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CK2 α' Drives Lung Cancer Metastasis by Targeting BRMS1 Nuclear Export and Degradation

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Abstract

Breast cancer metastasis suppressor 1 (BRMS1) is decreased in non-small cell lung cancer (NSCLC) and other solid tumors, and its loss correlates with increased metastases. We show that BRMS1 is posttranslationally regulated by tumor necrosis factor-induced casein kinase 2 catalytic subunit (CK2 α') phosphorylation of nuclear BRMS1 on serine 30, resulting in 14-3-3 ϵ -mediated nuclear exportation, increased BRMS1 cytosolic expression, and ubiquitin-proteasome-induced BRMS1 degradation. Using our *in vivo* orthotopic mouse model of lung cancer metastases, we found that mutation of serine 30 in BRMS1 or the use of the CK2-specific small molecule inhibitor CX4945 abrogates CK2 α' -induced cell migration and invasion *in vitro* and decreases NSCLC metastasis by 60-fold. Analysis of 160 human NSCLC specimens confirmed that tumor CK2 α' and cytoplasmic BRMS1 expression levels are associated with increased tumor recurrence, metastatic foci, and reduced disease-free survival. Collectively, we identify a therapeutically exploitable post-translational mechanism by which CK2 α -mediated degradation of BRMS1 promotes metastases in lung cancer.

Keywords

BRMS1; CK2; Phosphorylation; Degradation; Metastasis

Introduction

BRMS1 has been implicated in the suppression of breast, lung, and bladder cancer metastases without significantly affecting primary tumor growth (1, 2). Specific to non-small cell lung cancer (NSCLC), BRMS1 protein and transcript are differentially expressed in noncancerous lung tissue (high) and tumors (low) (2, 3). We previously demonstrated that transcriptional repression of BRMS1 occurs via RelA/p65-DNMT-1-mediated promoter

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methylation (4). Other groups have indicated that BRMS1 is also regulated via the Cul3-SPOP complex (5).

Casein kinase 2 (CK2) is a pleiotropic, highly conserved serine/threonine kinase that consists of two α (α or α') catalytic and two β regulatory subunits (6). CK2 controls the stability of both I κ B (7) and the tumor suppressor PML (8) and regulates Snail 1–induced epithelial mesenchymal transition (9) and promotion of NCoR-mediated cancer cell invasion (10). CK2 is a driver of malignant progression and a classic example of nononcogene addiction in tumors with high levels of CK2 (11). More recently, CK2 has been added to the human “druggable kinome,” as evidence exists that selective CK2 inhibitors enhance apoptosis in drug-resistant cancer cells and clinical trials have demonstrated the antitumor activity of these compounds (12).

We (2) and others (13) have observed that, although BRMS1 is primarily a nuclear protein, it is also present in the cytosol in cancer cells and primary human tumors. This suggests that BRMS1’s function may be modulated by intracellular compartmentalization. Although Rivera and colleagues identified a conserved nuclear exportation motif in BRMS1 (14), the mechanisms through which BRMS1 undergoes nuclear export and the biologic significance of this process to the development of metastases are unknown.

Herein, we report that BRMS1 protein is regulated by tumor necrosis factor (TNF)–induced activation of CK2 α' . CK2 α' -mediated phosphorylation of BRMS1 at serine 30 (S30) promotes nuclear exportation of BRMS1 via a 14-3-3–dependent mechanism. Using an orthotopic lung cancer model and computed tomographic (CT) visualization and quantification of tumor metastatic deposits, we show that CK2 phosphorylation of S30 on BRMS1 results in a significant increase in metastatic cells. Moreover, we observed abnormally elevated levels of CK2 α' in human NSCLC specimens, compared with adjacent noncancerous tissues. The increased expression of CK2 α' was significantly associated with nuclear exportation of BRMS1 and increased tumor recurrence. Collectively, our observations demonstrate that CK2-mediated phosphorylation of BRMS1 is an important posttranslational modification that regulates BRMS1 nuclear export and protein stability. We also identify BRMS1 as a new target of CK2 activity and provide mechanistic support and, thus, a strong rationale for the use of CK2-specific inhibitors in the treatment of lung cancer.

Materials and Methods

Cell culture, human NSCLC specimens, antibodies, and reagents

Normal human bronchial epithelial (NHBE) cells and NSCLC H1299, H1993, A549, and H157 cells were purchased from ATCC and grown as described (3). NHBE, H1299, H1993, and A549 cells were used within 6 months after purchase. H157 cells were authenticated in December 2015 by PCR and genomic DNA sequencing. Mycoplasma was tested routinely. Low-passage (<6) cell cultures were used for the experiments. Human NSCLC specimens were obtained following written, informed consent and approval by the Human Investigations Committee at Memorial Sloan Kettering Cancer Center. The antibodies used were BRMS1, phospho-serine/threonine, Myc-tag, and His-tag (Abcam); RNA Pol II, α -tubulin, CK2 α' , 14-3-3, and β -actin (Santa Cruz Biotechnology); and HA-epitope tag (BD

Biosciences). The reagents used were human recombinant TNF, cycloheximide (CHX), and 4,5,6,7-tetrabromobenzotriazole (TBB) (Sigma Aldrich); CX4945 (Silmisertib, Selleck Chemicals); MG132 (EMD Biosciences); human recombinant CK2 and substrate (Promega); ^{35}S -labeled methionine/cysteine and $[\gamma\text{-}^{33}\text{P}]\text{-ATP}$ (PerkinElmer); and antibiotics (Puromycin, Geneticin, Blasticidin, Zeocin, and Tetracycline) (Thermo Fisher Scientific). Small interfering RNA control and CK2 α' pool were purchased from Santa Cruz.

