

Combined genomic and phenotype screening reveals secretory factor SPINK1 as an invasion and survival factor associated with patient prognosis in breast cancer

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Secretory factors that drive cancer progression are attractive immunotherapeutic targets. We used a whole-genome data-mining approach on multiple cohorts of breast tumours annotated for clinical outcomes to discover such factors. We identified Serine protease inhibitor Kazal-type 1 (SPINK1) to be associated with poor survival in estrogen receptor-positive (ER+) cases. Immunohistochemistry showed that SPINK1 was absent in normal breast, present in early and advanced tumours, and its expression correlated with poor survival in ER+ tumours. In ER- cases, the prognostic effect did not reach statistical significance. Forced expression and/or exposure to recombinant SPINK1 induced invasiveness without affecting cell proliferation. However, down-regulation of SPINK1 resulted in cell death. Further, SPINK1 overexpressing cells were resistant to drug-induced apoptosis due to reduced caspase-3 levels and high expression of Bcl2 and phospho-Bcl2 proteins. Intriguingly, these anti-apoptotic effects of SPINK1 were abrogated by mutations of its protease inhibition domain. Thus, SPINK1 affects multiple aggressive properties in breast cancer: survival, invasiveness and chemoresistance. Because SPINK1 effects are abrogated by neutralizing antibodies, we suggest that SPINK1 is a viable potential therapeutic target in breast cancer.

INTRODUCTION

In breast cancer treatment, resistance to prevailing drug treatments prompts a need to find novel molecular targets for antibody-based or small molecule-based therapeutics. Towards this end, we mined the expression profiles of 760 primary breast cancers from multiple patient cohorts to select genes encoding extracellular proteins, secreted growth factors and cell surface receptors that are consistently associated with poor patient survival for potential therapeutic targets. The most promising candidate was the serine protease inhibitor Kazal-type 1 (SPINK1).

SPINK1 is a 6 kDa secreted factor and its primary function is trypsin inhibition in the pancreas and small intestines (Paju & Stenman, 2006). Multiple variants of SPINK1 have been

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previously shown to be a strong causal factor for chronic pancreatitis possibly due to inhibition of its trypsin inactivation function (Bagul et al, 2009; Derikx and Szmola, 2009). In addition, inactivation of SPINK1 in the pancreas results in autophagy of exocrine pancreatic cells, suggesting that SPINK1 may be important in cell survival. Interestingly, mice deficient in SPINK3, the mouse homolog of human SPINK1, die due to excessive autophagy of the pancreatic acinar cells and, similarly, autophagy has recently been demonstrated by silencing the SPINK1 homolog in Hydra. Autophagy is mediated by ATG5/12 and can be regulated by Bcl2 expression (Djavaheri-Mergny et al, 2010). In liver cells, Hepatitis B and C viruses induced expression of SPINK1, thus protecting liver cells from serine protease dependent cellular apoptosis (SPDCA; Lamontagne et al, 2010). Taken together, SPINK1 appears to play an important role in both cell survival and prevention of apoptosis by several different pathways in normal tissues.

In cancer, SPINK1 was first discovered in the urine of ovarian cancer patients, but has since been found to be strongly associated with poor prognosis in multiple cancers including that of the prostate, ovary, bladder, pancreas and colon (Gaber et al, 2009; Gouyer et al, 2008; Kelloniemi et al, 2003; Tomlins et al, 2008). In some cell lines derived from these tissue types, SPINK1 enhanced proliferation and cell invasion. It has been suggested that SPINK1 induces cellular invasion through pathways such as phosphoinositide 3-kinase (PI3K), Rho-like GTPases and protein kinase C (PKC). This process appears to require its trypsin inhibitory property as a single mutation at the trypsin binding site fails to induce cellular invasion of colon cancer cells (Gouyer et al, 2008). These data suggest that SPINK1 confers some oncogenic properties to cancer cells.

Since SPINK1 expression associated with poor survival outcome in breast cancer, we comprehensively altered SPINK1 levels using siRNA-mediated knockdown, stable overexpression and exogenous application of SPINK1 to characterize its molecular functions. We show that SPINK1 not only affects invasion, motility and cellular survival as reported previously, but, in addition, confers chemoresistance to multiple drugs by decreasing caspase-3 levels and increasing basal levels of anti-apoptotic factors, Bcl2 and phosphor-Bcl2. Moreover, the protease inhibitor activity of SPINK1 is essential for such chemoresistance but unlike in prostate and colon cancer, it is dispensable for cell invasion. Further, we show that both SPINK1 RNA and protein levels are predictive of patient survival specifically in ER+ cases. Our data identifies novel and breast cancer specific mechanisms that may affect cancer progression and suggest that SPINK1 is an important therapeutic target.

RESULTS

SPINK1 transcript levels are positively correlated with breast cancer metastasis

To discover novel targetable oncogenes in breast cancer, we developed a whole-genome data-mining approach that leverages large independent microarray studies for the systematic discovery of genes correlated with distant metastasis-

free survival (DMFS) of patients. In an initial screen involving 760 tumours derived from four independent breast cancer cohorts (Loi et al, 2007; Miller et al, 2005; van de Vijver et al, 2002; Wang et al, 2005), we performed Cox proportional hazards regression on each of 44,928 gene probe sets (Affymetrix U133A and U133B Genechips) to identify candidate oncogenes by their statistically significant and reproducible associations with metastatic recurrence. The genes were then cross-referenced with the Gene Ontology database (www.geneontology.org) to identify all metastasis-associated genes encoding known and predicted transmembrane receptors and secreted growth factors (Table S1 of Supporting information). Of the small number of candidate genes we identified, SPINK1 was positively correlated with metastatic recurrence in all four cohorts. We next extended our meta-analysis to all publicly available breast cancer expression array datasets (annotated for survival outcomes; Chin et al, 2006; Loi et al, 2007; Miller et al, 2005; Minn et al, 2005; Schmidt et al, 2008; van de Vijver et al, 2002; Wang et al, 2005; Zhang et al, 2009) as of 2009, and found that the expression of SPINK1 was reproducibly correlated with metastatic recurrence in 8 of 10 breast cancer studies (overall hazard ratio = 1.19; confidence interval [1.10, 1.28]; $p < 1E-05$; Fig 1). Therefore, SPINK1 and its clinical and biological effects were analysed further.

SPINK1 associates with poor prognosis mainly in ER+ LN- breast cancer

To study the association of SPINK1 with prognosis in specific subtypes of breast cancer classified by Perou et al (Perou et al, 2000), we extended the analyses to a 'mega cohort' of 2034 publicly available breast cancer expression profiles from 14 independent cohorts (Table S2 of Supporting information; Chin & Gray, 2008; Desmedt et al, 2007; Ivshina et al, 2006; Loi et al, 2007, 2008; Miller et al, 2005; Minn et al, 2005, 2007; Pawitan et al, 2005; Schmidt et al, 2008; Wang et al, 2005; Yau et al, 2007; Zhang et al, 2009). We found that SPINK1 expression was linearly associated with poor DMFS in ER+ patients ($p < 0.001$), but did not significantly correlate with the survival of the

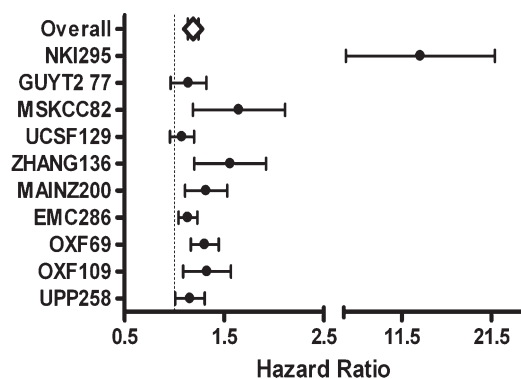


Figure 1. Meta-analysis displaying the association of SPINK1 expression with poor DMFS across multiple cohorts ($p < 1E-05$). Ten publicly available datasets were analysed and are listed on the Y-axis of the graph. The dotted line represents hazard ratio of 1.

