hygromycin (10687010) from Life technologies; Human TGF β 1 from Peprotech (AF-100-21C) or Miltenyi Biotec (130-095-067); IL1 β (PHC0814) and TNF α (PHC3015) from Fisher; SIS3 (566405) and pimelic diphenylamide 106 (PD106, SML0313) from Merck; IFN γ (12343534) and IL13 (12340131) from Immunotools; and IL4 (574304) from Biolegend; MC-1568 (4077) from TOCRIS. All were reconstituted in the solvent recommended by the datasheet provided by the manufacturer, unless specified. Conditioned media were generated by culturing a confluent monolayer of cells in DMEM 10% FBS for 48 h. The media was then recovered and filtered through a $0.22\text{ }\mu\text{m}$ filter and used as indicated. Where indicated, FBS content was modified.

Transfections and cell perturbations

Indicated cell lines were stably transfected using 3rd generation lentivirus generated in 293FT cells. Selection was performed using the appropriate antibiotic for 5 days. Doxycycline induction was performed in all cases for at least 5 days with 300 mg/mL (unless stated otherwise). For transient transfection (i.e. luciferase reporter), Lipofectamine-3000 was employed following manufacturer instructions. RAW 264.7 cells were polarised for 48 h in 100 ng/mL LPS and 20 ng/mL IFN γ (M1-like), 20 ng/mL IL4 and IL13 (M2-like), or in vehicle (M0). Unless stated otherwise, supplements and inhibitors were employed at this final concentration: NAA, $100\text{ }\mu\text{M}$; TGF β 1, 5 ng/mL ; IL1 β , 3 ng/mL ; TNF α , IFN γ , 10 ng/mL ; SB431542, $5\text{ }\mu\text{M}$; SIS3, $6\text{ }\mu\text{M}$; XAV939, $1\text{ }\mu\text{M}$; PD106, $25\text{ }\mu\text{M}$; TSA, 100 nM ; Tubastatin, 10 nM ; Romidepsin, 5 nM ; MC-1568, 250 nM ; AGK2, $4\text{ }\mu\text{M}$.

Animal studies

Xenograft experiments with PCa models were performed upon subcutaneous injection of 1×10^6 PC3 cells in both flanks of Athymic Nude-Foxn1^{nu} mice (male, 6-week-old). Once tumours were established (100 mm^3), animals were treated daily with a dosage of 25 mg/kg of NAA peritumorally or the equivalent volume of physiological saline as control. Mice reaching a maximum tumour volume of 1500 mm^3 were euthanized. The conditional tissue-specific *Pten* knock-out (males) was used for NAA supplementation studies. NAA was resuspended in physiological saline and injected intraperitoneally at a dose of 25 mg/kg on a daily basis. Treatment started when animals were 8 weeks old and finished when mice were 14 weeks old.

TS1 murine BCa cells were used to generate tumours in wild-type FVB/n. Briefly, 5×10^5 TS1 cells and 1.5×10^6 mNF1 (shC or sh4) were suspended in $100\text{ }\mu\text{L}$ of PBS: Matrigel (1:1) and injected subcutaneously into 6–8 week old females. Doxycycline was administered in water (1 mg/mL), changed every two days to avoid degradation. Tumour size was measured every other day using callipers. All mice were culled before any tumour reached the maximum allowed size ($1,500\text{ mm}^3$). To calculate tumour latency, mice with tumours reaching a minimum size of 0.5 mm^3 were considered as developing an observable tumour, and the day post-injection was recorded and used for the disease-free survival calculation.

Cell proliferation assays

For analyses of cancer cell proliferation in mono-culture, crystal violet staining⁴⁰ was performed. Alternatively, soft-agar colony formation was performed as previously described⁴⁰. For mNF1/mCAF1 co-culture analyses of cancer cell proliferation, 2×10^3 mNF1/mCAF1 and 1×10^3 GFP-labelled cancer cells were seeded in a 24-well plate with complete DMEM. When cells were attached, if needed, the growth conditions were changed as indicated. Cancer cell proliferation was monitored during 7 days by acquiring the GFP signal in different areas of the well with a $4\times$ objective with the fluorescent microscope Nikon Eclipse TS100 every 24 h. To calculate the cancer cell growth index, the area covered by cancer cells in each field of view was calculated.

Invasion assays

Relative invasive growth⁴¹ was assessed by plating 700 cells upside down in suspension with 20% methylcellulose DMEM drops. After 3 days, when the spheres were formed, they were embedded in a collagen-based medium (55% collagen I, 20% DMEM 5X, 21.3% H_2O_2 and 3% 0.1 N NaOH), and images of cell spheroids were taken after collagen polymerization (0 h time point). After 48 h, images were taken and the relative invasive growth was calculated with the relative area increase.

ECM-remodelling assay

To assess force-mediated matrix remodelling, 1×10^5 mNF1/mCAF1 were embedded in 100 μ L of a mixture of collagen I (BD Biosciences) and ECM gel mixture (Sigma) yielding a final collagen concentration of 4.6 mg/mL and a final ECM gel mix concentration of 2.2 mg/mL. The NF/CAF-containing gel was seeded in a 48-well plate and incubated at 37 °C. Once the gel was set, cells were maintained in complete medium and supplemented as indicated. Gel contraction was monitored daily by scanning the plates. Unless stated otherwise, the gel contraction value refers to the contraction observed after 2 days. To obtain the gel contraction value, the relative diameter of the well and the gel were measured using ImageJ software, and the percentage of contraction was calculated using the formula [(well area–gel area) / well area].

