Mechanotransduction and YAP-dependent matrix remodelling is required for the generation and maintenance of cancer-associated fibroblasts

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To learn more about cancer-associated fibroblasts (CAFs), we have isolated fibroblasts from different stages of breast cancer progression and analysed their function and gene expression. These analyses reveal that activation of the YAP transcription factor is a signature feature of CAFs. YAP function is required for CAFs to promote matrix stiffening, cancer cell invasion and angiogenesis. Remodelling of the ECM and promotion of cancer cell invasion requires the actomyosin cytoskeleton. YAP regulates the expression of several cytoskeletal regulators, including ANLN and DIAPH3, and controls the protein levels of MYL9 (also known as MLC2). Matrix stiffening further enhances YAP activation, thus establishing a feed-forward self-reinforcing loop that helps to maintain the CAF phenotype. Actomyosin contractility and Src function are required for YAP activation by stiff matrices. Further, transient ROCK inhibition is able to disrupt the feed-forward loop, leading to a long-lasting reversion of the CAF phenotype.

Tumours contain a complex mixture of cell types and matrix components¹,². Non-cancerous cells and matricellular molecules within the tumour can promote dissemination³. In addition, the physical characteristics of the extracellular matrix (ECM) enhance invasion and metastasis⁴-⁸. Increased matrix stiffness is a feature of most solid tumours⁹. Matrix stiffness can be sensed by many cell types. Force exerted on integrin-mediated adhesions can lead to the activation of FAK, Src family kinases and RhoA (refs 7,10,11). More recently, it has been shown that stiff matrices promote the activity of the YAP and TAZ transcriptional regulators¹². Elevated YAP and TAZ function is associated with increased cancer stem cell properties and metastasis¹³,¹⁴. These observations help to explain how matrix stiffness might be linked to more aggressive tumour phenotypes. However, the interplay between the tumour stroma and matrix stiffness is not well understood.

CAFs are found in many solid tumours, including breast and squamous cell carcinoma (SCC), and promote invasion and metastasis through the production of soluble factors and matrix remodelling¹⁵. In some cases, matrix remodelling leads to the generation of tracks through the ECM that enable subsequent cancer cell invasion⁶. Markers such as α-smooth muscle actin (αSMA) and S100A4 (also known as FSP1) are associated with CAFs; however, they are often not specific for CAFs and may not relate directly to the functional properties of CAFs (ref. 16). This has hampered understanding of the pro-tumorigenic signals in the stroma that are required to generate and maintain CAFs. In this study, we uncover a positive feedback loop involving YAP function and matrix stiffening that is critical for the function of CAFs.

RESULTS

Characterization of fibroblasts from different stages of mammary tumour progression

To learn more about CAFs we isolated fibroblasts from normal mouse mammary glands and hyperplastic tissue, mammary adenoma and mammary carcinoma in mice containing the MMTV-PyMT transgene¹⁷ (Supplementary Fig. S1a). This model was chosen because it contains a fibroblastic stroma with a high density of collagen fibres (Supplementary Fig. S1b), and its progression can be modulated by genetic manipulation of fibroblasts¹⁸,¹⁹. To facilitate further experiments, the fibroblasts we isolated were immortalized using human papilloma virus (HPV)-E6 (ref. 20). In total we isolated three

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sets of normal mammary fibroblasts (NFs), two hyperplasia-associated fibroblasts (HpAFs), two adenoma-associated fibroblasts (AdAFs) and four sets of CAFs. The multi-focal nature of the PyMT model enabled us to establish a hyperplasia-, an adenoma- and a carcinoma-associated fibroblast line from different regions of the same mouse; these were designated HpAF#1, AdAF#1 and CAF#1. We confirmed that they were positive for the expected fibroblast markers (Fig. 1a). Expression of CAF markers, αSMA and S100A4, was increased in the fibroblasts isolated from adenoma and carcinoma (Fig. 1b). Quantification revealed increased αSMA levels in all four CAF isolates, fibroblast-activation protein (FAP) levels were highest in CAF#1 and CAF#2, whereas S100A4 was elevated in CAF#1 and CAF#4 (Supplementary Fig. S2c). At the single-cell level there was significant heterogeneity in the expression of αSMA, S100A4 and FAP, both in vivo and in vitro; not all fibroblasts in tumours were αSMA positive, and various combinations of αSMA, S100A4 and FAP positivity were observed (Supplementary Figs S1b and S2d). Similar results were obtained with the human SCC CAF lines CerCAF (cervical SCC CAF), HNCAF (head and neck SCC CAF) and VCAF (vulval SCC CAF; Supplementary Fig. S2e). Thus, the diversity of CAF marker expression is represented in the CAFs that we isolated (Supplementary Fig. S2f). CAFs exhibited cytoskeletal changes that suggested increased

Figure 1 Isolation and characterization of fibroblasts from different disease stages. (a) Vimentin and αSMA staining of fibroblasts isolated from different disease stages. Scale bar, 50 μm. (b) Western blots of αSMA, fibronectin and S100A4 levels in fibroblasts isolated from different disease stages. (c) Upper panels show F-actin, paxillin and DAPI (magenta) staining in NFs and CAFs. Lower panels show F-actin, fibronectin and DAPI (blue) staining in NFs and CAFs. Scale bar, 10 μm. (d) Western blots showing pS19-MYL9, MYL9, MYH9, MYH10 and DIAPH1 levels in fibroblasts. (e) Images show gel contraction by fibroblasts and 4T1 breast cancer cells. Histogram shows mean ± s.e.m. (n = number of gels) (NF, 15; HpAF, 13; AdAF, 15; CAF, 35; 4T1, 4), assessed over multiple experiments (NF; 5; HpAF, 5; AdAF, 5; CAF, 10; 4T1, 2). (f) Images show F-actin staining of fibroblasts and collagen second-harmonic signal. Scale bar, 50 μm. Histogram shows elastic modulus of matrices remodelled by NFs or CAFs. Each data point represents a single measurement, n = 100 measurements in total. Line and error bars indicate mean ± s.d. (g) Representative images showing the invasion of 4T1 cells cultured in the presence of different fibroblasts. Scale bar, 50 μm. (h) Quantification of 4T1 invasion into matrices previously remodelled by different fibroblasts. Bars represent mean ± s.e.m. n = organotypic assays (-, 4; NF, 18; HpAF, 5; AdAF, 6; CAF, 24), assessed over multiple experiments (-, 2; NF, 4; HpAF, 2; AdAF, 2; CAF, 6). (i) Quantification of 4T1 invasion when co-cultured with different fibroblasts. Bars represent mean ± s.e.m. n = organotypic assays (-, 29; NF, 53; HpAF, 19; AdAF, 22; CAF, 56), assessed over multiple experiments (-, 6; NF, 12; HpAF, 4; AdAF, 5; CAF, 12). (j) Images show endomucin and vimentin staining of matrix plugs with or without different fibroblasts injected subcutaneously in mice. Scale bar, 50 μm. Graph shows quantification of endomucin staining relative to fibroblast number (vimentin staining). Each data point represents a different plug (NF, n = 7; CAF, n = 16). * P < 0.05; ** P < 0.001, unpaired t-test. Uncropped images of blots are shown in Supplementary Fig. S6.
We then performed global messenger RNA analysis on the fibroblasts (Fig. 1j; endomucin stains endothelial cells, vimentin stains fibroblasts). (Fig. 1g,h). NFs and HpAFs did not generate matrices permissive for the disease stage.