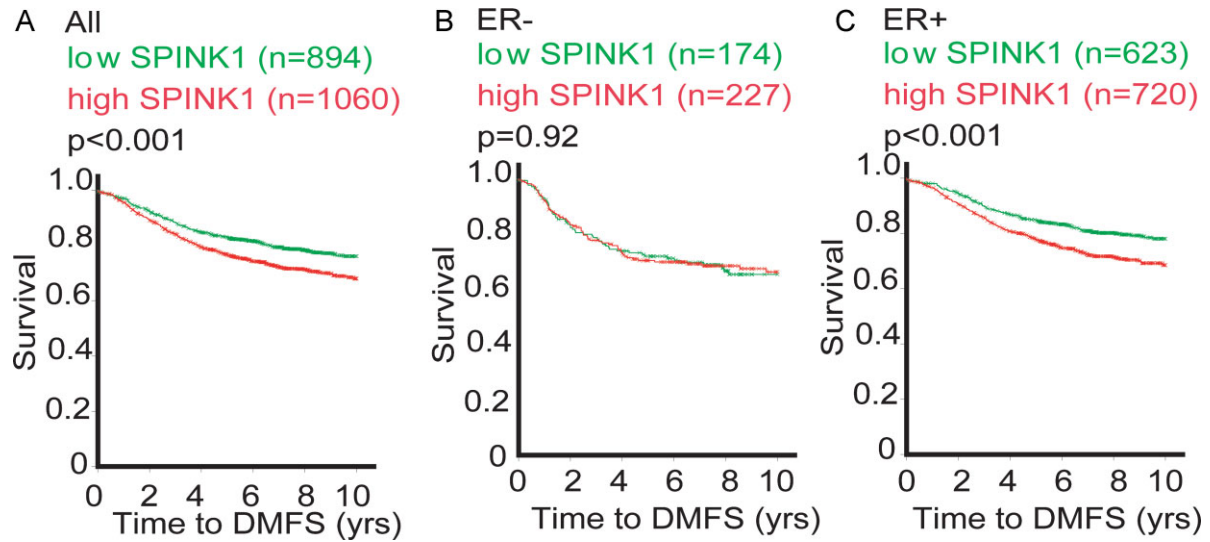


Figure 2. Kaplan–Meier curves demonstrating the association of SPINK1 expression with patient prognosis in ER+ and ER– cohorts. Samples are divided into two groups, above mean SPINK1 expression (red) and below mean SPINK1 expression (green). Prognosis is measured by disease and metastasis free survival (years).

- A. SPINK1 expression in all samples.
- B. ER-negative cases.
- C. ER-positive cases.

ER-negative (ER–) group (Fig 2). However, stratification of ER– patients into deciles based on the intensity of SPINK1 expression showed that very high SPINK1 levels marginally displayed a minor trend towards poor prognosis in ER– patients (Fig S1 of Supporting information). This suggests that SPINK1 exerts its greatest differential impact on ER+ cancers, with only marginal effect on ER– breast cancers. Interestingly, the impact of SPINK1 on prognosis was highest in the less aggressive clinical subsets of breast cancer (ER+/LN–, Table 1). Further, we studied if SPINK1 was a predictor of outcome or distinguished any intrinsic subtypes within the ER+ tumours. We analysed SPINK1 expression–survival associations in LumA, LumB, Normal-like, HER2-enriched, No Subtype and Basal-like groups, within ER+ breast cancer only (Table 2). By Cox analysis, in the Normal-like ER+ tumours, SPINK1 expression was significantly correlated with DMFS ($p=0.02$), while positive trends for

association were observed in both LumA and ‘No Subtype’ ER+ cases ($p=0.10$ and 0.13 , respectively). In the LumB ER+ subgroup, the Cox p -value was 0.62 which essentially argued no survival association. Together, these observations suggest that SPINK1 along with ER status was an important predictor of outcome irrespective of further sub-classification into intrinsic subtypes. SPINK1 did not show differential expression between ER+ and ER– samples, nor was its expression biased in any intrinsic molecular subtypes of breast cancer (Fig S2 of Supporting information). This is consistent with the notion that the SPINK1 association with survival is not a function of differential expression in the different tumour subtypes.

SPINK1 expression differs in normal and breast tumour tissue

To investigate the localization of the SPINK1 protein in both benign and malignant breast tissues, we immunostained 98

Table 1. SPINK1 and clinical outcome in breast cancer subgroups

Subgroups	No. of Cases	p-Value	Hazard ratio	95% Confidence interval
All	1954	0.008	1.10	1.026–1.179
ER–	401	0.945	1.01	0.874–1.155
ER+	1343	0.011	1.12	1.027–1.218
LN–	1404	0.004	1.13	1.041–1.225
LN+	377	0.262	1.09	0.937–1.271
G1	236	0.061	1.38	0.978–1.952
G2	652	0.218	1.08	0.960–1.206
G3	576	0.916	1.01	0.893–1.135
ER+, LN–	902	0.017	1.14	1.026–1.261
ER+, LN+	300	0.069	1.18	0.988–1.400
ER+, LN–, Endocrine Monotherapy	357	0.036	1.21	1.023–1.443
ER+, LN+, Endocrine Monotherapy	197	0.145	1.17	0.949–1.438

Table 2. SPINK1 and intrinsic subtypes of breast cancer

Subtype	#ER+	Cox pval	Cox HR	Cox 95% CI
LumA	471	0.1	1.19	0.97–1.45
LumB	328	0.62	1.03	0.91–1.17
Normal-like	228	0.02	1.34	1.06–1.69
No Subtype	69	0.13	1.36	0.91–2.04
HER2±like	106	0.72	0.96	0.76–1.21
Basal	141	0.67	0.94	0.71–1.24

clinically annotated cores on a tissue microarray (TMA) using an anti-SPINK1 antibody. Normal pancreas served as a positive control and specificity of the staining was evaluated by pre-neutralizing the antibody with recombinant SPINK1 protein (Fig S3 of Supporting information). The expression of SPINK1 was negligible in normal breast tissue, but most tumours were immunoreactive (95%; Fig 3, panels A–C). This suggested that SPINK1 was probably expressed early in tumour development and continued to be present in advanced disease. Of the 98 cores stained, 81 breast cancer samples were annotated for patient survival data. Analysis (see Materials and Methods Section) showed that high SPINK1 protein levels correlated with overall lower disease-free survival. Upon sub-division of patients into ER+ and ER– cohorts, this correlation was most significant in the subset of ER+ breast cancer cases (Fig 4), supporting our initial findings that the SPINK1 transcript displayed positive association with poor prognosis predominantly with ER+ cases. Moreover, SPINK1 expression did not associate with grade, age,

ethnicity, stage and *cERBB2* (Table S3 of Supporting information). Interestingly, SPINK1 subcellular localization seemed to vary across tumour tissues, with some expressing SPINK1 in the cytoplasm and others in the nuclei. To confirm that this nuclear localization is not an artefact of the immunohistochemical staining of paraffin processed tissue, we performed immunofluorescence on MDA-MB-231 cells treated with soluble recombinant SPINK1 bearing a 6Xhis tag. Using an antibody against the tag as well as an antibody against SPINK1, we confirmed that exogenously applied SPINK1 localized in the nucleus within 24 h of application (Fig 3D).

Loss of SPINK1 results in reduced cell survival

Although we observed significant correlation of SPINK1 to DMFS mainly in ER+ patients, we investigated the mechanistic effects of SPINK1 in both ER+ and ER– cells. We attenuated SPINK1 transcript levels by siRNA in the breast cancer cell lines MCF-7 and MDA-MB-231 and conducted assays to detect phenotypic changes in cell proliferation, motility, invasiveness and anchorage-independent growth. Each of the 3 SPINK1-targeted siRNA constructs reduced SPINK1 levels in MCF-7 and MDA-MB-231 cells by >50%, with C2 siRNA being the most efficient (>95%; Fig 5A). Consistent with a threshold effect, C2 siRNA led to the most significant reduction of proliferation for both MCF-7 and MDA-MB-231 (Fig 5B), whereas, C1 and C3 siRNAs displayed marginal phenotypes. We posited that the observed decrease in proliferation could reflect either cell cycle arrest or apoptosis. Therefore, we studied the activation of