Plasmid construction

HA-tagged BRMS1, glutathione S-transferase (GST)–fusion BRMS1, His₍₆₎-tagged ubiquitin, and shRNA *BRMS1* were described previously (3, 15, 16). Site-directed mutagenesis (S→A) was performed using the quick-change mutagenesis kit (Agilent). For pBabe-puro FLAG-tagged BRMS1, *BRMS1* was amplified from pCMV HA-tagged BRMS1 by PCR and inserted into *BamHI/EcoRI* sites. To construct shRNA-resistant BRMS1, the shRNA targeting sequence was synonymously mutated. Plasmids encoding subunits of CK2 were provided by Professor D. Litchfield (University of Western Ontario, Ontario, Canada). For construction of pcDNA 4/TO Myc/His₍₆₎-tagged CK2 α' (Thermo Fisher Scientific), CK2 α' from pRC/CMV-HA-CK2 α' was amplified by PCR and inserted into *BamHI/EcoRI* sites. pcDNA3-luciferase was purchased from Addgene.

Preparation of cellular fractions

Nuclear and cytoplasmic extracts were isolated as described previously (17).

Transfection

Cultured cells were transfected using Polyfect reagent for plasmid transfection and oligofectamine for siRNA transfection, as described previously (3).

Virus production and stable cell generation

Viruses were generated and H157 *BRMS1* knockdown (KD) cells were established, as described previously (16). These *BRMS1*^{KD} cells were cotransfected with pcDNA3-luciferase and Tet-on CK2 α' . Stable clones were selected by Geneticin (400 $\mu\text{g/mL}$), Blasticidin (5 $\mu\text{g/mL}$), and Zeocin (150 $\mu\text{g/mL}$). Myc/His₍₆₎-CK2 α' expression was confirmed by immunoblot after treatment with TCN (1 $\mu\text{g/mL}$) for 48 h. Then, the H157 *BRMS1*^{KD}/luciferase/Tet-on CK2 α' cells were infected with pBabe-shRNA-resistant FLAG-BRMS1 wild-type, S30A mutant, or empty vector and selected with puromycin (1 $\mu\text{g/mL}$). Flag-BRMS1 expression was confirmed by Western blot.

Generation of phosphospecific BRMS1 antibody

BRMS1 (pS30) antibody was generated by Open Biosystems. In brief, two rabbits were immunized with KHL-conjugated BRMS1 peptide centered on phospho-Ser30, and the phosphospecific antibody affinity was purified with phosphopeptide.

Protein half-life analysis

CHX blocking analysis was performed to determine the half-life of endogenous BRMS1. Cells were incubated with CHX (100 μM) for various times, and endogenous BRMS1 was

detected by Western blot. The densitometry of immunoblots for BRMS1 and tubulin was measured using the ChemIDoc MP system (Bio-Rad). The level of BRMS1 was quantified by normalization with tubulin, the percentage of remaining BRMS1 was plotted on a logarithmic scale over time, and half-life was determined using Prism 6.0 (GraphPad Software).

For detection of the turnover rate of HA-BRMS1, pulse-chase assays were performed (18). In brief, cells transfected with HA-BRMS1 were pulsed with 100 μ Ci of 35 S-labeled methionine/cysteine for 30 min and chased for various times. Immunoprecipitations were performed using antibody against HA-tag (5 μ g). Proteins were resolved by SDS-PAGE gel and visualized by autoradiography.

***In vitro* protein expression, purification, and kinase activity assays**

GST-fusion proteins were expressed and purified as described previously (3). For *in vivo* kinase activity assays, endogenous CK2 α' was immunoprecipitated by CK2 α' antibody (5 μ g), followed by incubation with GST-fusion BRMS1 (20 μ g) or CK2-specific substrate peptide (100 nmol) in the presence of [γ - 33 P]-ATP or regular ATP, respectively. For experiments using GST-BRMS1 as substrate, phospho-GST-BRMS1 was resolved by SDS-PAGE gel and visualized by autoradiography. For experiments using CK2-specific substrate peptide as substrate, the CK2 α' kinase activity was measured using the ADP-Glo Kinase Assay (Promega), according to the manufacturer's protocol.

For *in vitro* kinase assays, GST-BRMS1 (5 μ g) was incubated with recombinant CK2 with [γ - 33 P]-ATP for 30 min at 30°C.

Immunoprecipitation, Western blotting, and immunofluorescence

Immunoprecipitation, Western blotting, and immunofluorescence were conducted as previously described (3).

Ubiquitination assay

NSCLC cells were transfected with HA-CK2 α' , and ubiquitination assays were conducted as described previously (15).

Invasion chamber assays

H157 stable cells were pretreated with or without TCN (1 μ g/mL) for 48 h. Invasion chamber assays were performed as described previously (16).

