Cancer Research

The WASF3–NCKAP1–CYFIP1 Complex Is Essential for Breast Cancer Metastasis

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Abstract

Inactivation of the *WASF3* gene suppresses invasion and metastasis of breast cancer cells. WASF3 function is regulated through a protein complex that includes the NCKAP1 and CYFIP1 proteins. Here, we report that silencing NCKAP1 destabilizes the WASF3 complex, resulting in a suppression of the invasive capacity of breast, prostate, and colon cancer cells. In an *in vivo* model of spontaneous metastasis in immunocompromized mice, loss of NCKAP1 also suppresses metastasis. Activation of the WASF protein complex occurs through interaction with RAC1, and inactivation of NCKAP1 prevents the association of RAC1 with the WASF3 complex. Thus, WASF3 depends on NCKAP1 to promote invasion and metastasis. Here, we show that stapled peptides targeting the interface between NCKAP1 and CYFIP1 destabilize the WASF3 complex and suppress RAC1 binding, thereby suppressing invasion. Using a complex-disrupting compound identified in this study termed WANT3, our results offer a mechanistic proof of concept to target this interaction as a novel approach to inhibit breast cancer metastasis. *Cancer Res; 76(17); 5133–42. ©2016 AACR.*

Introduction

Tumor progression is associated with an accumulation of genetic changes that define the transition to more aggressive forms (1). Acquisition of the invasion and metastasis phenotypes has been associated with genes that promote metastasis in combination with the loss of metastasis suppressor genes (2–4). Where it has been studied, these two classes of metastasis-related genes operate independently of the genetic defects that lead to uncontrolled proliferation, survival, or cell death. The *WASF3* gene has been shown to be a promoter of cell invasion *in vitro* (5–9) and metastasis *in vivo* (10–12) in different cancer cell types. Inactivation of WASF3 in breast and prostate cancer cells, for example, leads to reduced cell migration and invasion and, in experimental metastasis models in mice (10, 11) and zebrafish (12), metastasis is suppressed. Upregulation of *WASF3* was part of the gene signature associated with the highly aggressive "claudin-low" subgroup of breast cancers (13). Immunohistochemical (IHC) data showed that *WASF3* is upregulated in high-grade breast tumors (10, 14). Similar analyses show the relationship between WASF3 expression levels and poor prognosis in non–small cell lung cancer (15), gastric cancer (16), hepatocellular carcinoma (17), and prostate cancer (11, 18). Thus, analysis of WASF3 expression levels in primary cancers, are consonant with the *in vitro* studies showing WASF3 promotes cancer cell invasion. In addition, the role of WASF3 in metastasis of osteosarcoma has recently been shown as a result of its downregulation by miRNA-217 (19).

The WASF3 protein contains a verprolin–cofilin–acidic (VCA) motif that binds monomeric actin and the Arp2/3 complex at the C-terminus (20, 21). In resting cells, the protein is held in an inactive conformation through association with a complex of proteins that bind to the WASF homology domain (WHD) at the N-terminus (22). These proteins include CYFIP1, NCKAP1, ABI1, and BRK1, collectively known as the WASF regulatory complex (WRC). Following stimulation of cells with cytokines or growth factors, for example, RAC1 binds to CYFIP1, initiating WASF3 phosphoactivation and leading to conformational changes in the WASF3 protein. As a result, the VCA domain is exposed, facilitating reorganization of the actin cytoskeleton through actin polymerization. This activation of WASF3 is dependent on its phosphorylation on tyrosine residues (5, 6, 9, 23) and the protein moves to the leading edge of the cell where the lamellipodia that are responsible for cell movement are being formed. In highly aggressive cancer cells, these lamellipodia also facilitate invasion through an artificial matrix *in vitro* and metastasis *in vivo* (24). WASF3 has also been shown to have a signaling function where, through suppression of KISS1, NFκB is released from its suppression by IκBα and moves to the nucleus where it activates invasion-promoting genes such as *MMP-9* and *ZEB1* (7, 25). We recently showed a feed-forward loop as a result of the IL6 activation of the JAK2/STAT3 where STAT3 acts as a promoter of *WASF3* transcription and JAK2 activates WASF3 (9, 23). WASF3 upregulation leads to downregulation of E-cadherin and members of the miR-200 family (25), both of which have been shown to suppress epithelial-to mesenchyme transition (EMT). WASF3 can also be upregulated by HIF1 as a result of hypoxia (26) and facilities communication between endoplasmic reticulum and mitochondria

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through its interaction with the ATAD3A mitochondrial protein and GRP78 (27).