Immunofluorescence (IF)

Cells. For IF experiments, cells were seeded in sterilized cover glass slides and 24 h later they were treated as indicated, and fixed in 4% paraformaldehyde (PFA) for 10 min. Cells were permeabilised with 0.2% Triton X-100 (Sigma) for 7 min, followed by a blocking step of 3% BSA 0.1% Tween for 1 h. Cells were then incubated with the corresponding primary antibody overnight at 4 °C in a humidified chamber. The following day, the primary antibody was washed with PBS (3 $\times$ 5 min washes) and cells were incubated with the fluorescent-conjugated secondary antibodies and DAPI for 1 h at RT. After washing the secondary antibody with PBS (3 $\times$ 5 min washes), glass covers were mounted in slides and imaged using a confocal microscope. IF images were obtained using a TCS SP5 Confocal Microscope (Leica).

Tissues. For histological analyses by IF, tissues were dissected into 10% neutral buffered formalin, embedded in paraffin blocks and serial sections were taken. Paraffin-embedded tissue sections were deparaffined by inclusion into xylene, rehydrated and subjected to antigen retrieval using pH 6 sodium citrate buffer. Sections were then incubated with 0.2% Triton X-100, 10% Goat serum (Sigma) blocking solution for 1 h at RT. Sections were incubated overnight at 4 °C with primary antibodies diluted in PBS. After washing, sections were incubated with fluorescent-conjugated secondary antibodies diluted in PBS for 1 h at RT. Sections were subjected to a treatment to reduce autofluorescence using $CuSO_4$ and NH_4Cl for 10 min and mounted on slides. Confocal images were captured using a Leica SP6 microscope. IF images were analysed using ImageJ, and data analysed in Prism software (GraphPad Software, Inc.). For each staining, the positive area was calculated and normalized to the total area for all slices processed. To analyse the stroma, fluorescence images were acquired using a CRI

Nuance Multispectral camera mounted on a Nikon 90i automated fluorescence microscope (at magnification $200\times$) and controlled by MetaMorph software. Each specimen was analysed in a similar manner, irrespective of tissue type. Briefly, five representative areas were randomly selected from stroma intermingled with tumour cells (Str Tum) and from the stroma located outside the tumour lesion (Str adj). For the metastatic samples, only five areas in the tumoral area were acquired (Str Met) since these samples consist essentially of tumour tissue. Image analysis was conducted using Fiji software. During the analysis, 8-bit images were thresholded at the same intensity value for a specific staining and converted to binary format. Each area analysed corresponded to 0.347 mm² of tissue. Analyses of macrophage infiltration were performed as previously described¹⁴, using a similar approach on serial sections of 5 μ m. Sections were mounted with ProLong Gold Antifade Mountant without DAPI (Invitrogen, P36930) and analyses performed with an Olympus BX41 microscope and Cell-Sense imaging software. In addition, representative images of ASPA staining from normal and cancerous breast, colon and prostate tissues were retrieved from the Protein Atlas (<https://www.proteinatlas.org/>). Antibodies are described in Supplementary Data 6.

Immunohistochemistry (IHC)

Analysis of FFPE human prostates. Tissues were deparaffined using standard protocols and unmasking/antigen retrieval was performed using 0.0005% Tween Tris-EDTA pH 9 solution. ASPA primary antibody was incubated overnight at RT. Hematoxylin/eosin was applied as counterstaining. Secondary antibody, 3,3'-diaminobenzidine (DAB) staining and imaging method were routinely used by the Department of Pathology of Cruces University Hospital. A qualitative scoring system was based on negative (0), low (1) or high (2) immunoreactivity.

Analysis of FFPE human and murine breast. For IHC analysis of murine and human breast tissue (normal and cancer) and rat brains, tissues were dissected into 10% neutral buffered formalin, embedded in paraffin blocks and serial sections were taken. Paraffin-embedded tissue sections were deparaffined by inclusion into xylene and rehydrated. Samples were then processed for H&E and Masson's Trichrome staining following standard procedures. Alternatively, paraffin-embedded tissue sections were rehydrated before blocking endogenous peroxidase (2% H_2O_2 in methanol for 10 min) and subjected to antigen retrieval using pH 6 sodium citrate buffer. Sections were incubated for 30 min at RT with blocking solution and 1 h at RT with primary antibodies. Sections were incubated with secondary antibodies for 50 min at RT, treated with DAB, counterstained with haematoxylin and mounted. Glass slides were scanned using Axio Scan.Z1 Slide Scanner (ZEISS) and analysed using QuPath, ImageJ and Prism. For each staining, the positive area was calculated and normalized to the total area (or indicated regions of interest) for all images processed, using ImageJ. For DAB images, Color Deconvolution (H DAB) in ImageJ was employed, DAB channel isolated and converted to 8 bit, images were thresholded at the same value, converted to binary format and % of positive area calculated. For Masson's trichrome staining images, pictures were processed in QuPath using the 'Normalize OD colors' command. Resulting images were quantified in ImageJ using the red channel to quantify collagen deposition, following a similar protocol employed for DAB analysis. Unless described otherwise, values represent the average of at least 4 representative images from independent tumours. Antibodies are described in Supplementary Data 6.