Orthotopic NSCLC xenograft model

All animal experiments were approved by the Animal Care and Use Committee at MSKCC (protocol #13-10-016). H157 stable cells (1×10^6) suspended in 100 μ L of DPBS were injected into the left lungs of 40 5-week-old female athymic nude mice (nu/nu, Taconic, Albany, NY), including *BRMS1*^{KD}/Tet-on CK2 α' /control (Control; N=10); *BRMS1*^{KD}/Tet-on CK2 α' /FLAG-BRMS1 S30A (BRMS1 S30A; N=10); and *BRMS1*^{KD}/Tet-on CK2 α' /FLAG-BRMS1 wild-type (BRMS1 WT; N=20). The BRMS1 S30A group and 10 mice from

the BRMS1 WT group were administrated a doxycycline diet (0.625 g/kg; Harlan TEKLAB) on the day of injection.

***In vivo* imaging and quantification**

Mice were anesthetized with 2.5% isoflurane and imaged after intraperitoneal injection of luciferin (150 mg/kg; Promega). Imaging was performed with an IVIS Spectrum-CT system (PerkinElmer) on day 8 after injection and repeated weekly (19). To determine the best time for imaging, a kinetic study was performed by continuously imaging at 5-min intervals for 40 min after luciferin injection. Three-dimensional reconstruction was accomplished by use of Living Image software (version 4.2; Caliper).

For quantification, H157 *BRMS1*^{KD}/luciferase cells were serially diluted into 96-well plates. After the addition of luciferin (15 mg/mL), the cells were continuously imaged at 2-min intervals for 30 min, to catch the peak luciferase signal. The standard curve of regions of interest (unit=radiance) versus cell numbers was drawn, and the cell numbers in primary tumors and metastatic sites were calculated using Living Image software.

Tissue microarray and immunohistochemistry

We created a tissue microarray (TMA) containing 160 NSCLCs and matched adjacent noncancerous tissue (SD Table). The immunohistochemical techniques used were described previously (2, 20). BRMS1 staining in the nucleus and cytoplasm were evaluated separately. The BRMS1 and CK2 α ' antibodies were used at a 1:200 dilution for 30 min.

Statistical analysis

The results of all experiments represent the mean $\pm$ SEM of three separate experiments performed in triplicate. Statistical analysis was performed using Prism. Student's *t* test, one-way ANOVA, Wilcoxon matched-pairs signed rank test, Mann-Whitney test, and Spearman correlation were used. Progression-free survival (PFS) was defined as the time from surgery to the development of metastasis and was assessed using the Kaplan-Meier method and compared using the log-rank test. The CK2 α ' immunoreactivity score cutoff value (3.3; $p=0.002$) was determined by receiver operating characteristic curve analysis using maximum sum of specificity and sensitivity. A 2-sided *P* value <0.05 was considered to indicate statistical significance for all calculations.

Results

TNF promotes CK2-mediated BRMS1 phosphorylation at S30

We observed that BRMS1 protein levels were decreased 5 to 10 times more than transcript in NSCLC, compared with NHBE cells or adjacent noncancerous tissues (3). This suggests that BRMS1 is posttranscriptionally regulated. To assess the stability of endogenous BRMS1 protein in NSCLC cells, we performed CHX blocking assays. As shown in Figure 1A, BRMS1 protein had a significantly shorter half-life in A549 and H1299 cells than in NHBE cells ($p<0.0001$). To exclude the possibility that a reduction of BRMS1 was secondary to transcriptional repression (4), we evaluated the half-life of ectopic HA-BRMS1. The half-life of HA-BRMS1 was also shorter in H1299 cells than in NHBE cells ($p<0.0001$) (Fig.

1B). Given the contributions of the tumor microenvironment and the associated proinflammatory cytokines to tumorigenesis and metastases (21, 22), we asked whether TNF accelerates BRMS1 protein degradation. TNF treatment resulted in a nearly 50% reduction in the half-life of BRMS1 in both NHBE and H1299 cells (Fig. 1B), confirming the importance of TNF in the posttranslational regulation of BRMS1. Using H157 cells, which stably express SR-I κ B, a dominant-negative inhibitor of NF- κ B (H157I), or a vector control (H157V) (4), subsequent experiments showed that TNF-induced BRMS1 protein degradation is independent of NF- κ B activation (SD Fig. 1A).

Analysis of BRMS1 protein (<http://scansite.mit.edu>) indicated the presence of three evolutionarily conserved CK2 consensus sites (Fig. 1C). We therefore hypothesized that BRMS1 may be a substrate of CK2. As shown in Figure 1D, GST-fusion BRMS1 was phosphorylated by recombinant CK2 *in vitro*. Of importance, introducing an S30A mutation in BRMS1 completely inhibited CK2-induced phosphorylation (Fig. 1D). These data indicate BRMS1 S30 is the primary site of CK2 phosphorylation. This is consistent with mass spectroscopy data identifying phosphorylation at S30, but not S45 or 46 (<http://www.Phosphosite.org>).

To elucidate whether BRMS1 S30 is the primary phosphorylation site in response to TNF, we developed a BRMS1 phosphospecific S30 (pS30) antibody. Stimulation with TNF significantly increased BRMS1 (pS30) in a time-dependent manner (Fig. 1E). Moreover, preincubation of lung cancer cells with a CK2-specific pharmacologic inhibitor TBB blocked TNF-induced BRMS1 phosphorylation (SD Fig. 1B). To confirm the specificity of our BRMS1 (pS30) antibody, we expressed either HA-tagged BRMS1 wild-type or S30A mutant in NSCLC cells. The level of BRMS1 (pS30) protein was robustly increased in cells expressing wild-type HA-BRMS1 following TNF treatment. However, no (pS30) protein was detected in cells expressing mutant BRMS1 (S30A) (Fig. 1F). These data confirm that TNF induces phosphorylation of BRMS1 at S30 through a CK2-dependent pathway. Additionally, similar to TNF, TGF β and IL-6 (but not EGF) are able to induce BRMS1 (pS30) (SD Fig. 1C)—suggesting that multiple inflammation-related cytokines in the tumor microenvironment are involved in CK2-induced BRMS1 phosphorylation.

