SPSB1 Promotes Breast Cancer Recurrence by Potentiating c-MET Signaling

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ABSTRACT
Breast cancer mortality is principally due to tumor recurrence; however, the molecular mechanisms underlying this process are poorly understood. We now demonstrate that the suppressor of cytokine signaling protein SPSB1 is spontaneously upregulated during mammary tumor recurrence and is both necessary and sufficient to promote tumor recurrence in genetically engineered mouse models. The recurrence-promoting effects of SPSB1 result from its ability to protect cells from apoptosis induced by HER2/neu pathway inhibition or chemotherapy. This, in turn, is attributable to SPSB1 potentiation of c-MET signaling, such that preexisting SPSB1-overexpressing tumor cells are selected for following HER2/neu downregulation. Consistent with this, SPSB1 expression is positively correlated with c-MET activity in human breast cancers and with an increased risk of relapse in patients with breast cancer in a manner that is dependent upon c-MET activity. Our findings define a novel pathway that contributes to breast cancer recurrence and provide the first evidence implicating SPSB proteins in cancer.

SIGNIFICANCE: The principal cause of death from breast cancer is recurrence. This study identifies SPSB1 as a critical mediator of breast cancer recurrence, suggests activation of the SPSB1–c-MET pathway as an important mechanism of therapeutic resistance in breast cancers, and emphasizes that pharmacologic targets for recurrence may be unique to this stage of tumor progression. Cancer Discov; 4(7); 1–14. ©2014 AACR.

See related commentary by Qin and McAllister, p. 760.

INTRODUCTION
Breast cancer is both common and treatable. As a consequence, it is by far the most prevalent cancer, with nearly 10 million women worldwide currently harboring this diagnosis. Because recurrent breast cancer is typically an incurable disease, the propensity of breast cancers to recur following surgery and adjuvant treatment is the most important determinant of clinical outcome. Recurrent breast cancers arise from the pool of local and disseminated residual tumor cells, termed minimal residual disease, that survive in a presumed dormant state following treatment of the primary cancer. However, although tumor dormancy and recurrence are responsible for the vast majority of breast cancer deaths, the mechanisms underlying these critical stages of cancer progression are largely unknown.

Because residual neoplastic cells constitute the reservoir from which recurrent cancers arise, the lack of therapeutic approaches targeting these cells, combined with our lack of understanding of their biology, constitutes a major obstacle to the successful treatment of breast cancer. Accordingly, the development of molecularly targeted therapies that are specifically designed to eliminate residual cancer cells, or maintain them in a dormant state, represents an attractive approach to preventing tumor recurrence. Such an approach, however, would require a far more detailed understanding of the biology of minimal residual disease, tumor dormancy, and recurrence than currently exists.

To better define the molecular and cellular events that contribute to breast cancer recurrence, we have developed a series of conditional mouse models for HER2/neu, c-MYC, and WNT1-overexpressing breast cancers that display key features of human breast cancer progression, including metastasis, minimal residual disease, and recurrence (1–5). Following oncogene induction with doxycycline, bitransgenic mice from each of these models develop invasive mammary adenocarcinomas in a manner that is highly penetrant, mammary-specific, and absolutely dependent upon transgene induction by doxycycline. Strikingly, the majority of these tumors regress to a nonpalpable state following oncogene downregulation induced by doxycycline withdrawal via a phenomenon referred to as oncogene addiction.

Notably, the majority of tumors in conditional transgenic mice that regress to a nonpalpable state following oncogene downregulation spontaneously recur over periods of up to a year in the absence of expression of the initiating oncogene (1–7). This observation is consistent with clinical studies demonstrating that neoadjuvant treatment with trastuzumab plus chemotherapy converts HER2/neu–amplified breast cancers to HER2-negative residual disease in more than 40% of cases (8). Thus, primary breast cancer cells likely have the ability to survive therapy and recur via HER2/neu–independent pathways in mice as well as humans. These parallels suggest that mouse models can enable mechanistic approaches to elucidating the molecular and cellular pathways that contribute to the survival and recurrence of residual cancer cells. This goal...
is of paramount importance for the development of more-effective therapies aimed at preventing—as well as treating—breast cancer recurrence.

The SPSB protein family is characterized by a central SPRY (sp1A/ryanodine receptor) protein-binding domain and a C-terminal suppressor of cytokine signaling (SOCS) box-containing domain. Although the biologic functions of these proteins have yet to be elucidated, some SPSB binding partners have been identified, including inducible nitric oxide synthase (iNOS), the c-MET receptor tyrosine kinase, and the prostate apoptosis response protein-4 (PAR-4), for which we have recently reported a role in mammary tumor recurrence (9–12). Although SPSB proteins have been reported to promote iNOS degradation, their functions with respect to PAR-4 and c-MET are unknown.

In the present study, we have identified a functional role for SPSB1 in breast cancer recurrence. We show that Spsb1 is spontaneously upregulated during mammary tumor recurrence in multiple genetically engineered mouse models and that SPSB1 is both necessary and sufficient to promote tumor recurrence. Furthermore, we demonstrate that the recurrence-promoting effects of SPSB1 are due to its ability to protect cells from apoptosis induced by chemotherapy as well as by HER2/neu pathway inhibition, and that the prosurvival effects of SPSB1 are attributable to its ability to potentiate hepatocyte growth factor (HGF)–induced c-MET signaling. Notably, the effects of SPSB1 and c-MET were found to be interdependent in that SPSB1 is required for c-MET activation and tumor recurrence in mice, whereas c-MET is required for SPSB1 to exert its antiapoptotic effects following HER2/neu downregulation or chemotherapy.

