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The Reprogramming of Tumor Stroma by HSF1 Is a Potent Enabler of Malignancy

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SUMMARY

Stromal cells within the tumor microenvironment are essential for tumor progression and metastasis. Surprisingly little is known about the factors that drive the transcriptional reprogramming of stromal cells within tumors. We report that the transcriptional regulator heat shock factor 1 (HSF1) is frequently activated in cancer-associated fibroblasts (CAFs), where it is a potent enabler of malignancy. HSF1 drives a transcriptional program in CAFs that complements, yet is completely different from, the program it drives in adjacent cancer cells. This CAF program is uniquely structured to support malignancy in a non-cell-autonomous way. Two central stromal signaling molecules—TGF-β and SDF1—play a critical role. In early-stage breast and lung cancer, high stromal HSF1 activation is strongly associated with poor patient outcome. Thus, tumors co-opt the ancient survival functions of HSF1 to orchestrate malignancy in both cell-autonomous and non-cell-autonomous ways, with far-reaching therapeutic implications.

INTRODUCTION

Cancer cells in a tumor mass are surrounded by a variety of other cell types, including immune cells, fibroblasts, and endothelial cells as well as extracellular matrix (ECM) components. Taken together, these comprise the tumor microenvironment. Cells of the tumor microenvironment contribute to the hallmarks of cancer, and their coevolution with cancer cells is essential for tumor formation and progression (Bissell and Hines, 2011; Hanahan and Weinberg, 2011).

In the majority of carcinomas, the most abundant cells in the tumor microenvironment are cancer-associated fibroblasts (CAFs) (Hanahan and Coussens, 2012; Hanahan and Weinberg, 2011). CAFs include myofibroblasts and reprogrammed variants of normal tissue-derived fibroblasts that are recruited by the tumor to support cancer cell proliferation, angiogenesis, invasion, metastasis, and drug resistance (Erez et al., 2010; Kalluri and Zeisberg, 2006; Olumi et al., 1999; Straussman et al., 2012; Wilson et al., 2012). CAFs support cancer cells in a non-cell-autonomous manner through secretion of ECM, chemokines, cytokines, and growth factors (Lu et al., 2012; Moskovits et al., 2006; Orimo et al., 2005; Pickup et al., 2013; Siegel and Massagué, 2003). The secretion of cytokines also feeds back to promote the fibroblast-to-CAF transition, through autocrine transforming growth factor β (TGF-β) and stromal-derived factor 1 (SDF1) signaling (Kojima et al., 2010).

Despite accumulating evidence for the non-cell-autonomous effects of CAFs on cancer cells, little is known about the transcriptional regulators that are responsible for stromal reprogramming to support tumorigenesis. That such reprogramming must occur is clear from evidence that normal fibroblasts usually constitute a tumor-restrictive environment (Bissell and Hines, 2011). In mouse models, tumor suppressors such as p53 and PTEN can act in the stroma to limit tumor growth (Lujambio et al., 2013; Moskovits et al., 2006; Trimboli et al., 2009). If tumor suppressors act in both the cancer cells and the stroma to inhibit malignancy, might there also be factors that actively support or enable malignancy in both cancer cells and in the stroma? Presumably, these would not be classical oncogenes because nonmalignant stromal cells are relatively stable genetically (Qiu et al., 2008). Instead, we wondered if tumors might hijack normal physiological pathways and programs in the stroma, subverting

them to enable neoplastic growth and metastatic dissemination. Here, we provide evidence for such a mechanism by investigating the stromal function(s) of heat shock factor 1 (HSF1) in tumor biology.

HSF1 is a ubiquitously expressed transcription factor best known for its activation by heat (Sakurai and Enoki, 2010; Shamovsky and Nudler, 2008). Recently, it has been shown to play a fundamental role in tumor biology (Dai et al., 2007; Jin et al., 2011). In a wide variety of human cancer cell lines, the depletion of HSF1 markedly reduces growth, survival, and metastatic potential (Mendillo et al., 2012; Meng et al., 2010; Santagata et al., 2012; Scott et al., 2011). *Hsf1* null mice develop normally but are profoundly resistant to tumorigenesis.

The transcriptional program that is activated by HSF1 in cancer cells is surprisingly different from the program activated by classical heat shock (Mendillo et al., 2012). In particular, it acts to support the malignant state by blunting apoptotic responses and promoting pathways that facilitate anabolic metabolism, protein folding, proliferation, invasion, and metastasis (Dai et al., 2012; Fang et al., 2012; Jin et al., 2011; Mendillo et al., 2012; Meng et al., 2010; Santagata et al., 2013; Scott et al., 2011). In humans, activation of this program by HSF1 in cancer cells is strongly associated with disease progression in patients with breast, colon, lung, and hepatocellular carcinomas (Fang et al., 2012; Mendillo et al., 2012; Santagata et al., 2011).

Clearly, HSF1 plays a central role in supporting the malignant transformation and progression of diverse cancer types. Here, we ask if it plays a complementary, and perhaps equally important, role in subverting the normally repressive activity of the stroma by converting it to a protumorigenic state. We also discuss the possible evolutionary origins of HSF1-mediated crosstalk between cancer and stromal cells in tumors, as well as its potential therapeutic implications.

RESULTS

HSF1 Is Activated in CAFs within Human Tumors

Under basal conditions in normal cells, HSF1 resides primarily in the cytoplasm. Upon activation, it accumulates in the nucleus (Morimoto, 2008; Santagata et al., 2011). To determine whether HSF1 is activated in cells of the tumor microenvironment, we scored the staining intensity of this transcription factor in the nuclei of tumor-associated stroma within patient-derived breast cancer samples. Stromal cells residing in the lobules of neighboring, normal breast tissue were used for comparison. These normal cells were almost invariably low or negative for nuclear HSF1. However, strong nuclear HSF1 staining was frequently present in stromal cells situated in close proximity to malignant cells (Figures 1A, upper panel, and 1B).

The morphology of the HSF1-positive stromal cells suggested that they were CAFs. To confirm, we costained tumor sections for HSF1 and smooth muscle actin (SMA). SMA stains normal myoepithelial cells (Figure 1A, lower-right panel). It is not present in normal fibroblasts, however, and is often used as a marker for stromal CAFs (Kalluri and Zeisberg, 2006; Quante et al., 2011). We also investigated markers of two other stromal components: leukocytes (LCA) and endothelial cells (CD31). Most of the HSF1-positive stromal cells in the tumors costained with SMA, suggesting that they are indeed CAFs (Figure 1A, lower-left panel; Figure S1 available online).

To test the generality of HSF1 activation in CAFs across different tumor types, we costained tumor sections from lung, skin, esophageal, colon, gastric, and prostate carcinomas with antibodies for HSF1 and SMA (Figure 1C). In all of these, most SMA-positive CAFs also had strong nuclear HSF1 staining.

Loss of *Hsf1* in Fibroblasts Reduces Xenograft Tumor Growth

To explore the function of stromal HSF1 activation in a tractable model system, we analyzed xenografts of human MCF7 breast cancer cells injected subcutaneously into immunocompromised (nonobese diabetic [NOD]-severe combined immunodeficiency [SCID]) mice. As expected, xenografts recruited endogenous stromal cells from their mouse hosts to support tumor formation. These SMA-positive CAFs exhibited strong nuclear HSF1 staining (Figure S2A).