CK2 α' is responsible for TNF-induced degradation of BRMS1

To address whether CK2 participates in BRMS1 degradation, we treated NSCLC cells with TNF and/or CX4945, a specific inhibitor of CK2 (23) currently in clinical trials. Treatment with TNF alone significantly reduced levels of BRMS1 protein. However, pretreatment with CX4945 abrogated TNF-induced phosphorylation and degradation of BRMS1 (Fig. 2A). These data indicate that CK2 activity is required for TNF-induced degradation of BRMS1.

To determine which subunit of CK2 is involved in BRMS1 degradation, wild-type and kinase-dead forms (CK2 α K68/m and CK2 α' K69/m) (24) of CK2 subunits were individually expressed in H1299 cells. Endogenous BRMS1 was clearly decreased in cells with overexpression of CK2 α' wild-type but not in cells expressing other isoforms (Fig. 2B). Coimmunoprecipitation assays revealed that BRMS1 physically interacts with ectopic and endogenous CK2 α' but not with CK2 α (Fig. 2C and SD Fig. 2A). TNF failed to induce BRMS1 degradation following siRNA knockdown of CK2 α' (Fig. 2D). Although CK2 α and

α' share an 86% similarity in their catalytic domain (25), Western blots indicated that only α' , not α , was specifically knocked down by siRNA (Fig. 2D). Collectively, these results indicate that CK2 α' is the key catalytic subunit mediating BRMS1 degradation.

To determine whether the ubiquitination-proteasome pathway is involved in CK2 α' -mediated degradation of BRMS1, *in vivo* ubiquitination assays were performed. The proteasome inhibitor MG132 was used to block ectopic CK2 α' -induced BRMS1 degradation. Ectopic CK2 α' significantly enhanced BRMS1 polyubiquitination in NSCLC cells (Fig. 2E). Additionally, treatment with MG132 rescued CK2 α' -induced BRMS1 degradation (SD Fig. 2B). The introduction of mutant BRMS1 (S30A) abrogated CK2 α' -induced BRMS1 degradation (Fig. 2F). Thus, phosphorylation of BRMS1 at S30 is required for CK2 α' -induced degradation.

TNF activates CK2 α' to phosphorylate BRMS1

To confirm that TNF induces CK2 α' kinase activity in NSCLC, we demonstrated that TNF induces the phosphotransferase activity of CK2 α' in a time-dependent manner (Figs. 3A and B). Importantly, pretreatment with CX4945 significantly decreased both basal and TNF-induced CK2 α' kinase activity (Fig. 3B). In addition, TNF-induced activation of CK2 α' resulted in robust phosphorylation of BRMS1 *in vitro* and *in vivo* (Figs. 3C and D) and was completely blocked following siRNA knockdown (Fig. 3D). To confirm that TNF induces phosphorylation of BRMS1 at S30 through activation of CK2 α' at a single-cell level, immunofluorescence analysis was performed. Stimulation with TNF significantly increased levels of BRMS1 (pS30) in NSCLC cells (Fig. 3E). Interestingly, unlike BRMS1, the majority of BRMS1 (pS30) is located in the cytoplasm, suggesting that phosphorylation alters subcellular localization of BRMS1. Preincubation with CX4945 abrogated TNF-induced BRMS1 (pS30) but did not affect the expression of CK2 α' , suggesting that CX4945 inhibits CK2 α' kinase activity, not expression. Similar to a recent study (26), we observed that TNF increased CK2 α' protein levels (Figs. 3C, D, and E). TNF increases the phosphotransferase activity of CK2 α' , resulting in phosphorylation of BRMS1 at S30.

TNF-induced phosphorylation promotes 14-3-3 ϵ -dependent BRMS1 nuclear export

BRMS1 is primarily a nuclear protein, whereas ubiquitin-dependent degradation occurs within the cytoplasm (27). To explore whether TNF alters the subcellular localization of BRMS1, we isolated nuclear and cytosolic extracts from NSCLC cells after stimulation with TNF. In the absence of stimulus, BRMS1 was primarily nuclear (Fig. 4A). Following TNF stimulation, there was a significant shift of BRMS1 protein from the nucleus to the cytosol of cells in a time-dependent manner (Fig. 4A). However, preincubation with CX4945 completely blocked TNF-induced nuclear exportation of BRMS1 (Fig. 4A). Subsequent immunofluorescence assays showed that stimulation with TNF dramatically increased cytosolic BRMS1, compared with vehicle treatment (SD Fig. 3A). Moreover, the BRMS1 S30 mutant was primarily intranuclear and did not undergo nuclear exportation, even with TNF stimulation (Fig. 4B). We therefore conclude that CK2-induced phosphorylation is required for the nuclear exportation of BRMS1.

