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Identification and characterization of a metastatic suppressor *BRMS1L* as a target gene of p53

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The tumor suppressor p53 and its family members, p63 and p73, play a pivotal role in the cell fate determination in response to diverse upstream signals. As transcription factors, p53 family proteins regulate a number of genes that are involved in cell cycle arrest, apoptosis, senescence, and maintenance of genomic stability. Recent studies revealed that p53 family proteins are important for the regulation of cell invasion and migration. Microarray analysis showed that breast cancer metastasis suppressor 1-like (*BRMS1L*) is upregulated by p53 family proteins, specifically p53, TAp63γ, and TAp73β. We identified two responsive elements of p53 family proteins in the first intron and upstream of *BRMS1L*. These response elements are well conserved among mammals. Functional analysis showed that ectopic expression of BRMS1L inhibited cancer cell invasion and migration; knockdown of BRMS1L by siRNA induced the opposite effect. Importantly, clinical databases revealed that reduced *BRMS1L* expression correlated with poor prognosis in patients with breast and brain cancer. Together, these results strongly indicate that BRMS1L is one of the mediators downstream of the p53 pathway, and that it inhibits cancer cell invasion and migration, which are essential steps in cancer metastasis. Collectively, our results indicate that BRMS1L is involved in cancer cell invasion and migration, and could be a therapeutic target for cancer.

Since its discovery in 1979, *p53* has been one of the best studied tumor suppressor genes.[1,2] A large number of studies involving p53 were undertaken because of the unparalleled frequency of deletions and mutations of this gene in the majority of the human cancers. Considering all the genes involved in the p53 network, nearly all human cancer types show one or more aberration of the p53 tumor suppressive pathway.[2] Therefore, understanding the complex intertwined relationships of the p53 family members would facilitate better understanding of cancer biology and development of antitumor mechanisms.

In response to DNA damage or other cellular stresses such as oncogene activation, p53 is activated post-transcriptionally through phosphorylation, acetylation, and sumoylation.[3] It regulates downstream signaling pathways as a transcriptional factor, and it works in a sequence-specific manner,[4–6] consequently behaving as a hub in the tumor suppressive machinery of the cell. The downstream signals are transmitted through various molecules associated with cell cycle arrest, apoptosis, and senescence. [3,4] Recently, apart from its tumor-suppressive functions, the role of p53 as a metastatic suppressor is becoming more critical,[7,8] underscoring its importance as a prognostic factor.[9] Our group has recently revealed that the p53 family transactivates anti-invasion genes such as *CLCA2*,[10] *ICAM2*,[11] and *LIMA1*.[12] However, the role of the p53 downstream pathway in cancer invasion, migration, and metastasis remains unclear.

To identify novel direct targets of the p53 family members, we carried out cDNA microarray analyses using H1299 human lung cancer cells. We found that expression of breast cancer metastasis-suppressor 1-like (*BRMS1L*) was markedly upregulated by introducing adenovirus encoding the p53 family proteins. A previous report clarified that BRMS1L functions as a metastatic suppressor in breast cancer cells and patients.[13] The present study indicates that BRMS1L is a novel downstream target of the p53 family and could possibly be a suppressor of cancer cell invasion and migration.

Materials and Methods

Cell culture, plasmids, and recombinant adenoviruses. The human osteosarcoma cell lines Saos2 and U2OS, lung cancer cell lines H1299 and A549, glioma cell lines U373 and A172, neuroblastoma cell line SH-SY5Y, breast cancer cell lines MDA-MB-231, MDA-MB-468, and ZR75-1, gastric cancer cell lines SNU638 and SNU1, and esophageal cancer cell line ECGI-10 were obtained from either ATCC (Manassas, VA, USA) or Japanese Collection of Research Bioresources (Osaka, Japan). Colorectal cancer cell line HCT116 (p53 wild-type, p53(+/+)) and its derivative cell line HCT116-p53(−/−) lacking p53 were kindly provided by Dr. Bert Vogelstein (Howard Hughes Medical Institute, Johns Hopkins University, Baltimore, MD, USA). Adriamycin (ADR) was purchased from Sigma (St. Louis, MO, USA).

The construction, purification, and infection procedures of replication-deficient recombinant adenoviruses encoding the

human p53 family proteins fused to an amino-terminal FLAG epitope (Ad-p53, Ad-p73β, and Ad-p63γ) or the bacterial *lacZ* gene (Ad-lacZ) were carried out as previously described.[14–16] The relative efficiencies of the adenovirus infections in each cell line were determined by subjecting the cells infected with a control Ad-lacZ to X-gal staining, and 90–100% of the cells were infected at an MOI of 25–100. To construct a BRMS1L-expressing plasmid, the entire coding region of the BRMS1L cDNA was inserted in-frame into the pF5K-CMV-neo vector (Promega, Madison, WI, USA). The resulting construct was sequenced and designated as pF5K-BRMS1L.

Real-time RT-PCR. Real-time RT-PCR was undertaken using TaqMan Gene Expression assays and a 7900HT real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's protocol. Relative gene expression levels were quantified using the ΔΔCt method by normalizing transcript levels to the expression of the housekeeping gene *GAPDH*. The data are shown as the mean ± SE of three independent experiments and were normalized to 1 based on their respective controls. The primer/probe sets used were as follows: *BRMS1L*, Hs00260608_m1; *CDKN2A (p21)*, Hs00355782_m1; and *GAPDH*, Hs99999905_m1.

Immunoblot analysis. The primary antibodies that were used for immunoblotting were as follows: rabbit anti-BRMS1L polyclonal antibody (pAb) (GeneTex, Irvine, CA, USA), mouse anti-human p53 mAb (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human p73 mAb (5B429; Santa Cruz Biotechnology), mouse anti-human p63 mAb (4A4; Oncogene Research, Boston, MA, USA), rabbit anti-human E-cadherin pAb (H-108; Santa Cruz Biotechnology), rabbit anti-human ZEB1 pAb (H-102; Santa Cruz Biotechnology), mouse anti-murine double minute 2 (MDM2; HDM2 in humans) mAb (SMP14; Santa Cruz Biotechnology), rabbit anti-survivin pAb (Sigma), mouse anti-human p21 mAb (F-5; Santa Cruz Biotechnology), mouse anti- poly(ADP-ribose) polymerase 1 (PARP1) mAb (BD Pharmingen, Franklin Lakes, NJ, USA), and mouse anti-β-actin mAb (C4; Merck Millipore, Darmstadt, Germany). The proteins were transferred onto Immobilon-P membranes (Millipore, Billerica, MA, USA) by electroblotting, and an immunoblot analysis was carried out as previously described.[14]

Other methods are included in Document S1.