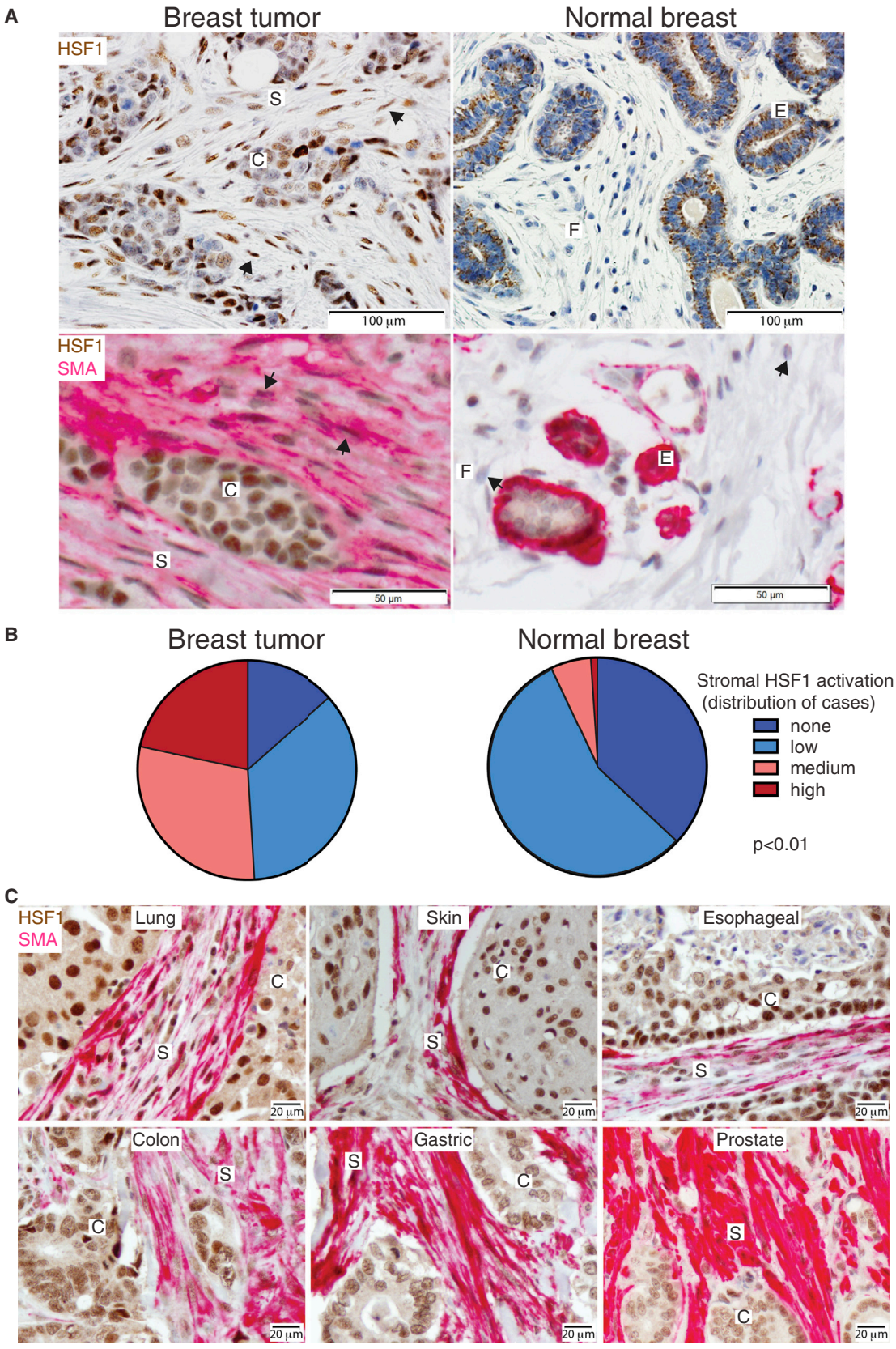
To test whether HSF1 activation in stromal cells plays a role in supporting malignant cells, we mixed primary mouse embryonic fibroblasts (MEFs)—either wild-type (WT) or *Hsf1* null—with the MCF7 cancer cells and coinjected them subcutaneously into NOD-SCID mice (Figure 2A). Tumors arising from MCF7 cells coinjected with *Hsf1* null MEFs (Figure 2A, blue) grew significantly more slowly than those of mice coinjected with WT MEFs (Figure 2A, red).

MCF7 cells injected without MEFs formed tumors more slowly than MCF7 cells coinjected with WT MEFs (Figure 2A). With time, cells injected without MEFs recruited WT host stroma, and the tumors grew to the same size as those formed by coinjection with WT MEFs. However, throughout the experiment, tumors formed by MCF7 cells coinjected with *Hsf1* null MEFs remained significantly smaller.

To better understand this result, we excised tumors at the end of the experiment and examined their histology (Figure 2B, upper panels). Tumors from mice injected with MCF7 cells only and tumors from mice coinjected with WT MEFs shared a poorly differentiated and sheet-like morphology typical of high-grade tumors. In contrast, coinjection of MCF7 cells and *Hsf1* null MEFs produced tumors with a more differentiated, stromal-rich architecture, indicative of a less malignant phenotype. Notably, some of the stromal fibroblasts were HSF1 positive, indicating that in addition to the injected MEFs, the tumors had recruited host fibroblasts (Figure S2B). (This might explain why, even though slower, these tumors still grew.) Masson's trichrome staining indicated that stroma-rich regions are mostly comprised of fibrous tissue and deposits of collagen (Figure 2B, lower panels). These results suggest that, in response to cancer cells, HSF1 is activated in stromal CAFs to support tumor growth. Moreover, in the absence of this HSF1-driven response, fibroblasts actually exert an inhibitory effect on tumor expansion.

Stromal HSF1 Regulates Cancer Cell Growth In Vitro

To learn how activation of HSF1 in stromal fibroblasts supports cancer cells, we plated fluorescently labeled breast cancer cells onto feeder layers of either WT or *Hsf1* null MEFs (Figures 3A and S3A). We found a higher number of cancer cells in cocultures with WT MEFs than with *Hsf1* null MEFs. This held true for several



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different mammary cancer cell lines (mouse D2A1, Figures 3A and 3B; human MCF7, human HCC38, and mouse 4T7; Figures S3B and S3C).

To confirm true HSF1 dependence, we used MEFs that were deleted for WT *Hsf1* but expressed a tetracycline-repressible *Hsf1* transgene (Bi-TetO-*Hsf1*). We repressed *Hsf1* expression for 5 days and then cocultured the MEFs with D2A1 cancer cells. Repression of *Hsf1* resulted in decreased accumulation of cancer cells (Figure S3D). Thus, even short-term loss of *Hsf1* impairs the ability of fibroblasts to support the growth of cocultured cancer cells.

Stromal HSF1 Drives a Transcriptional Program in Cancer Cells that Promotes Malignant Phenotypes

To test the effects of coculture on gene expression, we separated cancer cells from fibroblasts by fluorescence-activated cell sorting (FACS), extracted RNA, and hybridized it to gene expression arrays. As a point of comparison, each cell type was grown alone (without coculture), treated, and analyzed in a similar manner.

In D2A1 cancer cells, regardless of the *Hsf1* status of the cocultured MEFs, the expression of ~700 genes was altered by ≥2-fold following coculture (Figure 3C, group b; Table S1). Of these, ~400 genes were upregulated, and ~300 genes were downregulated. The upregulated set was enriched for genes involved in cellular differentiation, migration, and ECM organization. No significant functional enrichment was found in the downregulated set.

With specific regard to the *Hsf1* status of the MEFs, approximately 200 genes were upregulated in cancer cells cocultured with WT, but not with *Hsf1* null MEFs (Figure 3C, group a). This set was enriched for genes involved in ECM organization, development, and adhesion (e.g., *Dmp1*, *Dkk3*, *Thy1*, *Grem1*, *Sparc*, *Mmp2*, and *Mmp3*; Figure 3D; Table S1). In cancer cells cocultured with *Hsf1* null MEFs, ~750 genes were uniquely upregulated (Figure 3C, group c). Proinflammatory cytokines (e.g., *Ccl5* and *Ccl8*) and immune responses (e.g., the response to type 1 interferon) were most significantly enriched in this group (Figure 3D; Table S1). Thus, activation of HSF1 in the stroma helps to reprogram cancer cells in at least two important ways. In a non-cell-autonomous manner, it upregulates genes in cancer cells that enhance their malignant potential and downregulates genes that would trigger host immune defense responses.

Stromal HSF1 Drives a Transcriptional Program in Fibroblasts that Supports Malignant Cells

Next, we examined a complementary question: how does coculture with cancer cells affect HSF1-dependent gene expression in stromal fibroblasts? Profiling of FACS-sorted MEFs showed that even in the absence of cancer cells, HSF1 regulated many genes involved in development, cell adhesion, and proliferation (e.g., *Fgf*, *Igf*, *Col*, *Lama*, *Snail*, and *Sdf1*; Figure 3E, group 1; Tables S2 and S3). This suggests that HSF1 alters the basal phenotype of MEFs in culture, and these alterations enhance the growth of cancer cells. In an HSF1-dependent manner, coculture with cancer cells induced an additional cluster of genes involved in development, proliferation, and response to wounding (e.g., *Tgf-β1*, *Cxcl1*, *Cxcl3*, and *Vcam1*; Figure 3E, group 4; Tables S2 and S3). Also in an HSF1-dependent manner, cancer cells induced in MEFs a striking downregulation of genes involved in cellular immune responses (e.g., *Cxcl10*, *Bst2*, and *C3*; Figure 3E, group 3; Tables S2 and S3). Thus, WT MEFs respond to cancer cells in a manner that supports tumor growth, whereas *Hsf1* null MEFs respond in a manner likely to impede the process.

To further characterize the HSF1 stromal signature, we performed additional analyses of the genes that are differentially upregulated in WT versus *Hsf1* null MEFs cocultured with cancer cells (Figure 3E, groups 1 and 4). We compared this list to publicly available gene sets of stroma from human cancer patients, fibroblast wound-healing responses, and the heat shock response. Although some heat shock-related genes were enriched, this was not the most prominent response. Rather, the HSF1 stromal signature was most highly enriched for genes previously characterized by their upregulation in fibroblasts in response to wounding and in stromal cells isolated from human tumors (Beck et al., 2008; Chang et al., 2004; Dvorak, 1986; Karnoub et al., 2007) (Figure 3F; Table S4). We also compared this list to the HSF1-dependent gene expression signature in cancer cells (Mendillo et al., 2012) and found that these signatures were, if anything, anticorrelated (Table S4). Thus, in fibroblasts, HSF1 activates a transcriptional program likely to support tumor progression, which is profoundly different from the response activated by HSF1 in the cancer cells themselves or in cells exposed to heat.