Flow cytometry analysis and sorting

Immune cell analysis by flow cytometry was performed as indicated in other studies⁴². Fresh prostate tissue was mechanically disrupted with a scalpel and further digested with 3 mL of DMEM with collagenase type I (Sigma, C0130) for 2 h at 37 °C. Cell suspension was then filtered through a 70 μ m cell strainer and stained with the LIVE/DEAD fixable

green dead cell stain kit (Thermo Fisher Scientific L34970) (1:1,000 in PBS) for 30 min at 4 °C protected from light. Cells were washed in PBS and incubated with the fluorochrome-labelled antibodies described in Supplementary Data 6. Flow cytometry was performed with BD FACSymphony and data were analysed with FlowJo software. An example of the FACS gating strategy can be found in Supplementary Fig. 10. Tissue digestion and cell isolation of MMTV-PyMT tumours were performed as described²⁰. For gene expression analysis, cell populations were subjected to RT-qPCR. RNA expression data of human normal/PDAC epithelial, immune, fibroblasts and endothelial cells were obtained from fresh primary PDAC specimens and adjacent normal pancreas by cell sorting after cell death exclusion using propidium iodide: epithelia, immunostained for anti-human CD326-FITC; immune, anti-human CD45-VioBlue; endothelia, anti-human CD31-APC; fibroblasts, negative. RNA extraction, library preparation and RNA sequencing were performed as previously described⁴³. Antibodies are described in Supplementary Data 6.

Nanostring® analysis from FFPE prostates

RNA extraction of FFPE human prostates was performed by Basque Biobank for research (BIOEF, Basurto University Hospital, Spain) with miRNeasy FFPE kit (Qiagen, 217504). The extraction was performed following the instructions provided in the kit. The analysis of transcripts was performed by NanoString Enterprise (NanoString Technologies, Inc., WA, USA). The extracted RNAs were run on the NanoString nCounter Analysis System according to the manufacturer's instructions in Vall d'Hebron Instituto de Oncología (VHIO, Spain). *ASPA* targeting sequence is described in Supplementary Data 7. To normalize the data, the nSolver software from Nanostring was used.

Western blotting (WB)

To study protein expression by WB, cells after the indicated perturbation were washed with PBS, and protein lysates were obtained, mixed with Laemmli Sample Buffer and boiled. Lysates were run in NUPAGE gradient precast gels (Life Technologies) in MOPS buffer. Alternatively, lysates were resolved in sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) in running buffer. Resolved proteins were transferred to nitrocellulose membranes at 400 mA for 1 h in cold transfer buffer. Subsequently, the membrane was blocked with 5% of milk diluted in T-TBS for 1 h, shaking at RT. Finally, the membranes were incubated overnight with primary antibodies in a 5% milk T-TBS solution. Next day, membranes were washed with T-TBS (3 × 10 min) and incubated with peroxidase-conjugated secondary antibodies prepared in a 5% milk T-TBS solution for 1 h shaking at RT. After washing (3 × 10 min), Clarity Western ECL substrate kit was used at 1:1 ratio for protein detection using film or an Amersham ImageQuant 800 (Cytiva) imaging system. Antibodies used in this work and working dilutions are described in Supplementary Data 6. Uncropped WB scans are provided in the Data Source File or at the end of the Supplementary Information File.

Metabolomic analyses

NAA measurement in patient-derived fibroblasts (cNF, cCAF, pNF and pCAF) and *mNF1*. Intracellular metabolites were extracted with an aqueous solution of 50% LC-MS grade methanol (Fisher Scientific, 10284580) and 30% acetonitrile (Fisher Scientific, 10001334) in ultrapure water. Media samples were diluted 1:50 in an aqueous solution of 50% methanol and 30% acetonitrile and incubated with shaking at 4 °C. Samples were analysed using a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific) in combination with a Thermo Ultimate 3000 HPLC system. 5 µL of cell extract was injected and the metabolites were separated over a 15 min mobile phase gradient from an initial content of 80% acetonitrile with 20% ammonium bicarbonate (pH 9.2), decreasing to 20% acetonitrile with a flow rate of 200 µL min⁻¹. Metabolites were detected over a period of 25 min using the Q-Exactive

mass spectrometer across a mass range of 75–1000 *m/z* and at a resolution of 35,000 (at 200 *m/z*). Samples were randomized for data acquisition. Peak identification and area quantification of NAA were carried out using TraceFinder software (v4.1) by comparison of the retention time and exact ion mass to that of an authenticated standard. Peak area was normalized to the amount of protein (in µg) obtained from the cells used for each sample. To assess NAA consumption, *mNF1* were cultured with exogenous NAA (100 µM) for 24 h and intracellular and extracellular NAA content was analysed using the same approach.