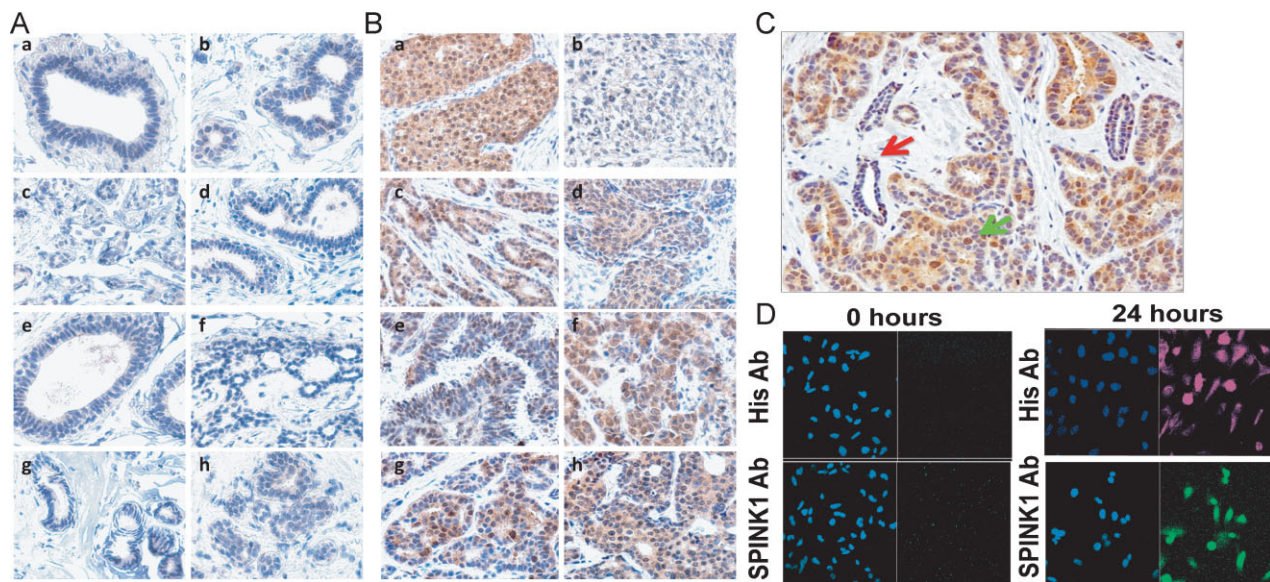


Figure 3. SPINK1 expression in normal breast and breast tumours.

- A. SPINK1 expression was negligible in 10 normal breast cores (panels a–h).
- B. This panel shows representative cores from the commercial breast TMA. As shown, invasive ductal carcinomas were positive but displayed various levels of SPINK1 (panels a–h).
- C. SPINK1 nuclear staining was largely restricted to the breast tumour cells (green arrow) as compared to adjacent normal cells (red arrow).
- D. Localization of SPINK1 *in vitro*. MDA-MB-231 cells were treated with SPINK1-CM and uptake of SPINK1 (if any) was studied using two antibodies. SPINK1 staining observed in cells after 12–24 h of SPINK1 treatment, recapitulating staining observed in primary tumours (Blue: DAPI; Green: anti-SPINK1 antibody; Red: anti-His antibody).

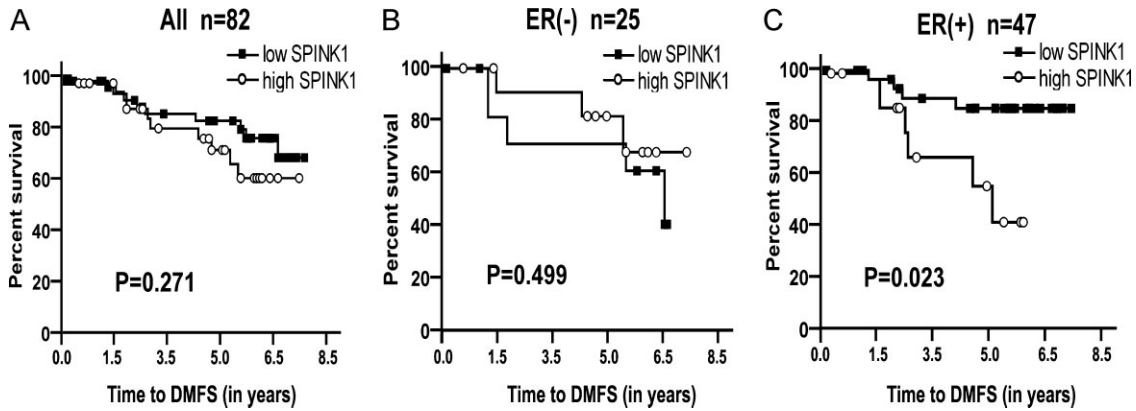


Figure 4. Kaplan–Meier curves demonstrating the association of SPINK1 protein levels with patient prognosis in ER+ and ER– cohorts. Prognosis is measured with DMFS (years).

- A. SPINK1 protein expression in all samples.
- B. ER-negative cases.
- C. ER-positive cases.

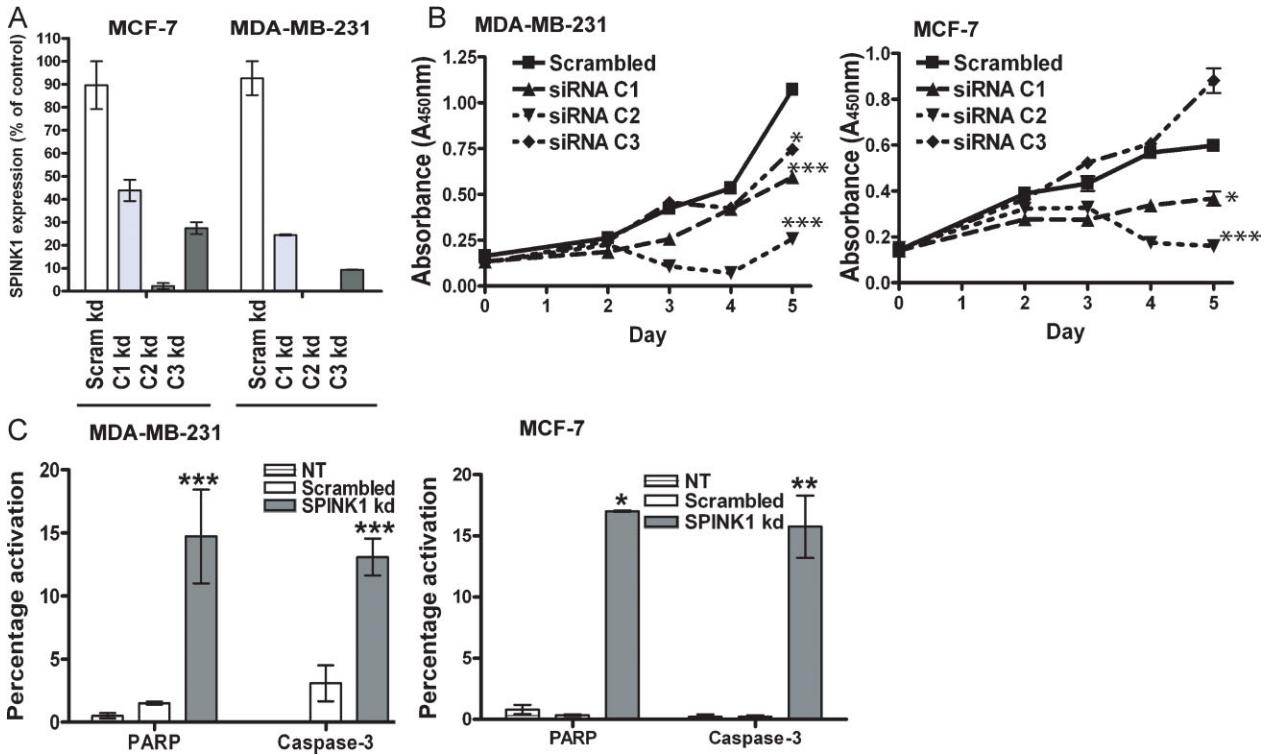


Figure 5. Phenotypic assays upon siRNA-knockdown of SPINK1.

- A. siRNA-mediated knockdown (kd) of SPINK1 using three different siRNAs in breast cancer cell lines MCF-7 and MDA-MB-231. SPINK1 expression was detected via RT-PCR.
- B. Effect of SPINK1-kd on cell proliferation in MDA-MB-231 (left panel) and MCF-7 (right panel).
- C. Activated PARP and caspase-3 upon siRNA-mediated knockdown of SPINK1 in MDA-MB-231 (left panel) and MCF-7 (right panel). (* $p < 0.005$; ** $p < 0.05$; *** $p < 1E-05$).

apoptotic markers PARP and caspase-3 using a cell-based immunofluorescence assay. Attenuation of SPINK1 induced activation of both PARP and caspase-3 in MDA-MB-231 ($p < 0.05$, Fig 5C, left panel) and MCF-7 cells (Fig 5C, right panel). Secondly, since loss of SPINK1 leads to excessive autophagy in animal model systems, we studied if siRNA mediated knockdown of ATG5/12 was able to rescue cell death caused by loss of SPINK1. As shown in Fig S4 of Supporting information, interfering with autophagy could not rescue cell death. Taken together, the apparent decrease in cell proliferation upon loss of SPINK1 was due to the induction of cellular apoptosis. Therefore, SPINK1 appears to be essential for survival of breast cancer cells lines regardless of estrogen receptor status.