Results

BRMS1L mRNA and protein levels are upregulated by p53 family members. Our previous microarray study identified several downstream target genes of p53 family proteins (Affymetrix GeneChip, Santa Clara, CA, USA) (NCBI Gene Expression Omnibus database, accession number GSE 13504; https://www.ncbi.nlm.nih.gov/geo/).[10,11] In the current study, we validated some of these upregulated genes by real-time RT-PCR and immunoblot analyses. One such gene is *BRMS1L*, a component of the mSin3/histone deacetylase 1 (HDAC1) repressive machinery.[17] BRMS1L mRNA and protein expressions were found to be upregulated in Ad-p53, Ad-p73β, and Ad-p63γ transfected human cancer cell lines regardless of p53 status (Fig. 1a,b). As a positive control, we undertook immunoblot analysis on a known p53 target, p21. TAp73β and TAp63γ were used as representatives among p73 and p63 isoforms, respectively, as they have been reported to have the strongest transcriptional activity. In addition, immunofluorescence staining revealed that endogenous BRMS1L expression was enhanced in p53-transfected cells compared with that of neighboring non-transfected cells (Fig. 1c).

To determine whether endogenous p53 can upregulate BRMS1L mRNA and protein expression levels, cancer cells with various p53 statuses were exposed to a DNA damaging agent. After cells were treated with ADR (0.5 μg/mL) for 24 h, BRMS1L mRNA and protein levels were measured by real-time RT-PCR and immunoblot analysis, respectively (Fig. 2). Results indicated that BRMS1L expression in p53 wild-type cell lines (SH-SY5Y, A172, and ZR75-1) was increased following ADR treatment, while that of p53 mutant (U373) and p53 null (Saos2) cells remained the same. We also found that Nutlin-3a, an inhibitor of MDM2, and a potent inducer of p53, increased the amounts of BRMS1L mRNA and protein in a wild-type p53-dependent manner (Fig. S1a). Treatment of the p53 isogenic cell lines HCT116-p53(+/+) and HCT116-p53(−/−) with Nutlin-3a upregulated the BRMS1L expression in HCT116-p53(+/+) but did not change BRMS1L expression in HCT116-p53(−/−) (Fig. S1b). Moreover, siRNA-mediated silencing of p53 prohibited ADR-dependent induction of BRMS1L in A172 and ZR75-1 cells (Fig. S2a). The expression of BRMS1L was decreased after silencing of p53 in HCT116-p53(+/+) cells (Fig. S2b), indicating that BRMS1L is regulated by endogenous p53 expression.

BRMS1L is a transcriptional target of p53 family. The p53 protein family are known to function as transcription factors by binding to the p53 consensus binding sequence, which is classically defined as two copies (half-binding sites) of PuPuPuC (A/T)(A/T)GPyPyPy separated by a spacer 0–13 nucleotides in length.[6] We therefore searched for consensus p53-binding sequences around the *BRMS1L* gene by the *in silico* approach. Three putative p53-binding sites were identified (data not shown). We then used ChIP assays to verify direct binding of the p53 protein to candidate sequences using Saos2 cells transfected with p53 family genes. As shown in Figure 3(a), DNA fragments containing the two candidate sites were present in the immunoprecipitated complexes containing p53, TAp73β, and TAp63γ proteins. We designated these sites as RE1-BRMS1L and RE2-BRMS1L, which were located 3241 bp upstream and 469 bp downstream (first intron) of the first exon, respectively (Fig. 3b). The known p53-binding site in the *p21* gene was used as a positive control for the ChIP assay (Fig. 3a).

To determine whether these binding sequences confer transcriptional activity in a p53 family member-dependent manner, we undertook a heterologous promoter–reporter assay using luciferase vectors containing RE1- or RE2-BRMS1L sequences (pGL3-RE1-BRMS1L or pGL3-RE2-BRMS1L). Saos2 and U373 cells were transiently cotransfected with each luciferase vector together with a p53-, p73β-, or p63γ-expressing plasmid. Luciferase activity from pGL3-RE1-BRMS1L and pGL3-RE2-BRMS1L was induced by each of the p53 family members compared with the control vector (Fig. 3c). In contrast, mutations in these constructs (pGL3-RE1-BRMS1L-mut or pGL3-RE2-BRMS1L-mut) abolished transactivation by p53 family members. Although TAp73β did not increase luciferase activity of the RE1 reporter, these observations agreed with previous expression studies, and support the hypothesis that p53 family members directly transactivate the *BRMS1L* gene by binding to two distinct p53 response elements.

When we reviewed our previously published next-generation ChIP-seq data,[18] RE2-BRMS1L showed a very strong p53-binding peak (Fig. S3). Moreover, RE1-BRMS1L and RE2-BRMS1L are well-conserved among species at nearly