Intracellular and extracellular NAA in prostate cell lines. Intracellular metabolite extraction was carried out using a pre-chilled extraction solution containing 50% LC-MS grade methanol, 30% LC-MS grade acetonitrile, 20% of ultrapure water, supplemented with 5 µM valine-d8 (CK isotopes, DLM-488) as internal standard. All extraction steps were performed in a dry ice-methanol bath to ensure metabolite stability. Extracellular metabolites were extracted by mixing cell culture media samples with the extraction solution at a 1:3 (v/v) ratio. The mixtures underwent homogenization for 15 min in a Thermomixer at 4 °C at maximum speed. Chromatographic separation was achieved using a Millipore SeQuant ZIC-pHILIC analytical column (5 µm, 2.1 × 150 mm) coupled to a guard column (5 µm, 2.1 × 20 mm) with a binary solvent system. Solvent A consisted of 20 mM ammonium carbonate with 0.05% ammonium hydroxide, and Solvent B was pure acetonitrile. The chromatographic gradient was run at a flow rate of 0.200 mL/min as follows: 0–2 min: 80% B; 2–17 min: linear gradient from 80% B to 20% B; 17–17.1 min: linear gradient from 20% B to 80% B; 17.1–22.5 min: hold at 80% B. Sample injections (5 µL each) were randomized to minimize variability and analysed with LC-MS in a blinded manner. Pooled samples were generated from an equal mixture of all individual samples and analysed interspersed at regular intervals within the sample sequence as a quality control. Metabolites were measured with a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap Mass spectrometer (HRMS) coupled to a Dionex Ultimate 3000 UHPLC. The mass spectrometer was operated in full-scan, polarity-switching mode, with the spray voltage set to +4.5 kV/–3.5 kV, the heated capillary held at 320 °C, and the auxiliary gas heater held at 280 °C. The sheath gas flow was set to 55 units, the auxiliary gas flow was set to 15 units, and the sweep gas flow was set to 0 units. HRMS data acquisition was performed in a range of *m/z* = 70–900, with the resolution set at 70,000, the AGC target at 1×10^6 , and the maximum injection time (Max IT) at 120 ms. Metabolite identities were confirmed using two parameters: (1) precursor ion *m/z* was matched within 5 ppm of the theoretical mass predicted by the chemical formula; (2) the retention time of metabolites was within 5% of the retention time of a purified standard run with the same chromatographic method. Peak integration and chromatogram review were performed using TraceFinder software (v5.0, Thermo Fisher Scientific). The half minimum method was used to fill in missing values. Peak areas were normalized to the total ion count (TIC) of each sample, and these adjusted values (adj value) served as input for subsequent statistical analyses.

DUI45 and mCAF1 intracellular and extracellular NAA measurements. Metabolite extraction from cells was performed by adding 500 µL an ice-cold solvent extraction liquid (methanol/water 50/50 % v/v) to each well of 6-well culture plates. Culture plates with extraction liquid were left on dry ice for 15 minutes. Next, 400 µL the obtained solutions were transferred to new Eppendorf tubes and centrifuged for 15 min at 4 °C and 20,000 × *g*. Supernatants (300 µL) were evaporated with SpeedVac for 2 h, and pellets were resuspended in 150 µL water/acetonitrile/formic acid (39.9/60/0.1 % v/v/v). Metabolite was measured with an ultra-performance liquid chromatography system (Acquity, Waters Inc., Manchester, UK) coupled to a Time-of-Flight mass spectrometer (SYNAPT G2, Waters Inc.). A BEH-AMIDE column (2.1 × 100 mm, 1.7 µm, Waters Inc. Manchester, UK) thermostated at 40 °C, was used to separate the analytes before entering the MS.

Mobile phase solvent A (aqueous phase) consisted of 99.5% water, 0.5% formic acid (FA) and 20 mM ammonium formate, while solvent B (organic phase) consisted of 29.5% water, 70% ACN, 0.5% FA and 1 mM ammonium formate. Separation of the analytes was performed using the following gradient: from 5% A to 50% A in 2.4 min in curved gradient (#8, as defined by Waters); from 50% A to 99.9% A in 0.2 min, constant at 99.9% A for 1.2 min, back to 5% A in 0.2 min. The flow rate was 0.250 mL/min and the injection volume was 2 μ L. All samples were injected randomly. After every 5 injections, a QC sample was injected. The MS was operated in positive electrospray ionization mode in full scan (50 Da to 1200 Da). Capillary voltage was 500 V, sampling cone voltage was 25, source offset was 80 V, desolvation gas flow was 800 L/h, cone gas flow was 5 L/h and nebulizer gas flow was 6 L/h. Source temperature was 120 °C and desolvation temperature was 450 °C. A 2 ng/mL leucine-enkephalin solution in water/acetonitrile/formic acid (49.9/50/0.1 v/v/v%) was infused at 10 μ L/min and used for a lock mass, which was measured every 32 s for 0.5 s. Spectral peaks were automatically corrected for deviations in the lock mass. Extracted ion chromatograms for the analytes of interest were extracted in their expected mass channels. Peak areas were integrated with TargetLynx software (Waters Inc.) to obtain raw signals. To correct for differences in biological material, signal drift and variation between extractions and injection volumes, median-fold change normalization (also called quotient normalization)^{44,45} was performed as follows. Features, defined as retention time mass-to-charge pairs, were extracted from full scan data using automatic peak detection in MarkerLynx software (Waters Inc.). Each injected sample yielded about 2000 features. The sample with the most detected features was chosen as the reference. For each feature, the signal in a sample was divided by the signal in the reference sample, resulting in about 2000 fold-change values per sample (with all values in the reference sample equal to 1). The median of these fold-change values was then calculated per sample and used to normalize the raw signals of targeted analytes.

NAA analysis in frozen tissues. For frozen tissues metabolic analysis, 20 mg of prostate lobes were used. Samples were homogenized in 500 μ L ice-cold extraction buffer (methanol: water, 50:50, v/v) with a tissue homogenizer for 40 s at 6000 rpm. A volume of 400 μ L of the homogenate was transferred to a new clean Eppendorf and a new homogenization cycle of 30 min at 1400 rpm at 4 °C was performed. To eliminate debris, samples were centrifuged for 15 minutes at 20,000 $\times g$ at 4 °C and 300 μ L of the supernatant was collected. Supernatants were evaporated with Speedvac for 2 h. Pellets were resuspended in 150 μ L of water: MeCN in a volume proportion of 40:60. The metabolite spectrum analysis was performed as previously described in DU145 and mCAF1 intracellular and extracellular NAA measurements.