The 14-3-3 family of proteins regulate the subcellular localization of multiple proteins via direct binding. Isoforms ϵ and ζ are the most abundant in lung tissue (28). As shown in SD Figure 3B, the 14-3-3 isoform ϵ , and not ζ , endogenously associates with BRMS1. TNF significantly enhanced the interaction of BRMS1 and 14-3-3 ϵ (Fig. 4C), whereas the BRMS1 S30 mutant failed to bind to 14-3-3 ϵ (Fig. 4D). Furthermore, knockdown of 14-3-3 ϵ not only blocked cytoplasmic translocation of BRMS1 but also increased BRMS1 protein within the cell, suggesting that 14-3-3 ϵ is required for BRMS1 nuclear export (Fig. 4E). Additionally, we observed that 14-3-3 ϵ knockdown blocked TNF-induced nuclear export of BRMS1 (Fig. 4F). Collectively, these data suggest that phosphorylation of BRMS1 by CK2 enhances 14-3-3 ϵ interaction, which promotes BRMS1 nuclear export and degradation.

BRMS1 S30A mutant tumors have a lower rate of metastases

To determine the significance of CK2 α' -mediated degradation of BRMS1 in NSCLC, we created H157 cell lines in which endogenous BRMS1 was stably knocked down. These cells were then used to establish H157 lines that ectopically expressed shRNA resistant BRMS1 wild-type or S30A mutant. Additionally, these cells stably expressed Myc-CK2 α' construct (Tet-on-Myc-CK2 α') under the control of the tetracycline (TCN) responsive operator. Treatment with tetracycline induced expression of Myc-tagged CK2 α' and reduced the level of BRMS1 wild-type (SD Fig. 4A). Cells expressing BRMS1 wild-type had significantly less invasion of NSCLC cells, compared with BRMS1 knockdown (control) cells. As expected, the S30A mutation did not affect the capacity of BRMS1 to repress invasion of NSCLC. TCN-induced CK2 expression significantly increased the NSCLC invasion in cells expressing BRMS1 wild-type, but not the S30A mutant (SD Fig. 4B).

To examine the contribution of CK2 α' -mediated degradation of BRMS1 to the progression of NSCLC *in vivo*, we used an orthotopic xenograft lung cancer model. The position of primary tumors in the lung tissue was confirmed by bioluminescence IVIS Spectrum-CT scan (SD Fig. 5A), and ectopic expression of BRMS1 and CK2 α' was verified by Western blot analysis (SD Fig. 5B). No significant difference was observed for growth of the primary tumors among groups (SD Fig. 5C). As expected, animals that received H157 cells expressing BRMS1 wild-type had significantly fewer metastases, compared with the H157 control group in which endogenous BRMS1 was reduced (Figs. 5A, 5B, and 5C). Administration of doxycycline induced Myc-CK2 α' expression, which abrogated BRMS1 wild-type-mediated suppression of metastasis, but was unable to drive metastases in the BRMS1 S30A mutant group (Figs. 5A, 5B, and 5C). Mutation of S30 in BRMS1 resulted in a 60-fold reduction of CK2-driven metastatic tumor burden (Fig. 5B and 5C). Collectively, these data show that BRMS1 suppresses NSCLC metastasis *in vitro* and *in vivo* and that CK2 α' -mediated phosphorylation of S30 results in degradation of BRMS1 and a robust increase in metastasis.

CK2 α' correlates with intracellular BRMS1 localization and metastases in human NSCLC

Having demonstrated the importance of BRMS1 in suppressing CK2 α' -driven metastases in our *in vivo* model, we wanted to determine whether similar correlations exist in human lung cancer. Immunohistochemical analysis was performed on a TMA of 160 human NSCLC and matched-adjacent noncancerous tissues (SD Table). As shown in Figure 6A, CK2 α' is

overexpressed in human NSCLCs, compared with adjacent noncancerous tissues (mean $\pm$ SD, 4.07 ± 3.4 vs. 2.99 ± 2.92 ; $p=0.004$). Importantly, CK2 α' expression levels were significantly higher in patients who developed distant metastases, compared to patients who remained progression free ($p=0.022$) (Fig. 6B). We also observed that high expression of CK2 α' in NSCLC is an independent factor associated with decreased PFS ($p=0.04$) (Fig. 6C).

To explore the relationship between CK2 α' activity and cytosolic localization of BRMS1 *in vivo*, we examined the protein levels of BRMS1 in the same TMA. Immunohistochemistry scores for cytosolic BRMS1 were lower than those for nuclear BRMS1 (1.37 ± 1.94 vs. 3.61 ± 3.83 ; $p<0.0001$)—confirming that BRMS1 was primarily nuclear in human tumors. Cytosolic BRMS1 was commonly found in tumors with higher levels of CK2 α' ($p=0.0001$) (Fig. 6D). Strikingly, protein levels of cytosolic BRMS1 positively correlated with increased levels of CK2 α' protein in NSCLC (Spearman $R=0.31$ [95% confidence interval, 0.145–0.452]; $p=0.0002$). These data provide important correlative confirmation that CK2 α' promotes nuclear exportation of BRMS1 in NSCLC and begin to lay the foundation for the use of CK2 α' inhibitors in selected individuals with lung cancer.