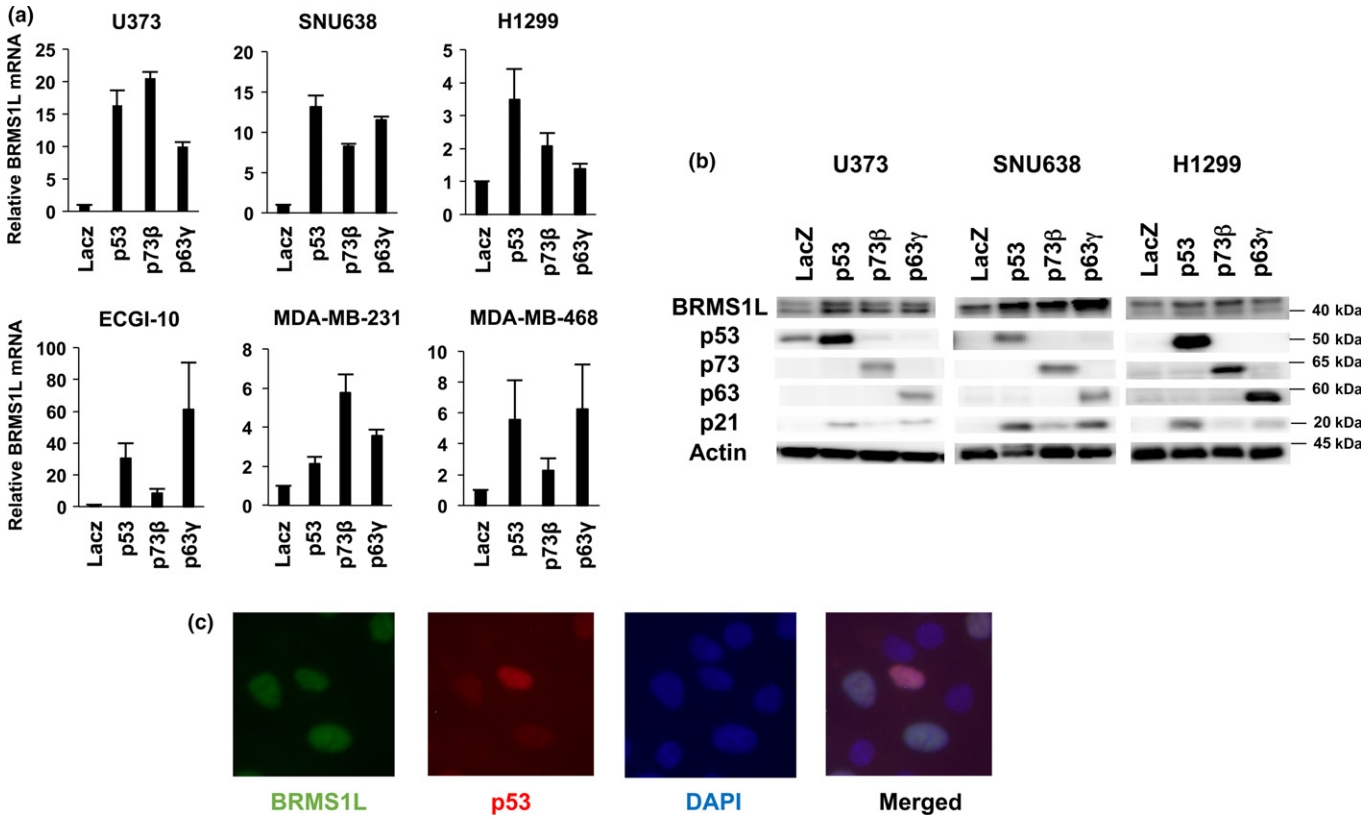

Fig. 1. Breast cancer metastasis suppressor 1-like (BRMS1L) mRNA and protein levels are upregulated by p53 family members. (a) Upregulation of *BRMS1L* mRNA by p53 family members in human cancer cell lines. Cells were infected with replication-deficient recombinant adenoviruses encoding human p53 family proteins or the bacterial lacZ at an MOI of 25–100 for 24 h. Relative gene expression levels were quantified by the ΔΔCt method, and results are normalized to *GAPDH* expression. Data are shown as the mean ± SE, $n = 3$. (b) Upregulation of BRMS1L protein by p53 family members in human cancer cell lines. Cells were infected with adenoviruses as described above; immunoblot analysis was carried out using an anti-BRMS1L antibody. (c) Immunostaining showing BRMS1L subcellular distribution following p53 transfection. U373 cells were transfected with either p53-expressing plasmids containing a FLAG epitope tag (lower row) or an empty vector (upper row) for 24 h. Cells were fixed and stained with BRMS1L (green) and FLAG epitope (transfected p53, red) antibodies, followed by appropriate secondary antibodies. Nuclei were detected by DAPI staining (blue).

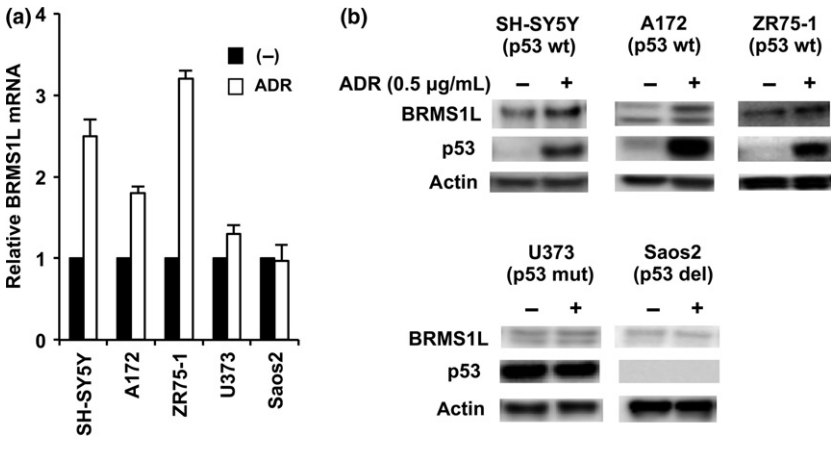

Fig. 2. Breast cancer metastasis suppressor 1-like (BRMS1L) mRNA and protein expression was induced by the DNA damaging agent adriamycin (ADR) in a p53-dependent manner. (a) Cells were treated with 0.5 μg/mL ADR for 24 h. *BRMS1L* mRNA level was measured by real-time RT-PCR. Endogenous status of p53 is wild-type for SH-SY5Y, A172, and ZR75-1, mutant for U373, and deleted for Saos2. (b) Cells were treated as described above, and immunoblot analyses were carried out on BRMS1L, p53, and β-actin.

identical positions within each ortholog, implying that these sequences are of functional importance (Fig. 3b). We found that only RE2-BRMS1L is conserved in mouse. We then undertook luciferase reporter assays and observed that luciferase activity was significantly increased after cotransfection of the mouse p53 expression vector and a pGL3-promoter vector containing the binding site RE2 in the mouse *Brms1 l* gene (RE2-mBrms1 l) (Fig. S4a). Induction of *Brms1 l* mRNA was found in ADR- or Nutlin-3a-treated normal (p53+/+) mouse embryonic fibroblasts (MEF), but not in p53 knockout (p53−/−) MEF (Fig. S4b,c). Overexpression of p53 also induced *Brms1 l* mRNA expression in MEF (Fig. S4d,e), indicating that BRMS1L is a direct target of p53 in human and mouse cells.