Rescue of siRNA-mediated phenotype(s) by exogenous SPINK1

To determine the specificity of our SPINK1 siRNA constructs by a phenotypic rescue, we generated two sources of recombinant SPINK1 by over-expressing SPINK1 in MCF-7 and SF9 insect cells (Fig S5, panels A–C of Supporting information). Conditioned media (CM) was collected from both sources (referred to as SPINK1 CM and SF9spink1 CM), as well as from cells transfected with empty vector as controls (vector-CM and SF9vecCM, respectively). Treatment of C2siRNA cells with SPINK1-CM resulted in 5-bromodeoxyuridine (BrDU) incorporation showing that these cells survived and could subsequently divide (Fig 6). The control Vec-CM was unable to rescue this cell death. This confirms the specificity of the siRNA used as well as the functionality of the recombinant SPINK1. Interestingly, neither MCF-7 cells over-expressing SPINK1 (MCF-7 SPINK1OE) nor various breast cancer cell lines treated with SPINK1-CM showed increased proliferation (Fig S6 of Supporting information). This further supports the notion that SPINK1 is probably an important survival factor rather than a potent growth inducer in breast cancer.

SPINK1 augments drug resistance

Given the evidence that SPINK1 is a survival factor in breast cancer cell lines, we asked whether SPINK1 could increase cellular resistance to anti-cancer drugs in either an autocrine or a paracrine fashion. First, we treated MCF-7 SPINK1 overexpressing cells (SPINK1OE) with increasing concentrations of five commonly used chemotherapeutic drugs, 5-fluorouracil (5-FU), suberoylanilide hydroxamic acid (SAHA), tamoxifen (TAM), adriamycin (ADR) and etoposide (VP16). SPINK1OE cells were significantly more resistant to chemically induced apoptosis as compared to control cells, as shown by decreased PARP (left panel) and caspase-3 (right panel) activation levels (Fig 7). Next, we pretreated MCF-7 parental cells with exogenous SPINK1-CM before inducing apoptosis with 5-FU. Strikingly, 5-FU-mediated cell death was abrogated by pretreatment with SPINK1-CM but not by control media (vector-CM), however to a degree lesser than that observed in MCF-7 SPINK1OE cells (Fig S7 of Supporting information). Therefore, increased levels of SPINK1, both by autocrine and paracrine mechanisms, were capable of conferring increased drug resistance in breast cancer cells.

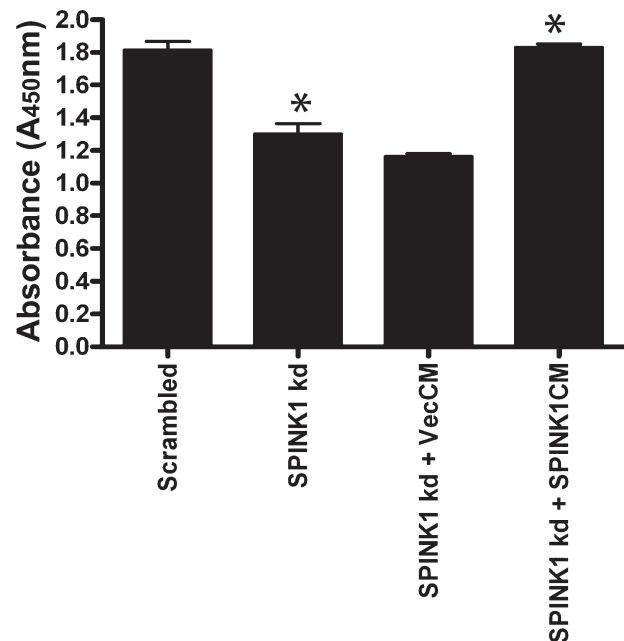


Figure 6. Proliferation rescue assay of SPINK1 in MDA-MB-231.

Incorporation of BrDU in MDA-MB-231 cells upon SPINK1-kd, and rescue with bioactive SPINK1-CM. Significance of knockdown was measured against scrambled control, while significance of SPINK1CM was measured against VecCM as a control (* $p < 0.005$).

SPINK1 induces resistance to apoptosis through regulation of main apoptotic players

Since SPINK1 protected cells from drug-induced apoptosis, we studied the expression of a panel of pro- and anti-apoptotic genes including caspase-3, caspase-8, PARP, BID, Bcl2 and phospho-Bcl2 in MCF-7 cells treated with SPINK1-CM for 2 h. As shown, apoptosis-inhibitor Bcl2 levels were upregulated without changing caspase-3, BID, PARP and caspase-8 levels (Fig 8A and S8 of Supporting information). Interestingly, in different clones of MCF-7 SPINK1 OE cells, endogenous Bcl2 and phospho-Bcl2 increased and caspase-3 levels decreased with increasing expression of SPINK1 (Fig 8B). The decrease in caspase-3 was, however, not evident in cells treated with SPINK1-CM (Fig 8A), perhaps indicating that prolonged high expression of SPINK1 may lead to higher chemoresistance. Since previous studies showed that the protease inhibitor function of SPINK1 is necessary for some oncogenic properties of SPINK1 (Lamontagne et al, 2010), we generated a SPINK1 K18Y mutant lacking this function (Gouyer et al, 2008; Fig S5D of Supporting information). As shown in Fig 9, pretreatment of cells with SPINK1-K18Y-CM (hatched bars) was unable to protect cells from 5 FU- (panel A) or SAHA- (panel B) induced apoptosis. Together, these data show that high SPINK1 induces chemoresistance and its protease inhibitor function is critical to mediate this effect.

SPINK1 leads to increased invasion of breast cancer cells

As part of our cellular screen for SPINK1 induced phenotypes, we had found that knockdown of SPINK1 attenuated invasion of

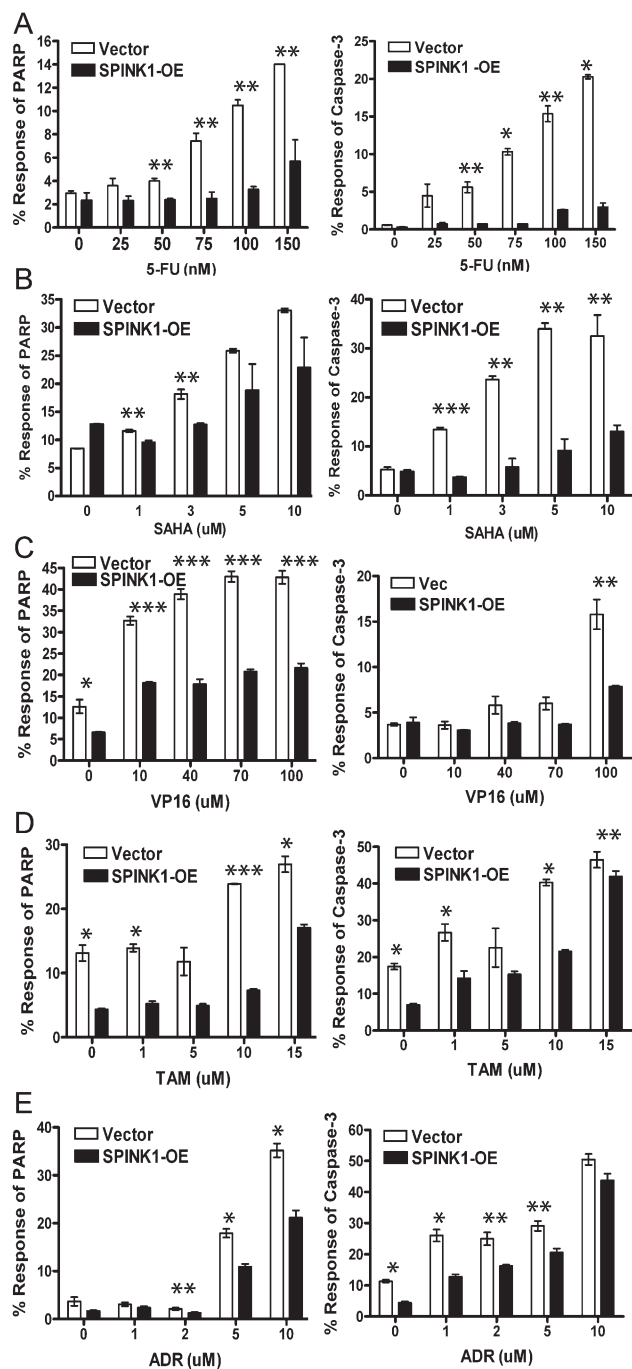


Figure 7. Induction of resistance to chemically induced apoptosis.