The central role for WASF3 in invasion and metastasis, combined with its selective overexpression in high-grade tumors, suggests that directly targeting its function may serve as a strategy for controlling metastasis. Here, we investigated using stapled peptides (SP) to disrupt WASF3 function and suppress invasion. SPs are synthetically designed to stabilize and constrain an α-helical structure through N-methylation and macrocyclic ring formation. Stabilization of the secondary structure introduces an entropically favorable, preordered binding state where key interacting residues are spatially poised for target binding. Further, these locked peptides exhibit drug-like properties, including enhanced cell permeability, non-immunogenicity, increased binding affinity and resistance to cellular degradation (28, 29). In this report, we demonstrate that NCKAP1 is required for WASF3 function and its regulation of invasion, and that targeting the interaction between two members of the WRC, CYFIP and NCKAP1, using SPs, leads to suppression of invasion. Thus, targeting this complex may serve as a means to inhibit metastasis and the lead compound identified from this study, WANT3, may offer a therapeutic approach for selectively targeting metastasis.

Materials and Methods

Cell culture and standard assays

MDA-MB-231, Hs578T, and T47D breast cancer cell lines were obtained directly from ATCC. MDA-MB-231 and T47D and have been verified using SNP-CGH (7, 27) for characteristic cytogenetic changes. The ATCC Cell Authentication Testing service confirmed the identity of Hs578T, PC3, and SW620 (August 2015) using STR DNA fingerprinting analysis (30). Standard cell culture, transient transfections, RT-PCR, Western blotting, immunoprecipitation (IP), flow cytometry, biotin–avidin pulldown, lentiviral transduction, cell proliferation, and Transwell invasion assays were carried out as described previously (7–9, 30).

DNA constructs, antibodies, and other reagents

Lentiviral pCDH-CMV-MCS-EF1-PURO-HA-WASF3 (pCDH-HA-WASF3) was generated as described previously (9). To construct the HA-NCKAP1 overexpression vector, the full-length human NCKAP1 was amplified from the template NCKAP1 cDNA clone (OriGene) and inserted into the pCDH-CMV-MCS-EF1-GFP lentiviral vector (System Biosciences) as described previously (25). To stably knock down NCKAP1, pLKO.1 lentiviral vectors harboring shRNA-targeting NCKAP1 were obtained from Open Biosystems. pcDNA3-EGFP-RAC1-T17N (RAC1DN) was a gift from Dr. Gary Bokoch (Addgene plasmid #12982). The RAC1 NSC24766 inhibitor was obtained from Selleckchem. For Western blot and IP assays, the following primary antibodies were used: NCKAP1, WASF1 (Abcam), WASF2, WASF3 (Cell Signaling Technology), HA, GST, RAC1, β-Actin (Sigma).

Glutathione *S*-transferase fusion protein interaction assays

To determine the interaction between NCKAP1 and WASF3, glutathione *S*-transferase (GST)-fusion protein pulldown assays were performed as described previously (5, 31). A GST–WASF3 (GST-W3) fusion protein was expressed in BL21 bacteria and purified using MagneGST glutathione particles (Promega). Once the correct size protein was confirmed, using Coomassie Brilliant Blue staining following SDS-PAGE, the immobilized fusion protein was used immediately. Cell lysates from MDA-MB-231 cells that had been transfected with a pCDH-CMV-MCS-EF1-PURO-NCKAP1 construct were incubated in 500 μL of binding buffer (20 mm Tris–HCl, pH 7.5, 140 mm NaCl, 1% Nonidet P-40 and 0.5% BSA) with the GST fusion protein tethered to the glutathione particles for 4 hours at 4°C. Precipitates were resolved by SDS-PAGE and analyzed by Western blotting.

Protein complementation assays

To identify the interaction between NCKAP1 and WASF3 in live cells, protein complementation assays (PCA) were performed as described previously (32). In brief, expression vectors encoding NCKAP1 and WASF3 fused to N- and C-terminal fragments of GFP were constructed respectively. The NCKAP1-venus1 (NCKAP1-v1) and WASF3-venus2 (WASF3-v2) constructs were either transiently transfected individually or cotransfected into MDA-MB-231 cells, and 12 hours after transfection, GFP was detected by fluorescence microscopy (Carl Zeiss).