Next, we examined the levels of CK2 α' and BRMS1 (pS30) in selected human NSCLC tumors. CK2 α' is overexpressed and BRMS1 is decreased in tumors, compared with adjacent noncancerous tissues. BRMS1 (pS30) was also overexpressed in most tumors, compared with adjacent tissues (9 of 12) (Fig. 6E). In more than half of these tumors (7 of 12), levels of BRMS1 (pS30) were consistent with elevated levels of CK2 α' (Fig. 6E). In addition, we observed significantly higher CK2 α' kinase activity in primary human NSCLC tumors than in adjacent tissues (SD Fig. 6A). Tumors with nodal metastasis had higher increased activity of CK2 α' , compared with tumors without nodal metastasis, indicating that elevated CK2 α' activity is a strong predictor of the nononcogene addiction observed in NSCLC (SD Fig. 6B). Collectively, our data demonstrate that both protein levels and kinase activity of CK2 α' are increased in NSCLC, compared with adjacent tissues. In addition, elevated levels of CK2 α' strongly correlate with BRMS1 S30 phosphorylation, cytoplasmic localization, increased metastases, and poor clinical prognosis.

Discussion

Herein, we have shown that BRMS1 is regulated posttranslationally via TNF-induced activation of CK2 α' , which phosphorylates BRMS1 on S30 and initiates 14-3-3-mediated nuclear export and proteasome-mediated degradation of BRMS1. Using an orthotopic NSCLC mouse model, we established the biological significance of CK2 α' -mediated regulation of BRMS1 as a major driver of increased metastases (Fig. 7A).

Whereas CK2 kinase activity is constitutively active, CK2 substrate recognition is regulated by cytokines, inflammatory mediators, and growth factors, such as EGF, TNF, and TGF β (29), as well as by p38 MAPK-mediated CK2 activation (12, 30). The role played by CK2 in cancer is illustrated in multiple myeloma, where CK2 phosphorylates the PIKK regulatory proteins Tel2/Tti1, which facilitates their proteasomal degradation by Fbxo9 in the mTORC1 complex. This CK2-initiated, Fbxo9-mediated deregulated ubiquitylation is associated

with myelomagenesis (31). Other groups have shown that inositol pyrophosphates mediate DNA-PK/ATM-p53 cell death via CK2 phosphorylation (32) and that CK2-mediated phosphorylation of prostate apoptosis response-4 reduces its apoptotic function and increases cell survival (33). These reports, as well as our own observations regarding BRMS1, provide evidence that CK2 is a potential therapeutic target for tumors with high CK2 activity.

Protein expression of the CK2 catalytic subunit α or regulatory subunit β is elevated in various cancers, and overexpression of these subunits is correlated with poor prognosis (34, 35). In NSCLC, we found that CK2 α' led to BRMS1 degradation, affirming the specificity of select CK2 catalytic subunits (36). Prior studies demonstrated that the free form of CK2 α' phosphorylates NKX3.1 in prostate tumor cells (37). Recently, the Litchfield group revealed distinct functions of CK2 isoforms in promoting cancer cell survival by identifying isoform-specific phosphorylation of caspase-3 by CK2 α' , not CK2 α (25). Confirming our observations that CK2 α' is overexpressed in human NSCLC, a recent Oncomine (www.oncomine.org) analysis of lung cancer and normal lung tissue demonstrated that CK2 α' was expressed at significantly higher levels in lung cancer tissue (38). Although the functions of CK2 α and β are well-studied, the functional role of CK2 α' in tumor biology remains unclear. Herein, we show that CK2 α' significantly enhances distant tumor metastasis *in vivo* and that expression of BRMS1 S30A mutant significantly diminishes CK2 α' -enhanced metastasis *in vitro* and *in vivo*.

Although BRMS1 is a predominantly nuclear protein, it does shuttle between the nucleus and cytoplasm (14). Nuclear export of BRMS1 is not CRM-1 dependent (14), and 14-3-3 can transport proteins (e.g. HDAC7) independent of CRM1 (39). 14-3-3 proteins are capable of regulating intracellular localization of their binding partners, such as Raf, Cdc25, BAD, and FKHL1 (40). We observed that CK2 α' -mediated phosphorylation induced nuclear exportation of BRMS1 in a 14-3-3 ϵ -dependent manner which is enhanced in the presence of TNF. Simultaneously, we observed that CK2 α' -mediated phosphorylation is required for nuclear export of BRMS1, as BRMS1 S30A mutant failed to interact with 14-3-3 ϵ and undergo nuclear exportation, even in the presence of TNF. Importantly, our observation of a positive correlation between the protein levels of CK2 α' and cytoplasmic BRMS1 expression in human NSCLC specimens confirms that phosphorylation by CK2 α' is a prerequisite for nuclear exportation of BRMS1.

Our data indicate that the fate of phosphorylated BRMS1/14-3-3 complex, once it enters the cytosol, is to be degraded. Whereas the ubiquitin-proteasome components can be found in the nucleus, full degradation of targeted proteins occurs through cytoplasmic ubiquitin-proteasome machinery. This occurs for many proteins, including the nuclear proteins p53 (41), cyclin D1 (42), p27^{kip1} (43), and I κ B α (44). There have been conflicting observations regarding the clinical significance of cytosolic BRMS1, with some reports suggesting it is a favorable prognostic indicator (45) and others suggesting the opposite (13). We show that increased CK2 α' is observed in tumors with BRMS1 localized to the cytoplasm; this was associated with increased metastasis, tumor recurrence, and reduced PFS in patients with NSCLC.