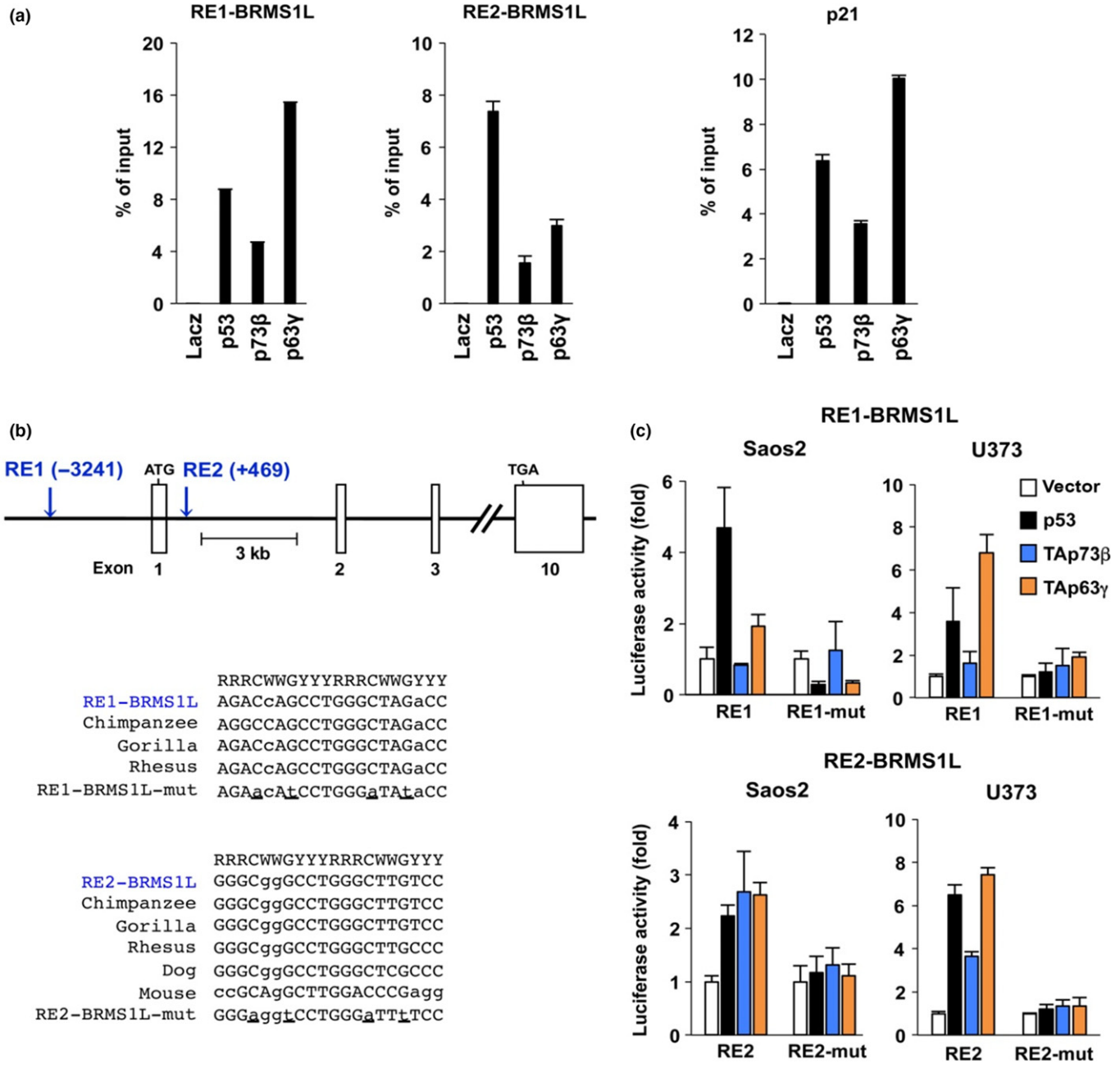

Fig. 3. Breast cancer metastasis suppressor 1-like (BRMS1L) is transcriptionally regulated by p53 family members. (a) p53 family proteins bind to the RE1-BRMS1L and RE2-BRMS1L *in vivo*. Saos2 cells were infected with adenovirus encoding FLAG-tagged p53, TAp73β, and TAp63γ at an MOI of 25 for 24 h. Crosslinked chromatin was extracted, and the cell lysates were then immunoprecipitated with an anti-FLAG antibody. Real-time PCR amplifications were performed in triplicate for each precipitation with primers surrounding each site. Data are normalized to the signal from input DNA (mean ± SE). (b) Position of responsive elements for p53 family in the *BRMS1L* gene. RE1-BRMS1L is located 3241 bp upstream of the *BRMS1L* transcription start site (TSS), and RE2-BRMS1L is located in the first intron of the *BRMS1L* gene. Both response elements consist of two copies of the 10-bp motif of the p53 binding sequence, indicated by uppercase letters. Lowercase letters identify mismatches with the consensus sequence. Alignment of the conserved *BRMS1L* binding sites in other species is also shown. Mutated sequences corresponding to potentially critical nucleotides of RE1-BRMS1L and RE2-BRMS1L used in the luciferase assay are shown in the lowest row. R, purine; W, adenine or thymine; Y, pyrimidine. (c) RE1-BRMS1L and RE2-BRMS1L are responsive to p53 family members. Saos2 and U373 cells were transiently transfected with the pGL3-promoter vector containing RE1-BRMS1L (pGL3-RE1-BRMS1L), RE2-BRMS1L (pGL3-RE2-BRMS1L), or mutants (pGL3-RE1-BRMS1L-mut and pGL3-RE2-BRMS1L-mut), along with the transfection control plasmid expressing *Renilla* luciferase (phRG-TK). Cells were cotransfected with either a control pCMV-Tag2 vector or a vector that expresses the p53 family members 24 h prior to the luciferase assay. Luciferase activity was measured using the dual luciferase reporter system with the *Renilla* luciferase activity as an internal control. All experiments were carried out in quadruplicate; bars indicate mean and SD.

Overexpression of BRMS1L inhibits cancer cell invasion and migration. To investigate the impact of BRMS1L on tumor cell proliferation, we transfected a BRMS1L expression vector into U373 and Saos2 cells (Fig. S5a), and measured cell viability. We did not observe any difference in proliferation between BRMS1L-overexpressing cells and the control, as assessed by