In vivo tumor growth and metastasis analysis

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Augusta University. Six-week-old female NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ) mice were purchased from The Jackson Laboratory and maintained in accordance with IACUC guidelines. The animal experiments were performed using the NSG mouse model as described previously (27). The mice were euthanized on day 56 after injection and dissected tumors were individually weighed. The lungs were also removed from these mice and the number of nodules on the surface of the lungs was counted. For histological analyses, the lungs were fixed in 10% neutral buffered formalin, embedded in paraffin blocks, sectioned at 5 μm, and subjected to hematoxylin and eosin (H&E) staining.

Peptide synthesis

Peptides were synthesized on rink amide MBHA resin using 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis in 1-methyl-2-pyrolidinone (NMP). Fmoc protecting groups were removed using 25% (v/v) piperidine in NMP for 20 to 30 minutes. For couplings using standard N-α-Fmoc protected amino acids, 10 equivalents were added (0.25 mol/L final concentration) along with 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU, 0.23 mol/L final concentration) and 8% (v/v) N,N-diisopropyl ethylamine (DIEA) in NMP. (S)-N-Fmoc-2-(4′-pentenyl) alanine couplings were performed using four equivalents. The ring-closing metathesis (RCM) reaction was performed prior to the addition of N-terminal labeling using 0.4 equivalents bis(tricyclohexylphosphine) benzylidine ruthenium(IV) dichloride (first-generation Grubbs Catalyst, Sigma) in 1,2-dichloroethane (DCE) for two 1-hour reaction periods with agitation.

Prior to N-terminal labeling, β-alanine was added to the N-terminus of all peptides before the addition of 5(6)-carboxyfluorescein. N-terminal fluorescein labeling was performed using two equivalents of 5(6)-carboxyfluorescein (Acros) in DMF along with 0.046 mol/L HCTU and 2% (v/v) DIEA. Resin cleavage was performed using a solution containing 95% trifluoroacetic acid, 2.5% water, and 2.5% triisopropylsilane (Sigma) for 4 hours at room temperature. Peptides were precipitated in methyl-*tert*-butyl ether at 4°C and lyophilized. Peptides were purified by high-

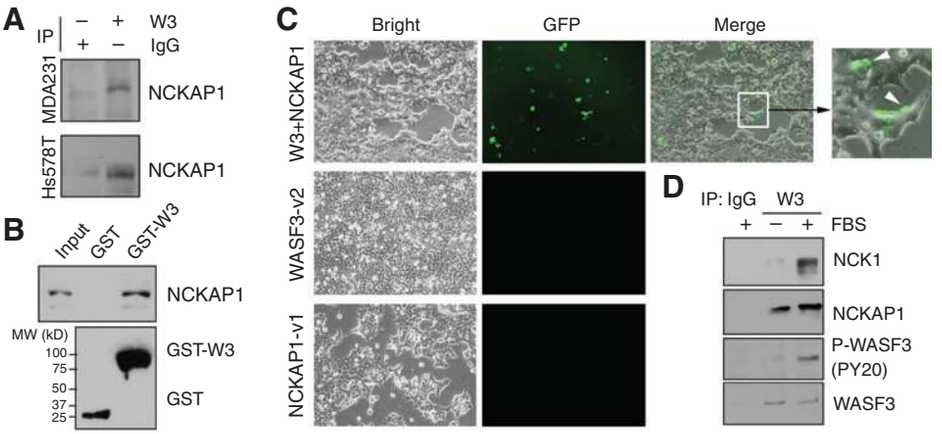

Figure 1.

NCKAP1 interacts with WASF3. **A,** following immunoprecipitation (IP) of WASF3 from MDA-MB-231 and Hs578T breast cancer cells, Western blot analysis identified NCKAP1 in the IP. **B,** the interaction between NCKAP1 and WASF3 was further demonstrated in a GST fusion–protein pulldown assays. Lysates from MDA-MB-231 cells were incubated with the GST-tagged WASF3 prepared in BL21 bacterial cells, where the correct size fusion protein was confirmed using anti-GST antibodies (below). The presence of NCKAP1 was then demonstrated in the WASF3-GST (GST-W3) complex using anti-NCKAP1 antibodies. **C,** interaction between NCKAP1 and WASF3 was also demonstrated *in vivo* following transfection of the NCKAP1-venus1 (NCKAP1-v1) and WASF3-venus2 (WASF3-v2) constructs into MDA-MB-231 cells. After 12 hours, GFP was detected by fluorescence microscopy in cells where both constructs were expressed but not in cells where either of the constructs was expressed alone. In the cotransfected cells, a membrane localization of the GFP signal could be seen (arrows). When the WASF3 complex was recovered using immunoprecipitation from MDA-MB-231 cells grown in the presence or absence of FBS (**D**), NCKAP1 was detected in the complex whether FBS was present or not. The presence of NCK1, however, was seen only in cells treated with FBS, where WASF3 (P-WASF3) was activated.