The Effects of Stromal HSF1 Activation on Cancer Cells Are Mediated by TGF-β and SDF1 Signaling

Unbiased analysis of gene set enrichment established that TGF-β signaling was one of the top categories regulated by HSF1 in MEFs cocultured with cancer cells (group 4 in Figure 3E; Table S5). Because TGF-β, along with SDF1, was previously found to promote CAF phenotypes (Kojima et al., 2010), we further interrogated both signaling pathways. We extracted RNA from both immortalized and three separate sets of primary WT or *Hsf1* null MEFs and performed quantitative PCR (qPCR) with primers

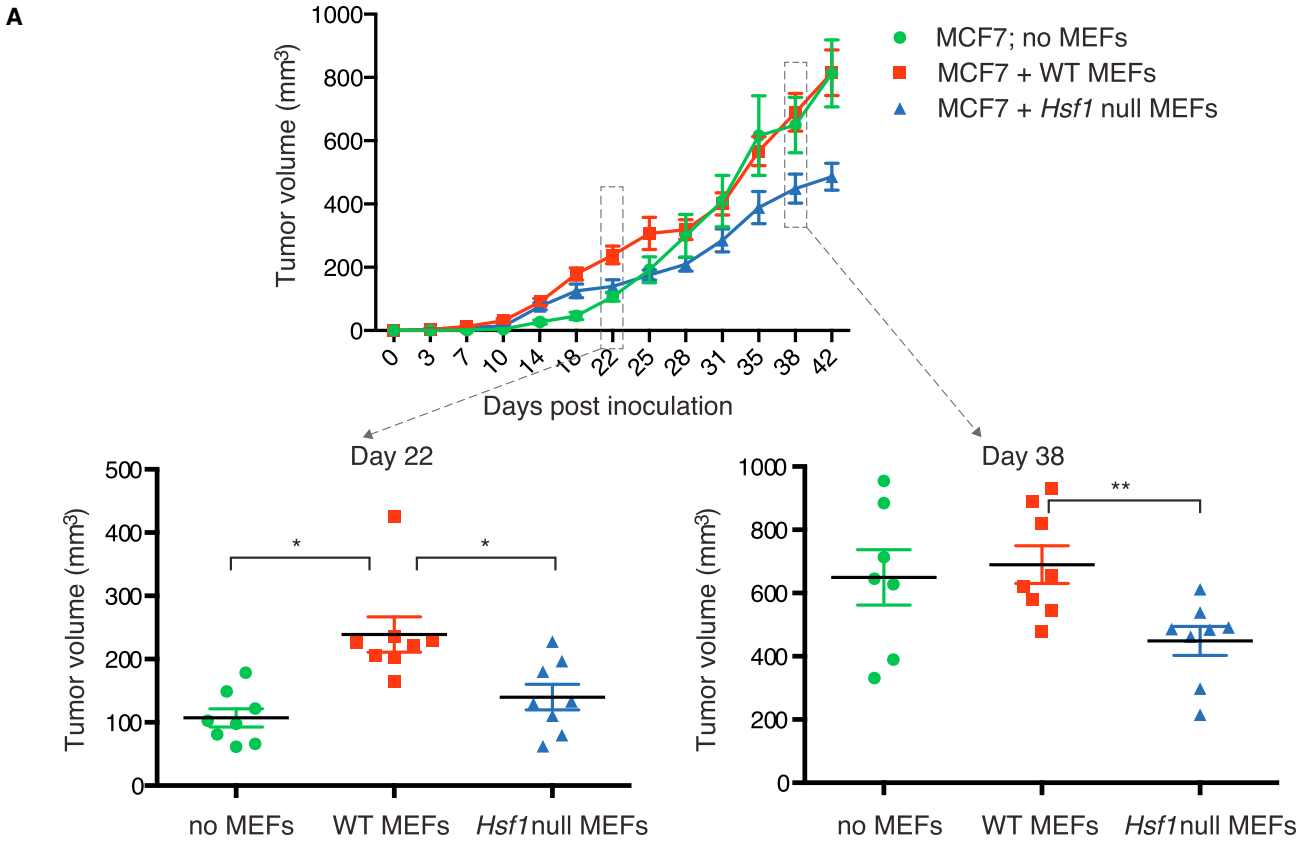
Figure 1. HSF1 Activation in CAFs within Human Tumors

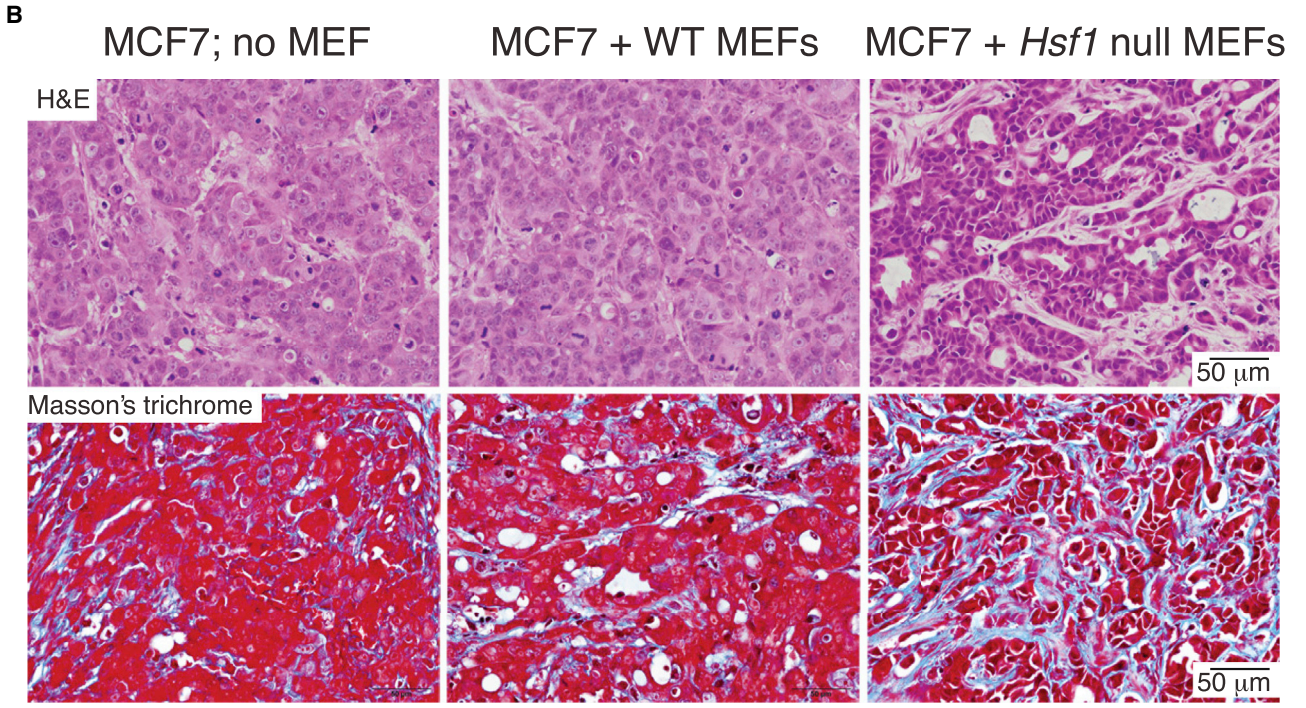
(A) Tissue sections of breast resection specimens from 12 patients encompassing both invasive ductal carcinoma and neighboring normal breast lobules (in the same section) were immunostained with anti-HSF1 antibodies (brown, upper panels) or costained with anti-HSF1 and anti-SMA (pink) antibodies (lower panels). Representative images are shown. Arrows indicate HSF1-positive CAFs in the left panels and HSF1-negative normal fibroblasts in the lower panels. C and S indicate cancer- and stroma-rich regions, respectively. For normal tissue, E and F indicate regions rich with epithelial cells or fibroblasts, respectively.

(B) Pie charts depict the distribution of relative nuclear HSF1 staining intensity in the stroma among 12 breast resection specimens with matching controls. For each specimen, four regions of tumor or normal tissue were evaluated. Statistical significance of the differences between normal and tumor was assessed using repeated measures ANOVA ($p = 4 \times 10^{-13}$), as well as paired t tests, followed by Bonferroni correction ($p < 0.01$).

(C) Representative images of tumor sections from patients with the indicated types of cancer costained for HSF1 (brown) and SMA (pink).

See also Figure S1.