whether CAF-remodelled matrices were rendered permissive for the stiffness of the matrix from 120 Pa to >1 kPa (Fig. 1f). This is highly consistent with the change in tissue stiffness observed between normal mammary tissue and tumours. We next investigated whether CAF-remodelled matrices were rendered permissive for the invasion of breast cancer cells. Fibroblasts were allowed to remodel the matrix for five days before being killed. The ability of cancer cells with predominantly nuclear YAP is shown in Supplementary Fig. S2b. These results were confirmed using the syngeneic breast cancer cell line MMTV-PyMT TS2 (ref. 22 and Supplementary Fig. S1c). Finally, we demonstrated that CAFs were able to promote angiogenesis in the absence of any cancer cells in vivo (Fig. 1j; endomucin stains endothelial cells, vimentin stains fibroblasts). These data demonstrate the successful isolation of fibroblasts from different stages of a murine breast cancer model. Further, we show that their matrix-remodelling- and invasion-promoting abilities increase with the disease stage.

YAP is activated in CAFs

We then performed global messenger RNA analysis on the fibroblasts isolated from different disease stages. RNA samples were prepared from the fibroblasts cultured on the collagen-rich matrices used for the functional assays. Gene set enrichment analysis (GSEA) was performed to identify changes in coordinated gene expression programs (Supplementary Table S1). Strikingly, we noted that the genes upregulated in our murine CAFs showed significant overlap with gene expression in the stroma of human breast cancer with poor prognosis (Supplementary Fig. S3a). These observations suggest that our experimental system is likely to have significant relevance for human disease. As expected, GSEA revealed changes in inflammatory signalling, although these changes were typically more pronounced in HpAFs and AdAFs (Supplementary Table S1). We also noted significantly increased expression of genes associated with two mechano-sensitive pathways, YAP/TAZ (refs 12,29–31; Table 1 and Supplementary Fig. S2b) and SRF (refs 32–34 and Supplementary Table S1). We chose to investigate whether YAP or TAZ, closely related paralogues, were involved in CAF function.

YAP/TAZ are negatively regulated by phosphorylation by LATS1&2, which are in turn regulated by MST1&2 (refs 35,36). Phosphorylation of YAP and TAZ is associated with their sequestration in the cytoplasm. Reduced MST1/2 and LATS1/2 activity leads to nuclear accumulation, binding to TAZ and other proteins, and activation of transcription. YAP activation is well known to be oncogenic within epithelial cells, but its role in the tumour stroma has not been explored. Recently, YAP/TAZ activation has been reported in response to mechanical stress and perturbation of the actin cytoskeleton (refs 38–41). Figure 2a shows that YAP is predominantly cytoplasmic in NFs but accumulates in the nucleus in CAFs; HpAFs and AdAFs show intermediate levels of nuclear YAP (alternative quantification of the percentage of cells with predominantly nuclear YAP is shown in Supplementary Fig. S3c). Nuclear localization of YAP was a universal feature of CAFs, regardless of their expression levels of αSMA, FAP or S100A4 (Supplementary Fig. S3d, also compare Supplementary Figs S2c and S3c). The localization of TAZ showed more subtle differences between normal mammary fibroblasts and CAFs (Fig. 2a). Interestingly, the activity of the upstream negative regulators, MST1/2, was not different between NFs and CAFs. Further, the activity of LATS kinases and phosphorylation of Ser 127 on YAP was even elevated in CAFs (Fig. 2c).

Table 1: Enrichment analysis of YAP gene sets in different fibroblasts.

| Gene set | HpAF | AdAF | CAF |
|----------|------|------|-----|
| YAP/TAZ (ref. 12) | NS | 0.026915* | <0.001* |
| YAP liver31 | 0.003521* | <0.001* | <0.001* |
| YAP common MCF10 and 3T3 (ref. 30) | 0.001919* | <0.001* | 0.040493* |
| Induced by YAP in 3T3 (ref. 30) | 0.004505† | <0.001† | 0.00266* |
| Induced by YAP (ref. 29) | 0.004796† | <0.001† | 0.010526* |
| Induced by YAP and TAZ (ref. 29) | 0.038379† | <0.001† | 0.021866* |
| TEAD-dependent YAP and TAZ (ref. 29) | NS | NS | <0.001* |