performance liquid chromatography (HPLC) and verified by ESI mass spectrometry (ESI-MS). Fluorescein-labeled peptides were quantified by measuring the absorbance of 5(6)-carboxyfluorescein at 495 nm. Absorbance values were measured using a Synergy 2 microplate reader (Bio-Tek). The masses of the purified peptides are as follows: WANT1 = 1849.5 (expected mass = 1849.0), WANT2 = 2014.5 (expected mass = 2015.2), WANT3 = 1848.6 (expected mass = 1849.1), WANT3 scr = 1848.6 (expected mass = 1849.1).

Results

The NCK/NCKAP1 complex interacts with WASF3

WASF3 is essential for invasion and metastasis in different cancer cell types (10, 11). By mass spectrometry, NCK-associated protein 1 (NCKAP1) was found to associate with the WASF3 immunocomplex (Supplementary Fig. S1). Immunoprecipitation (IP) analysis of the WASF3 immunocomplex from MDA-MB-231 and Hs578T cells confirmed the presence of NCKAP1 (Fig. 1A). To further validate the interaction between NCKAP1 and WASF3, a GST–WASF3 fusion protein was immobilized on glutathione–agarose and incubated with lysates from NCKAP1-overexpressing MDA-MB-231 cells. In these pulldown assays, NCKAP1 was recovered using the GST–WASF3 fusion protein, but not GST alone (Fig. 1B), indicating that NCKAP1 indeed interacts with WASF3. This interaction was confirmed using protein-fragment complementation (PCA) assays, based on split green fluorescent protein (GFP). Interestingly, the GFP signal was identified at the plasma membrane when NCKAP1-v1 and WASF3-v2 were cotransfected into MDA-MB-231 cells, while no fluorescence was observed when either construct was transfected alone (Fig. 1C). IP analysis showed that serum starvation suppressed WASF3 phosphorylation, without affecting the engagement of NCKAP1 in the WASF3 complex (Fig. 1D).

The NCK1 protein contains multiple SH2/SH3 domains (33) and is the target of several cell surface tyrosine kinase receptors. It was shown that ligand binding activates NCK1 by phosphorylation, and that this event triggers downstream effectors to activate cell motility (34–36). As we have shown, growth factors such as PDGF and cytokines such as IL6 activate WASF3, thereby leading to increased migration and invasion and WASF3 is recruited to the cell membrane to facilitate actin reorganization at leading edges of cells (6, 9, 10, 23). However, NCK1 was not found in the WASF3 immunocomplex in starved cells as shown by IP analysis (Fig. 1D).

NCKAP1 is required for WASF3 protein stability and invasion potential

To investigate this relationship between NCKAP1 and WASF3, we used two different shRNA constructs to suppress NCKAP1 expression in MDA-MB-231 and Hs578T cells, which led to a reduction in WASF3 protein levels (Fig. 2A), while not affecting its transcript levels (Supplementary Fig. S2), supporting the idea that NCKAP1 protects WASF3 from degradation. Because NCKAP1 is also found in complex with other members of the WASF family (36–38), we analyzed the effect of its knockdown on their protein levels, where levels of both WASF1 and WASF2 were reduced in MDA-MB-231 and Hs578T cells (Fig. 2A). Thus, NCKAP1 is also required for the stability of these proteins. Invasion potential, however, is not affected by knockdown of WASF1 and WASF2 in MDA-MB-231 cells (30).