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targeting *Tgf-β1*, *Tgf-β2*, *Tgf-β3*, and *Sdf1*. This confirmed that expression levels of *Sdf1*, *Tgf-β1*, and *Tgf-β2* were significantly lower in *Hsf1* null MEFs than in WT MEFs, even without coculture with cancer cells (Figures 4A and S4A).

Next, we asked if TGF-β and SDF1 mediate HSF1's stromal support of cancer cells. We added these factors as purified recombinant proteins to cocultures of D2A1 cancer cells with *Hsf1* null MEFs. Combined addition of TGF-β1 and SDF1 restored cancer cell growth to levels achieved by coculture with WT MEFs (Figures 4B and 4C). Partial effects, which did not reach statistical significance, were achieved by addition of either factor alone (Figure S4B).

As a further functional test, we repressed TGF-β signaling in cocultures by adding a TGF-β receptor type I (TβRI)/TβRII dual inhibitor, LY2109761, to the media (Dituri et al., 2013). To control for direct effects on cancer cells themselves, we treated cancer cells with the inhibitor in the absence of MEFs. Treatment with LY2109761 did not affect cancer cells grown alone (Figure S4C). It did, however, significantly reduce their growth in coculture with WT MEFs (Figure 4D; $p = 0.008$). A smaller effect, which did not reach statistical significance, was seen in coculture with *Hsf1* null MEFs (Figure 4D; $p = 0.1$). Taken together with the increased expression of *Tgf-β* and *Sdf1* in WT MEFs compared to *Hsf1* null MEFs (Figure 4A), these results suggest that TGF-β and SDF1 are produced and secreted by fibroblasts in an HSF1-dependent manner.

Once secreted, TGF-β and SDF1 could activate the fibroblasts themselves, the cancer cells, or both. To investigate, we knocked down the expression of several signaling molecules downstream of TGF-β and SDF1 in either cancer cells or MEFs (see Experimental Procedures). Knockdown of *Smad2*, a key downstream mediator of TGF-β signaling, in WT MEFs impaired the growth of cocultured D2A1 cancer cells (Figures 4E and S4E). This growth defect could not be rescued by addition of recombinant TGF-β1 and SDF1 (Figure 4E). Notably, *Smad2* knockdown was only effective in the MEFs. Knockdown of the same gene in the D2A1 cells themselves had no effect on cell number (Figures S4D and S4E). We conclude that HSF1 supports an autocrine TGF-β signaling loop in MEFs. As for SDF1, although we cannot discriminate whether it signals to the cancer cells or to the stroma, SDF1 expression is clearly upregulated by HSF1 in fibroblasts. Taken together, our data indicate that TGF-β and SDF1 are key mediators of the tumor-promoting activity of stromal HSF1.

HSF1 Directly Binds HSEs of the *Sdf1* Gene in Stromal Cells

Next, we asked whether TGF-β and SDF1 are direct transcriptional targets of HSF1. A bioinformatic search for HSF1-binding elements (HSEs) in genes of the TGF-β and SDF1 signaling pathways confirmed that the *Tgf-β2* and *Sdf1* genes themselves contain canonical HSEs. No HSEs were found in *Tgf-β1* or any of the downstream signaling molecules mentioned above. To determine whether HSF1 directly regulates *Tgf-β2* and *Sdf1* expression in CAFs, we performed chromatin immunoprecipitation (ChIP) using anti-HSF1 antibodies and extracts prepared from MCF7 tumor xenografts. To focus specifically on the supporting mouse stromal cells, and not the human cancer cells, we performed qPCR using primers flanking potential HSF1-binding sites that were specific to the mouse genes (Figure S4F). Primers for an intergenic region served as a negative control. *Sdf1* was significantly amplified from stromal (mouse) DNA bound by HSF1 (Figure 4F). No significant amplification was detected for *Tgf-β2*. Together with the effects of HSF1 seen on expression of these genes, these data suggest that regulation of *Tgf-β* by HSF1 may be indirect. However, HSF1 directly binds and activates *Sdf1*.

HSF1 Activation in Breast Cancer Stroma Is Associated with Poor Patient Outcome

Our findings in mouse xenografts and in vitro coculture models indicate that stromal HSF1 contributes to tumor progression. To evaluate whether stromal HSF1 contributes to disease progression in human cancers, we first asked whether *HSF1* mRNA levels in the stroma correlate with disease outcome. We looked for this association in a publicly available mRNA data set from 53 pure tumor stroma samples obtained from patients with primary breast tumors (stromal cells were separated from cancer cells by laser microdissection; Finak et al., 2008).

In this data set, high *HSF1* levels significantly correlated with increased tumor grade (Figure 5A) and poorer patient outcome (Figure 5B). We further asked whether high stromal *Hsf1* expression is associated with a specific breast cancer subtype. No significant association was found with estrogen receptor (ER; Figure S5A) or progesterone receptor (PR; Figure S5B) expression. (The number of triple-negative tumors in this cohort was too small to determine a possible association with *HSF1* expression.) *HSF1* expression was, however, significantly higher in HER2-positive tumors as compared to HER2-negative tumors (Figure 5C).

HSF1 is often activated posttranscriptionally without a change in its mRNA level. To provide an independent assessment of the importance of its activation in breast cancer stroma, we assembled a breast cancer cohort to evaluate HSF1 activation at the protein level by immunohistochemistry (IHC). We examined a total of 46 samples from patients with early-stage breast cancer (Table S6), for whom we had both appropriate tissue sections as well as a minimum of 8 years of continuous clinical follow-up.

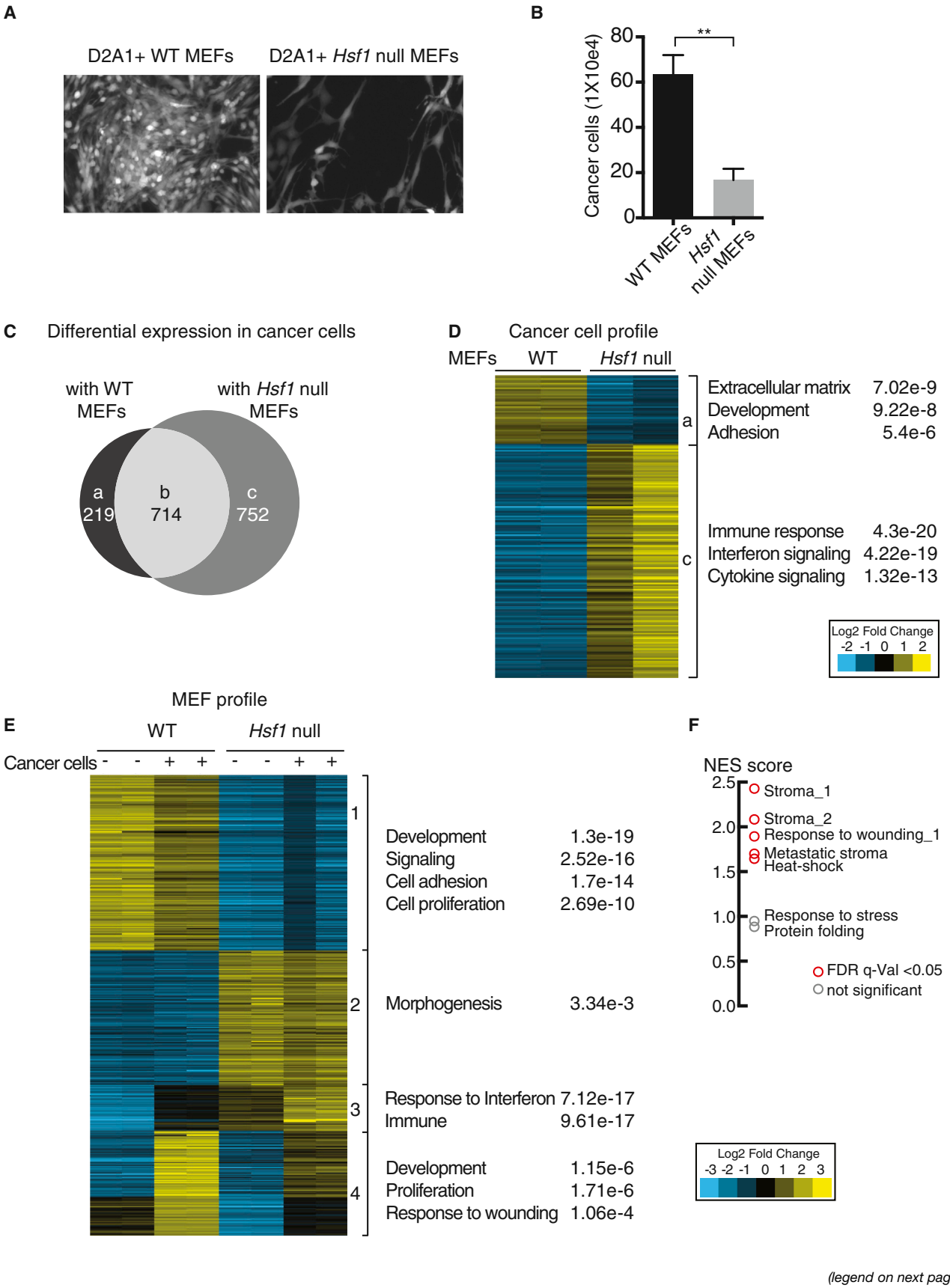
Figure 2. Stromal *Hsf1* Status Alters Tumor Progression and Histology in Human Breast Xenografts

MCF7 breast cancer cells alone or mixed with WT or *Hsf1* null primary MEFs were injected subcutaneously into NOD-SCID mice. The experiment was repeated twice, with four mice per group in each experiment.