Luciferase reporter assays

The following plasmids were used for luciferase reporter assays: SMAD2/3-reporter construct (pGL3-CAGA-luc) and pEGFP-empty. Cells previously treated with vehicle or doxycycline were seeded in 6-well plates at 70% confluency. Cells were transfected with the indicated plasmids and treated as described. Cells were lysed 24 h after transfection in 100 μ L of lysis buffer (Promega). Aliquots of the cell lysates were used to read luciferase emission using Dual-Glo® Luciferase Assay System (Promega) according to the manufacturer's instructions. A WB against GFP expression was performed with the same lysates. Firefly luciferase activities were normalized to GFP expression. Fold changes against control conditions were calculated.

RNA isolation and qRT-PCR

RNA was isolated using the NZY Total Isolation Kit according to the manufacturer's instructions. Reverse transcription was performed using NZY First-Strand cDNA Synthesis Kit, and qPCR using NZY-Speedy qPCR Green Master Mix 2X ROX plus, according to the

manufacturer's instructions in a StepOnePlus real-time PCR system. Expression levels of indicated genes were normalized to *Gapdh/GAPDH*. Primers used and their corresponding sequence are described in Supplementary Data 7. Alternatively, RNA was extracted using NucleoSpin® RNA isolation kit from Macherey-Nagel (ref: 740955.240 C) or automatically extracted using Maxwell RSC instrument (Promega) according to manufacturer's instructions. Complementary DNA (cDNA) from 0.5 or 1 μ g of the extracted RNA was synthesised using Maxima™ H Minus cDNA Synthesis Master Mix (Invitrogen Ref: M1682). QS6 or ViiA7 (Life Technologies) systems were used for RT-qPCR analysis. TaqMan probes and primers with their corresponding probes from Universal Probe Library (Roche) are described in Supplementary Data 7.

RNA-sequencing processing and analysis

Samples were first quality checked and transformed into a library of stranded mRNA molecules using the TrueSeq® Stranded mRNA library preparation kit (Illumina). The stranded mRNA library was then sequenced at a depth of 40 million reads per sample, with a total read length of 150 paired-ended base pairs (bp), using a NovaSeq 6000 S4 system (Illumina). RNA-Seq data was pre-processed to perform quality check, removal of contaminants, alignment (Genome Reference: GRCh38) and quantification. Subsequent analyses were performed using either 'count' or 'tpm' (Transcript Per Million) matrix datasets. Principal Component Analysis (PCA) and hierarchical clustering were performed using pre-filtered datasets after rlog transformation. DESeq2 software was used to identify the Differentially Expressed Genes (DEGs, cutoff: $P_{adj} < 0.05$) and fold changes between experimental conditions, following the program guidelines. This dataset was also employed to derive gene signatures (gene sets) associated with Aspa silencing in mNF1, which were employed in subsequent analyses. Unless stated otherwise, expression values for each gene represented in the figures or tables were z-score normalized from the 'tpm' matrix dataset. Genes downregulated in murine NF1 after Aspa silencing with both shRNA treatment ($\log_2FC < -1.5$, $P_{adj} < 0.005$ threshold) were identified, transformed to human orthologs and employed to generate an ASPA gene signature described in Supplementary Data 8.

ChIP-Seq and Remap analysis

mNF1 and mCAF1 were subjected to ChIP-Seq analyses as previously described⁴⁶. Briefly, $7-10 \times 10^6$ cells at subconfluency were fixed in 1% formaldehyde, washed, lysed and sonicated using the Bioruptor Pico sonication device (Diagenode; B01060001) using 10 cycles (30 sec on; 30 sec off) at maximum intensity. Purified chromatin was then separated for: (i) immunoprecipitation using Dynabeads Protein G (Life Technologies: 10003D) coated with 10 μ g of Rabbit anti-Histone H3 acetyl K27 antibody (Abcam, ab4729) per ChIP experiment; (ii) non-immunoprecipitated chromatin, used as an input control; and (iii) assessment of sonication efficiencies using a 1% agarose gel. ChIP DNA was washed, eluted and treated with 2.7 μ L of 1 mg/mL Ribonuclease A (RNaseA) for 30 min at 37 °C and subsequently incubated with 1.3 μ L of 20 mg/mL proteinase K (Invitrogen) for 1 h at 55 °C. Then, DNA extraction was performed using SPRI magnetic beads (Beckman Coulter, B23318). After elution in TE buffer, DNA was quantified using Qubit (ThermoFisher Scientific; Qubit 3.0 Fluorometer; #Q33216) high-sensitivity assay (ThermoFisher Scientific; #33216). Prior to sequencing, ChIP samples were library prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, NEB-Next Ultra II DNA library prep kit for Illumina, #E7770, NEBNext Multiplex Oligos for Illumina, #E7335L). Adaptor ligated DNA was size selected with SPRI magnetic beads (Beckman Coulter, B23318) for the Illumina sequencer (#NextSeq500). After library preparation, we performed qPCR, high-sensitivity DNA quantification and size selection measurement (Agilent Bioanalyzer 2100 system + High-sensitivity DNA

measurement assay; 5067–4626) before sending samples for sequencing. Raw sequencing files processed by the Illumina NextSeq500 sequencer were obtained in 'FASTQ' format. Raw data was pre-processed to perform a quality check and removal of contaminants. The raw sequencing files were then aligned using the mouse reference genome 38 (GRCm38) as a reference. MACS2 was used for peak calling, and Bedtools for overlap and subsetting analysis. Tracks were visualized using the UCSC Genome Browser (<https://genome.ucsc.edu/>), focusing on the indicated areas. To explore TF binding regions at the *Aspa* locus, we employed ReMap (<https://remap.univ-amu.fr/>). Using the ReMap UCSC genome browser link (https://remap.univ-amu.fr/hsap_tracks_page) for the mouse genome, we explored the presence of Smad and HDAC1,2,3 Cis Regulatory Modules (CRMs) in established promoter and enhancer regions near the *Aspa* locus.