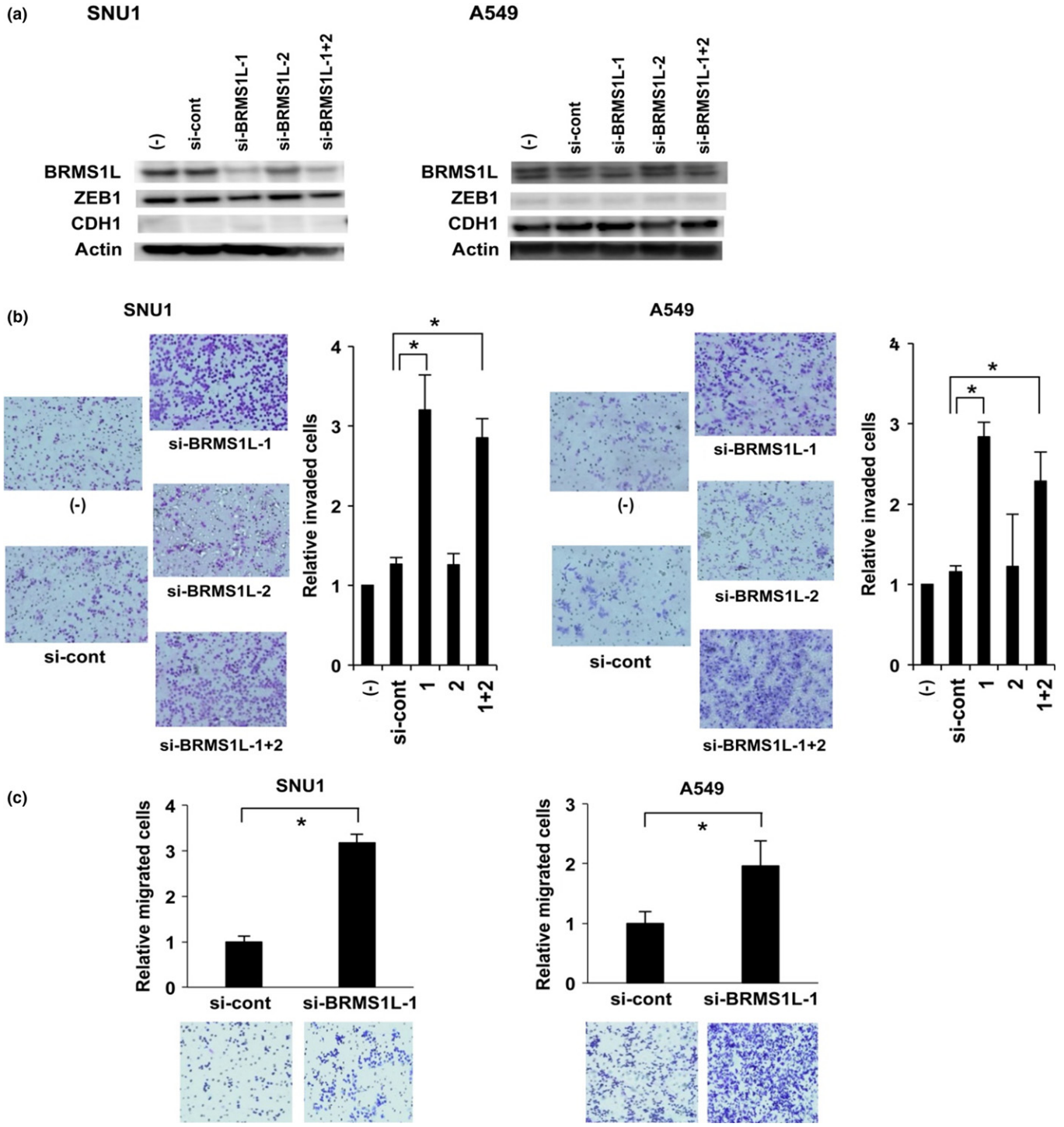

Fig. 4. Breast cancer metastasis suppressor 1-like (BRMS1L) siRNA promotes cancer cell invasion and migration. (a) SNU1 and A549 cells were transfected with either siRNAs targeting BRMS1L (si-BRMS1L-1, si-BRMS1L-2, or both) or a negative control (si-cont) every 24 h for 2 consecutive days. Twenty-four hours after the last transfection, cells were harvested, and cell lysates were then blotted with the indicated antibodies. (b) Under the same conditions as described above, cell invasion was measured by the Matrigel invasion assay. Experiments were repeated three times with similar results. Relative cell invasion was normalized to control cells. $*P < 0.01$, Student's *t*-test. (c) Cell migration was measured by the Transwell migration assay. Relative cell migration was normalized to control cells. $*P < 0.05$, Student's *t*-test. (-), untransfected control.

MTT (Fig. S5b) and colony formation assays (Fig. S5c). This suggested that BRMS1L has minimal effect on cell proliferation. We then investigated whether BRMS1L regulates cell invasion and migration using Matrigel invasion and Transwell migration assays, respectively. We showed that BRMS1L alone has strong capability to suppress cancer cell invasion and migration (Fig. S6).

BRMS1L siRNA promotes cancer cell invasion and migration. To confirm the anti-invasive capacity of BRMS1L, we designed two specific siRNAs targeting BRMS1L, si-

BRMS1L-1 and -2, and confirmed their inhibitory effects on BRMS1L in SNU1 and A549 cells, which express relatively high level of endogenous BRMS1L. As shown in Figure 4(a), si-BRMS1L-1, but not si-BRMS1L-2, reduced the expression of BRMS1L protein. We found that BRMS1L silencing significantly reduced the invasion and migration capabilities of both types of cells (Fig. 4b,c). BRMS1L knockdown in U2OS cells also yielded similar effects on cell invasion and migration (Fig. S7). Together, these data suggest that BRMS1L is a strong determinant of invasion and migration, both of which are critical events during cancer metastasis.

Epithelial–mesenchymal transition (EMT) is a metastasis-promoting phenomenon by which cancer cells acquire invasive and migratory properties.[19,20] As changes in BRMS1L expression levels resulted in functional alterations, it is possible that EMT program was involved in the process. Therefore, we investigated the expression levels of EMT markers, such as CDH1 and ZEB1. However, we did not observe any changes in these EMT markers (Fig. 4a; Figs S5a and S7a), indicating that BRMS1L-related functional changes are independent of EMT. Interestingly, BRMS1L-silencing in U2OS cells resulted in significant morphological changes, such as the appearance of actin-rich plasma membrane protrusions (Fig. S7d). It is possible that BRMS1L may have an effect on actin filament remodeling.

BRMS1L fortifies p53 transcriptional activity. BRMS1L carries out its function through several mechanisms, including regulation of gene expression by binding to the mSin3/HDAC complex. We postulated that p53 and BRMS1L work together in cancer cells, as several studies reported that transcriptional regulation by p53 is mediated by interactions with the mSin3 co-repressor.[21–23] As shown in Figure 5(a), p53 was found to be colocalized with BRMS1L in both the nucleus and the cytoplasm. In addition, immunoprecipitation assays revealed interactions between p53 and BRMS1L (Fig. 5b).

To clarify the effect of BRMS1L on p53-induced tumor suppression, we infected BRMS1L knockdown U373 and Saos2 cells with a p53-expressing adenovirus. BRMS1L siRNA partially blocked p53-mediated induction of p21 at the level of mRNA (Fig. S8a) and protein (Fig. 5c). It also blocked reduction of survivin, a known direct target that is repressed by p53.[24,25] Importantly, levels of PARP cleavage, an indicator of apoptosis, were inhibited by BRMS1L knockdown (Fig. 5c), suggesting that BRMS1L may facilitate p53-mediated apoptosis. In addition, BRMS1L siRNA partially blocked p21 induction (mRNA levels) and survivin reduction (both mRNA and protein levels) by activated endogenous p53 following ADR treatment (Fig. 5d; Fig. S8b). Although wild-type p53 significantly increased the luciferase activity of vectors containing a p53 consensus binding site located in the *p21* gene, the activity of p53 seemed to be alleviated by BRMS1L silencing (Fig. S9). These results suggested that BRMS1L could fortify p53 transcriptional activity.