In support of the relevance of this mechanism to breast cancer relapse in humans, we find that elevated SPSB1 expression in primary tumors is associated with an increased risk of recurrence in patients with breast cancer independent of classical prognostic factors. Consistent with our biochemical observations, SPSB1 expression was positively correlated with c-MET activity in human breast cancers, and SPSB1 was associated with an increased risk of relapse specifically in women whose tumors expressed high levels of c-MET. Finally, although the association of SPSB1 expression with decreased relapse-free survival was independent of classical prognostic factors, it was dependent on the association between SPSB1 and c-MET activity, suggesting that c-MET pathway activation mediates the effect of SPSB1 on relapse in patients with breast cancer. Taken together, our observations provide the first evidence for a role for the SPSB family of proteins in cancer and identify a novel pathway involved in breast cancer recurrence.

RESULTS

SPSB1 Is Spontaneously Upregulated during Mammary Tumor Recurrence

To explore the potential function of SPSB proteins in tumor progression, we performed quantitative real-time RT-PCR (qRT-PCR) for Spsb1, Spsb2, Spsb3, and Spsb4 in primary and recurrent tumors harvested from MMTV-rtTA;TetO-HER2/neu, MMTV-rtTA;TetO-MYC, and MMTV-rtTA;TetO-Wnt1;Trp53+/− mice (1–4). Expression of Spsb1 was consistently upregulated in recurrent tumors compared with primary tumors in each of the models tested (Fig. 1A). No alterations in mRNA expression were detected for Spsb2 or Spsb3 in any of the mouse models, and Spsb4 expression was decreased in recurrent tumors from MMTV-rtTA;TetO-HER2/neu mice, but unchanged in recurrent tumors from MYC and Wnt1;Trp53+/− mice (Supplementary Fig. S1A–S1C).

Consistent with its upregulation at the mRNA level, SPSB1 protein expression was also elevated in recurrent HER2/neu tumors (Fig. 1B and C), recurrent MYC tumors (Fig. 1D and E), and recurrent Wnt1;Trp53+/− tumors (Fig. 1F and G). In addition, Spsb1 mRNA levels were strongly correlated with SPSB1 protein levels, suggesting that mRNA levels are generally indicative of protein levels (Supplementary Fig. S1D).

SPSB1 Is Necessary and Sufficient to Promote Mammary Tumor Recurrence In Vivo

Our finding that SPSB1 is upregulated in mouse models for breast cancer recurrence induced by three different oncogenic pathways suggested the possibility that this protein might functionally contribute to tumor recurrence. To address this possibility, we first transduced doxycycline-dependent mammary tumor cells derived from an MMTV-rtTA;TetO-HER2/neu primary tumor with a retrovirus expressing HA-tagged SPSB1 (SPSB1) or an empty vector control (pK1). We previously reported that the transcription repressor SNAIL promotes epithelial-to-mesenchymal transition (EMT) and breast cancer recurrence in a HER2/neu–independent manner (5). Unlike SNAIL, SPSB1-expressing primary tumor cells retained an epithelial morphology indistinguishable from control cells (Supplementary Fig. S2A) and expressed HER2/neu, E-cadherin, and Cytokeratin 8 (KRT8) at levels comparable with controls (Fig. 2A). Furthermore, SPSB1 expression did not induce endogenous Snail expression in primary mammary tumor cells, and SNAIL expression, if anything, modestly downregulated endogenous SPSB1 (Supplementary Fig. S2B and S2C). Thus, SPSB1 does not induce SNAIL expression or EMT in mouse primary mammary tumor cells.

Next, we used a previously validated orthotopic model (5) to determine whether SPSB1 expression is sufficient to promote mammary tumor recurrence in vivo, as we hypothesized might be the case based on the spontaneous upregulation of SPSB1 in recurrent mammary tumors. H2B-EGFP-labeled control (pK1) or SPSB1-expressing primary tumor cells were injected into the mammary fat pads of nu/nu mice maintained on doxycycline to permit tumor outgrowth in the presence of HER2/neu expression. Similar to the behavior of tumors in intact transgenic mice, doxycycline withdrawal–induced downregulation of HER2/neu resulted in orthotopic tumor regression to a nonpalpable state irrespective of SPSB1 expression status. Strikingly, within 120 days of doxycycline withdrawal, 93% (14 of 15) of SPSB1-transduced tumors had recurred, whereas only 33% (5 of 15) of control tumors had recurred (HR, 5.3; P < 0.0001; Fig. 2B). All recurrent tumors were GFP-positive, confirming that they were derived from injected primary tumor cells, and recurrent tumors derived from SPSB1-transduced cells maintained SPSB1 expression (Supplementary Fig. S2D and S2E). Neither pK1 nor SPSB1-derived recurrent tumors exhibited HER2/neu upregulation based on qRT-PCR or Western blot analysis, indicating that recurrent tumors did not arise...
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Figure 1. SPSB1 is spontaneously upregulated in recurrent mammary tumors. A, qRT-PCR analysis of Spsb1 mRNA expression in primary (open bar) and recurrent (closed bar) tumors from MMTV-rtTA;TetO-HER2/neu (primary, n = 7; recurrent, n = 15), MMTV-rtTA;TetO-MYC (primary, n = 7; recurrent, n = 7), and MMTV-rtTA;TetO-Wnt1;Trp53+/- (primary, n = 10; recurrent, n = 10) mouse models. Relative Spsb1 expression was normalized to the expression of Tbp, which serves as a loading control. Western blot analysis of SPSB1 protein expression in primary and recurrent tumors from MMTV-TetO-HER2/neu (B), MMTV-rtTA;TetO-MYC (D), and MMTV-rtTA;TetO-Wnt1;Trp53+/- (F) mice. β-Tubulin is shown as a loading control. NMuMG parental and SPSB1-overexpressing cell lysates are shown as negative (−) and positive (+) controls, respectively. Quantification of SPSB1 protein expression normalized to β-tubulin in primary (open bar) and recurrent (closed bar) tumors from MMTV-TetO-HER2/neu (C), MMTV-rtTA;TetO-MYC (E), and MMTV-rtTA;TetO-Wnt1;Trp53+/- (G) mice. *, P < 0.05; **, P < 0.01; ***, P < 0.001. P values are calculated by Mann–Whitney test (A); and Student t test (C, E, and G).