Gene-set enrichment analyses (GSEA)

For transcriptomic analyses by GSEA, gene count RNA-Seq data were first normalized using DESeq2. Pre-processed data was then run through the GSEA software (Broad Institute of MIT and Harvard, USA) following the program guidelines. The specific settings applied in all analyses were: Number of Permutations (1000); Permutation Type (Gene set); Enrichment statistic (Weighted); Metric for ranking genes: (*t*-test). Analysed genesets were retrieved from the Molecular Signatures Database (MSigDB, <https://www.gsea-msigdb.org/gsea/msigdb>) and Comparative Toxicogenomics Database (CTD, <https://ctdbase.org/>). In addition, in-house manually curated gene sets or gene sets from specific publications were employed and described in Supplementary Data 8. Results were represented using an in-house adapted script from GSEA Multi-sample Running Enrichment plots. Values represent the False Discovery Rate (FDR) and the Nominal Enrichment Score (NES) of each gene set. To calculate the gene-signature score in each individual sample, we used single-sample Gene Set Enrichment Analysis (ssGSEA) Projection Software from the GenePattern platform, following the programs guidelines. Each ssGSEA enrichment score represents the degree to which the genes in a particular gene set are co-ordinately up- or downregulated within a sample. ssGSEA scores were z-score normalized. Similar approaches (GSEA and ssGSEA) were employed to investigate the enrichment and/or expression of particular gene signatures in different datasets

Expression analyses of public datasets and survival analyses

All the bioinformatics analysis were performed with available information downloaded from Gene Expression Omnibus (GEO), The Cancer Genome Atlas (TCGA), CANCER TOOL⁴⁷ or xCell⁴⁸ (Supplementary Data 9). For microarray-based datasets, probe-to-gene mapping was performed using the highest variance probe. Unless otherwise stated, expression values were z-score normalized.

To identify consistent differentially expressed genes (DEGs) in cancer stroma *vs* normal stroma, we studied the up- and down-regulated DEGs in the indicated datasets (unpaired heteroscedastic *t*-test, cutoff *p* value < 0.05) and identified common DEGs. This analysis was represented using the difference in mean z-score between cancer and normal stroma gene expression. GSE20100 was employed to generate gene signatures of genes repressed by HDAC1 and 2 in MEFs (Supplementary Data 8).

For mRNA and protein analyses of ASPA expression in human specimens (normal, tumour and stage), we retrieved data from ProteinAtlas (<https://www.proteinatlas.org/ENSG00000108381-ASPA>) for the indicated datasets, employing provided units (mRNA, TPMs; protein, nRPX). This resource was also employed to investigate ASPA expression in IHC tissues.

For additional analyses based on clinical material, PCa-TCGA and BCa-METABRIC gene expression and clinical information were retrieved from cBioportal. For TCGA, mRNA data were normalized in RSEM values and employed in downstream bioinformatics analyses.

For the PCa-TCGA, samples with no disease-free survival data were excluded from further analyses. In addition, duplicated samples from an individual patient were also excluded. For the BCa-METABRIC dataset, non-classified molecular subtypes, normal samples and samples with missing tumour size, grade, ER, HER2, or PR status were removed from the analysis. In all cases, survival was censored at 120 months. These two datasets were employed in downstream analyses of gene expression, digital pathology analysis using xCell or survival analyses. Curated mRNA matrices from PCa-TCGA and BCa-METABRIC were subjected to single-sample GSEA (ssGSEA) to calculate the gene expression score of different gene sets in each individual sample, which were z-score normalized for representation. For the ssGSEA analysis, genes with no values (N/A) in any of the analysed patient samples were removed from the analysis.

Curated PCa-TCGA and BCa-METABRIC datasets were also employed for prognosis analyses, together with additional PCa and BCa datasets retrieved from CANCER TOOL or KMPLOTTER⁴⁹ online applications, as well as information generated in this study from Basurto and Champalimaud cohorts. For mRNA-based analyses of the prognostic value of *ASPA* expression in BCa stroma, the Finak dataset was employed⁵⁰.

Digital pathology analyses to assess cell type composition in tumours

To infer macrophage content from bulk RNA datasets of solid tumours, we employed the online tool xCell⁴⁸ following standard guidelines on dataset pre-processing and analysis. Unless indicated otherwise, the M2 macrophage content was calculated as the ratio between raw scores of the M2 macrophage and the monocyte signatures. Samples were then stratified based on ASPA expression, ASPA signature expression or NAA content.