(A) The mean tumor volume (total eight per treatment group) is shown. The distribution of individual measurements is shown in the lower panels, in scatterplots for days 22 and 38 postinjection. Error bars, SEM. *$p < 0.05$; **$p < 0.01$.

(B) Mice were sacrificed when tumor burden reached size limit, and the tumors were excised, fixed, and stained with hematoxylin and eosin (H&E; upper panels) or Masson's trichrome stain (lower panels). All images were collected at the same magnification. Scale bar, 50 μm.

See also Figure S2.



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Tumor sections were scored in a blinded manner for nuclear HSF1 staining intensity in the cancer cells and in the stromal cells.

We found markedly reduced disease-free survival, as well as overall survival, in patients whose tumors had high stromal HSF1 activation (Figures 5D and S5C). In this cohort, HSF1 activation in stromal cells was correlated with HSF1 activation in the cancer cells ($p = 0.01$, chi-square test). Indeed, high HSF1 activation in the cancer cells also correlated with lower overall survival, consistent with our previous findings (Mendillo et al., 2012; Santagata et al., 2011). However, the association with patient outcome was weaker in cancer cells than in the stroma (Figures S5D and S5E). Moreover, in a multivariate model considering the independent contributions of HSF1 activation in the cancer cells and in the stroma to overall survival, only stromal HSF1 (and not cancer cell HSF1) was a significant predictor of survival (Table S6). Stromal HSF1 was also an independent, significant predictive factor in a multivariate model considering various clinicopathologic factors (Table S6). The significant association of stromal HSF1 activation with poor patient outcome seen in two independent cohorts using very different methodologies suggests that stromal HSF1 could be a useful, independent prognostic indicator in breast cancer.

HSF1 Activation in Early-Stage Lung Cancer Stroma Is Associated with Poor Outcome

Might stromal HSF1 serve as a potential prognostic marker in other tumor types? Our initial survey of human cancers showed that HSF1 is activated in the CAFs of many tumor types, including lung, colon, skin, esophageal, gastric, and prostate (Figures 1C and 6A). Of these tumor types, we had access to a cohort of lung cancer patients with appropriate tissue samples for stromal assessment of HSF1, together with clinical follow-up data. Encouraging the analysis of this data set, pilot testing of human non-small cell lung cancer (NSCLC) lines (A549 and H1703) showed that these lines grew more robustly when cocultured with WT MEFs than when cocultured with *Hsf1* null MEFs (Figure S6A).

A total of 72 samples from patients with stage I non-small cell lung adenocarcinoma (Table S7) (Sholl et al., 2010) were scored in a blinded manner for HSF1 activation (nuclear staining intensity) in cancer cells and stromal cells. Patients with stage I NSCLC have a 5-year survival of 60%–70% (Goldstraw et al., 2007). Stromal HSF1 activation did not correlate with demographic factors such as age, sex, or smoking status (Table S7). It did, however, show a significant correlation with patient outcome.

Disease-free survival was significantly shorter in lung cancer patients whose tumors had either high or intermediate HSF1 activity in the stroma (Figure 6B). A similar trend was found for survival of patients with high HSF1 in the cancer cells (Figure S6B). In this cohort, HSF1 activation in the cancer cells did not correlate with HSF1 activation in the stroma ($p = 0.28$, chi-square test). We therefore asked if evaluation of HSF1 activation in both cell types could improve our ability to predict patient outcome. Although the number of patients is small, it is striking that there was not a single recurrence in any of the patients that had low HSF1 activity in both the cancer cells and in the stroma over the course of follow-up (Figure 6C).

To assess the independent contributions to outcome of increased HSF1 activation in cancer cells versus stromal cells, we fitted a multivariate Cox proportional hazards regression model to recurrence-free survival, considering stromal HSF1 activation separately from cancer cell activation. Cancer cell HSF1 was not independent from stroma in its association with disease progression. However, as in our breast cancer cohort, stromal HSF1 activation was significantly and independently associated with disease-free survival (Table S7).

To further refine our analysis, we genotyped the collection of tumor samples for the most commonly mutated oncogenes in lung adenocarcinoma, *KRAS* and *EGFR* (Pillai and Ramalingam, 2014), and tested the association of HSF1 activation and disease outcome with different mutations. In the 52 samples from our cohort that were successfully genotyped, *KRAS* mutations, but not *EGFR* mutations, correlated with poor disease-free survival (Figures S6C and S6D). We found no correlation between HSF1 activation (in either cancer cells or stroma) and *KRAS* or *EGFR* mutations per se. We did, however, find a significant association between high activity of HSF1 in the stroma and poor outcome in patients with *KRAS* mutant tumors (Figure 6D). Moreover, stromal HSF1 (but not cancer cell HSF1) was an independent predictor of progression-free survival in several multivariate

Figure 3. HSF1 in Fibroblasts Supports Cancer Cell Growth by Activating Gene Expression Programs Both in Cancer Cells and in Fibroblasts

(A and B) WT or *Hsf1* null immortalized MEFs were treated with 10 μg/ml mitomycin C. D2A1 mouse mammary tumor cells stably expressing dsRed (D2A1-dsRed) were seeded on top of the MEFs and allowed to grow for 72–96 hr, after which cancer cells were either visualized by fluorescent microscopy (A) or trypsinized and quantitated by flow cytometry (B). The mean of three independent experiments is shown. Error bars, SEM. $^{**}p < 0.005$.

(C and D) Total RNA was purified from duplicate cultures of D2A1 cancer cells grown with or without WT or *Hsf1* null MEFs and sorted as described above. RNA was hybridized to Agilent microarrays, and relative gene expression levels were analyzed using cluster 3.0. For each gene, expression in D2A1 cells grown alone was set to 1, and the relative change in expression upon coculture with WT or *Hsf1* null MEFs was calculated.

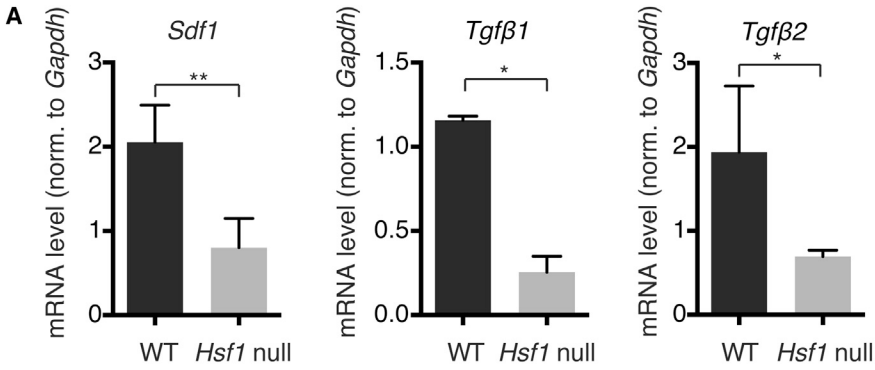
(C) Overlap of genes differentially expressed in D2A1 cancer cells in the presence of WT or *Hsf1* null MEFs.

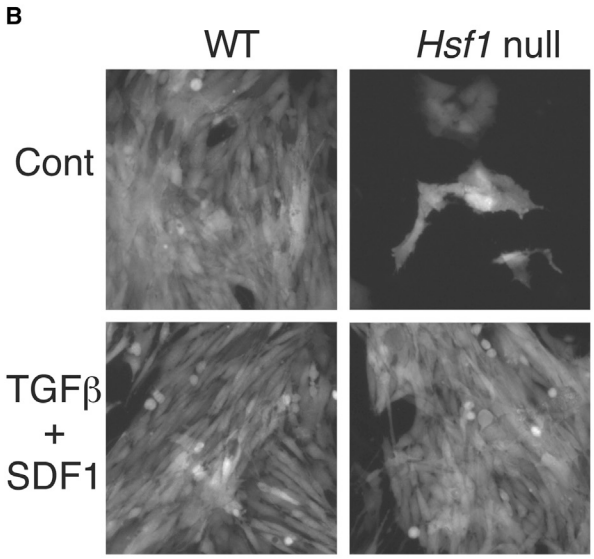
(D) Heatmap depicting fold change in mRNA levels of genes differentially expressed in D2A1 cells grown in coculture with WT versus *Hsf1* null MEFs (in duplicate). Gene Ontology (GO) enrichment is shown to the right of the panel. Groups a and c correspond to groups a and c in (C).