Interestingly, we also found that ectopic BRMS1L expression inhibited survivin in p53 mutant U373 cells, although survivin expression was not altered in p53-deleted Saos2 cells following the same treatment (Fig. S5a). Conversely, endogenous survivin proteins were activated in p53 wild-type U2OS

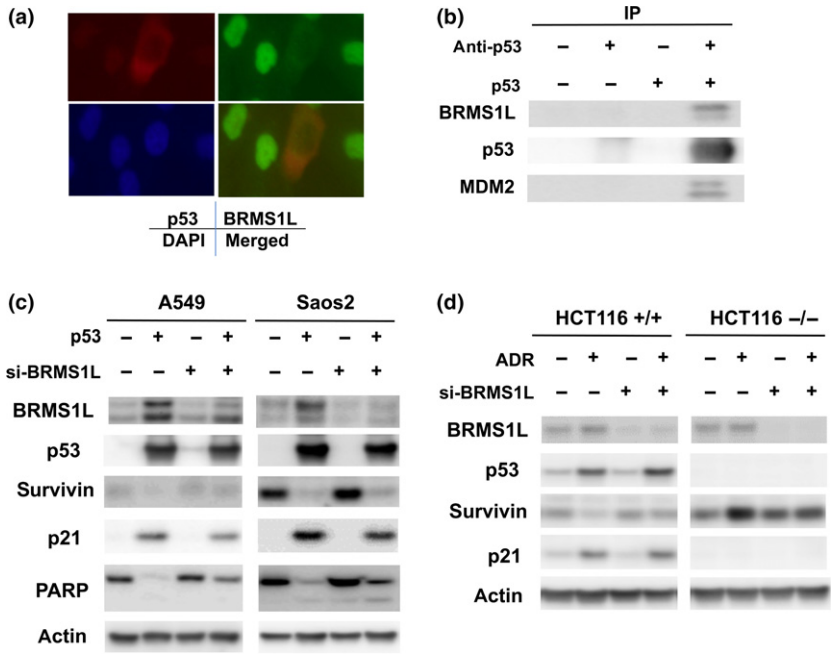

Fig. 5. Breast cancer metastasis suppressor 1-like (BRMS1L) siRNA partially blocked p53 activity. (a) p53 and BRMS1L colocalize in cancer cells. p53 wild-type A549 cells were fixed and double stained for BRMS1L (green) and p53 (red). Colocalization of BRMS1L and p53 are shown (yellow) in merged images. Nuclei were detected by DAPI staining (blue) (b) p53-deleted Saos2 cells were infected with adenoviral vectors expressing p53 at an MOI of 25 for 24 h. Whole-cell extracts (250 μg) were immunoprecipitated (IP) with Dynabeads Protein G that had been pre-incubated with 5 μg anti-p53 antibody (DO-1) in PBS. The Dynabeads antigen–anti-p53 complex was resuspended in 40 μL loading buffer and incubated at 70°C for 5 min. Twenty microliters of each sample was separated, transferred to Immobilon P membranes, and immunoblotted. (c) BRMS1L siRNA partially blocked p21 induction and survivin reduction by p53 at the protein level. Saos2 and A549 cells were transfected with control or BRMS1L siRNA every 24 h for 2 consecutive days. Cells were then infected with adenoviral vectors expressing p53 for 24 h. Immunoblot analysis was carried out using the indicated antibody. (d) BRMS1L siRNA partially blocked the p21 activation and survivin reduction by endogenous p53 at the protein level. HCT116-p53(+/+) and HCT116-p53(−/−) cells were transfected with control or BRMS1L siRNA every 24 h for 2 consecutive days. Cells were then treated with 0.5 μg/mL adriamycin (ADR) for 24 h. Immunoblot analysis was carried out using the indicated antibodies. MDM2, murine double minute 2; PARP, poly(ADP-ribose) polymerase.

cells following BRMS1L knockdown (Fig. S7a), suggesting that BRMS1L exerts its tumor-suppressive role, at least partly, through modulation of survivin level.

Clinical significance of BRMS1L. To assess the clinical importance of BRMS1L, we used a collection of human cancer microarray data (Oncomine)[26] (Fig. S10). Downregulation of *BRMS1L* expression in human breast and brain cancer tissues was compared to that of normal tissues observed in four gene expression datasets.[27–29] Finally, to examine whether *BRMS1L* expression affects prognosis in cancer patients, we surveyed the PrognoScan database,[30] and constructed survival curves using the Kaplan–Meier method (Fig. S11). In the three datasets assessed, poor overall survival, relapse-free survival, and distant metastasis survival were all correlated with low *BRMS1L* expression levels.[31,32] Taken together, we suggest that *BRMS1L* may be a useful biomarker of cancer prognosis.

Furthermore, we determined endogenous BRMS1L protein levels and p53 mutational status in a panel of cancer cell lines. As shown in Figure S12, an anti-BRMS1L antibody detected two bands at approximately 40 kDa in various cell types. The relative band intensity differed from each other, and each sample showed either the upper band only or both upper and lower bands. When p53 was not induced in the cell lines, we identified no significant correlation between the p53 status and the basal level of BRMS1L protein (Fig. S12). We also found no relationship between *BRMS1L* expression and p53 mutation in cancer tissues using Oncomine (Fig. S13), suggesting that BRMS1L is regulated by both p53-dependent and -independent pathways.

Discussion

Metastasis is a sequential event in cancer progression that eventually leads to human death. Therefore, targeting metastasis is very important for reducing cancer-associated deaths.[33–35] In order to metastasize to surrounding tissues, cancer cells need to be equipped with certain invasive features.[33,35] Metastasis suppressor genes inhibit key steps in the metastatic cascade, which includes invasion, migration, dissemination, nodal metastasis, and colonization of distant organs.[36] For example, accumulating evidence suggests that BRMS1 is one of the key players involved in metastasis suppression.[37] BRMS1 functions as a transcriptional regulator, and is a component of the mSin3/HDAC1 repressor complex. The effect of BRMS1 is mediated by its target genes such as *OPN*,[38] *uPA*,[39] and *CXCR4*.[40] These genes are also downstream targets of p53 family proteins,[41–43] suggesting that BRMS1 and the p53 family proteins work together to suppress metastatic events. In our study, expression of BRMS1L, which shares 79% overall homology with BRMS1, is induced not only by overexpression of p53 family members, but also by activation of endogenous p53 through ADR or Nutlin-3a in several types of cancer cells (Figs 1 and 2; Fig. S1).

Originally, BRMS1L was identified as a component of the same complex as BRMS1.[17] Structurally, BRMS1L is part of a family that includes BRMS1 and suppressor of defective silencing 3 (mSds3 or SUDS3). Although it is clear that *BRMS1* is a metastasis suppressor gene, mSds3 does not suppress metastasis.[44] A previous study showed that mSds3 has tumor-suppressive effects by facilitating the establishment of pericentric heterochromatin, and that knockdown of mSds3 results in chromosome missegregation, leading to earlier tumor formation.[45] Interestingly, mSds3 knockdown-mediated tumor growth was exacerbated by p53 depletion, and a single copy of p53 was sufficient to reverse this effect.[45] Compared with BRMS1 and mSds3, the role of BRMS1L was poorly understood.