Knockdown of NCKAP1 in both MDA-MB-231 and Hs578T cells leads to a significant reduction in invasion (Fig. 2B), but does not affect cell proliferation (Supplementary Fig. S3). The Rho-

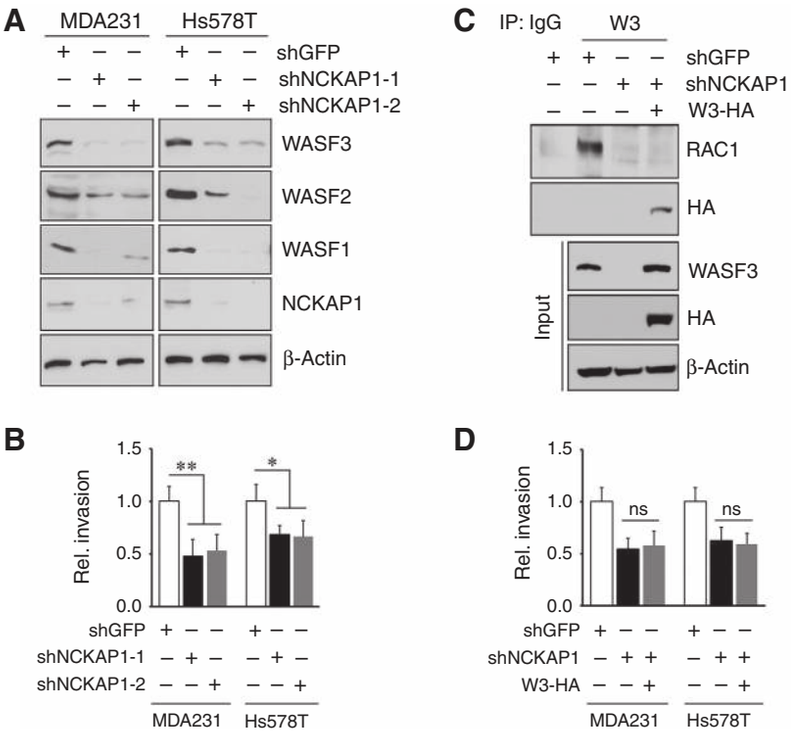

Figure 2.

Molecular and cell invasion analysis following NCKAP1 knockdown. Breast cancer MDA-MB-231 and Hs578T cells in which NCKAP1 had been stably knocked down (shNCKAP1-1 and shNCKAP1-2) show significantly reduced levels of WASF3 (**A**) compared with cells carrying a control shRNA (shGFP). Similarly, reduced levels of the WASF1 and WASF2 proteins were also seen in the NCKAP1 knockdown cells. When NCKAP1 knockdown cells were analyzed using Transwell invasion assays (**B**), their invasion potential was suppressed. Immunoprecipitation of HA-tagged WASF3 from MDA-MB-231 cells in which NCKAP1 had been knocked down shows the absence of RAC1 in the WASF3 immunocomplex (**C**), compared with parental cells expressing the control shRNA (shGFP). When WASF3 was overexpressed in NCKAP1 knockdown MDA-MB-231 and Hs578T cells, there was no recovery of invasion potential (**D**). *, $P < 0.05$; **, $P < 0.01$; ns, no statistical significance.

GTPase, RAC1, relays signals to WASF proteins, leading to activation of Arp2/3-mediated actin polymerization (38, 39). To determine whether depletion of NCKAP1 suppresses the interaction of RAC1 with the WASF3 complex, we transfected an HA-tagged WASF3 construct into NCKAP1-knockdown MDA-MB-231 cells. IP analysis in these cells using WASF3 antibodies showed that RAC1 was not coimmunoprecipitated with the exogenous WASF3 protein (Fig. 2C), in the absence of NCKAP1. Moreover, suppression of invasion in NCKAP1 knockdown cells was not reversed by forced expression of WASF3 (Fig. 2D). Thus, NCKAP1 is essential for the RAC1 interaction with the WASF3 complex in order to promote cell invasion.

Knockdown of NCKAP1 suppresses metastasis in breast cancer cells *in vivo*

To relate the *in vitro* observation that links NCKAP1 expression with invasion to clinical parameters, we evaluated the correlation between NCKAP1 expression and survival of patients with breast cancer. Using an online gene profiling database we related *NCKAP1* expression levels with relapse-free survival data from 3,554 cancer patients based on relative NCKAP1 expression levels as described (40). Univariate survival analysis (Kaplan–Meier method and log-rank test) revealed that high *NCKAP1* expression significantly correlates with poor, relapse-free, survival (Fig. 3A), which is likely related to its involvement in metastasis.