Figure 2. SPSB1 is necessary and sufficient to promote mammary tumor recurrence in vivo. A, Western blot analysis of HA-tagged SPSB1, HER2/neu, E-cadherin, KRT8, and β-tubulin expression in pK1 and SPSB1, doxycycline-dependent HER2/neu–induced primary tumor cells grown in the presence of doxycycline. B, recurrence-free survival for mice harboring fully regressed orthotopic tumors derived from pK1 or SPSB1-expressing cells. The experiment was repeated three times and a representative result is shown. C, qRT-PCR analysis of endogenous Spsb1 mRNA expression in primary mouse HER2/neu–induced tumor cells expressing Spsb1–specific hairpins (shSpsb1a and shSpsb1b) or a nonsilencing control. Error bars, standard error of the mean. **, P < 0.01. P values are calculated by Student t test. D, recurrence-free survival of mice bearing fully regressed tumors derived from Spsb1 knockdown or control cells. The experiment was repeated three times and a representative result is shown. P values and HR as determined by Kaplan–Meier survival analysis.
as a consequence of doxycycline-independent reactivation of the HER2/neu transgene or activation of endogenous HER2/neu (data not shown). This result demonstrates that Spsb1 upregulation can promote mammary tumor recurrence in a HER2/neu-independent manner.

Next, to determine whether SPSB1 is required for mammary tumor recurrence following HER2/neu downregulation, we knocked down endogenous Sphb1 expression in HER2/neu-induced mouse primary tumor cells using either of two hairpins, shSphb1a or shSphb1b, that downregulated endogenous Sphb1 expression by 60% to 75% (Fig. 2C). Next, shSphb1 and control shRNA-expressing doxycycline-dependent primary tumor cells were injected into the mammary fat pads of nu/nu mice maintained on doxycycline to permit primary tumor outgrowth in the presence of HER2/neu expression.

As before, downregulation of HER2/neu induced by doxycycline withdrawal resulted in the rapid regression of orthotopic tumors to a nonpalpable state irrespective of whether they were derived from control or Sphb1 knockdown cells (data not shown). However, consistent with the spontaneous upregulation of SPSB1 observed in recurrent mammary tumors, the rates of recurrence of mammary tumors derived from Sphb1 knockdown cells were dramatically reduced compared with the control tumors (Fig. 2D, shSphb1a vs. control HR, 0.17, P < 0.01; shSphb1b vs. control HR, 0.34, P < 0.05). These results demonstrate that endogenous SPSB1 is required for mammary tumor recurrence following HER2/neu downregulation. Consistent with this conclusion, Sphb1 expression levels were restored in recurrent tumors derived from shSphb1 knockdown cells to levels comparable with those seen in recurrent tumors derived from control cells (Supplementary Fig. S2F), suggesting that SPSB1 upregulation in tumor cells is strongly selected for during the process of recurrence. In the aggregate, our findings indicate that SPSB1 is both necessary and sufficient to promote mammary tumor recurrence.

SPSB1 Protects Tumor Cells from Apoptosis Induced by HER2/neu Downregulation

To delineate the cellular mechanism by which SPSB1 promotes mammary tumor recurrence, we stably transduced pK1 and SPSB1 primary tumor cells with H2B-ECFP and H2B-EGFP, respectively. Equal numbers of ECFP-labeled pK1 and EGFP-labeled SPSB1 cells were admixed and injected into the mammary fat pads of nu/nu mice maintained on doxycycline. Primary orthotopic tumors, regressing tumor grafts (96 hours after doxycycline withdrawal), residual lesions (32 days after doxycycline withdrawal), and recurrent tumors were harvested, and the number of EGFP- and ECFP-positive cells was determined as a percentage of the total number of fluorescently labeled tumor cells present at each time point (Fig. 3A and B).

Despite being injected in equal numbers, SPSB1 cells comprised only 30% of labeled tumor cells in primary orthotopic tumors, suggesting that SPSB1 cells are at a selective disadvantage compared with control cells in the presence of HER2/neu expression (Fig. 3A and B). However, within 96 hours of doxycycline withdrawal, this trend reversed as SPSB1 cells were markedly enriched compared with control cells. Selection for SPSB1 cells was further accentuated in residual tumor lesions 32 days following doxycycline withdrawal, at which time EGFP-positive SPSB1 cells constituted approximately 65% of tumor cells. Strikingly, recurrent tumors were composed almost exclusively of SPSB1-transduced cells (Fig. 3A and B). To rule out differential growth effects of H2B-EGFP and H2B-ECFP, the experiment was repeated after swapping fluorescent protein labels between pK1 and SPSB1 cells. Similar results were observed (data not shown). These data demonstrate that SPSB1 expression confers a strong selective advantage to tumor cells in which HER2/neu activity has been downregulated.

To investigate the basis for the observed selection for SPSB1-expressing cells following HER2/neu downregulation, we determined proliferation rates in tumors derived from control cells or SPSB1-expressing cells during the course of recurrence. Consistent with the observed selection against SPSB1-expressing cells during primary tumor outgrowth, SPSB1 cells in primary tumors expressing HER2/neu exhibited lower proliferation rates compared with control cells (Fig. 3C). Following doxycycline withdrawal, proliferation rates fell dramatically and did not differ between SPSB1 and control pK1 cells (Fig. 3C). In residual lesions, proliferation rates were extremely low, although SPSB1 cells exhibited slightly elevated proliferation rates compared with pK1 control cells (Fig. 3C). Proliferation rates for SPSB1 cells in recurrent tumors were restored to levels similar to those observed in primary tumors. Too few pK1 control cells were present in recurrent tumors to ascertain their proliferation rates. Repeat experiments conducted after swapping fluorescent protein labels between pK1 and SPSB1 cells yielded similar results (data not shown).