Activation of PARP (left panel) and caspase-3 (right panel) was measured in MCF-7 vector control (white bars) and SPINK1 overexpressing MCF-7 cells (black bars) following 24 h of exposure to varying concentrations of drugs. Effects of SPINK1 were measured using the student's *t*-test with vector as control (**p* < 0.005; ***p* < 0.05; ****p* < 1E-05).

- A. 5-FU (5-fluorouracil)
 B. SAHA (suberoylanilide hydroxamic acid)
 C. VP16 (etoposide)
 D. TAM (tamoxifen)
 E. ADR (adriamycin)

breast cancer cell lines and that this effect could be rescued using SPINK-CM but not by Vec-CM (Figs S9 and 10A of Supporting information). We extended this analysis using recombinant SPINK1 and found that in two invasive breast cancer cell lines, MDA-MB-231 and BT549, SPINK1-CM significantly increased invasion by 2–3 fold (Fig 10B). Non-invasive MCF10A and MCF-7 cells do not invade in 24 h. After 48 h, these cells show a relative increase in invasion, however, the total number of cells was extremely low (Fig S10 of Supporting information). Importantly, we found that induction of cellular invasion in MDA MB 231 and BT-549 cells could be specifically immuno-neutralized by anti-SPINK1 antibodies (Fig 10C, Fig S11 of Supporting information). We then tested whether the invasive property of SPINK1 was due to its activity as a trypsin/serine protease inhibitor. We exposed K18YCM to MDA-MB-231 cells, and found that K18YCM retained the ability to induce cellular invasion like wild-type (WT) SPINK1-CM, suggesting that active protease inhibitor function is not necessary (Fig 10D). Similar effects were observed in BT549 cells (data not shown). Thus, the anti-apoptotic and the pro-invasive activity of SPINK1 are separable by its protease inhibitor function.

SPINK1 induces metastasis *in vivo*

To test if the *in vitro* invasiveness of MDA-MB-231 cells was similarly affected *in vivo*, we injected MDA-MB-231 cells overexpressing SPINK1 (MB231 SPINK1OE) into the tail-vein of Balb/c nu/nu mice. Figure 11 shows that SPINK1 expression led to increase in both the number and size of metastatic lesions in the mouse lung. Mice injected with MB231-vector cells had only $4.1\% \pm 2.7$ lesions larger than 200 μm , while MB231 SPINK1OE cells gave rise to significantly larger lesions with $24.8\% \pm 8.5$ larger than 200 μm (*p* < 0.05). The MB231 SPINK1OE cells also formed almost threefold more lesions than that of the control cells (*p* < 0.05). In concordance with our *in vitro* data, MCF-7 SPINK1OE cells did not show lung metastases *in vivo* (data not shown). These data suggest that in breast tumour cells with prior capacity to invade, high SPINK1 expression may lead to increased metastasis as compared to the same cells expressing relatively lower amounts. However, due to unavailability of a suitable invasive model system, SPINK1 effects in ER+ cells remain unexplored.

DISCUSSION

In this study, using genome-wide profiling of breast tumours from multiple cohorts as well as validation by cell-based phenotypic assays, we identified for the first time that SPINK1 is a biological driver of aggressiveness in breast cancer. All breast cancer cohorts analysed display a positive association of SPINK1 expression with poor outcome. Further analysis of SPINK1 in 2034 breast tumours from 14 independent cohorts revealed that SPINK1 is significantly associated with poor prognosis only in ER+ tumours but not in ER-. In intrinsic subtype-based analysis, SPINK1 was prognostic in Normal-like and LumA groups. Though LumB is clinically the most relevant subtype for poor prognosis, we found no SPINK1-survival association in this

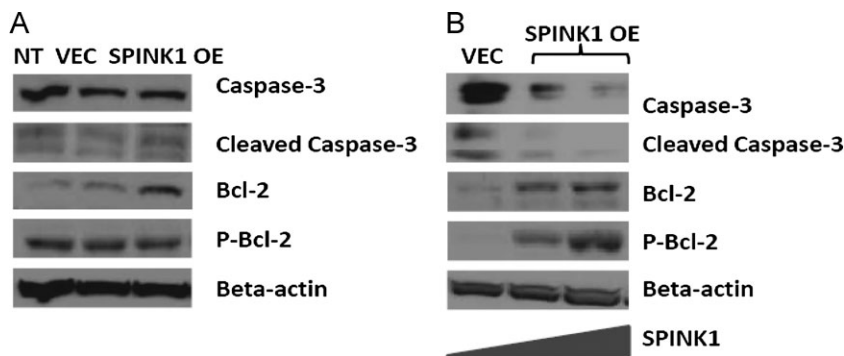


Figure 8. Protein levels of apoptotic genes in the presence of SPINK1.

A. Protein levels of main apoptotic regulators were measured via Western blot 2 h after SPINK1CM (SH9), vecCM or no CM was added to MCF-7 cells.
B. Expression of main apoptotic regulators was measured in MCF-7 cells that overexpress SPINK1 at varying levels (SH5 and SH9), along with empty vector overexpression as a control.

group. Careful analysis of our cohorts showed that ER status was not synonymous with LumA/LumB classification, the ER+ group contained variable numbers of all five intrinsic subtypes as well as a ‘no subtype’ group (i.e. <10% correlation with any subtype). Secondly, ER- tumours and those whose clinical ER status was unknown, occasionally mapped to LumA and LumB. We suggest that these factors potentially confound the impact of SPINK1 in these subtypes as it relates to clinical ER positivity. However, SPINK1-survival association was strongest in less aggressive ER+ LN- tumours.

This prognostic association is recapitulated at the protein level: in a panel of clinically annotated breast tumours, high SPINK1 expression was consistently and significantly associated with poor prognosis of ER+ rather than ER- tumours. More detailed analysis of meta-analytical data, however, showed a small effect by SPINK1 on prognosis of ER- patients: ER- individuals in the highest SPINK1 expressing deciles have a slightly poorer survival than the lowest expressing deciles. However, the numbers of ER- patients in the meta-analysis were one third those of ER+ patients, thus limiting the power to

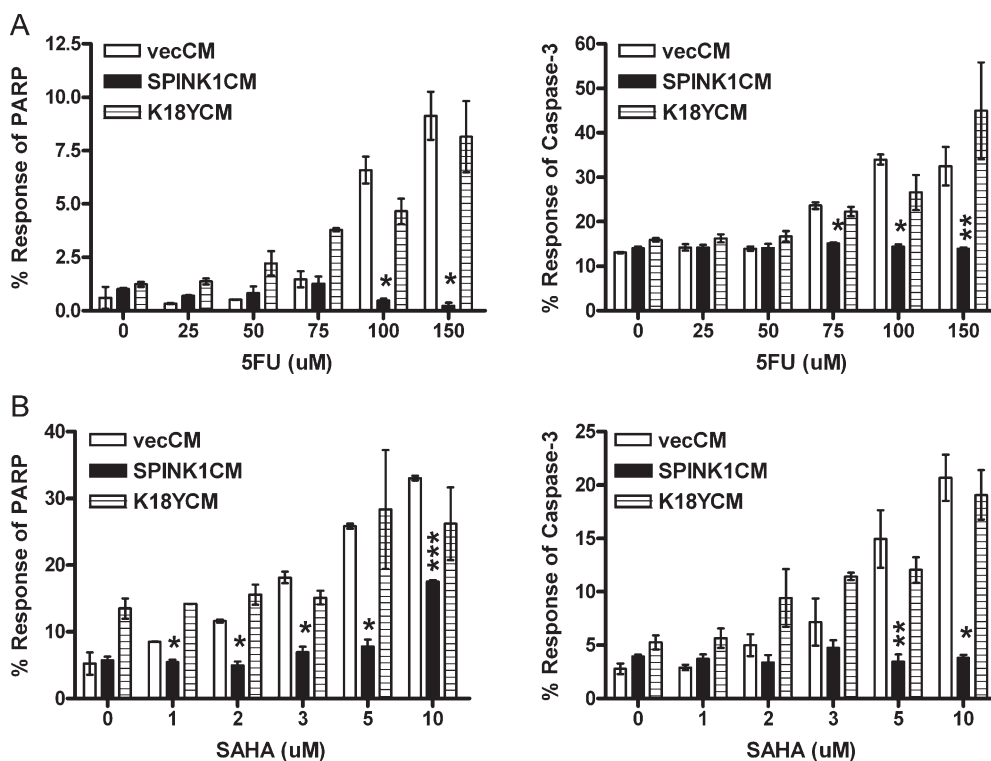


Figure 9. Induction of resistance to chemically induced apoptosis. Parental MCF-7 cells were pretreated with vecCM (white bars), WT SPINK1CM (black bars) or mutant K18YCM and then exposed to drugs for 24 h. Apoptosis was measured via activated PARP (left panels) and activated caspase-3 (right panels) levels. (**p* < 0.05; ***p* < 0.01; ****p* < 1E-05).