Table showing GSEA of multiple YAP and YAP/TAZ gene sets in different fibroblasts. Comparisons based on pooled gene expression profiles of 3 NFs, 1 HpAF, 1 AdAF and 4 CAFs. The table shows the P values for enrichment of different gene sets in hyperplasia, adenoma- and carcinoma-associated fibroblasts from MMTV-PyMT mice when compared with normal fibroblasts. *Indicates significant positive enrichment. †Indicates negative enrichment. Reference to each gene set is indicated. NS, not significant.
Figure 2 YAP is activated in CAFs. (a) Images show YAP or TAZ localization (greyscale and green) and DAPI (red) in different fibroblasts. Scale bars, 20 µm. (b) Quantification of nuclear relative to cytosolic fluorescent intensity of YAP or TAZ. Bars represent mean ± s.e.m for YAP data (NF, n = 4 experiments with ~15 20 × fields of view analysed in each experiment; HpAF, n = 3; AdAF, n = 3; CAF, n = 4). Bars represent mean for TAZ data (one representative experiment shown, with ~15 20 × fields of view analysed. Further data in Supplementary Table S2 (Statistics Source Data). (c) Western blots show the levels of active MST1/2 (judged by pT183/T180), MST1, MST2, active LATS1/2 (judged by pS909 and pT1079), LATS1/2, pS127-YAP, pY357-YAP, TAZ, YAP and tubulin loading control in NF#1, HpAF#1, AdAF#1 and CAF#1. (d) Western blots show immune-precipitation of TEAD1, TEAD4 and 14-3-3 with YAP in NF#1, HpAF#1, AdAF#1 and CAF#1. Graphs represent quantification of the blots showing the amount of TEAD1, TEAD4 and 14-3-3 proteins bound to YAP in HpAF#1, AdAF#1 and CAF#1 relative to NF#1. (e) Quantitative real-time PCR analysis of gene expression in different fibroblasts. Data from 3 independent experiments performed in triplicate (normalized to Gapdh). Bars represent mean ± s.e.m. of the average fold induction compared to NF#1 in each experiment. (f) Images show YAP localization and DAPI staining of normal and cancerous mammary tissue. White arrows indicate nuclear accumulation of YAP in the stroma. Scale bar, 20 µm. Charts show quantification from different stages of MMTV-PyMT disease and from different tumoral regions on carcinomas. n = number of images analysed (normal, n = 27; hyperplasia, n = 41; adenoma, n = 20; carcinoma, n = 18; central, n = 19; margin, n = 18). Images were taken from multiple tissue samples (normal, 3; hyperplasia, 2; adenoma, 7; carcinoma, 7; central, 7; margin 7). In the box-and-whisker plot, the central box represents values from the lower to upper quartile. The middle line represents the mean. The horizontal line extends from the minimum to the maximum value. Values represent the percentage of YAP-positive stromal cells per ×20 field of view. *P < 0.05; **P < 0.01; ***P < 0.001, unpaired t-test. Uncropped images of blots are shown in Supplementary Fig. S6.

YAP is required for CAF function

We next investigated whether YAP or TAZ function was important for the functional properties of CAFs. Depletion of YAP with three different short interfering RNAs (siRNAs) reduced the ability of the CAFs to physically contract collagen-rich matrices (Fig. 4a). YAP-depleted cells also had fewer focal adhesions, essential structures for force...
We then sought to identify YAP-regulated genes that were required (Fig. 4c). In contrast, depletion of TAZ had little effect (Fig. 4a,b). The YAP also reduced the ability of CAFs to form fibrous collagen networks (Supplementary Fig. S4a). We confirmed these observations in three different human CAF lines derived from SCC (Fig. 4e). Depletion of YAP or TAZ localization (green) and DAPI (red) in human CerCAFs, HNCAFs and VCAFs. Scale bars, 20 μm. (d) Histogram shows the percentage of CerCAF, HNCAF and VCAF with predominantly nuclear YAP. Bars represent mean ± s.e.m. of 5 independent experiments. **P < 0.01; ***P < 0.001, unpaired t-test.

transmission between the cytoskeleton and matrix (Fig. 4b). These defects were associated with fewer thick collagen fibres within matrices containing YAP-depleted cells together with a lower elastic modulus (Fig. 4c). In contrast, depletion of TAZ had little effect (Fig. 4a,b). The ability of CAFs to promote cancer cell invasion was also significantly dependent on YAP function, but TAZ was not required (Fig. 4d and Supplementary Fig. S4a). We confirmed these observations in three different human CAF lines derived from SCC (Fig. 4e). Depletion of YAP also reduced the ability of CAFs to form fibrous collagen networks and promote angiogenesis in vivo (Fig. 4f—Masson’s trichrome and endomucin staining, respectively). Conversely, activation of YAP signalling by either overexpression of an active mutant or depletion of MST1&2 increased invasion-promoting ability of NFs (Supplementary Fig. S4b,g). Together, these data demonstrate that YAP is critical for many aspects of the tumour-promoting function of CAFs.

YAP regulates the contractile actomyosin cytoskeleton

We then sought to identify YAP-regulated genes that were required for matrix remodelling by CAFs. We focused on 14 genes that our microarray analysis had indicated were upregulated in CAFs (see Fig. 2e and Supplementary Fig. S3e for validation of specific genes) and had been implicated as YAP targets in genomic analyses. We also tested DIAPH1 and MYL9 because we had noted their elevated protein levels in CAFs (Fig. 1d). Figure 5a shows the effect of YAP siRNA on the mRNA expression levels of the indicated genes (based on YAP siRNA in CAF#1, CAF#2, CerCAF, HNCAF and VCAF). This revealed that 6 out of the 14 genes with elevated mRNA levels in CAFs were found to be consistently transcriptionally regulated by YAP (AMOTL2, ANKR1, ANLN, CTGF, DIAPH3 and SDPR). Others were YAP targets in the minority of the five CAF isolates that we tested (DIAPH1, FLNA and THBS1). MYL9 and MYH10 were not transcriptionally regulated by YAP. Amongst these genes, we identified several that are required for CAF-driven matrix remodelling and invasion (Fig. 5b,c; see Supplementary Fig. S4c for knockdown efficiency). Significantly, ANLN, DIAPH3 and FLNA were required for CAFs to both remodel the ECM and promote invasion (Fig. 5b,c, invasion data shown with multiple siRNA in Supplementary Fig. S4d). This establishes a role for at least two YAP transcriptional targets in
enabling the CAF phenotype. This is in agreement with previous reports showing YAP and TEAD binding to the ANLN promoter. We also found that MYL9 and MYH10 are required for CAF functionality (Fig. 5b, c and see Supplementary Fig. S4e for multiple siRNA). These genes are not transcriptionally regulated by YAP; nonetheless we explored whether their protein levels depend on YAP. Figure 5d shows that depletion of YAP, but not TAZ, reduces both the total levels of MYL9 and MYH10 and the level of active Ser 19 phosphorylated MYL9. Similar data were obtained in CAF#2 and human vulval SCC CAFs (Supplementary Fig. S4f). The critical importance of MYL9 for CAF function was confirmed by overexpressing either wild-type MYL9 or an inactive mutant in NFs. Only wild-type MYL9 overexpression resulted in a gain of matrix-remodelling- and invasion-promoting functions (Fig. 5e, f). Finally, blockade of MYL9 function (using blebbistatin) reduced matrix remodelling by CAFs (Fig. 5g). Together, these data establish regulation of MYL9 by YAP as a critical factor for generating CAFs with matrix-remodelling- and invasion-promoting abilities.