Integrated analyses of gene expression and metabolites in BCa patient samples

Normalized mRNA gene expression, metabolite level data and clinical information from cancerous and normal adjacent BCa tissues were retrieved from GSE37751 and from the Supplementary Data from Terunuma et al²⁵. For independent analyses (mRNA or metabolites separately) all profiled samples (cancer or normal) were included, with the exception of metabolic samples with no available data/no detection of NAA levels, that were excluded. For paired normal-cancer analyses, samples with no associated pair were excluded. For correlative analyses of RNA or RNA-based (i.e. xCell) and metabolite levels, only samples with both types of data were analysed. One sample appeared as normal in the metabolic dataset and as cancer in the RNA dataset, so it was excluded. Spearman correlation coefficient and simple linear regression with 95% confidence bands are shown. RNA data were z-score normalized for each gene in all analyses. NAA data was log2-normalized and used in the analyses. RNA data was employed to calculate M2 macrophage content employing xCell. M2 macrophage score was obtained for cancer specimens and samples stratified based on the mean NAA expression of samples with NAA data available. For survival analyses, patients were stratified based on NAA content between the lowest quartile and the rest; for this analysis, samples with undetected NAA levels were considered within the lowest quartile.

Single-cell RNA-Seq data collection, processing and analysis

For analysis of prostate tissues, a tumour adjacent (contralateral lobe) benign prostate specimen from a PCa patient was collected at Basurto University Hospital (Spain) at the time of robotic prostatectomy. Sequencing data was processed using 10X Cell Ranger with default parameters (version 7.0.1), aligned to the GRCh38 human reference genome. In total, we obtained 5,032 cells. The R package Seurat V4.3.0.0 was used to import the raw count data. Cells that were considered of low quality were removed from the raw data and the

following exclusion criteria were applied: (i) cells with less than 200 genes expressed; (ii) cells with more than 20% of genes derived from mitochondrial genes; and (iii) cells with less than 80% of complexity index (calculated as the number of genes detected per UMI). The DoubletFinder R package was utilized to identify the doublets from the raw counts data. Data was normalized, highly variable genes were identified, and raw counts data were scaled using the `NormalizeData`, `FindVariableFeatures` and `ScaleData` functions. Using the `ElbowPlot` function, the appropriate number of principal components was obtained. Afterward, the dimensionality reduction projection (UMAP) was performed using the `RunUMAP` function to visualize and explore the dataset. The `FindClusters` function was implemented to identify potential cell clusters using 0.8 resolution. Cell clusters were annotated by the average expression of the well-established genes for prostate cancer cells: T cells (CD3D and CD3E), B cells (CD79A and IGKC), macrophages (CD14, CD68, CSF1R and LYZ), mast cells (KIT and MS4A2), fibroblasts (FN1 and PDGFRA), pericytes (ACTA2 and RGS5), endothelial cells (ENG, PECAM1 and VWF), and epithelial cells (AR, EPCAM, KRT5 and KRT8).

In addition, sc-RNA-Seq analysis was performed from public datasets of breast and bladder human cancers. Breast sc-RNA-Seq data were obtained from the European Genome-Phenome Archive, reference E-MTAB-1060. This dataset consists of 14 fresh human breast tumour tissue samples. Bladder sc-RNA-Seq data were obtained from the China National Center for Bioinformation, reference GSA-Human HRA000212, and consists of tumour and normal mucosa samples processed immediately after being obtained from bladder cancer patients of the study. Our analyses included only the ones with publicly uploaded metadata, remaining 5 tumour samples and 2 mucosae: ('BC1', 'BC2', 'BC4', 'BC5', 'BC7', 'Mucosal', 'Mucosa3').

Each sample was pre-processed individually following the methodology of their original publication, with some changes in software versions in R. Once all individual cells were assigned to specific clusters/cell types, we assigned the expression of normalized gene counts to each cell. Individual cell gene expression was calculated with `Seurat`'s `AddModuleScore` function. Either the target gene or the gene signature was projected on the UMAP graphs to obtain a visualization of gene expression per cluster. In addition, matrices of gene/gene signature expression per cell were further processed for additional analyses. Briefly, for correlation analyses, the Pearson coefficient between the expression of particular genes/gene signatures was calculated for each identified cell type. For analyses of expression in each cell cluster, the average expression per cell type was calculated and then scaled within a 1 to 0 range (1 high expression, 0 no expression). For genes, where the lowest value was always 0, average values were scaled by dividing by the highest average value. For gene signatures, where certain cell types might contain negative values, average values were scaled by subtracting the minimum average value and dividing by the total range (maximum average value minus minimum average value). For certain analyses, cell types were further stratified depending on the tissue of origin (mucosal or tumour in the BLCA dataset).

Statistical analysis

No statistical method was used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. *n* values represent the number of independent experiments performed, the number of individual mice, or the number of patient specimens. Statistical analyses were performed using GraphPad Prism. When *n* permitted, values were tested for Gaussian distribution using the D'Agostino-Pearson normality test. For Gaussian distributions, paired or unpaired Student's *t* test and one-way ANOVA with Tukey post-test (for multiple comparisons) were performed. For non-Gaussian distributions, Mann-Whitney's test and Kruskal-Wallis test with Dunn's post-test (for multiple comparisons) were performed. Unless stated

otherwise, mean values and standard errors (SEM) are shown. For in vivo experiments, outlier analysis was performed to evaluate their potential influence on statistical outcomes. Samples identified as statistical outliers were excluded from the final analysis to ensure robustness of the results. Survival curves were estimated based on the Kaplan-Meier method and compared using a Mantel-Cox test, unless stated otherwise. Contingency studies were performed by Chi-square analysis. The confidence level used for all statistical analysis was 95% (*p*-value = 0.05). In general, this level was distributed in two tails (for experiments without predicted results). Where indicated, statistical test was one-tailed (for hypothesis-driven studies, i.e. in vivo analyses). Where indicated, individual *p*-values are shown; alternatively, the following symbols were used to describe statistical significance: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001; n.s., non-significant (also when no *p* value is shown).