A. 5-FU
B. SAHA

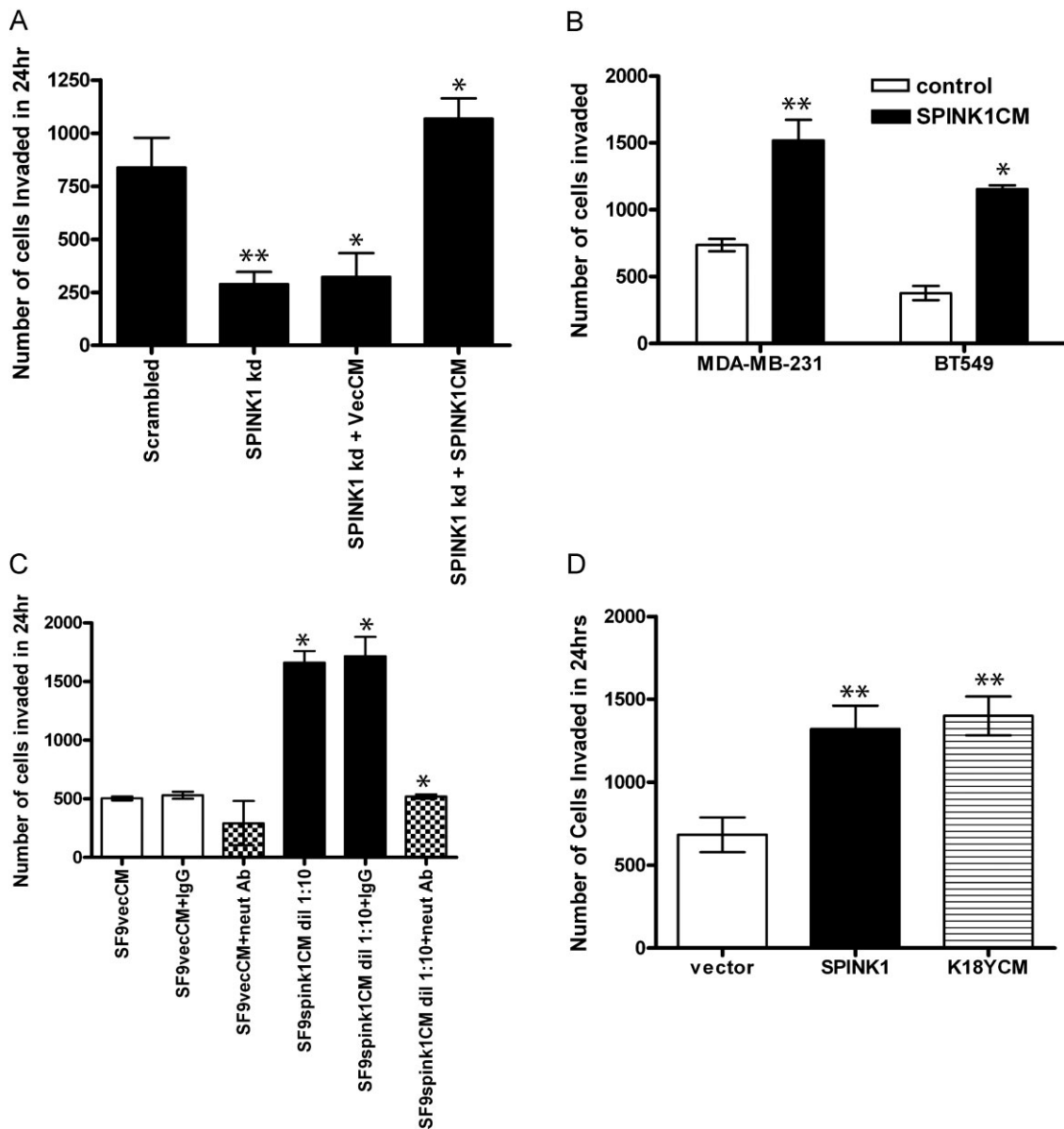


Figure 10. SPINK1 induces cellular invasion *in vitro* and *in vivo*.

- A.** Cell invasion assay with MDA-MB-231 cells. Loss of invasion upon SPINK1 siRNA treatment, and rescued with SPINK1-CM.
B. Effect of SPINK1-CM on invasion in two breast cell lines. The total number of cells that invaded was measured after 24 h (MB231) or 48 h (BT549) in response to SPINK1-CM and vector control CM.
C. Effect of SF9spink1CM and immunoneutralization on MDA-MB-231 cells. (White bars—SF9vecCM; black bars—SF9spink1CM diluted 10×; checkered bars—CM with addition of neutralizing antibody against SPINK1).
D. Invasion of MDA-MB-231 treated with WT SPINK1 and mutant SPINK1. Number of cells invaded were measured 24 h after assay setup. (* $p < 0.005$; ** $p < 0.05$).

discern SPINK1 effects in the ER⁻ group. That SPINK1 may be operative in both ER⁺ and ER⁻ conditions is confirmed by the enforced overexpression of SPINK1 or the addition of exogenous recombinant SPINK1 protein, which induced the same cellular effects in ER⁺ and ER⁻ breast cancer cell lines. Such dramatic and non-physiologic overexpression of SPINK1 in cell culture systems serves to exaggerate phenotypic outcomes. We suggest that any association of SPINK1 with DMFS in ER⁻ tumours

could be masked by more dominant oncogenic pathways operative in ER⁻ tumours.

With the exception of gastric malignancies, high levels of SPINK1 expression have been associated with poor prognosis in multiple cancer types such as prostate cancer, liver cancer, ovarian cancer and bladder cancer (Gaber et al, 2009; Gkialas et al, 2008; Gouyer et al, 2008; J-P Wiksten, 2005; Kelloniemi et al, 2003; Shariat et al, 2005; Tomlins et al, 2008). SPINK1

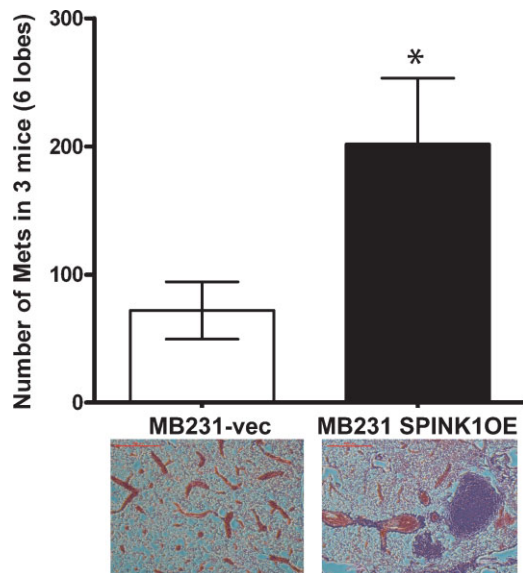


Figure 11. SPINK1 enhances metastases *in vivo*. MDA-MB-231 vector-control cells or SPINK1-overexpressing cells were injected intravenously into three mice each, and total lung metastases were counted 12 weeks later. (* $p < 0.005$).

expression is high in ETS-rearrangement negative prostate cancer and loss of SPINK1 attenuates invasiveness and tumour growth (Ateeq et al, in press; Tomlins et al, 2008). Recombinant SPINK1 has been described to have opposing effects that are cell type specific, augmenting cell proliferation in gastric and pancreatic (Marchbank et al, 2009; Ozaki et al, 2009) but not in prostate cancer cell lines (Tomlins et al, 2008). Functionally, SPINK1 serves as a motility factor in various colon, prostate and gastric cancer cell lines and this effect can be attenuated in colon cancer cell lines by PI3K, PKC and Rho GTPase inhibitors (Gouyer et al, 2008; Marchbank et al, 2009; Tomlins et al, 2008).