Because selection for SPSB1 cells within 96 hours following HER2/neu downregulation could not be explained by differences in proliferation rates between these two populations of cells, we next compared apoptosis rates in orthotopic tumors derived from pK1 or SPSB1 cells 48 hours following doxycycline withdrawal. As predicted on the basis of prior results (4), pK1 control tumor cells exhibited a marked increase in cleaved caspase-3 staining 48 hours after doxycycline withdrawal (Fig. 3D). In contrast, apoptotic rates for SPSB1-expressing tumor cells remained low despite oncogene downregulation (Fig. 3D). These findings suggest that SPSB1 expression protects tumor cells from apoptosis induced by acute HER2/neu downregulation and, more broadly, that this survival advantage forms the basis for their selection relative to control cells in the context of HER2/neu downregulation.

To confirm the cell-intrinsic survival advantage of SPSB1 cells observed in vivo, we withdrew doxycycline from the above pK1 and SPSB1 cells cultured in vitro. Consistent with our in vivo observations, acute HER2/neu downregulation resulted in a dramatic increase in apoptotic rates in pK1 cells, as evidenced by immunofluorescence for cleaved caspase-3, whereas apoptotic rates in SPSB1 cells remained low (Fig. 3E). This finding was confirmed by immunoblotting analysis for cleaved caspase-3 (CASP3) and cleaved poly (ADP-ribose) polymerase 1 (PARP1; Fig. 3F). In contrast, proliferation rates did not differ between SPSB1 and control cells regardless of HER2/neu expression status (Supplementary Fig. S3A). As a result, there was a net growth advantage for SPSB1 cells compared with control cells when grown in the absence of doxycycline (Supplementary Fig. S3B). In addition, SPSB1 cells were far more efficient than control cells at forming colonies.
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SPSB1 Protects Human Breast Cancer Cells from Apoptosis Induced by HER2/neu Inhibition or Treatment with Chemotherapeutic Agents

To extend our observations from mouse mammary tumors to human breast cancer, we overexpressed SPSB1 in BT474 cells, a HER2/neu-amplified human breast cancer cell line (13) that expresses relatively low levels of SPSB1 (Supplementary Fig. S3C). In agreement with our observations in mouse tumor cells, SPSB1-expressing BT474 cells treated with lapatinib exhibited lower Caspase-3/7 activity and less cell death than control cells (Fig. 4A and Supplementary Fig. S3D).

Next, we tested whether SPSB1 could also protect human breast cancer cells from apoptosis induced by treatment with chemotherapeutic agents, which are more commonly used as antineoplastic agents in patients with breast cancer than targeted therapies directed against HER2. We used two different hairpins targeting human SPSB1 (Supplementary Fig. S3E; shSPSB1a and shSPSB1b) to knockdown endogenous SPSB1 expression in Hs578T cells, a HER2/neu-negative human breast cancer cell line that expresses high levels of endogenous SPSB1 (Supplementary Fig. S3C). Hs578T cells expressing SPSB1 hairpins exhibited increased levels of apoptosis following treatment with the chemotherapeutic agents Adriamycin and etoposide, as indicated by increased staining for cleaved Caspase-3 (Fig. 4B and C and Supplementary Fig. S3F). Similarly, Hs578T cells with SPSB1 knockdown were less efficient at forming colonies in a clonogenic assay (Supplementary Fig. S3G). Conversely, ectopic expression of SPSB1 in MDA-MB-231 cells, which are HER2/neu-negative and express low levels of SPSB1 (Supplementary Fig. S3C), reduced the rate of apoptosis following Adriamycin treatment by almost 50% (Fig. 4D and E). Together, our data indicate that SPSB1 plays an essential role in protecting human breast cancer cells from...
apoptosis induced by either HER2/neu downregulation or treatment with chemotherapeutic agents.

**SPSB1 Potentiates c-MET Signaling In Vitro**

To explore the molecular mechanism underlying SPSB1’s antiapoptotic effects, we considered two proteins reported to bind to SPSB1, PAR-4 and the c-MET receptor tyrosine kinase, each of which has been implicated in breast cancer. Although human PAR-4 has been reported to bind to multiple SPSB proteins, mouse PAR-4 does not contain an SPSB-binding motif (11). Consistent with this, we were unable to detect an interaction between SPSB1 and PAR-4 in mouse primary tumor cells by communoprecipitation, nor did we observe a difference in PAR-4 expression between pK1 and SPSB1-expressing tumor cells (Supplementary Fig. S4A). Like PAR-4, c-MET has been reported to bind to all four SPSB proteins, but only SPSB1 has been reported to potentiate HGF-induced ERK signaling when transiently overexpressed in HEK293 cells (14). Given that SPSB1, but not other SPSB family members, is upregulated in recurrent tumors and that recurrent mammary tumors in our mouse models frequently activate c-MET (Supplementary Fig. S4B), we considered the possibility that the antiapoptotic effects of SPSB1 might be mediated by c-MET.