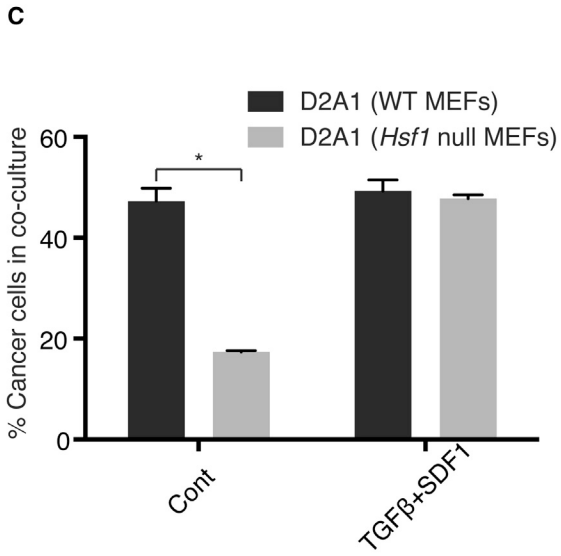
(E) WT or *Hsf1* null MEFs were cocultured with D2A1-dsRed cells as described in (A), but not treated with mitomycin C. After 72–96 hr, cultures were sorted, and mRNA was extracted and hybridized to Agilent microarrays. MEFs cultured without D2A1 cells and processed in the same manner served as controls. Gene expression was analyzed using Cluster 3.0, and the differentially expressed genes were clustered into four groups. GO enrichment is shown to the right of the panel.

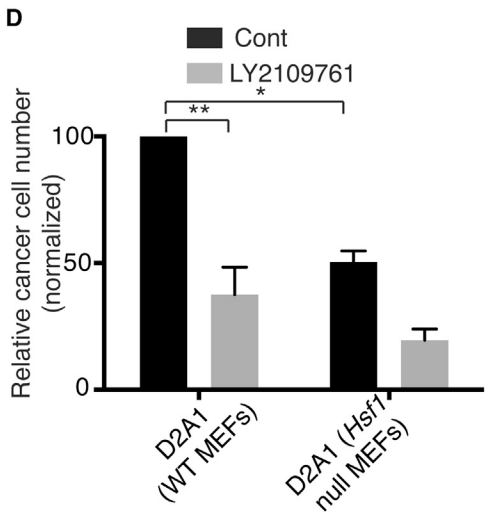
(F) Gene set enrichment analysis of genes upregulated in WT versus *Hsf1* null MEFs cocultured with cancer cells (groups 1 and 4 in E). Enrichment was calculated for the indicated gene sets and is presented as normalized enrichment score (NES). Statistically significant enrichment (false discovery rate [FDR] q value < 0.05) is shown in red; nonsignificant enrichment is shown in gray.

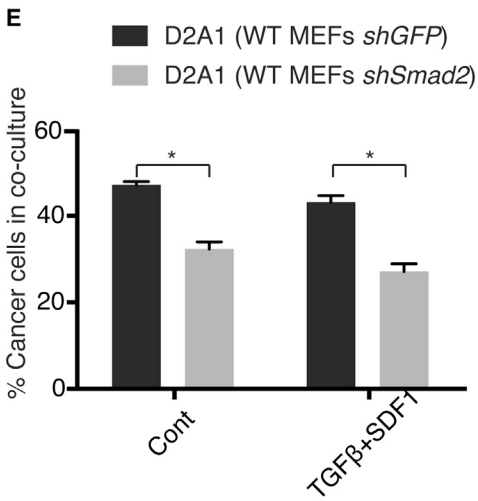
See also Figure S3 and Tables S1, S2, S3, S4, and S5.

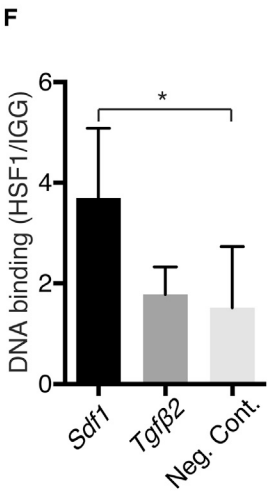












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models considering *KRAS* and *EGFR* mutational status as well as clinicopathologic factors (Table S7). These clinical association data suggest that HSF1 status could serve as a promising independent prognostic marker in lung cancer as well as breast cancer.

DISCUSSION

For cancer cells to proliferate, invade, and metastasize, they must recruit and reprogram nonmalignant stromal cells. We find that HSF1 activation is a key factor in the transcriptional reprogramming of the stroma from a tumor-repressive environment to a supportive one. At least two central signaling pathways in the tumor microenvironment are empowered by HSF1: pathways mediated by TGF-β and by SDF1. Establishing the relevance of our experimental findings to human disease, HSF1 was activated in the stroma of a wide variety of human cancers, and this activation correlated strongly with poor outcome in both lung and breast cancer.

Our work establishes a role for stromal HSF1 in tumor biology that is distinct, yet highly complementary, to its recently established role in malignant cells. HSF1 has historically been viewed as a stress-activated transcription factor. In tumors, stromal and cancer cells alike must cope with a variety of potentially lethal challenges, including oxidative stress, nutrient deprivation, and protein misfolding. Yet, neither the cancer HSF1 program we previously reported in malignant cells (Mendillo et al., 2012) nor the stromal HSF1 program we report here is a simple reflection of these inevitable stresses.

The cancer HSF1 program supports the malignant lifestyle of cancer cells in a multitude of ways, including direct effects on cell cycle, DNA repair, anabolic metabolism, and proliferation (Jin et al., 2011; Mendillo et al., 2012; Meng et al., 2010; Santagata et al., 2013). The stromal HSF1 program drives pathways that are of specific benefit to the malignant elements within the tumor. These pathways facilitate angiogenesis, ECM organization, adhesion, and migration.

Clearly, HSF1 is capable of driving highly divergent transcriptional programs depending on the cellular context. One feature of these programs, which we have begun to unravel, is the way HSF1 responses are coordinated between cancer cells and stroma. We have found that TGF-β and SDF1 are two extracellular mediators of the HSF1 program in CAFs. Although it was previously recognized that these proteins, when secreted by CAFs, enhance the protumorigenic phenotype (Kojima et al., 2010), the factors responsible for their upregulation were unknown. HSF1 has been shown to directly bind to HSEs in the genes of several chemokines (Henderson and Kaiser, 2013; Maity et al., 2011) during heat shock. Conversely, HSF1 can be activated by exposure to cytokines such as TGF-β and interleukin-1β in vitro (Sasaki et al., 2002). Taking these observations together, we suggest that reciprocal interactions between secreted cytokines and intracellular HSF1 programs that are normal responses to fever and infection have been co-opted by diverse cell types in tumors to fuel the malignant state.

But how did such non-cell-autonomous HSF1 programs evolve? The HSF1-dependent heat shock response has traditionally been conceived as an internally driven cellular response to proteotoxic stress. However, recent work in *C. elegans* has established that HSF1 can be activated in a non-cell-autonomous manner. Acute stresses detected by thermosensory neurons can orchestrate HSF1-dependent heat shock responses throughout the animal. This coordinated response benefits the organism as a whole (Prahlad et al., 2008). Similarly, in tumors, cancer cells induce the activation of HSF1 in the stroma, and this activation benefits the tumor as a whole (albeit to the detriment of the patient). But, in addition to this, in stroma, the HSF1-regulated program itself is non-cell autonomous. It results in secretion of factors that act to enhance the survival and proliferation of neighboring cancer cells. We suggest that the interplay between HSF1 responses in cancer cells and stroma has its origins in ancient biological mechanisms that act to promote the survival of multicellular organisms in a non-cell-autonomous way.

The complementary but distinct roles of HSF1 in cancer and stromal cells of tumors have both diagnostic and therapeutic implications. From a diagnostic perspective, assessing HSF1 in both stromal and cancer cells might help to guide treatment choices in early-stage cancers, especially lung cancer, where currently there are no reliable markers for gauging malignant potential other than tumor size. The increased surveillance of

Figure 4. TGF-β and SDF1 Mediate the Support of Cancer Cell Growth by Stromal HSF1

(A) The relative expression of *Sdf1*, *Tgf-β1*, and *Tgf-β2* in WT or *Hsf1* null immortalized MEFs was measured by qPCR and normalized (norm.) to *Gapdh*. The mean of three experiments is shown. Error bars, SEM.

(B and C) WT or *Hsf1* null immortalized MEFs were cocultured with D2A1-dsRed cells as explained in Figure 3A, in the presence or absence of 10 ng/ml TGF-β1 and 100 ng/ml SDF1. After 96 hr, cells were either visualized by fluorescent microscopy (B) or quantitated by flow cytometry (C). The percentage of cancer cells in coculture is presented. The experiment was repeated three times, in triplicate. Representative results of one experiment are shown as mean ± SEM. Cont, control.