A recent paper showed that reduced BRMS1L expression in breast cancer tissues is associated with metastasis and poor survival; functional experiments revealed that BRMS1L inhibits breast cancer cell invasion and migration by inhibiting EMT. [13] The BRMS1L-mediated inhibition of cell invasion and migration was attributed to epigenetic silencing of FZD10, as well as downregulation of Wnt signaling.[13] However, the overall function of BRMS1L as a metastatic suppressor is yet to be determined. In the present study, we found that BRMS1L can modulate expression of survivin in cancer cells regardless of p53 status. However, the biological importance of survivin regulation by BRMS1L is not clear.

Cancer cell motility is a critical factor that affects cancer progression.[46] We recently identified two genes, *FOXF1*[47] and *CRKL*,[48] which are downstream of p53 and are associated with cancer cell invasion. The current study showed that BRMS1L also suppresses cancer cell motility downstream of the p53 pathway. In addition, BRMS1L can alter cancer cell morphology by inducing the formation of spindle-shaped cells with protruding membranes. Recent studies indicated that p53 controls EMT to suppress cancer cell invasion by transactivation of its target genes.[49] Contrary to the reported results in breast cancer, we found no alteration of EMT markers, suggesting that BRMS1L may regulate cell invasion and migration in a cell-type specific manner. Accordingly, BRMS1L may have anti-invasive features by controlling the expression of actin remodeling genes.

We found that p53 and BRMS1L are colocalized in cancer cells, and interact with one another, suggesting they may have synergistic effects. Previous reports indicated that p53 interacts with mSin3a, which not only contributes to stabilization of p53 expression, but also acts as a repressor complex to downregulate target genes. Forced expression and siRNA knockdown analysis showed that BRMS1L may work to fortify transcriptional intensity by p53. Overall, our results propose a novel positive feedback loop for transcriptional function of p53.

Clinical data from publicly available databases indicate that *BRMS1L* expression level is correlated with cancer patient survival (Fig. S10). In addition, we found that *BRMS1L* is significantly underexpressed in breast and brain cancer tissues (Fig. S11), suggesting that BRMS1L may be a promising prognostic marker for these two tumor types. A previous report showed that nuclear localization of BRMS1 correlates with better prognosis of melanoma patients due to BRMS1 regulating antimetastatic gene expression in the nucleus.[50] From the experimental fact that BRMS1 and BRMS1L work in the same transcriptional complex, subcellular localization of BRMS1L may be a useful novel biomarker.

In summary, our present study showed that metastasis-suppressing BRMS1L is a novel target of tumor-suppressor p53, and supports a novel biomolecular pathway by which the p53 family suppresses tumor metastasis. We also provided additional insights into p53-mediated tumor suppression and gene regulation.

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Disclosure Statement

The authors have no conflict of interest.