**A positive feedback loop involving actomyosin contractility sustains YAP activation**

To understand what drives YAP activation in CAFs, we investigated how YAP activity was induced in normal fibroblasts. Conditioned media from cancer cells promotes matrix remodelling by normal fibroblasts (Fig. 6a) and increases their ability to support cancer cell
invasion (Supplementary Fig. S5a–c). This change in behaviour was also associated with the nuclear translocation of YAP and the activation of some YAP/TAZ target genes (Fig. 6b,c). The degree of YAP nuclear localization triggered by conditioned media, and the range of target genes activated was less extensive than in CAFs (compare Figs 2 and 6b,c). We also noted that other pro-contractile stimuli, such as t-α-lysophosphatidic acid (LPA) and transforming growth factor-β (TGF-β), promoted nuclear accumulation of YAP (Fig. 6a,b). YAP can respond to matrix stiffness12; therefore, the ability of soluble factors to activate YAP could be the indirect result of their ability to promote matrix remodelling and concomitant stiffening. Such a mechanism of YAP activation would be reduced by blockade of matrix remodelling. We prevented matrix remodelling by chronically blocking actomyosin function (using blebbistatin). Actomyosin inhibition blocked the matrix remodelling and nuclear accumulation of YAP induced by conditioned media, TGF-β and LPA (Fig. 6a,b). These data show that YAP can be activated by various soluble factors; however the mechanism may be indirect because a functional contractile cytoskeleton is required in all cases. We next focused on the high steady-state level of YAP signalling in CAFs. This elevated level of YAP signalling could be prevented if the stiffening of the ECM was blocked. Figure 6d shows that ROCK inhibition reduces the elastic modulus of matrices containing CAFs from >500 Pa to 100 Pa. This is associated with reduced nuclear localization of YAP and reduced expression of several, but not all, YAP target genes (Fig. 6e,f). Similar results were obtained if actomyosin function was blocked using blebbistatin. Finally, we confirmed previous findings24 that increasing collagen matrix stiffness is sufficient to drive the nuclear accumulation of YAP in NFs (Fig. 6g).

The data above establish a link between the contractile cytoskeleton, matrix stiffness and YAP activity. However these phenomena could be linked in a number of different, but not mutually exclusive ways. Actomyosin function could simply be required to generate a stiff ECM, but not to respond to it. Similarly, the contractile actin network might be required for sensing of matrix rigidity. To explicitly test the latter hypothesis, we allowed CAFs three days to remodel the collagen-rich matrix and then acutely treated with inhibitors (Fig. 7a). These experiments reveal that ROCK, myosin...
and Src function (blocked by PP2 and dasatinib) are required to maintain the nuclear localization of YAP in CAFs, even after the ECM has been remodelled. Consistent with this, treatment of mice with Y27362 reduced the nuclear localization of YAP in CAFs, and prevented the CAFs from promoting angiogenesis (Fig. 7b,c). Examination of cell morphology revealed that ROCK inhibition disrupted contractile stress fibres, whereas Src inhibition did not (Supplementary Fig. S5d). Further, ROCK inhibition reduced Src activity (Supplementary Fig. S5e). Thus, we propose that Src acts downstream from cytoskeletal tension to regulate YAP. Inhibition of ROCK and Src did not affect phosphorylation of MST1/2 and S127-YAP (Supplementary Fig. S5e). However, blebbistatin, Y27362 and dasatinib all reduced the association of YAP with TEAD1 and TEAD4, but increased its association with 14-3-3 proteins (Fig. 7d). Together, these data suggest that matrix stiffness promotes actomyosin-dependent regulation of Src-family kinases leading to a switching of YAP away from binding 14-3-3 proteins in favour of association with TEAD1 and TEAD4. This change does not seem to be linked to changes in MST activity or S127-YAP phosphorylation (Fig. 2c and Supplementary Fig. S5e).

Figure 6 Actomyosin- and Src-dependence of YAP activation in CAFs. (a) Effects of 4T1-conditioned media, LPA and TGF-β on matrix remodelling by NFs. One of two independent experiments is shown. (b) Effects of 4T1-conditioned media, TGF-β and LPA on YAP localization in NFs. Grey bars indicate presence of blebbistatin. One of two independent experiments is shown. (c) Effect of 4T1-conditioned media on gene expression in NFs normalized to Gapdh. Mean and ± s.e.m. n = 3 experiments. (d) Elastic modulus of matrices remodelled by CAF#1 transfected with control siRNA with or without Y27632 for 3 days. Central box represents the lower and upper quartiles, middle line represents the mean, and horizontal lines show the minimum to the maximum value (n = 100 independent elastic modulus measurements). (e) YAP localization in CAF#1 with or without Y27362 and blebbistatin for 3 days. Mean ± s.e.m. n = 3 experiments. (f) Effects of Y27632 and blebbistatin on gene expression in CAF#1 normalized to Gapdh expression. Mean of n = 4 experiments, ± s.e.m. (g) YAP localization in NFs cultured on 1.5 mg ml⁻¹ collagen I gel (~100 Pa), 8 mg ml⁻¹ collagen (~300 Pa) and collagen-coated glass (~10 kPa). Mean ± s.d. n = 8 experiments. (h) YAP localization in CAF#1 with or without Y27362, blebbistatin, PP2 and dasatinib for 2 h. Mean ± s.e.m. n = 4 experiments. (i) YAP and vimentin staining in CAF#1 matrix plugs grown subcutaneously in mice, where the indicated mice received 50 mg kg⁻¹ Y27632. Scale bar, 100 μm. Average number of YAP-positive nuclei shown for each mouse as an individual data point nuclei (4 control and 5 Y27632-treated mice in one cohort). Bars represent mean ± s.e.m. (j) Quantification of endomucin. Each point is an independent plug (3 control and 5 Y27632-treated mice in one cohort). Bars represent mean ± s.e.m. (k) Immune-precipitation of TEAD1, TEAD4 and 14-3-3 with YAP in control, blebbistatin-, Y27632- and dasatinib-treated CAF#1. (l) Matrix remodelling by CAF#2 previously cultured with Y27632, or cultured with Y27632 during the assay. Data from two experiments, distinguished in black and grey. *** P < 0.001, unpaired t-test. Further data for a,e,l in Supplementary Table S2 (Statistics Source Data). Uncropped images of blots are shown in Supplementary Fig. S6.
YAP is required for elevated levels of MYL9 and MYH10 and matrix stiffening by CAFs (Fig. 5). Conversely, stiff matrices and the contractile actin cytoskeleton work together to activate YAP (Fig. 6). Together these elements generate a positive feedback loop. Depending on the level of damping, this loop of matrix stiffening and YAP activation might be able to be broken by inhibition of the loop for a period of time. We explored this hypothesis by culturing CAFs in the presence of Y27632 for a prolonged period and then assayed their phenotype after removal of the inhibitor. Figure 7e shows that Y27632 effectively reduced matrix remodelling when included during the assay. More notably, cells that had previously been cultured in Y27632, but were assayed in normal media, showed significantly defective matrix remodelling abilities 3–5 days after removal of the drug (Fig. 6l). This demonstrates that if the feed-forward loop between the matrix and YAP can be blocked, then a stable reversion in cell behaviour arises.