To determine cellular functions of SPINK1 in breast cancer, we analysed the phenotypic effects of SPINK1 attenuation, recombinant SPINK1 treatment and SPINK1 over expression. Our results strongly suggest that SPINK1 does not induce proliferation in breast cancer cells, but rather is required for survival of cancer cells. Further, SPINK1 led to increased invasion of cancer cells without affecting cell proliferation both *in vitro* and in *in vivo* tail vein metastasis assays. However, unlike prostate and colon cancer, we found that the protease inhibitor function of SPINK1 was not important for invasion in breast cancer; however, inhibitors of PI3K, PKC and Rho GTPase pathways attenuated this response (data not shown). This suggests that SPINK1 may initiate a slightly distinct molecular pathway in breast cancer cells. Such decoupling of growth from the conjoint phenotype of invasion and survival has been well documented in another cancer-associated ligand–receptor system such as the GAS6 ligand with its cognate receptor, the tyrosine kinase AXL (McCloskey et al, 1997).

Since SPINK1 was important for cell survival, and SPINK1 expression was associated with poor patient prognosis, we studied if SPINK1 could induce chemoresistance in cancer cells. We show that recombinant SPINK1 not only enhances cell survival, but also induces resistance to apoptosis induced by common cytotoxic agents: SAHA, 5-FU, TAM, ADR and VP16. Mechanistically, we demonstrate that the presence of SPINK1 greatly reduces caspase-3 expression and consequent activation upon drug treatment. In addition, SPINK1 also leads to high basal expression of anti-apoptotic Bcl2 and phospho-Bcl2. This is compelling as Bcl2 is a strong prognostic factor in node-negative, ER+ breast cancers that respond poorly to chemotherapy (Paik et al, 2006). Thus a molecular relationship between SPINK1, Bcl2 and chemoresistance may explain the association of SPINK1 with poor outcome in ER+ LN– patients. Whether SPINK1 expression can be utilized to predict response/benefit from chemotherapy in ER+ node negative patients needs further study.

The K18Y mutant fails to protect cells or induce anti-apoptotic signalling in cells. Taken together, these data suggest that SPINK1 with intact protease inhibitor activity alters ‘set point’ of the apoptotic machinery towards one favouring survival. Conceivably, attenuation of SPINK1 expression in breast cancer may promote the efficacy of anticancer therapies (Ghobrial et al, 2005; Kasibhatla & Tseng, 2003; Sun et al, 2004). Further experiments are required to identify the downstream effector that links SPINK1 to invasion and survival and explains this differential requirement for protease modification.

Lu et al (Lu et al, 2008) suggested that SPINK1 inhibits only serine protease-dependent apoptosis (SPDCA) owing to its protease inhibitor function but has no role to play in caspase-dependent apoptosis (CDCA) in human hepatoma cells. In breast cancer, we demonstrate that SPINK1 is capable of interfering with caspase-3 induced apoptosis suggesting additional mechanisms of chemoresistance.

Our work also found some intriguing new observations about SPINK1 biology. Despite SPINK1 being a secreted extracellular protein, we unexpectedly observed nuclear localization of SPINK1, not only in a time-dependent manner in cell lines *in vitro*, but also in the nuclei of primary tumours by immunohistochemistry. Soluble secreted growth factors such as the epidermal growth factor and fibroblast growth factor, are transported into the nucleus and are known to mediate transcription (Planque, 2006). However, the implications of such nuclear localization, in breast cancer progression remain unclear, and will need further study.

These data suggest that though SPINK1 may not be a ‘driver’ oncogene in breast cancer, its high expression critically influences key processes in cancer progression including cell survival, drug response and cell invasion ultimately leading to poor clinical outcome. Its association with ER+ tumours makes SPINK1 a plausible combinatorial therapeutic target in early breast cancer. That most of these aggressive phenotypes—chemoresistance, invasion and anti-apoptosis—can be reversed with attenuation of SPINK1 levels suggest that SPINK1 may be a reasonable therapeutic target for neutralizing antibodies.

MATERIALS AND METHODS

Breast cancer cohorts

SPINK1 expression characteristics were analysed in breast cancer using an integrated 'super cohort' of 2116 breast tumours profiled using Affymetrix GeneChip technology. The super cohort is comprised of 15 distinct breast cancer cohorts for which corresponding microarray data and clinical annotation were publicly accessible from microarray databases including the Gene Expression Omnibus (National Center for Biotechnology Information, USA), ArrayExpress (European Bioinformatics Institute, UK) and caArray (National Cancer Institute, NIH, USA). General cohort details and literature references are presented in Table S1 of Supporting information. Original discovery and metaanalysis were performed on a subset of this 'super cohort'.

Microarray data processing and case selection

In all cases, raw data (CEL files) were pre-processed and normalized using the R software package (R development Core Team, 2010) and library files provided via the Bioconductor project (Gentleman et al, 2004). In order to preserve a consistent normalization strategy across all cohorts, raw data were MAS5.0 normalized on a per-cohort basis using the justMAS function in the simpleaffy library from Bioconductor (no background correction, target intensity of 600). The specific array platforms employed here were the HG-U133A, HG-U133plus2 and HG-U133A2 gene chips. To ensure equal information content from each chip type, only probe sets common to all chip types were utilized in subsequent analysis. This resulted in the use of 22,268 probe sets that were common to all microarrays in all cohorts. Cross-cohort batch effects were corrected using the COMBAT empirical Bayes method (Johnson et al, 2007). Of the initial 2116 tumour profiles, 2034 represent primary invasive breast cancers with no exposure to neoadjuvant therapy prior to array analysis. Of these, 1954 cases are annotated with DMFS time and event. Of note, other clinical annotation such as treatment type, estrogen receptor status, nodal status, tumour size, histologic grade and patient age are available for the majority of cases.

Subtype assignment

Intrinsic subtypes were assigned to samples using the Single Sample Predictor (SSP) algorithm described by Hu et al (2006) and utilized by Fan et al (2006). Affymetrix probe sets were matched to the genes comprising the SSP centroids using UniGene annotation. Prior to batch correction, the expression data for each gene were mean centered, and Spearman correlation was used to find the centroid most closely associated with each tumour sample. In cases where a correlation greater than 0.1 was not achieved with at least one centroid, a subtype was not assigned to that sample ($n = 92$ cases).

Immunohistochemistry and immunofluorescence

TMA blocks containing cores from 98 breast cancer patients were constructed as described previously (Das et al, 2008; Salto-Tellez et al, 2007) under institutional ethics committee approval for the tissue array program (NUS-IRB 05-017). Staining was done with anti-SPINK1 monoclonal antibody (MoBiTec: PSKAN2-0100) at a dilution of 1:1000 for immunohistochemistry and 1:50 for immunofluorescence. Other antibodies used for immunofluorescence include anti-His (Delta Biolabs), Alexa 488 goat anti-mouse and Alexa 647 goat anti-rabbit

(Invitrogen) at a dilution of 1:500. The TMA slides were digitized using the Ariol SL-50 platform (Applied Imaging Inc., San-Jose, CA). Each slide was scanned at 20 \times objective with three filters: red, green and blue (RGB). The Ariol software then converted these RGB-channel scans into colour reconstructions for image analysis. For SPINK1 expression, the Ariol analytical algorithm was trained using the positive controls. The positivity of the SPINK1 expression was initially confirmed by two senior pathologists (BP and MST). The training of the Ariol algorithm was then performed by a pathological assistant (OCW) to ensure that stromal matrix and adipose cells are excluded from the image analysis. After the initial program training, the rest of the analysis was performed without human supervision. Absolute values of expression were subsequently provided by the software for all the TMA cores. Previously, we have reported a high level of concordance ($\kappa > 0.9$) between the Ariol scoring and human observer scoring methods for immunohistochemistry in a technical journal (Ong et al, 2010). The positivity of SPINK expression was determined by rank-transforming all the positive scores of SPINK1 expression into two groups based on a 50th percentile cutoff. Associations between SPINK1 expression and clinicopathological features were assessed using the student's *t*-test. All statistical analyses were performed using the SPSS package.