CX4945 is a potent and specific ATP-competitive inhibitor of both CK2 α and α' . As the first orally bioavailable small-molecule inhibitor of CK2, CX4945 exhibits antitumor activity in multiple solid and hematopoietic tumor models, and its safety and efficacy have been established by a phase I/II clinical trial (23). A recent *in vitro* study demonstrated that CX4945 suppresses epithelial-mesenchymal transition and metastasis in A549 lung cancer cells through inhibition of multiple signaling pathways, including MAPK, AKT, FAK/Src, and MMPs (46). Our study demonstrates that CX4945 abrogates TNF-induced BRMS1 degradation through inhibition of CK2 α' -mediated phosphorylation. This suggests that treatment with CX4945, potentially with the addition of a proteasome inhibitor, may result in a robust suppression of NSCLC metastasis via inhibition of BRMS1 degradation (Fig. 7B). Moreover, mutation of S30 in BRMS1 prevents CK2 α' -mediated phosphorylation and degradation and leads to a 60-fold reduction in metastases; this illustrates the importance of BRMS1 as a primary regulator of metastases.

In summary, our work establishes a new, targetable mechanism through which BRMS1 is posttranslationally regulated. Given the increased expression of CK2 α' and the correlative low levels of BRMS1 in a number of different highly metastatic tumors, these observations may have broad implications in the clinical management of metastatic cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. CK2 phosphorylates BRMS1 at S30
(A) BRMS1 protein has a shorter half-life in NSCLC than in NHBE cells. NHBE and A549 and H1299 cells were treated with CHX (100 μ M) for the indicated times. BRMS1 expression was probed by immunoblot (**Upper**). The half-life ($t_{1/2}$) of BRMS1 protein was calculated (**Lower**). **(B)** TNF promotes BRMS1 degradation. NHBE and H1299 cells were transfected with HA-tagged BRMS1. Thirty-six hours posttransfection, pulse-chase analysis was performed with or without TNF (20 ng/mL) at the indicated times. BRMS1 was visualized by autoradiography, and $t_{1/2}$ was calculated. **(C)** Schematic representation of BRMS1 protein. Orange rectangles: Coiled-coil domains; red characters: putative CK2-phosphorylated sites. **(D)** BRMS1 is phosphorylated by CK2 *in vitro*. *In vitro* phosphorylation assays were performed by incubating GST-BRMS1 with recombinant CK2 and subjection to SDS-PAGE gel. Phospho-GST-BRMS1 was visualized by autoradiography. **(E)** TNF induces BRMS1 phosphorylation at S30. H157 and H1299 cells were treated with TNF (20 ng/mL) for the indicated times. BRMS1 (pS30) was detected by immunoblot. **(F)** S30A mutant abrogates TNF-induced BRMS1 phosphorylation. H157 cells were transfected with HA-BRMS1 wild-type or S30A mutant and stimulated with or without TNF (20 ng/mL) for an additional 2 h. Immunoprecipitation (IP) assays were performed using anti-HA antibody, and BRMS1 (pS30) was detected.

Figure 2. CK2 catalytic subunit α' is required for TNF-induced BRMS1 degradation

(A) CX4945 abrogates TNF-induced BRMS1 degradation. H1299 cells were pretreated with or without CX4945 (30 μM) for 2 h, followed by TNF (20 ng/mL) for 16 h. The indicated proteins were evaluated. (B) CK2α' induces BRMS1 degradation. H157 cells were transfected with the indicated CK2 subunits. Protein levels of BRMS1 and CK2s were evaluated. BRMS1 expression was quantified by densitometry (normalized with tubulin) and labeled under each corresponding band. (C) CK2α', but not α, interacts with BRMS1. H1299 cells were transfected with HA-tagged CK2α, α', or empty vector.

Coimmunoprecipitation (IP) assays were performed following treatment with MG132 (5 μ M) for 16 h, and BRMS1 was detected. **(D)** Knockdown *CK2 α '* inhibits TNF-induced BRMS1 degradation. H1299 cells were transfected with siRNA *CK2 α '* or scramble and treated with TNF (20 ng/mL). The indicated proteins were evaluated. **(E)** *CK2 α '* enhances BRMS1 polyubiquitination. NSCLC cells were transfected with HA-*CK2 α '* and treated with MG132 (5 μ M) for 16 h. Ubiquitination assays were conducted. **(F)** S30A mutation abrogates *CK2 α '*-induced BRMS1 degradation. H1299 cells were cotransfected with HA-BRMS1 wild-type or S30A, and HA-*CK2 α '*. The indicated proteins were assessed.

Figure 3. TNF induces BRMS1 phosphorylation by activation of CK2α'

(A) TNF increases CK2α' activity. H1299 and H157 cells were treated with TNF (20 ng/mL) for the indicated times. Endogenous CK2α' was immunoprecipitated, and kinase activity assays were performed. * $p < 0.05$ and ** $p < 0.01$, compared with time 0. (B) CX4945 blocks TNF-induced CK2α' activation. H1299 cells were pretreated with or without CX4945 (30 μ M) for 2 h, followed by TNF (20 ng/mL) for an additional 2 h. Endogenous CK2α' kinase activity was determined. * $p < 0.05$ and ** $p < 0.01$, compared to vehicle; # $p < 0.01$, compared to TNF alone. (C) TNF activates CK2α' to induce BRMS1 phosphorylation. Cells were treated

with TNF (20 ng/mL) for the indicated times. Immunoprecipitated CK2 α' was incubated with GST-BRMS1, and phospho-GST-BRMS1 was visualized by autoradiography. **(D)** Knockdown CK2 α' abrogates TNF-induced BRMS1 phosphorylation. H1299 cells were transfected with siRNA CK2 α' or scramble and treated with or without TNF (20 ng/mL) for 30 min. Phospho-BRMS1 was assessed. **(E)** Pretreatment with CX4945 blocks TNF-induced phosphorylation of BRMS1. H157 cells were pretreated with CX4945 (30 μ M) for 2 h, followed by stimulation with TNF (20 ng/mL) for an additional 1 h. Immunofluorescence assays were performed using antibodies against BRMS1 (pS30) (red)/CK2 α' (green)/DAPI (blue).