**DISCUSSION**

Here we demonstrate a new role for the YAP oncoprotein in the tumour stroma. YAP function is critical for the establishment and maintenance of CAFs. We propose that soluble factors secreted by cancer cells initially promote an intermediate level of YAP and TAZ activity. YAP activation occurs as a consequence of two inter-related events: first, the matrix remodelling and stiffening induced by factors such as LPA and TGF-β, and, second, changes in the contractile actin cytoskeleton within fibroblasts. Stiff matrices require actomyosin function for their generation; however they also enable actomyosin to generate isometric tension within cells (Fig. 7). This leads to stress fibre formation, and the activation of Src-family kinases at focal adhesions. Src-family kinase function is required for YAP activation. YAP function is required for the expression of MYL9, matrix stiffening and many pro-tumorigenic properties of fibroblasts. The matrix stiffening resulting from YAP activation generates a positive feedback loop, leading to the more robust and sustained activation of the YAP pathway observed in CAFs. Once this feedback is established it can become self-sustaining, and this could explain the stability of the CAF phenotype in the absence of co-culture with cancer cells. In vivo, it is likely that this mechanism cooperates with epigenetic changes induced by soluble factors in the tumour micro-environment. It is also possible that YAP activation could be initiated by soluble factors in the absence of cancer cells; these may act by triggering matrix stiffening or through more direct G-protein-coupled receptor signalling to YAP (ref. 44). It will be interesting to determine whether YAP plays a role in matrix stiffening in pre-cancerous lesions or, more generally, in fibrosis. YAP-dependent matrix stiffening driven by CAFs may also lead to pro-tumorigenic YAP activation in cancer cells in close proximity to the stiff matrix. Consistent with this, we and others have noted high levels of nuclear YAP in cancer cells adjacent to the stroma. Similarly, matrix stiffening may lead to the propagation of YAP activity to nearby normal fibroblasts. To conclude, we identify a key role for YAP in the tumour stroma. It is required for many of the pro-tumorigenic functions of CAFs, including matrix stiffening, invasion and angiogenesis. High levels of YAP function in the stroma are sustained through positive feedback between CAF-driven matrix stiffening and mechanotransduction leading to YAP activation.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGEMENTS**

F.C., N.E., S.H., R.P.J., S.I.C., K.H. and E.S. are financially supported by Cancer Research UK. A.G-G. was financially supported by a Royal Society Newton Fellowship, E.M. is in receipt of a Dorothy Hodgkin Postgraduate Award (DHPA) from the Engineering and Physical Sciences Research Council. G.C. is in receipt of a Royal Society University Research Fellowship. We thank N. Tapon, B. Thompson and laboratory members for help and advice throughout this work.

**AUTHOR CONTRIBUTIONS**

F.C. carried out all the experiments except those noted otherwise. N.E. performed all the quantitative real-time PCR analyses and generated data for Fig. 7e. E.S. generated data for Figs 3, 6g and 7b. E.M. and G.C. performed all the AFM analyses. A.G-G. and S.H. isolated and immortalized several breast and human CAFs. R.P.J. wrote the script for organotypic invasion quantification and helped analyse data for Supplementary Fig. S2c,e. S.I.C., K.H. and P.W. provided clinical material. F.C. and E.S. conceived the study and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at www.nature.com/doifinder/10.1038/ncb2756

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**Figure 7** Model outlining the role of YAP in the generation and maintenance of CAFs. A range of soluble factors can promote matrix remodelling by normal fibroblasts. This requires some level of ROCK and actomyosin function. As the matrix becomes stiffer, isometric tension within the cell increases, leading to Src activation and the association of YAP with TEAD transcription factors. Active YAP then promotes the expression of ANLN and DIAPH3 mRNA and stabilizes actomyosin proteins. This leads to further matrix stiffening, thereby generating a positive feedback loop. NF, normal fibroblast.
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METHODS

Mouse strains. Transgenic FVB/n mice expressing the polyoma middle T antigen oncogene under the mouse mammary tumour virus promoter (MMTV-PyMT; ref. 17) were kept in accordance with UK regulations under project licence 70/6962. 6-8-week-old female mammary carcinomas (from 16-week-old females) and mammary carcinoma/late carcinoma/invasive carcinoma tissue (older than 12 weeks) were dissected out and part taken for histological analysis and part for fibroblast isolation. Two approaches were followed for fibroblast isolation. To isolate fibroblasts from normal tissue, which is very soft and fatty, we placed the tissue into dishes where it was compressed under a 20 mm coverslip to prevent fatty tissue floating. We then covered it with DMEM (Invitrogen) with 10% FCS (PAA Labs) and 1% ITS (insulin-transferrin selenium; #41400-045; Invitrogen) supplement and changed the media daily. After 7–10 days, fibroblasts started to grow out of the tissue into the coverslip and the dish. Then, the tissue was removed and the fibroblast population expanded. For hyperplastic, adenoma and cancerous tissue, the sample was cut into small pieces and collagenase/dispase digested. After filtering the undigested tissue, the solution was serial centrifuged and the final pellet re-suspended in DMEM (Invitrogen) with 10% FBS and 1% ITS and seeded on a culture dish. After 30 min the fibroblasts had already adhered to the dish whereas other cellular types remained in suspension. In both cases, fibroblasts were subsequently grown and immortalized with HPV-E6 retrovirus20. They were then selected using 2.5 μg ml⁻¹ puromycin.