The suppression of invasion seen *in vitro* following knockdown of WASF3 correlates with suppression of metastasis *in vivo* in both zebrafish (12) and mouse models (10, 11). We have previously used the NSG mouse model for *in vivo* metastasis studies where, unlike SCID mouse models, primary tumor formation and metastasis occurs coincidentally within 2 months (27). MDA-MB-231 cells with NCKAP1 knockdown were injected into the mammary fat pads of NSG mice, and tumor development and metastasis was monitored over 8 weeks. There was no significant difference in primary tumor size (Fig. 3B) between the mice injected with either the NCKAP1 knockdown or knockdown control cells. When pulmonary metastasis was examined at the conclusion of the experiment, mice injected with the control cells showed multiple surface tumors. In contrast, those animals injected with NCKAP1 knockdown cells showed a significantly reduced number of metastases (Fig. 3C). Histological analysis further demonstrated that, while the mice injected with the control cells show multiple large tumors throughout the lungs, there are relatively few, small metastases in the mice injected with the NCKAP1 knockdown cells (Fig. 3D). Thus, loss of NCKAP1 expression inhibits *in vivo* metastasis, confirming that disrupting its interaction with the WASF3 complex may be a means of suppressing this aggressive stage of cancer.

RAC1 binding to the WASF3 complex is required for the NCKAP1–WASF3 invasive signaling axis in breast cancer cells

Knockdown of NCKAP1 does not affect *WASF3* transcription levels, but leads to a destabilization of the WASF3 protein (see above). To further investigate whether NCKAP1 is functionally associated with WASF3, we overexpressed *NCKAP1* in invasive MDA-MB-231 and noninvasive T47D cells, and analyzed cell invasion potential. Overexpression of NCKAP1 did not increase WASF3 protein levels in either cell line (Fig. 4A) but significantly increased the invasion potential in MDA-MB-231 cells while making no difference in T47D cells, which lack WASF3 expression (Fig. 4B). Thus, WASF3 is essential for NCKAP1-mediated invasion in breast cancer cells. IP analysis of WASF3 from MDA-MB-231 cells demonstrated increased levels of NCKAP1 and RAC1 in the WASF3 immunocomplex, concomitant with increased WASF3 activation levels (Fig. 4C). These observations suggest that overexpressing NCKAP1 leads to increased engagement of

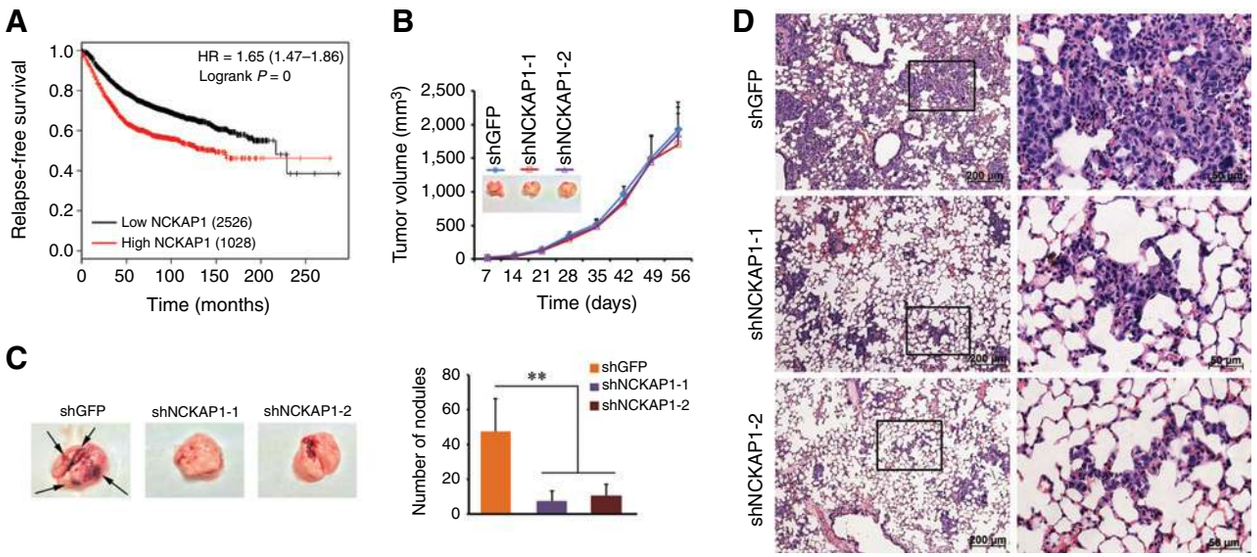

Figure 3.