First, we determined that SPSB1 can stably bind to endogenous c-MET in mouse mammary tumor cells in the presence of HER2/2 expression (Fig. 5A). Next, we investigated the effect of SPSB1 on c-MET activity. In cells expressing HER2/2, c-MET phosphorylation was low irrespective of SPSB1 expression status (Fig. 5A and B). However, following HER2/2 downregulation, c-MET phosphorylation was markedly upregulated in SPSB1-expressing cells, but remained low in pK1 control cells (Fig. 5A and B). Consistent with the observed increase in c-MET phosphorylation following HER2/2 downregulation, downstream effectors of the c-MET pathway, phospho-AKT (Ser473) and phospho-STAT3 (Tyr705), were also upregulated in SPSB1-expressing cells compared with control cells following doxycycline withdrawal (Fig. 5C–E). However, contrary to a prior report (10), no difference in phospho-ERK activity was detected between SPSB1 cells and pK1 control cells under the same conditions (Supplementary Fig. S4C).

Next, we wished to determine whether SPSB1 could potentiate HGF-induced c-MET signaling. In mouse mammary tumor cells subjected to acute HER2/2 downregulation in vitro, SPSB1-expressing cells exhibited more pronounced c-MET activation in response to HGF treatment than pK1 control cells (Fig. 5F and G). Similarly, ectopic expression of SPSB1 in human MDA-MB-231 breast cancer cells also enhanced HGF-induced c-MET activation (Supplementary Fig. S4D). Conversely, knockdown of endogenous SPSB1 impaired HGF-induced c-MET activation in human Hs578T breast cancer cells and in mouse HER2/2 primary mammary tumor cells subjected to HER2/2 downregulation (Fig. 5H and I and Supplementary Fig. S4E). In aggregate, these data confirm that SPSB1 is a c-MET binding partner and demonstrate that SPSB1 can potentiate c-MET signaling in both mouse and human breast cancer cells.

**c-MET Is Required for SPSB1-Mediated Tumor Cell Survival**

Given that c-MET signaling is a well-documented cell survival pathway and that SPSB1 potentiates c-MET signaling, we reasoned that the antiapoptotic effects exerted by SPSB1 might be mediated by c-MET. Consistent with this hypothesis, we found that mouse tumor cells with impaired HGF-induced c-MET activation due to Spsb1 knockdown were less efficient at forming colonies than control cells when grown in the presence of HGF following HER2/2 knockdown (Fig. 6A, P < 0.01). In an analogous manner, HGF-mediated rescue from Adriamycin-induced apoptosis was less efficient in Hs578T cells in which SPSB1 had been knocked down (Fig. 6B).

To determine whether c-MET is required for SPSB1-mediated cell survival, we knocked down endogenous c-MET expression in pK1 and SPSB1-expressing cells and subjected
cells to acute HER2/neu downregulation. Consistent with our findings above, SPSB1-expressing cells exhibited elevated levels of phospho-MET, phospho-AKT, and phospho-STAT3, as well as diminished levels of cleaved caspase-3, compared with control cells following acute HER2/neu downregulation (Fig. 6C–F). In comparison, SPSB1-expressing cells in which c-MET had been knocked down exhibited decreased levels of phospho-MET, phospho-AKT, and phospho-STAT3, as well as a corresponding increase in levels of cleaved caspase-3, following acute HER2/neu downregulation (Fig. 6C–F). Consistent with these findings, c-MET knockdown in SPSB1-expressing cells resulted in decreased cell viability as well as a decrease in cell number following HER2/neu downregulation (Supplementary Fig. SSA and SSB).

To demonstrate directly that c-MET is required for SPSB1-mediated cell survival following HER2/neu downregulation,
Figure 6. c-MET is required for SPSB1-mediated tumor cell survival. A, clonogenic assay for survival and outgrowth of control and Spsb1 knockdown cells treated with 10 ng/mL HGF growing in the absence of doxycycline. The numbers of colonies in each plate was quantified using CellProfiler (24). Experiments were performed in triplicate and repeated. A representative result is shown. B, HGF-mediated rescue of Adriamycin induced apoptosis in Hs578T cells expressing control and SPSB1-specific hairpins. Maximum levels of apoptosis were defined as caspase-3/7 activity in the same cells treated with Adriamycin in the absence of HGF. C–F, immunoprecipitation and Western blot analysis (A) and quantification (B–D) of c-MET (T1234/1235) phosphorylation, AKT (S473) phosphorylation, STAT3 (T706) phosphorylation and cleaved caspase-3 (cCASP3) levels in pK1 and SPSB1 cells expressing a c-MET-specific shRNA (shMet) or nonsilencing control in the absence of doxycycline. Results represent the average of four independent experiments. Quantification of c-MET, AKT, or STAT3 activity was performed by normalizing the levels of each phospho-protein to its corresponding total protein. The experiment was performed in triplicate for each condition and repeated twice. Representative results are shown. Clonogenic assay (G) and quantification (H) of pK1 and SPSB1 cells growing in the absence of doxycycline and treated with c-MET inhibitor for 14 days. I and J, photomicrographs (I) and quantification (J) of pK1-H2B-mCherry (red) or SPSB1-H2B-EGFP (green) cells harvested from primary orthotopic tumors growing in the presence of doxycycline and regressing tumor grafts 96 hours after oncogene deinduction treated with vehicle or c-MET inhibitor. On Dox, primary orthotopic tumor (n = 4), off Dox (96 hours after deinduction) with vehicle, n = 4; off Dox with c-MET inhibitor, n = 6. Error bars, standard error of the mean. P values are calculated by one-sample t test (B–D), Student t test (E and F), one-way ANOVA (H), or Kruskal–Wallis test with Dunn multiple comparison test (J). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
we first performed a clonogenic assay in which pK1 or SPSB1-expressing cells were treated with either a c-MET small-molecule inhibitor, compound 22 (Supplementary Fig. S6A–S6D; ref. 15), or vehicle control. Consistent with our previous finding, SPSB1-expressing cells formed colonies far more efficiently than control cells following HER2/neu downregulation (Fig. 6G and H). Supporting the notion that SPSB1’s recurrence-promoting effect is MET-dependent, treatment with the c-MET inhibitor compound 22 markedly reduced the number of colonies formed by SPSB1-expressing cells in the absence of doxycycline (Fig. 6G and H). This observation is consistent with a model in which c-MET activity is required for SPSB1-mediated tumor cell survival following HER2/neu downregulation in vitro.