Clinical samples and human CAFs. The tumour specimens and tissue samples used in this study were obtained with informed consent from all subjects. Human SCC CAFs were isolated following similar approaches to murine CAFs and were grown and immortalized with htert lentivirus. They were selected using 400 μg ml⁻¹ puromycin. Western blotting. Unless stated otherwise, all protein lysates were obtained from cells seeded on top of a thin layer of a mixture of collagen I/Matrigel matrices. Generally, 100 μl of the gel was spread evenly on a 6-well-plate dish. Apart from that, protein lysates were processed following standard procedures. Antibody description and working dilutions used can be found in Supplementary Table S3.

Immunoprecipitation. Endogenous YAP immunoprecipitations were performed as described previously47. Briefly, cells were lysed in 50 mM Tris (pH 8), 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM MgCl₂, 2 mM phenylmethyl sulphonyl fluoride, and protease and phosphatase inhibitors. The supernatants were pre-cleaned by incubating for 30 min at 4 °C with protein-G beads. The YAP antibody was then added overnight. Immunoprecipitates were washed three times with NET buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.25% gelatine and 0.1% Nonidot P-40) and analysed by immunoblotting. Exposures within the dynamic range were quantified by densitometry using the program NIH Image 1.60.

ECM-remodelling assay. To assess force-mediated matrix remodelling, 75 × 10⁶ fibroblasts were embedded in 100 μl of collagen I/Matrigel and seeded on a 35-mm glass-bottom MatTek dish (P35-1.3-14-C, MatTek). Once the gel was set, cells were maintained in DMEM + 10% FCS (unless otherwise stated) + 1% ITS. Gel contraction was monitored daily by taking photographs of the gels. Unless stated otherwise, the gel contraction value refers to the contraction observed after 3 days. To obtain the gel contraction value, the relative diameters of the well and the gel were measured using an optical microscope and the percentage of contraction was calculated using the formula 100 × (well diameter – gel diameter)/well diameter.

Atomic force microscopy. To assess the elastic modulus of gels remodelled by fibroblasts, 75 × 10⁶ fibroblasts were embedded in 100 μl of collagen I/Matrigel and seeded on an ultra low-attachment 96-well plate well (#3474, Costar). Once the gel was set, cells were maintained in DMEM + 10% FCS + 1% ITS. After 3 days, the elastic modulus of the gels was measured. On the day of the experiment, gels were gently lifted from their well and fixed in the centre of 50-mm glass-bottom Petri dishes using cyanoacrylate superglue. Once glued, Leibovitz L-15 medium (Invitrogen) supplemented with 10% FCS was added to the dish.

Atomic force microscopy (AFM) measurements were performed with a JPK NanoWizard-I (JPK instruments) interfaced to an inverted optical microscope (IX-81, Olympus). AFM cantilevers with pyramidal tips (MLCT, Bruker) and nominal spring constants of 0.07 Nm⁻¹ were modified by gluing 35-μm-radius glass beads to the cantilever underside with ultraviolet curing glue (ultraviolet curing, Locite). Cantilever spring constants were determined before modification using the thermal noise method implemented in the AFM software (JPK SPM). Before any indentation tests, the sensitivity of the cantilever was set by measuring the slope of force–distance curves acquired on glass regions of the Petri dish. Using the optical microscope, the tip of the cantilever was aligned over regions in the middle of the gel and, for each gel, measurements were acquired in 30–40 locations ~100 μm apart. Force–distance curves were acquired with an approach speed of 5 μm s⁻¹ until reaching the maximum set force of 3 nN. After the experiment, the elastic moduli were extracted from the force–distance curves by fitting the contact portion of curves to a Hertz contact model58. For each force–distance curve, the goodness of fit was evaluated by calculating r² values and only fits with r² > 0.80 were retained for further analyses (representing on average 80% of the acquired force curves).

Invasion assays. The human CAF organotypic culture system was set up as previously described17. The mouse mammary CAF organotypic culture system was set up with some adaptations. Briefly, 10⁶ fibroblasts, unless stated otherwise, were embedded in a mixture of collagen I/Matrigel matrix. After the gel was set at 37 °C for 1 h, DMEM + 10% FCS + 1% ITS was added on the top. Sixteen hours later, 5 × 10⁴ 4T1/410.4/T2 cells were seeded on top in DMEM + 10% FCS + 1% ITS. After 8 h, a thin layer of gel was added covering the breast cancer cells. The gel was then mounted on a metal bridge and fed from underneath with DMEM + 10% FCS + 1% ITS. After 6 days, the cultures were fixed using 4% paraformaldehyde plus 0.25% glutaraldehyde in PBS and processed by standard methods for haematoxylin and eosin staining. For assays involving the removal of fibroblasts (fibroblast-conditioned ECs), the fibroblasts were left to remodel the gel for 5 days, after which the gels were incubated in DMEM + 10% FCS + 1% ITS plus hygromycin (400 μg ml⁻¹) for 48 h to kill the fibroblasts and then washed three times with DMEM + 10% FCS + 1% ITS (>30 min per wash). Then, 5 × 10⁴ 4T1/410.4/T2 cells were plated on top and the assay proceeded as usual. The invasion index was calculated by measuring the total area over which breast cancer cells had dispersed (including invading and non-invading cells) and the area of non-invading cells. The value shown is the average of the measurement (1 – (non-invading
area/total area) of at least ten measurements from two or more independent experiments and the error bars represent the s.e.m. MATLAB script was used to automatically identify the total cell area and the non-invading area. The M files are available on request, email robert.jenkins@cancer.org.uk.