(D) Immortalized WT or *Hsf1* null mitomycin-treated MEFs were pretreated, or not, with LY2109761 for 30 min before coculture with D2A1-dsRed cells. Cultures were continued for 72 hr, with daily supplementation of LY2109761 (or not, as control), and then analyzed as in (C). The experiment was repeated three times, in triplicate. Results are expressed as the mean relative number of cancer cells, normalized to nondrug-treated cocultures with WT MEFs. Error bars, SEM.

(E) Immortalized WT MEFs stably expressing small hairpin RNAs targeting *Smad2* (*shSmad2*) or *GFP* (*shGFP*) were cocultured with D2A1 cells, treated and analyzed as in (C). The percentage of cancer cells in the coculture is presented.

(F) ChIP was performed with anti-HSF1 antibodies using material prepared from MCF7 tumor xenografts. Normal rat IgG served as a negative IP control. IPs were analyzed by qPCR with primers targeting potential heat shock elements in mouse *Sdf1* and *Tgf-β2*. Primers targeting an intergenic region in the mouse DNA, not expected to be amplified, were used as a negative control (Neg. Cont.). The experiment was repeated twice; tumors from three mice were used for each experiment. Representative results from one experiment are shown as mean ± SEM.

*$p < 0.05$; **$p < 0.01$. See also Figure S4.

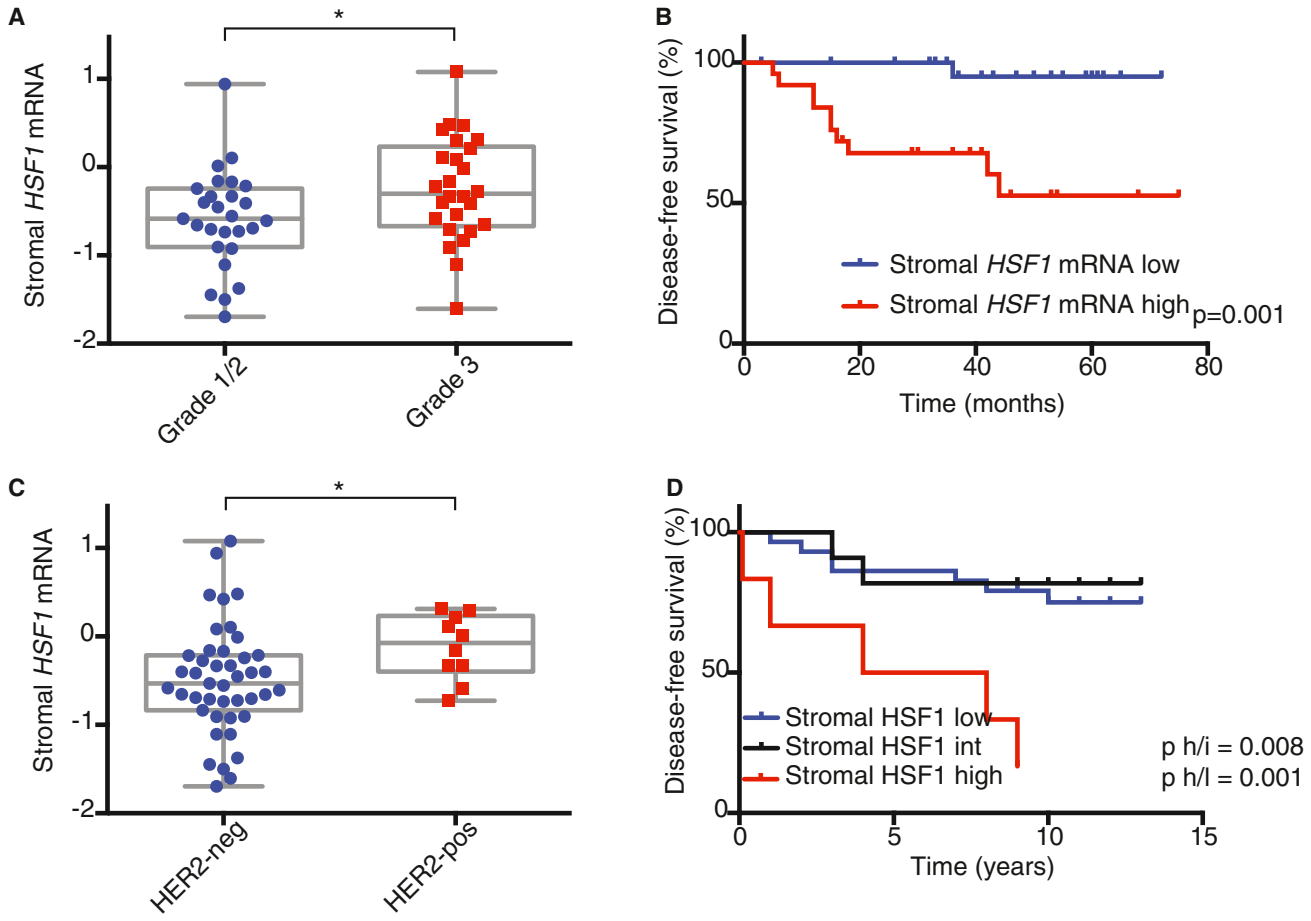

Figure 5. Increased HSF1 Activation in the Stroma Is Associated with Decreased Survival in Breast Cancer Patients

(A–C) Analysis of *HSF1* mRNA expression levels in the stroma of 53 breast cancer patients from Finak et al. (2008).

(A) The association between *HSF1* expression and tumor grade is presented in a box and whiskers plot.

(B) KM analysis of patients stratified by *HSF1* expression.

(C) The correlation between *HSF1* expression and HER2 status is presented in a box and whiskers plot. HER2-neg, HER2 negative; HER2-pos, HER2 positive.

(D) Breast cancer resections from 46 early-stage patients were stained with anti-HSF1 antibodies and scored for HSF1 protein activation (relative nuclear staining intensity) in the stroma by IHC. Association of stromal HSF1 activation with disease-free survival was assessed by KM analysis. int, intermediate; h/i, high/intermediate; h/l, high/low.

*$p < 0.05$. See also Figure S5 and Table S6.

patients at high risk of developing lung cancer is creating an acute need for markers that can predict which early-stage tumors are most likely to progress, in order to avoid overtreatment and its associated morbidities. The widespread activation of stromal HSF1 in diverse cancers suggests that it might be a useful biomarker in other tumor types as well, as we have shown for breast cancer. From a therapeutic perspective, the dependence of even the most robust cancers on supporting stromal cells, and the relative genetic stability of the stroma, make HSF1 an attractive target for intervention in both cancer cells and stroma (Bissell and Hines, 2011; Luo et al., 2009; Place et al., 2011; Saturno et al., 2013; Whitesell and Lindquist, 2009). As we and others have suggested, the nearly unthwartable ability of advanced cancers to evolve resistance to virtually every available therapy makes it attractive to target normal biological networks that have been co-opted to support malignancy, rather than relying solely on the targeting of mutated malignant drivers.

EXPERIMENTAL PROCEDURES

Ethics Statement

All clinical data were collected following approval by the Rabin Medical Center institutional review board (breast cohort) and the Partners Health Care institutional review board (lung cohort). All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH. Mice were maintained according to the guidelines of the MIT Committee on Animal Care (CAC), and study designs were approved by the MIT CAC (protocol # 0612-055-15).