Figure 4. TNF induces nuclear exportation of BRMS1 in a 14-3-3-dependent manner

(A) CK2 mediates TNF-induced BRMS1 nuclear exportation. H1299 cells were pretreated with MG132 (5 μ M) alone or with CX4945 (30 μ M) for 2 h, followed by TNF (20 ng/mL). Nuclear/cytosolic BRMS1 expression was probed. (B) S30A mutant abrogates TNF-induced nuclear exportation of BRMS1. H157 cells were transfected with HA-tagged BRMS1 wild-type or S30A mutant. Transfected cells were treated with or without TNF (20 ng/mL) for 2 h; HA-BRMS1 in nuclear extract (NE) or cytosolic extract (CE) was probed separately. (C) TNF enhances the interaction of BRMS1 and 14-3-3 ϵ . H157 cells were pretreated with MG132 (5 μ M) for 2 h and then treated with or without TNF (20 ng/mL) for an additional 2 h. Coimmunoprecipitation (IP) assays were performed using the indicated antibodies. The

presence of 14-3-3 ϵ was detected. **(D)** S30A mutant abrogates BRMS1 binding to 14-3-3 ϵ . H1299 cells were transfected with HA-tagged BRMS1 wild-type or S30A. Co-IP assays using NE were performed, and the presence of 14-3-3 ϵ was probed. **(E)** BRMS1 is primarily located in the nucleus following loss of 14-3-3 ϵ . Immunofluorescent merged micrographs show subcellular localization of BRMS1 (red)/DAPI (blue) in H1993 cells infected with shRNA 14-3-3 ϵ or scramble. Western blots indicate that 14-3-3 ϵ was specifically knocked down. **(F)** Knockdown 14-3-3 ϵ inhibits TNF-induced nuclear exportation of BRMS1. H157 cells were infected with shRNA 14-3-3 ϵ or scramble, pretreated with MG132 (5 μ M) for 2 h, and then treated with or without TNF (20 ng/mL) for an additional 2 h. HA-BRMS1 in NE or CE was detected.

Figure 5. CK2 α' -induced BRMS1 degradation promotes metastasis of NSCLC *in vivo*

(A) Induction of CK2 α' promotes distant metastasis of NSCLC. H157 stable cell lines were orthotopically injected into the left lung of mice. Mouse bioluminescent CT images show the growth of tumor and metastatic sites in 3 representative mice in each group (1–3, control; 4–6, BRMS1WT without doxycycline; 7–9, BRMS1WT with doxycycline; 10–12, BRMS130A with doxycycline). The scale indicates the signal intensity–cell quantification.

(B) S30A mutant blocks CK2 α' -induced metastasis of NSCLC. The logarithmic graph represents average total cell numbers of all metastatic sites in each group. \$ $p < 0.05$, compared to control; # $p < 0.05$, compared to BRMS1 wild-type; * $p < 0.05$, compared to BRMS1 wild-type with doxycycline. (C) Activation of CK2 α' induces metastasis of tumors with BRMS1 wild-type. The graph represents fold changes of metastasis burden over control group 43 days postinjection.

Figure 6. CK2α' overexpression is associated with cytoplasmic localization of BRMS1 and metastases in human lung cancer

(A) CK2α' is overexpressed in NSCLC. The graph represents immunohistochemistry scores of CK2α' in tumors and adjacent noncancerous tissues in our NSCLC TMA (N=160) ($p=0.004$). Photomicrographs show CK2α' staining in two pairs of representative samples. (B) CK2α' is increased in patients with distant metastasis. Immunohistochemistry scores of CK2α' in patients with or without tumor recurrence ($p=0.022$, patients with distant metastasis compared to those without recurrence). (C) High CK2α' is associated with poor progression-free survival (PFS). Kaplan-Meier PFS plot based on tumor CK2α' protein levels ($p=0.04$). (D) Higher levels of CK2α' correlate with cytosolic BRMS1 staining. Immunohistochemistry scores of CK2α' in patients with positive (>0) and negative (0) cytosolic BRMS1 staining ($p=0.0001$). Photomicrographs show staining for CK2α' and BRMS1 in two representative patient samples. (E) Increased BRMS1 (pS30) in NSCLC,

compared with matched adjacent tissues. The indicated proteins were detected in NSCLC (T) and adjacent noncancerous tissues (N): (1–6, squamous cell carcinoma; 7–12, adenocarcinoma). Immunoblot bands were quantified by densitometry and normalized with actin. Fold changes of T vs. N are labeled under each corresponding blot.

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Figure 7. Putative therapeutic relevance of the mechanisms controlling BRMS1 phosphorylation, nuclear exportation, and degradation

(A) In response to TNF, activated CK2 α' phosphorylates BRMS1 at S30, which is required for the interaction with 14-3-3 ϵ , resulting in nuclear exportation and ubiquitin-proteasome-mediated degradation of BRMS1. This reduction of intratumoral BRMS1 promotes lung cancer metastasis. (B) Hypothetic model: Treatment with a specific CK2 inhibitor (CX4945 or others) preserves BRMS1 expression and inhibits lung cancer metastasis.