Next, we tested the functional importance of c-MET activity for SPSB1-mediated tumor cell survival in vitro. Consistent with our observations in fluorescent cell competition assays (Fig. 3), SPSB1-expressing cells comprised only 30% of fluorescently labeled cells in primary orthotopic tumors in the presence of HER2/neu expression, and HER2/neu downregulation resulted in rapid selection for SPSB1-expressing cells within regressing tumor grafts within 96 hours following doxycycline withdrawal (Fig. 6I and J). However, treatment of tumor-bearing mice with compound 22 impaired the selection for SPSB1-expressing cells following HER2/neu downregulation, such that in the presence of the c-MET inhibitor significant enrichment of SPSB1-expressing cells relative to primary tumors growing in the presence of HER2/neu was no longer observed (Fig. 6I and J). Together, these findings suggest that c-MET is required for SPSB1-mediated tumor cell survival following HER2/neu downregulation.

**SPSB1 Is Associated with Increased Risk of Relapse in Breast Cancer Patients Independent of Classical Prognostic Factors**

In light of our finding that SPSB1 is necessary and sufficient to promote mammary tumor recurrence in mice, we hypothesized that women with primary breast cancers expressing high levels of SPSB1 would relapse at a faster rate than women whose breast cancers expressed lower levels of SPSB1. To test this hypothesis, we assembled 1,710 unique patient profiles from nine published human primary breast cancer microarray datasets of mixed estrogen receptor (ER) status, each of which contained more than 100 patients and provided information on relapse-free survival as well as SPSB1 mRNA expression.

For each dataset, the effect size of the association between SPSB1 mRNA expression and relapse-free survival was estimated using two different methods: the HR from Cox proportional hazard regression in which SPSB1 expression was modeled as a continuous variable; and the concordance index (c-index; ref. 16). To derive an overall estimate and statistical significance of the association between SPSB1 expression and relapse-free survival while accounting for heterogeneity among datasets, each type of effect size estimate was combined across datasets by meta-analysis using the inverse-variance weighting method (17). In the presence of significant heterogeneity (P value for Cochran Q statistic < 0.05), a random-effect model (18) was used for meta-analysis. Otherwise a fixed-effect model (19) was used.

Among the 1,710 patients studied, information on relapse-free survival was available for 1,644. Across all patients, a significant positive association was observed between elevated SPSB1 expression and increased risk of relapse within 5 years of diagnosis [Fig. 7A; HR, 1.32 (1.08–1.61), P = 0.0066; c-index, 0.54 (0.51–0.57), P = 0.01; plot not shown].

Next, we assessed the association between SPSB1 expression and common clinicopathologic prognostic factors for breast cancer, including ER status, progesterone receptor (PR) status, HER2/neu status, tumor size, tumor grade, lymph node status, and intrinsic molecular subtype. Among these, SPSB1 expression was positively and significantly associated only with ER-negative status, PR-negative status, and basal-like subtype (Supplementary Fig. S7A–S7C).

Because ER-negative, PR-negative, and basal-like subtype tumors are each generally associated with a worse prognosis in patients with breast cancer (20), we wished to determine whether the poor prognosis observed in patients with breast cancers expressing high levels of SPSB1 was an indirect reflection of the association between elevated SPSB1 expression and any of these pathologic variables. To address this question, we performed multivariate Cox proportional hazards regression in combination with meta-analysis. The result demonstrated that the association between elevated SPSB1 expression and decreased relapse-free survival was unaffected, and remained significant, after adjusting for ER status and the basal-like subtype using multivariate Cox proportional hazard regression in combination with meta-analysis (Supplementary Fig. S8A and S8B).

Together, these data indicate that SPSB1 is preferentially expressed in aggressive subsets of human breast cancer, but that the association between elevated SPSB1 expression and decreased relapse-free survival is not attributable to this pattern of expression. Rather, it raises the intriguing possibility that the aggressive behavior of these breast cancer subsets may be due, at least in part, to SPSB1.

**SPSB1 Expression Is Correlated with Increased c-MET Pathway Activity in Human Breast Cancers, Which Mediates Its Association with Increased Risk of Relapse**

In light of our genetic and pharmacologic experiments indicating that c-MET is required for SPSB1-mediated tumor cell survival, we hypothesized that the association of SPSB1 expression with relapse-free survival in patients with breast cancer would be more pronounced in tumors expressing high levels of c-MET than in those expressing low levels of c-MET. After stratifying samples by median c-MET mRNA expression, we found that SPSB1 mRNA expression in primary human breast tumors was significantly associated with relapse-free survival in patients whose tumors expressed levels of c-MET mRNA higher than the median [Fig. 7B; HR, 1.51 (1.13–2.00), P = 0.005; c-index, 0.56 (0.51–0.60), P = 0.0078; plot not shown], but not in patients whose tumors expressed levels of c-MET mRNA lower than the median (Supplementary Fig. S9). This observation was consistent with our in vitro findings that the biochemical effects of SPSB1 on tumor cell survival require the presence of c-MET, and that c-MET knockdown or c-MET pharmacologic inhibition
abrogate the effects of SPSB1 on tumor cell survival. These findings further suggest that c-MET expression levels may be rate limiting for the effects of SPSB1.