Cell Culture

D2A1, 4T7, MCF7, and MEFs were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS). HCC38, A549, and H1703 cells were cultured in RPMI with 10% FBS. For coculture, immortalized MEFs were plated

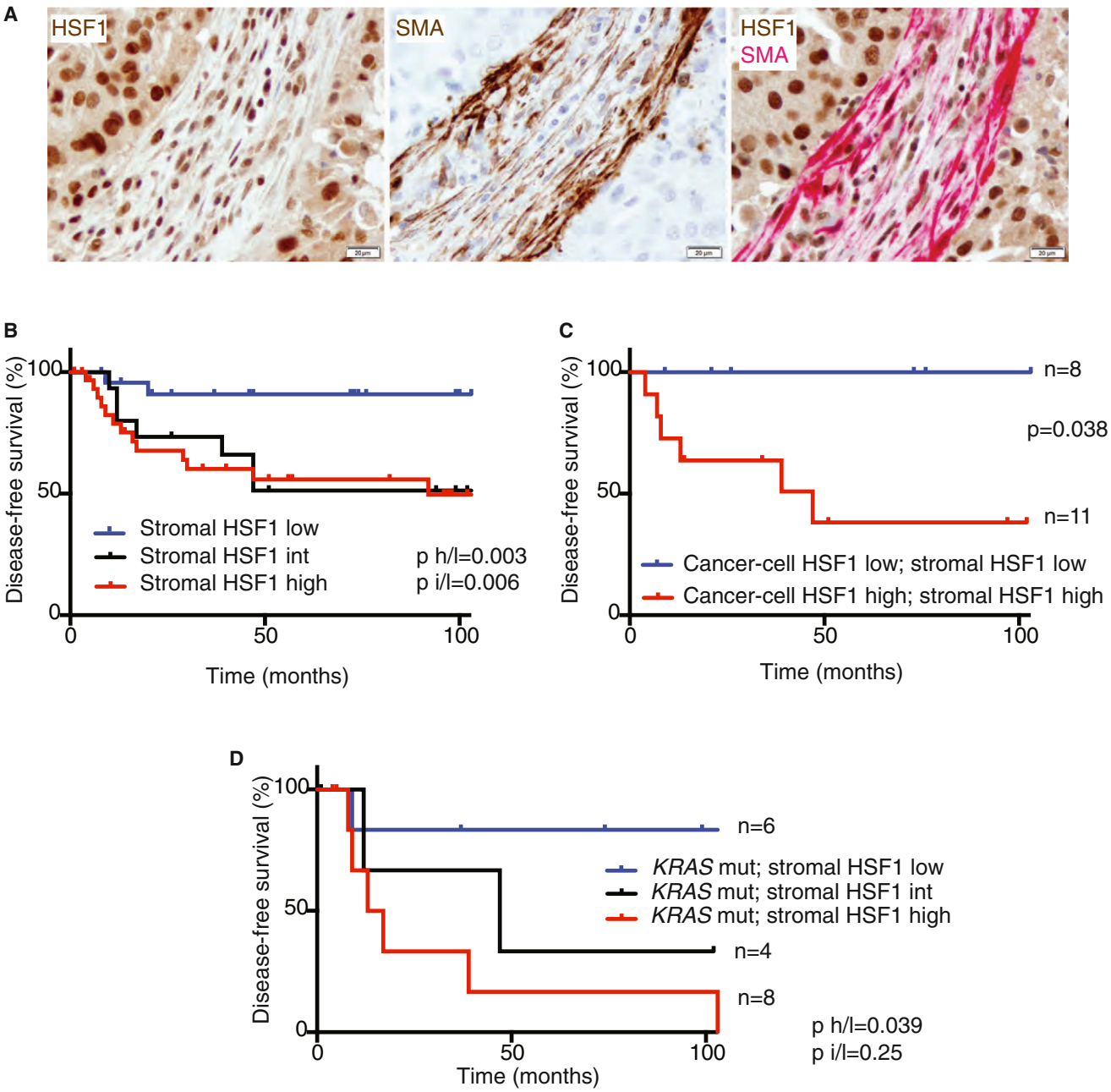

Figure 6. Increased HSF1 Activation in the Stroma Is Associated with Decreased Survival in Lung Cancer Patients

(A) Lung cancer resections from five patients were stained with anti-HSF1 (brown), anti-SMA (brown), or a combination of both antibodies (HSF1 in brown; SMA in red). Representative images are shown.

(B and C) Lung cancer resections from 72 patients with stage I disease were stained with anti-HSF1 antibodies and scored for HSF1 activation in the stromal cells and in the cancer cells.

(B) HSF1 stromal scores are correlated with disease-free survival by KM analysis.

(C) KM analysis of disease-free survival for patients with concordant high or low HSF1 scores in both stromal cells and cancer cells.

(D) Stromal HSF1 levels in *KRAS* mutant tumors (n = 18) from the lung cancer cohort correlate with disease-free survival by KM analysis.

See also Figure S6 and Table S7.

at near confluency and 24 hr later, treated, where indicated, with 10 μg/ml mitomycin C (Sigma-Aldrich) for 2 hr and washed with PBS. Cancer cells were seeded on top of the MEFs (1:5 ratio of cancer cells:MEFs) and allowed to grow for 72–96 hr. Where indicated, MEFs were incubated with LY2109761 (1 μM; Selleck Chemicals) for 30 min before seeding of cancer cells. The same concentration of inhibitor was then added daily. Recombinant TGF-β1 (R&D Systems; 240-B-002) and SDF1 (R&D Systems; 460-SD-010) were added to cocultures at the indicated concentrations once, when coculture was started.

Bi-Tet-*Hsf1* MEFs

Bi-Tet-*Hsf1* MEFs were constructed as explained in Extended Experimental Procedures. Where indicated, cells were treated with 2 μg/ml doxycycline to inhibit *Hsf1* expression.

Flow Cytometry

For expression profiling, cocultures were sorted using a FACSAria (BD Biosciences) instrument, as explained in Extended Experimental Procedures. For all other experiments, a Guava EasyCyte (Millipore) cytometer was used, 10,000 cells/sample were analyzed, and the fraction of cancer cells was calculated using FlowJo 8.8.7 software.

Gene Expression Analysis

Duplicate RNA samples were reverse transcribed and hybridized to duplicate SurePrint Agilent microarrays (Agilent Technologies; G4852A). Data were analyzed using Cluster, GOrilla, and MSigDB and visualized using Java TreeView (details in Extended Experimental Procedures).

Small Hairpin RNA Knockdown of Genes in the TGF-β and SDF1 Signaling Networks

The following genes were stably knocked down in D2A1 cells and in MEFs: *Smad2*, *Smad3*, *Smad4*, and *Tgf-βR2* (details in Extended Experimental Procedures).

Xenografts

MCF7 cells (1×10^6) were inoculated subcutaneously in the right inguinal region of each mouse. Where indicated, 1×10^6 MCF7 cells were mixed with 3×10^6 WT or *Hsf1* null primary MEFs prior to injection. Tumor growth was monitored by caliper measurements twice weekly. Mice were sacrificed, and tumors were excised when volume reached 1.5 cm^3 or overlying skin became ulcerated. Half the resected tissue was flash frozen for ChIP and half fixed in 10% formalin, processed using standard methods, cut into 5 mm sections, and immunostained as described below.

ChIP-qPCR

Flash-frozen tumor xenografts (0.5 cm^3 each) were pulverized, fixed in formalin, and processed as described previously (Lee et al., 2006; Mendillo et al., 2012). Anti-HSF1 (Thermo Scientific; RT-629-PABX) was used to immunoprecipitate (IP) HSF1, and normal rat immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories; 012-000-003) was used as control. qPCR was performed using the primers listed in Extended Experimental Procedures.

IHC of Tissues, Scoring, and Patient Outcome Analysis

Paraffin blocks and tissue microarrays were retrieved, processed, stained, and scored as described in Extended Experimental Procedures. Outcome analysis was performed on 46 breast cancer patients and 72 lung cancer patients. Time to progression of disease and overall survival were estimated by the Kaplan-Meier (KM) method using GraphPad Prism 6 software. Unless indicated otherwise, the log rank test was used to assess statistical significance. All statistical tests were two sided; $p < 0.05$ was considered statistically significant. Multivariate Cox proportional hazards regression analysis was performed using the coxph function in the survival package in R (http://www.r-project.org/).

EGFR and *KRAS* Genotyping

Total nucleic acid was extracted from formalin-fixed, paraffin-embedded surgical specimens of the lung cohort described above using a modified FormaPure System (Agencourt Bioscience). SNaPshot mutational analysis of a panel of cancer genes that included *EGFR* and *KRAS* was performed using primers listed in the Extended Experimental Procedures as previously described (Dias-Santagata et al., 2010).

Stromal *HSF1* mRNA Profiling and Patient Outcome Analysis

Stromal gene expression-profiling data and clinical outcome for 53 breast cancers were obtained from Gene Expression Omnibus (GEO) (GSE9014) and the Finak et al. study (Finak et al., 2008). Analysis was performed as explained in Extended Experimental Procedures. Survival distributions in the patients with low stromal *HSF1* and high stromal *HSF1* were compared by KM analysis. The associations between *HSF1* expression, tumor grade and molecular subtype are presented by box and whisker plots. Statistical significance was assessed with the log rank test using GraphPad Prism 6. All statistical tests were two sided; $p < 0.05$ was considered significant.

ACCESSION NUMBERS

Microarray raw data were deposited in a public database (GEO accession GSE56252).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.05.045.

AUTHOR CONTRIBUTIONS

R.S.-S., S.L., and L.W. conceived the project, designed experiments, and wrote the paper. R.S.-S. performed all mouse and cell culture experiments, analyzed data, and made the figures. S.S. performed all patient-sample staining and scoring and provided clinical guidance. L.M.S. assembled the lung cancer patient cohort, performed sample scoring, and provided clinical outcome data. I.B.-A. and S.M.S. assembled the breast cancer patient cohort and provided clinical outcome data. M.L.M. analyzed microarray data, and M.K. assisted. A.H.B. performed statistical analysis of patient data. D.D.-S. performed genotyping of lung cancer patient samples.

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