Matrigel plugs. Fibroblasts (2 × 10⁶) were resuspended in 100 µl of PBS and mixed in 300 µl of Matrigel. Then, 300 µl of the mixture was injected subcutaneously on the mammary fat pad of a 12-week-old FVB/n female. After 7 days, the plugs were surgically removed and taken for histological and immunofluorescence microscopy analyses. Blood vessel density was calculated by normalizing the area positive for endomucin staining to the area positive for vimentin staining of all the slides processed. Each value in the plot corresponds to the blood vessel density of an individual plug.

RNA isolation and arrays. To obtain RNA from the different fibroblast populations, cells were seeded on top of the collagen I/Matrigel matrix and fed with complete media. After 5 days, RNA was isolated using RNeasy Kit (#74104, Qiagen). RNA was then processed in collaboration with the Bart’s and London Centre for Genome Research (Illumina bead array platform—details available on request).

GSEA. Array data were processed and analysed using the GSEA software, developed by the Broad Institute of MIT and Harvard (USA) and available at www.broadinstitute.org, following the program guidelines. The specific settings applied in all analyses are: number of permutations (1,000), permutation type (gene set), enrichment statistic (weighted), metric for ranking genes (t-value or symbols describing it (*, **, ***). P values were obtained from t-tests with paired or unpaired samples, with significance set at P < 0.05. Graphs show either the actual P value or symbols describing it (*, P < 0.05; **, P < 0.01; ***. P < 0.001). Source data for the indicated figures can be found in Supplementary Table S2.

Quantitative real-time PCR. Sequences of the oligonucleotides used are described in Supplementary Table S5.

Transfections, inhibitors, siRNA and cDNAs. Fibroblasts were cultured in standard conditions and transfected using DharmaFECT 1 (#T-2001-03; Dharmacon, Perbio Science). Briefly, cells were plated at 60% confluence and subjected to transfection the following day using 100 nM final concentration of siRNA. siRNA was purchased from Dharmacon and sequences are listed in Supplementary Table S6. The following growth factors and drugs were used: TGF-β, 2 ng ml⁻¹ (Pepro-Tech); LPA, 2 µg ml⁻¹ (L7260, Sigma); Y27632, 10 µM (#1254; Tocris Bioscience); blebbistatin, 25 µM (#20391; Calbiochem/Merck); dasatinib, 300 nM (LC Labs); and PP2, 10µM (Calbiochem/Merck). Conditioned medium from 4T1 cells was obtained by conditioning plain DMEM in a 75% confluent culture for 24–48 h. pEGFP-MYL9 wild-type and pEGFP-MYL9 T18A, S19A (TASA) mutant were a gift from M. Olson (Beatson Institute, Glasgow, UK). pCDNA-FLAG-YAP S5A plasmid was a gift from B. Thompson (London Research Institute, London, UK).

Accession numbers. Primary accessions: data files are available at the NCBI Gene Expression Omnibus (GEO) under GSE45256.
**SUPPLEMENTARY INFORMATION**