Given our biochemical findings that SPSB1 potentiates c-MET signaling, we hypothesized that c-MET pathway activity would be more likely to be elevated in human breast cancers expressing high levels of SPSB1. Because c-MET activity data are not available for these datasets, we generated a 24-gene c-MET signature with a previously described scoring algorithm (21). Using this system, we assigned each of 1,710 human breast cancer samples a relative c-MET activity score; consistent with our in vitro biochemical findings that SPSB1 expression potentiates c-MET activation, we observed a highly significant positive correlation between SPSB1 mRNA expression and estimated c-MET activity in human breast cancers (Fig. 7C; $r = 0.27; P = 3.1e-29$). The magnitude of this correlation was comparable with that observed for c-MET mRNA and estimated c-MET activity in human breast cancers from the same dataset (data not shown).

On the basis of our in vivo and in vitro findings to this point, particularly those in mouse models for mammary tumor recurrence, we hypothesized that the association of SPSB1 with relapse-free survival in human breast cancers might be attributable to its potentiation of c-MET pathway activity. Consistent with our hypothesis, the association between SPSB1 expression and relapse-free survival in patients with breast cancer was dependent upon the association between SPSB1 expression and c-MET activity. Consistent with our hypothesis, the association between SPSB1 expression and relapse-free survival in patients with breast cancer was dependent upon the association between SPSB1 expression and c-MET activity. Consistent with our hypothesis, the association between SPSB1 expression and relapse-free survival in patients with breast cancer was dependent upon the association between SPSB1 expression and c-MET activity.
survival was no longer evident. These results suggest that the recurrence-promoting effects of SPSB1 are mediated by its ability to potentiate c-MET signaling.

In the aggregate, our analyses demonstrate that SPSB1 expression in human breast cancers is associated with decreased relapse-free survival in patients with breast cancer independent of the fact that SPSB1 is preferentially expressed in aggressive subsets of human breast cancers. Moreover, using a c-MET gene expression signature as a surrogate marker for c-MET pathway activity, our findings identified a positive correlation between SPSB1 expression and c-MET activity in human breast cancers that seemed to account for the observed association between SPSB1 expression and relapse-free survival. Together, these findings are compatible with a model in which SPSB1 promotes breast cancer recurrence in humans and does so by virtue of its ability to potentiate c-MET pathway activity.

**DISCUSSION**

For many women with breast cancer, even those with early-stage disease, disseminated tumor cells are already present at the time of diagnosis. These residual cells, termed minimal residual disease, have the ability to survive in a presumed dormant state following treatment and linger unrecognized for more than a decade before emerging as recurrent disease. As a result, breast cancers that seem cured by surgery, radiation, and adjuvant therapy may resurface as local or distant recurrences 10 or 20 years later.

In light of these considerations, understanding the biology of minimal residual disease and elucidating the molecular pathways that contribute to tumor dormancy and recurrence is a critical priority in breast cancer research. Using genetically engineered mouse models for breast cancer recurrence, human breast cancer cell lines, and interrogation of multiple patient datasets, we have identified SPSB1 as a critical mediator of breast cancer recurrence. In aggregate, our findings identify a novel molecular pathway that contributes to the survival and recurrence of residual cancer cells, define a subset of human breast cancers with a high likelihood of recurrence, and provide the first evidence for a role for the SPSB family of proteins in cancer.

Using three different genetically engineered mouse models, we found that SPSB1 is upregulated during mammary tumor recurrence. Consistent with its upregulation in recurrent tumors, enforced expression of SPSB1 in primary mammary tumor cells promoted tumor recurrence, whereas knockdown of endogenous SPSB1 expression inhibited recurrence, demonstrating a requirement for SPSB1 in this process. Cellular analyses indicated that the recurrence-promoting effects of SPSB1 were due to its ability to promote survival in human and mouse breast cancer cells subjected to chemotherapy or HER2/neu pathway downregulation, and that therapy selected for preexisting Spsb1-overexpressing cells in primary tumors. Biochemical analyses revealed that the requirement for SPSB1 in tumor cell survival was attributable to, and dependent upon, its ability to potentiate c-MET signaling. In agreement with these findings, endogenous SPSB1 was also required for the survival of human breast cancer cells treated with chemotherapeutic agents.

In support of the potential functional importance of SPSB1 in human breast cancer recurrence, SPSB1 expression was associated with a high risk of relapse in patients with breast cancer, and SPSB1 expression was associated with aggressive subtypes of human breast cancer, including ER-negative, PR-negative, and basal-like tumors. However, SPSB1 expression was associated with a high risk of recurrence in women independent of its association with these clinicopathologic parameters. This suggests that SPSB1 expression is an independent prognostic factor for human breast cancer recurrence and raises the possibility that SPSB1 itself may contribute to the aggressive behavior of human breast cancers.

In agreement with our findings in preclinical models that SPSB1 promotes recurrence by potentiating c-MET pathway activity, the association between SPSB1 expression and relapse-free survival was evident in patients whose breast cancers expressed high levels of c-MET, but not in those whose breast cancers expressed low levels of c-MET. Also consistent with our findings in preclinical models, SPSB1 expression in human primary breast cancers was positively correlated with estimated c-MET pathway activity, and this association was primarily responsible for mediating the association of SPSB1 expression with risk of relapse in patients with breast cancer.

Tumor heterogeneity poses a major stumbling block to the effective treatment of human cancers as therapeutic interventions frequently result in the selection and outgrowth of preexisting resistant variants. Data from fluorescent cell competition assays indicated that SPSB1-expressing cells exhibit a pronounced survival advantage following HER2/neu downregulation and are further selected for during recurrent tumor outgrowth. On the basis of these findings, we propose that cells with elevated SPSB1 expression preexist in HER2/neu–induced mouse primary tumors and are selected for in the context of HER2/neu downregulation and recurrent tumor outgrowth. If true, this model would explain the observed upregulation of SPSB1 expression in recurrent mouse mammary tumors and could also explain the association of elevated SPSB1 expression in human primary breast cancers with increased rates of relapse, because primary tumors with higher SPSB1 expression may harbor a greater number of apoptosis-resistant, SPSB1-expressing cells, which are more likely to survive therapy and recur. Together, these findings emphasize the fact that clinically important properties of cancers—namely those that determine resistance and recurrence—may arise from small, yet biologically critical, subsets of cells.