Diagrams and artwork

All diagrams and artwork were generated using Microsoft PowerPoint, unless indicated. The following images (Public Domain licenses) were downloaded from [NIAID Visual & Medical Arts](#). (10/7/2024), NIAID NIH BIOART Source references: 281, 232, 506, 231, 212, 371.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Data generated during the course of this study includes: (1) Gene expression datasets of murine NF1 after ASPA modulation (silencing and over-expression), deposited at NCBI GEO under [GSE304425](#); (2) scRNA-Seq data of human PCa (*n* = 1 patient), available at Array Express under [E-MTAB-13764](#); (3) ChIP-Seq data (H3K27ac) of untreated murine NF1 and CAF1, deposited at NCBI GEO under [GSE327372](#). Sorting-based RNA-seq data from human PDAC tumours is available at EGA under accession code [EGAS00001004660](#). Raw and normalized counts for ASPA are provided in the Source data file. Access to the dataset is restricted due to patient data protection, but can be requested through the DKFZ-HIPO Data Access Committee of Heidelberg Center for Personalized Oncology (http://hipo_daco@dkfz-heidelberg.de). The following public datasets have been employed in this study and acquired through NCBI GEO. Human tumour stroma: Karnoub ([GSE8977](#), Breast), Yeung ([GSE40595](#), Ovary), Nishida ([GSE35602](#), Colon), Navab ([GSE22863](#), Lung) and Planche ([GSE26910](#), Prostate); Breast cancer stroma with clinical information (Finak, [GSE9014](#)); expression data from human PCa patients and associated clinical information: Grasso ([GSE35988](#)), Lapointe ([GSE3933](#)), Taylor ([GSE21034](#)), Varambally ([GSE3325](#)), Fraser ([GSE84042](#)); expression data from human BCa patients and associated clinical information: Lu ([GSE5460](#)); Terenuma ([GSE37751](#)); expression profiles of murine and human NF/CAFs: [GSE20086](#) (human breast NF and CAFs), [GSE70468](#) (human colon NF and CAFs), [GSE35250](#) (human ovarian NF and CAFs), [GSE22862](#) (human lung NF and CAFs), GSE68164 (human prostate NF and CAFs and NFs subjected to IL6 and TGFβ treatments), expression profile of MEFs after HDAC1&2 depletion ([GSE20100](#)). BCa-METABRIC and PCa-TCGA gene expression data and the associated clinical information were obtained from cBioPortal. Other datasets were donated by MSKCC (Glinsky). [CANCERTOOL](#) and [KMLOTTER](#) online applications and associated data were used for prognostic assessment of ASPA expression. Protein and mRNA expression were also assessed using [ProteinAtlas](#). Metabolomics data from BCa patient tissues associated to [GSE37751](#) were retrieved from the Supplementary Information of the associated publication (PMID: 24316975). Motif analysis was performed using the [ReMap](#) database and associated data. Breast scRNA-Seq data were obtained from the European Genome-Phenome Archive/Array Express ([E-MTAB-10607](#)). Bladder scRNA-Seq data were

obtained from the China National Center for Bioinformation, reference GSA-Human [HRA000212](#). All this information is described in Supplementary Data 9. Source data are provided with this paper.

Code availability

Scripts employed for RNA-Seq and scRNA-Seq analysis have been deposited at GitHub: <https://github.com/MiguelJulia/Astobiza2026>.

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Author contributions

I.A., C.C.-S. and C.V. performed most in vitro and in vivo assays, performed the data analysis and prepared the figures of the manuscript. I.A. co-supervised with A.C. the work of C.V. J.R. contributed to in vitro and in vivo assay design, execution and data analysis. A.M., CO-S, PC and MS provided technical support with the in vitro experiments. C.P.-L. provided support for in vivo assays and performed IHC-IF analyses. S.G.-L., I.M., M.J. and F.C. performed bioinformatics studies with public transcriptomics datasets and single-cell analyses. EJK and SZ contributed to the sample preparation and analysis of matched human breast NF and CAFs. C.R.-D., S.R. and M.M. performed macrophage assessment using IF. O.C., S.F.-R., J.R.C., V.T., N.M.-M. and M.P.-V. provided technical support with in vivo experiments. N.E. and F.J.V. provided scientific input and key reagents. L.E.-M. and A.P. contributed with the flow cytometry analysis. S.C. and M.C.-M. performed the multiplex IF analysis in the Champalimaud cohort. E.E. and A.T. performed and analyzed sorting-based pancreatic cancer cell type RNA-Seq experiment. A.V., and J.S. performed the nanostring analysis. D.G., S.R., A.S.-M., A.U.-O., M.U. and A.L.-I. generated the Basurto cohort and provided biological specimens. D.C., S.M.V.L. and J.M.F.-P. performed the metabolomics analyses at CIC bioGUNE. C.M. and J.I.L. contributed with I.H.C. staining, pathological analysis and immunoscore. R.R.-G. provided guidance and training on the in vivo experiments. C.F. contributed with metabolomics analysis. A.C. and F.C. conceived the study, supervised the execution of the project and wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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