References

1 Levine AJ, Oren M. The first 30 years of p53: growing ever more complex. *Nat Rev Cancer* 2009; **9**: 749–58.
2 Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000; **408**: 307–10.
3 Kruse JP, Gu W. Modes of p53 regulation. *Cell* 2009; **15**(137): 609–22.
4 Menendez D, Inga A, Resnick MA. The expanding universe of p53 targets. *Nat Rev Cancer* 2009; **9**: 724–37.
5 Tokino T, Nakamura Y. The role of p53-target genes in human cancer. *Crit Rev Oncol Hematol* 2000; **33**: 1–6.
6 el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B. Definition of a consensus binding site for p53. *Nat Genet* 1992; **1**: 45–9.
7 Powell E, Piwnica-Worms D, Piwnica-Worms H. Contribution of p53 to metastasis. *Cancer Discov* 2014; **4**: 405–14.
8 Adorno M, Cordenonsi M, Montagner M *et al.* A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. *Cell* 2009; **137**: 87–98.
9 Soussi T, Beroud C. Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer* 2001; **1**: 233–40.
10 Sasaki Y, Koyama R, Maruyama R *et al.* CLCA2, a target of the p53 family, negatively regulates cancer cell migration and invasion. *Cancer Biol Ther* 2012; **13**: 1512–21.
11 Sasaki Y, Tamura M, Takeda K *et al.* Identification and characterization of the intercellular adhesion molecule-2 gene as a novel p53 target. *Oncotarget* 2016; **7**: 61426–37.
12 Tomoko O, Masashi I, Yasushi S, Tokino T. p53 mediates the suppression of cancer cell invasion by inducing LIMA1/EPLIN. *Cancer Lett* 2017; **390**: 58–66.
13 Gong C, Qu S, Lv XB *et al.* BRMS1L suppresses breast cancer metastasis by inducing epigenetic silence of FZD10. *Nat Commun* 2014; **5**: 54060.
14 Fomenkov A, Zangen R, Huang YP *et al.* RACK1 and stratifin target DeltaNp63alpha for a proteasome degradation in head and neck squamous cell carcinoma cells upon DNA damage. *Cell Cycle* 2004; **3**: 1285–95.
15 Stiewe T. The p53 family in differentiation and tumorigenesis. *Nat Rev Cancer* 2007; **7**: 165–8.
16 Wu N, Rollin J, Masse I, Lamartine J, Gidrol X. p63 regulates human keratinocyte proliferation via MYC-regulated gene network and differentiation commitment through cell adhesion-related gene network. *J Biol Chem* 2012; **287**: 5627–38.
17 Nikolaev AY, Papanikolaou NA, Li M, Qin J, Gu W. Identification of a novel BRMS1-homologue protein p40 as a component of the mSin3A/p33 (ING1b)/HDAC1 deacetylase complex. *Biochem Biophys Res Commun* 2004; **323**: 1216–22.
18 Idogawa M, Ohashi T, Sasaki Y *et al.* Identification and analysis of large intergenic non-coding RNAs regulated by p53 family members through a genome-wide analysis of p53-binding sites. *Hum Mol Genet* 2014; **23**: 2847–57.
19 Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; **139**: 871–90.
20 Puisieux A, Brabletz T, Caramel J. Oncogenic roles of EMT-inducing transcription factors. *Nat Cell Biol* 2014; **16**: 488–94.
21 Zilfou JT, Hoffman WH, Sank M, George DL, Murphy M. The corepressor mSin3a interacts with the proline-rich domain of p53 and protects p53 from proteasome-mediated degradation. *Mol Cell Biol* 2001; **21**: 3974–85.
22 Peinado H, Ballestar E, Esteller M, Cano A. Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. *Mol Cell Biol* 2004; **24**: 306–19.
23 Wilkinson DS, Tsai WW, Schumacher MA, Barton MC. Chromatin-bound p53 anchors activated Smads and the mSin3A corepressor to confer transforming-growth-factor-beta-mediated transcription repression. *Mol Cell Biol* 2008; **28**: 1988–98.
24 Mirza A, McGuirk M, Hockenberry TN *et al.* Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. *Oncogene* 2002; **21**: 2613–22.
25 Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J Biol Chem* 2002; **277**: 3247–57.
26 Rhodes DR, Kalyana-Sundaram S, Mahavisno V *et al.* Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. *Neoplasia* 2007; **9**: 166–80.
27 Sun L, Hui AM, Su Q *et al.* Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain. *Cancer Cell* 2006; **9**: 287–300.
28 Bredel M, Bredel C, Juric D *et al.* Functional network analysis reveals extended gliomagenesis pathway maps and three novel MYC-interacting genes in human gliomas. *Cancer Res* 2005; **65**: 8679–89.
29 Curtis C, Shah SP, Chin SF *et al.* The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* 2012; **486**: 346–52.
30 Mizuno H, Kitada K, Nakai K, Sarai A. PrognoScan: a new database for meta-analysis of the prognostic value of genes. *BMC Med Genomics* 2009; **2**: 18.
31 Phillips HS, Kharbanda S, Chen R *et al.* Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 2006; **9**: 157–73.
32 Loi S, Haibe-Kains B, Desmedt C *et al.* Predicting prognosis using molecular profiling in estrogen receptor-positive breast cancer treated with tamoxifen. *BMC Genom* 2008; **9**: 239.
33 Chiang AC, Massague J. Molecular basis of metastasis. *N Engl J Med* 2008; **359**: 2814–23.
34 Steeg PS, Theodorescu D. Metastasis: a therapeutic target for cancer. *Nat Clin Pract Oncol* 2008; **5**: 206–19.
35 Eccles SA, Welch DR. Metastasis: recent discoveries and novel treatment strategies. *Lancet* 2007; **369**: 1742–57.
36 Smith SC, Theodorescu D. Learning therapeutic lessons from metastasis suppressor proteins. *Nat Rev Cancer* 2009; **9**: 253–64.
37 Seraj MJ, Samant RS, Verderame MF, Welch DR. Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. *Cancer Res* 2000; **60**: 2764–9.
38 Wu Y, Jiang W, Wang Y *et al.* Breast cancer metastasis suppressor 1 regulates hepatocellular carcinoma cell apoptosis via suppressing osteopontin expression. *PLoS One* 2012; **7**: e42976.
39 Cicek M, Fukuyama R, Welch DR, Sizemore N, Casey G. Breast cancer metastasis suppressor 1 inhibits gene expression by targeting nuclear factor-kappaB activity. *Cancer Res* 2005; **65**: 3586–95.
40 Yang J, Zhang B, Lin Y, Yang Y, Liu X, Lu F. Breast cancer metastasis suppressor 1 inhibits SDF-1alpha-induced migration of non-small cell lung cancer by decreasing CXCR4 expression. *Cancer Lett* 2008; **269**: 46–56.
41 Morimoto I, Sasaki Y, Ishida S, Imai K, Tokino T. Identification of the osteopontin gene as a direct target of TP53. *Genes Chromosom Cancer* 2002; **33**: 270–8.
42 Eren M, Boe AE, Klyachko EA, Vaughan DE. Role of plasminogen activator inhibitor-1 in senescence and aging. *Semin Thromb Hemost* 2014; **40**: 645–51.
43 Mehta SA, Christopherson KW, Bhat-Nakshatri P *et al.* Negative regulation of chemokine receptor CXCR4 by tumor suppressor p53 in breast cancer cells: implications of p53 mutation or isoform expression on breast cancer cell invasion. *Oncogene* 2007; **26**: 3329–37.
44 Silveira AC, Hurst DR, Vaidya KS, Ayer DE, Welch DR. Over-expression of the BRMS1 family member SUDS3 does not suppress metastasis of human cancer cells. *Cancer Lett* 2009; **276**: 32–7.
45 David G, Dannenberg JH, Simpson N *et al.* Haploinsufficiency of the mSds3 chromatin regulator promotes chromosomal instability and cancer only upon complete neutralization of p53. *Oncogene* 2006; **25**: 7354–60.
46 Friedl P, Alexander S. Cancer invasion and the microenvironment: plasticity and reciprocity. *Cell* 2011; **147**: 992–1009.
47 Tamura M, Sasaki Y, Koyama R, Takeda K, Idogawa M, Tokino T. Forkhead transcription factor FOXF1 is a novel target gene of the p53 family and regulates cancer cell migration and invasiveness. *Oncogene* 2014; **33**: 4837–46.
48 Tamura M, Sasaki Y, Kobashi K *et al.* CRKL oncogene is downregulated by p53 through miR-200s. *Cancer Sci* 2015; **106**: 1033–40.
49 Puisieux A, Brabletz T, Caramel J. Oncogenic roles of EMT-inducing transcription factors. *Nat Cell Biol* 2014; **16**: 488–94.
50 Slipicevic A, Holm R, Emilsen E *et al.* Cytoplasmic BRMS1 expression in malignant melanoma is associated with increased disease-free survival. *BMC Cancer* 2012; **12**: 73.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Murine double minute 2 (MDM2) inhibitor upregulates breast cancer metastasis suppressor 1-like (BRMS1L) in a p53-dependent manner.

Fig. S2. Knockdown of p53 by siRNA downregulates breast cancer metastasis suppressor 1-like (BRMS1L) expression.

Fig. S3. Chromatin immunoprecipitation sequencing peaks in the human *BRMS1L* gene.

Fig. S4. *Brms1 l* is a transcriptional target of p53 in mouse cells.

Fig. S5. Effects of breast cancer metastasis suppressor 1-like (BRMS1L) expression on cancer cell growth.

Fig. S6. Overexpression of breast cancer metastasis suppressor 1-like (BRMS1L) inhibits cancer cell invasion and migration.

Fig. S7. Breast cancer metastasis suppressor 1-like (BRMS1L) knockdown by siRNA alters cancer cell morphology.

Fig. S8. Breast cancer metastasis suppressor 1-like (BRMS1L) siRNA attenuates mRNA expression of p53 target genes.

Fig. S9. Breast cancer metastasis suppressor 1-like (BRMS1L) partially blocked p53-mediated p21 transcription.

Fig. S10. *BRMS1L* expression in human breast and brain cancer tissues.

Fig. S11. Correlation between *BRMS1L* expression and prognosis of human cancers.

Fig. S12. Expression of endogenous *BRMS1L* in human cancer cell lines.

Fig. S13. No association between *BRMS1L* mRNA level and p53 status in human cancer tissues.

Doc. S1. Supplementary materials and methods.