DOI: 10.1038/ncb2756

**Figure S1** Histochemical analysis of MMTV-PyMT stroma. A) Diagram illustrating the workflow. B) Images show S100A4, vimentin, αSMA, fibronectin, endomucin (blood vessels), and Masson’s (collagen fibres) staining of normal mouse mammary gland, and hyperplasia, adenoma, and carcinoma from MMTV-PyMT mice. Panels are 500x300μm, except Masson’s staining is 200x140μm. C) Representative images showing the invasion of MMTV-PyMT-TS2 cells cultured in the presence of different fibroblasts. Scale bar is 50μm. Chart shows quantification of TS2 invasion when co-cultured with different fibroblasts. Bars represent mean ± s.e.m. of multiple experiments (NF, n=5; HpAF, n=5; AdAF, n=3; CAF, n=4). ** P < 0.01 – unpaired t-test.
Figure S2 Characterisation of different fibroblast isolates. A) Histogram shows gel contraction by different isolates of normal mammary fibroblasts, and fibroblasts isolated from MMTV-PyMT mice at different stages of disease progression. Bars represent mean ± s.e.m. of multiple experiments (NF#1, NF#2, NF#3, HpAF#1, HpAF#2, AdAF#1, AdAF#2, n=6 gels; CAF#1, CAF#2, n=14 gels; CAF#3, n=11 gels; CAF#4, n=13 gels). B) Histogram shows invasion of 4T1 cells when co-cultured in organotypic models with normal mammary fibroblasts, and fibroblasts isolated from MMTV-PyMT mouse at different stages of disease progression. Bars represent mean ± s.e.m. of multiple experiments (no FIBs, NF#1, n=29; NF#2, n=4; NF#3, n=20; HpAF#1, n=14; HpAF#2, n=5; AdAF#1, n=12; AdAF#2, n=10; CAF#1, n=15; CAF#2, n=23; CAF#3, n=18; CAF#4, n=10). C) Charts indicate the expression of αSMA, FAP and S100A4 in the four different MMTV-PyMT derived CAFs. Data from one representative experiment from a total of 2 are shown. Each point represents quantification of an individual cell. Line and error bars indicate mean ± s.d. of the population. Thus the plots illustrate both the mean level and heterogeneity in marker expression in each CAF isolate. * P < 0.05; ** P < 0.01; *** P < 0.001 – ANOVA relative to NF#1 and NF#2. D) Left hand panel shows double immune-fluorescence for αSMA (red) and Fibronectin (green) with DAPI shown in blue. Scale bar is 100µm. Mid-left panel shows triple immune-fluorescence for αSMA (red), S100A4 (green), and FAP (blue) in tumour section. White arrows indicate αSMA and S100A4 double positive cells, red arrows indicate αSMA positive/S100A4 negative cells, and green arrow indicates S100A4 positive/αSMA negative cell. Scale bar is 50µm. Mid-right and right hand panels show the same staining on CAF#1 and CAF#2 cultured in vitro. E) Charts indicate the expression of αSMA, FAP and S100A4 in the three human SCC CAF isolate – CerCAF – cervical SCC, HNCAF – head and neck SCC, and VCAF – Vulval SCC. Data from one representative experiment from a total of 2 are shown. Each point represents quantification of an individual cell. Line and error bars indicate mean ± s.d. of the population. Thus the plots illustrate both the mean level and heterogeneity in marker expression in each CAF isolate. CerCAF, HNCAF, and VCAF have different αSMA expression, VCAF have different FAP expression from other CAFs and CerCAF have different S100A4 expression from other CAFs.* P < 0.05; ** P < 0.01; *** P < 0.001 – ANOVA. F) Chart summaries the average level of expression of different markers in the seven CAF isolates used in this study.
Figure S3  Increased YAP activity in CAFs. A) Plots show progressive enrichment of Finak SDPP25 and Reopman Up in Stroma when Mets24 gene sets in HpAF, AdAF and CAFs. B) GSEA plots show the progressive up-regulation of YAP/TAZ target genes with increasing disease stage. C) Histogram shows the % of NF, HpAF, AdAF, and CAF cells with predominantly nuclear YAP. Bars represent mean ± s.e.m. on 5 independent samples. * P < 0.05; ** P < 0.01; *** P < 0.001. D) Panels show YAP localisation (blue) in CAF#1 stained for the indicated marker (green) and F-actin (red). Scale bar is 20 µm. E) Histogram shows the expression of YAP, TAZ, CTGF and five genes verified to be YAP regulated in NF#1 and CAF#2. Average of three experiments performed in triplicate normalized against the expression of Gapdh. Bars represent mean ± s.e.m. E) Histogram shows the expression of five genes (previously reported to be either YAP or TAZ regulated) 72 hours after control, YAP or TAZ siRNA transfection of CAF#1. One representative experiment from a total of 2 performed in triplicate using Lamc2 as housekeeping gene. *** P < 0.001 – unpaired t-test.
**Figure S4** Role of YAP in CAFs. A) Histogram shows invasion of 4T1 cells when co-cultured in organotypic models with CAF#2 transfected with either control, YAP, TAZ or YAP and TAZ smart-pool siRNA. Bars represent mean ± s.e.m of 5 experiments. B) Histogram shows normalised invasion of 4T1 cells when co-cultured in organotypic models with NF#1 transfected with either control, MST1, MST2, or MST1&2 siRNA. Bars represent mean ± s.e.m of 5 experiments. C) Knock-down efficiency of the indicated siRNAs (related to Figures 5b&c) as measured by QRT-PCR normalised to Lamc2. Results from a single experiment. D) Chart shows quantification of 4T1 invasion when co-cultured with CAF#1 transfected with the indicated single siRNAs targeting ANLN, DIAPH3 and FLNA. Bars represent the mean ± s.e.m (n=8 organotypic assays) (AU: please define what n represents). E) Left chart shows quantification of 4T1 invasion when co-cultured with CAF#1 transfected with the indicated single siRNAs targeting MYL9/MLC2 and MYH10. Right chart shows matrix remodelling by CAF#1 following transfection with the same single siRNAs. Bars represent mean ± s.e.m of 3 experiments. Additional data in Table S2 (Statistics Source Data). Western blots show knock-down efficiency of MYL9 and MYH10 siRNAs. siRNA against YAP was included as a positive control. F) Western blots show levels of MLC2(MYL9), YAP, and TAZ in CAF#2 and VCAF transfected with either control, YAP, TAZ or YAP and TAZ smart-pool siRNA. * P < 0.05, ** P < 0.01, *** P < 0.001 – unpaired t-test.
**Figure S5** Effects of conditioned media and inhibitors. A) Representative images showing the invasion of 4T1 cells cultured in the presence of NF#1 previously cultured in normal media (NM) or 4T1 conditioned media (4T1 CM). Control experiment shows the invasion of 4T1 cells without fibroblasts. B) Quantification of 4T1 invasion when co-cultured with control (NM) and 4T1 CM NF#1. Bars represent mean ± s.e.m of 5 experiments. C) Quantification of 4T1 invasion into matrices previously remodelled by NF#1 cultured in NM or 4T1 CM. Bars represent mean ± s.e.m of 5 experiments. D) Panels show F-actin staining in CAF#1 treated with 10μM Y27632, 25μM blebbistatin, or 10μM PP2. Scale bar is 50μm. E) Panels show pY416-Src, pS19-MLC2, pT183/181-MST1/2, pS127-YAP and tubulin in CAF#1 following treatment with 25μM blebbistatin, 10μM Y27632, or 500nM Dasatinib. **P < 0.01 – unpaired t-test.
Blots from Figure 1

Fig 1b – αSMA
Fig 1b – FN1
Fig 1b – S100A4
Fig 1b – Tubulin

Fig 1d – pMLC2
Fig 1b – MLC2
Fig 1b – MYH9
Fig 1b – MYH10

Blots from Figure 2

Fig 2c – pMST1/2
Fig 2c – pS909-LATS
Fig 2c – pT1079-LATs
Fig 2c – pS127-YAP
Fig 2c – YAP

Fig 2d – TEAD4
Fig 2d – pan14-3-3
Fig 2d – YAP

Lysates
IP
αYAP
Lysates
IP
αYAP
Lysates
IP
αYAP
Lysates
IP
αYAP
Lysates
IP
αYAP
Lysates
IP
αYAP

Blots from Figure 5 & Supp Figure 4

Supp Fig 4g & Fig 5d – MLC2
Supp Fig 4g & Fig 5d – YAP
Supp Fig 4g & Fig 5d – TAZ
Supp Fig 4g & Fig 5d – Tubulin

Figure S6 Original un-cropped Western Blots.
Supplementary table legends

**Supplementary Table 1:** GSEA analysis of different fibroblasts. Table shows the p-values for enrichment of different gene sets in hyperplasia, adenoma and carcinoma associated fibroblasts from MMTV-PyMT mice when compared to normal fibroblasts. Comparison is based on 3 NFs vs. either 1 HpAF, 1 AdAF or 4 CAFs. Green boxes indicate significant positive enrichment and red text indicates negative enrichment. Reference to each gene set is indicated. n.s., non-significant.

**Supplementary Table 2:** Statistics Source Data. This file provides all the source data for all the experimental points of the indicated figures.

**Supplementary Table 3:** Antibodies. This table describes all the antibodies used in this study. This includes Name, company, catalogue number and working dilutions (for IF, WB and IHC).

**Supplementary Table 4:** Gene-sets for GSEA analysis. Name, reference and gene list of all the gene sets used in this study.

**Supplementary Table 5:** Primers. Gene, sequences (forward and reverse), product size and target gene full name of the genes analysed by qRT-PCR, both for human or murine genes.

**Supplementary Table 6:** siRNAs. Sequence, species and catalogue number of the single siRNA used in the study.