Cell lines, stable overexpression and conditioned media

Human breast cell lines, BT-549, MCF10A, MCF-7 and MDA-MB-231 were obtained from American Type Culture Collection (ATCC) and maintained at 37°C with 5% CO₂ with growth medium recommended by ATCC. Full-length SPINK1 was commercially obtained (Invitrogen: IOH10627). A 6XHis Tag was added using the primers (-F- 5'-ATGAAGGTAACAGGCATCTTTCTTCTCAGTGCCTT-3') and R2 5'-TCAGTGATGATGATGATGGTGGCAAGGCCAGAT-3'). Mutant SPINK1 K18Y was generated by site-directed mutagenesis kit according to the manufacturer's protocol (Calbiochem: QIA58). All clones were confirmed by sequencing on both strands. PCR products were shuttled into pCR[®] 2.1-TOPO[®] vector (Invitrogen: K4510-20) and further subcloned in mammalian expression vector pcDNA[™]3.1(+)(Invitrogen: V790-20) via restriction sites NheI and EcoRI. SPINK1 and empty control plasmids were independently transfected into MCF-7/MDA-MB-231 cells using Lipofectamine 2000. Stable colonies are selected by G418 selection (Sigma). The clones are referred to MCF-7 SPINK1OE and MB231 SPINK1OE, respectively, in this study. Insect SF9 cells were obtained from BD Biosciences and maintained in Grace's insect media (Invitrogen) supplemented with fungizone (Invitrogen) and 10% FBS. SPINK1 with a 3' 6XHis tag and K18Y mutant SPINK1 were cloned into pPK30x vector, and infected into SF9 cells with baculovirus (BD Biosciences) according to the manufacturer's protocol (Fig S4 of Supporting information).

Immunoblot analysis

For Western blot analysis, whole cell lysates were prepared using RIPA buffer. The proteins were separated by SDS-PAGE and transferred to 0.2 μ m PVDF membrane (Biorad). SPINK1 antibody (MoBiTec: PSKAN2-0100) was used to probe the membrane at 500 times dilution, and antibody-protein complex was detected by HRP-conjugated antibodies and ECL (Amersham Biosciences).

siRNA mediated knockdown

RNAi transfection on breast cancer cell lines was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's

The paper explained

PROBLEM:

In breast cancer treatment, resistance to prevailing drug treatments prompts a need to find novel molecular targets for antibody-based or small molecule-based therapeutics. Secretory factors that drive cancer progression are, therefore, attractive immunotherapeutic targets. Towards this end, we mined the expression profiles of 760 primary breast cancers from multiple patient cohorts to select genes encoding extracellular proteins, secreted growth factors and cell surface receptors that are consistently associated with poor patient survival for potential therapeutic targets. SPINK1 emerged as the most promising candidate.

RESULTS:

Integrated analysis of microarray profiles of clinically well-annotated breast tumours identified SPINK1 to be associated with poor survival in estrogen receptor-positive (ER+) cases. Immunohistochemistry showed that SPINK1 was absent in normal breast, present in early and advanced tumours, and its expression correlated with poor survival in ER+ tumours. Using

multiple breast cancer cells as model systems, we show that SPINK1 is essential for cell survival and its forced expression confers chemoresistance via reduction in pro-apoptotic caspase-3 protein and induction of the pro-survival gene, *BCL2*. Intact protease inhibitor function of SPINK1 is necessary for this function. Further, SPINK1 increased invasion of breast cancer cells *in vitro* and in mouse tail vein assays.

IMPACT:

Our data shows that SPINK1 significantly associates with poor prognosis in ER+ versus ER- patients. Further, high SPINK1 expression in the well-differentiated ER+ lymph node-negative (LN-) group is a highly significant predictor of poor outcome. On a molecular level, SPINK1 affects multiple aggressive properties in breast cancer: survival, invasiveness and chemoresistance. Because SPINK1 effects are abrogated by neutralizing antibodies, we suggest that SPINK1 is a viable potential therapeutic target in breast cancer.

protocol. 3 SPINK1 specific siRNAs were used, C2siRNA (5'-CCAA-GAUUAUGACCCUGUTT-3') gave the optimal knockdown of SPINK1, (C1-5'-CCAAGUUUUUGAAAUCCATT-3'; and C3-5'-GAGGCCAAU-GUUACAAUGTT-3'). Knockdown efficiency of the siRNAs were checked using Polymerase Chain Reaction (after reverse transcription) (RT-PCR) with (SPINK1-F 5'-CATCTGCCATATGACCCTTC-3'; SPINK1-R 5'-GATGCCTGTTACCTTCATGG-3'; SPINK1-H 5'-FAM-GACGTGG-TAAGTGGGTGCAG-BHQ1-3'), GAPDH was internal housekeeping control. ATG 5 and ATG12 specific siRNAs were purchased from Applied Biosystems (#s18158-s18160; s17464-s17466).

Cell proliferation and apoptosis assays

Cells were transfected with siRNAs at 100 nM for 48 h. Cells were plated at a density of 5000 cells/well in 96-well plates, and cell proliferation was measured at various time points in quadruplicate using WST-1 (Roche: 11644807001) or BrDU (CalBiochem: QIA58) according to the manufacturer's protocol. *p*-Values were obtained by doing a two-tailed *t*-test comparing proliferation rates between loss of gene and scrambled control. For the apoptosis PARP and caspase-3 assay, 5000 cells were plated into each well in a 96-well plate. 5-FU was then added for a period of 24 h, with eight replicates per condition. Cells were fixed and blocked with goat serum. Antibodies anti-cleaved PARP (BD: 552596), anti-active caspase3 (BD:559565), goat anti-mouse IgG Alexa 488 (Invitrogen: A21121) and goat anti-rabbit IgG Alexa 647 (Invitrogen: 21244) were used for staining. Rescue experiments were carried out using SPINK1CM (see below).

Conditioned media

A total of 8–10 ml of serum-free DMEM was added to each 15 cm plate of MCF-7 SPINK1 OE cells (Supporting information Fig 1,

clone 9). Twenty-four hours later, SPINK1CM was collected, centrifuged for 5 min at 800 rpm at 4°C. The CM was warmed to 37°C before use. For SF9 cells, the cells were infected with virus (SPINK1 or SPINK1-K18Y) at the optimal titer. Three days later, CM was collected and centrifuged for 3 min at 1000 rpm at 4°C to remove cells. Vec-CM was collected in a similar manner from empty vector transfected cells served as a control. Appropriate volumes of these CM were used in relevant assays. To neutralize SPINK1, CM was pre-incubated for 2 h at room temperature with 50× molar excess of the anti-SPINK1 antibody OR IgG. The latter served as another control.

In vitro invasion and migration

Transwell migration and invasion assays were performed using Falcon FluoroBlok 24-Multiwell inserts (BD Biosciences) with 8 µm pores. For invasion assays, the inserts were coated with 20 µg Matrigel (BD Biosciences) in 80 µl serum-free growth medium. For both assays, 30,000 cells in 200 µl serum-free growth medium were loaded into each transwell insert with 750 µl complete growth medium or CM or CM with neutralizing antibody in the lower chamber. After 24 or 48 h, cells that had migrated or invaded through the pores of the inserts were fixed with 3.7% formaldehyde, stained with 2.5 µg/ml Hoechst 33342 (Invitrogen) for 15 min, washed with PBS and counted using the Target Activation Bioapplication on an ArrayScan VTI (Cellomics). Field size was 1 mm². For both invasion and migration assays, experiments were performed with fourfold replication and 10 fields were scanned per experiment.

Tail vein assay

5 × 10⁵ MB231 SPINK1OE cells or empty vector control cells were resuspended in 300 µl of PBS and injected into the tail vein of Balb/c

nude mice 12-week-old. Four mice were injected per cell line. Mice were euthanized 12 weeks later and lung sections were stained with haematoxylin and eosin. IACUC protocol #070256 was approved by the Biological Resource Centre, Agency of Science, Technology and Research.

Author contributions

This study was designed and written by KVD, ETL and WWS; WWS performed all experiments with assistance from XBC in certain areas; Breast cohort analysis was done by LDM and MAB; CD provided biostatistical support for immunohistochemistry; BP, CWO and MST provided the NUH tissue arrays, performed and scored the immunostaining.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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