Our laboratory recently reported that PAR-4 downregulation can promote mammary tumor recurrence and PAR-4 has been reported to bind to SPSB1 (9). However, consistent with the fact that mouse PAR-4 lacks the protein motif responsible for mediating this interaction (11), we did not observe an interaction between PAR-4 and SPSB1 in mouse mammary tumor cells. Instead, we found that activation of another SPSB1 binding protein, c-MET, is enhanced by the presence of SPSB1 and is required for SPSB1-mediated tumor cell survival. As it is likely that multiple mechanisms of therapeutic resistance contribute to tumor recurrence, further investigation will be required to determine whether PAR-4 and SPSB1 escape pathways are operative within the same tumors, or in the same cells within those tumors.
Tumor recurrence in the conditional transgenic HER2/neu mouse model used here takes place following targeted downregulation of the oncogenic pathway driving tumor growth. In contrast, the recurrence of HER2/neu-amplified breast cancers in patients is presumed to occur in the presence of continued HER2/neu signaling. Nevertheless, as it has become increasingly evident that neoadjuvant therapy with trastuzumab may convert HER2-positive breast cancers to HER2-negative residual disease (8), the molecular mechanisms of tumor escape underlying tumor recurrence in these mouse models may be clinically relevant. Indeed, based on our observations that SPSB1 is spontaneously upregulated in recurrent mouse mammary tumors induced by oncogenic pathways other than HER2/neu, that SPSB1 provides a survival advantage to non-HER2/neu-amplified human breast cancer cells treated with chemotherapeutic agents, and that SPSB1 is associated with recurrence-free survival across all patients with breast cancer (i.e., not simply those that are HER2/neu-amplified), SPSB1-mediated activation of survival pathways may constitute a general mechanism of therapeutic resistance in patients with cancer. As such, although conditional mouse models cannot accurately model all biologic contexts relevant to tumor recurrence as it occurs in patients, we anticipate that pharmacologic agents that target pathways activated by SPSB1 may have clinical utility.

Small-molecule inhibitors of EGFR are effective in treating non–small cell lung cancers (NSCLC) harboring activating mutations in EGFR. However, their clinical utility is limited by the near-universal development of acquired resistance to these agents. Beyond secondary mutations in EGFR itself, amplification of c-MET has been identified as an important mechanism of resistance (22). In addition, HGF-induced c-MET activation has been shown to lead to drug resistance by selecting for preexisting c-MET-amplified clones in NSCLC cell lines (23). As described here, we have found that c-MET activation induced by SPSB1 upregulation constitutes a potent in vivo mechanism of escape for mammary tumors in which activity of the EGFR family member, HER2/neu, has been downregulated. However, distinct from these prior examples, c-MET pathway activation does not arise via amplification of c-MET, but rather by upregulation of a binding partner that potentiates ligand-induced c-MET signaling.

Finally, it is interesting to note that although our findings reveal that SPSB1 plays a critical role in the survival and recurrence of residual tumor cells, it does not seem to play an important role in the growth of primary HER2/neu-induced mammary tumors. In fact, SPSB1-expressing cells were selected against during primary tumor outgrowth. This observation raises the important possibility that pharmacologic targets for preventing or treating cancer recurrence may be unique to this stage of disease progression. If true, this would necessitate that such targets be identified and their roles elucidated in the specific clinical context of tumor dormancy and recurrence. In this regard, our findings suggest that the use of genetically engineered mouse models to identify compensatory pathways of tumor escape from targeted therapies and chemotherapy in mice will enable a better understanding of the molecular underpinnings of cancer recurrence.

METHODS

Animals, Tissues, Immunostaining, and Molecular Analyses
All mice were housed and treated in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania (Philadelphia, PA). MMTV-rTA;TetO-HER2/neu (MTB/TAN), MMTV-rTA;TetO-MYC (MTB/TOM), and MMTV-rTA;TetO-Wnt1;Trp53−/− (MTB/TWNT;Trp53−/−) mice were housed, induced with 2 mg/mL doxycycline, monitored for tumor development, and sacrificed as described previously (2, 4). Details of tissue fixation, immunofluorescence, immunohistochemistry, Western blotting, and quantitative RNA analyses are provided in the Supplementary Experimental Procedures.

Cancer Cell Lines
Hs578T, BT474, and MDA-MB-231 were obtained as part of the NCI60 panel available at the ATCC and were grown under recommended conditions at 37°C in 5% CO2. Overexpression of HER2 was confirmed by Western blot analysis in BT-474 cells. Primary tumor cells harvested from tumors arising in MMTV-rTA;TetO-HER2/neu mice maintained on doxycycline were cultured as described previously (4).

Tumor Grafting and Retroviral Transduction
Details for culture of tumor cells harvested from MMTV-rTA;TetO-HER2/neu mice, retroviral transduction, and tumor grafting are provided in the Supplementary Experimental Procedures.

Human Breast Cancer Datasets
Descriptions of human breast cancer microarray datasets that were interrogated and methods used for data analysis, including survival analysis, calculation of HRs, and estimation of the association between SPSB1 expression and established prognostic factors, are described in detail in the Supplementary Experimental Procedures.

Statistical Analyses
Details for statistical analysis are included in the Supplementary Experimental Procedures.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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