Metastasis *in vivo* is suppressed following NCKAP1 knockdown. **A,** Kaplan–Meier plot analyses with the log-rank test shows that higher NCKAP1 expression was associated with lower relapse-free survival rates compared with low NCKAP1 expression. When MDA-MB-231 cells were implanted subcutaneously into 6-week-old female NSG mice (**B**), primary tumor growth was not affected by knockdown of NCKAP1 (shNCKAP1-1 and shNCKAP1-2), compared with control knockdown (shGFP) cells. When the lungs were removed from these mice, however, the number of nodules on the surface of the lungs was significantly reduced in the NCKAP1 knockdown cells (**C**). Histological analysis of these lungs demonstrated that, while animals receiving the control cells showed extensive tumor infiltration throughout the lung (**D**), the NCKAP1 knockdown cells showed relatively few, small tumor foci. Images on the right derived from the insets on the left. **, $P < 0.01$.

RAC1 with the WASF3 complex. Treating MDA-MB-231 and Hs578T cells with NSC23766, an inhibitor of RAC1 (41), led to a dose-dependent reduction in invasion in both cell lines (Fig. 4D), without affecting cell proliferation (Supplementary Fig. S4) or affecting the protein levels of either WASF3, NCKAP1, or RAC1 (Fig. 4E). IP analysis shows that NSC23766 does not disrupt engagement of NCKAP1 with the WASF3 complex, although high dose of NSC23766 (50 μmol/L) significantly inhibited both WASF3 phosphorylation and RAC1 binding to the WASF3 complex (Fig. 4F). This is likely due to the reduced levels of RAC1 expression. To further determine the role of RAC1 on the NCKAP1–WASF3 complex, we transfected a T17N dominant–negative RAC1 construct (RAC1DN) into MDA-MB-231 cells overexpressing NCKAP1. Similar to NSC23677 treatment, expression of RAC1DN disrupted the WASF3 interaction with RAC1 and subsequently impaired WASF3 phosphoactivation (Fig. 4G). Moreover, both NSC23677 treatment (Fig. 4H) and overexpression of RAC1DN (Fig. 4I) led to a significant reduction of invasion potential in cells expressing NCKAP1, suggesting that inhibition of RAC1 activation attenuates NCKAP1-mediated cell invasion. Taken together, these data demonstrate that RAC1 binding to the WASF3 complex is critical for promoting invasion in breast cancer cells.

NCKAP1 engagement with the WASF3 complex is required for metastasis of breast cancer cells

Knockdown of WASF3 in MDA-MB-231 and Hs578T cells (Fig. 5A) led to a significant reduction in invasion potential (Fig. 5B), which is consistent with our previous reports (5–12, 25–27). However, when NCKAP1 is overexpressed in WASF3 knockdown cells, there was no significant increase in invasion potential compared with the parental cells (Fig. 5A and B). When *in vivo* metastasis assays were performed using NSG mice, increased numbers of tumor nodules were seen on the surface of the lungs in the knockdown control cells compared with either the WASF3 knockdown cells or the WASF3 knockdown cells overexpressing NCKAP1 (Fig. 5C). In addition, there is no significant change in the number of tumor nodules on the surface of the lungs when NCKAP1 was overexpressed in WASF3 knockdown cells (Fig. 5C). Histological examination of the lungs of these mice shows large tumor foci in the knockdown control cells, compared with the small tumor foci seen in WASF3 knockdown cells (Fig. 5D). Thus, there was no increased metastasis potential to the lung in the WASF3 knockdown MDA-MB-231 cells expressing NCKAP1, compared with the WASF3 knockdown cells (Fig. 5D). This is consistent with the *in vitro* invasion assays. Collectively, these data demonstrate that the NCKAP1–WASF3 complex is essential for cell invasion and metastasis in breast cancer cells.

Suppression of invasion by targeting the CYFIP1–NCKAP1 interaction using SPs

Because the NCKAP1–WASF3 interaction is required for invasion and metastasis, we explored whether targeting this complex could suppress invasion. Knockdown of NCKAP1 in highly invasive cancer cells leads to suppression of invasion (Fig. 2B), which is accompanied by decreased WASF3 levels. Reduced WASF3 levels are also seen after knockdown of CYFIP1 (30), which associates with WASF3 as a dimer with NCKAP1 (42, 43). Loss of either member of this trimeric complex leads to loss of WASF3 protein levels (Supplementary Fig. S5) and suppresses invasion. These observations suggest that disrupting the engagement of

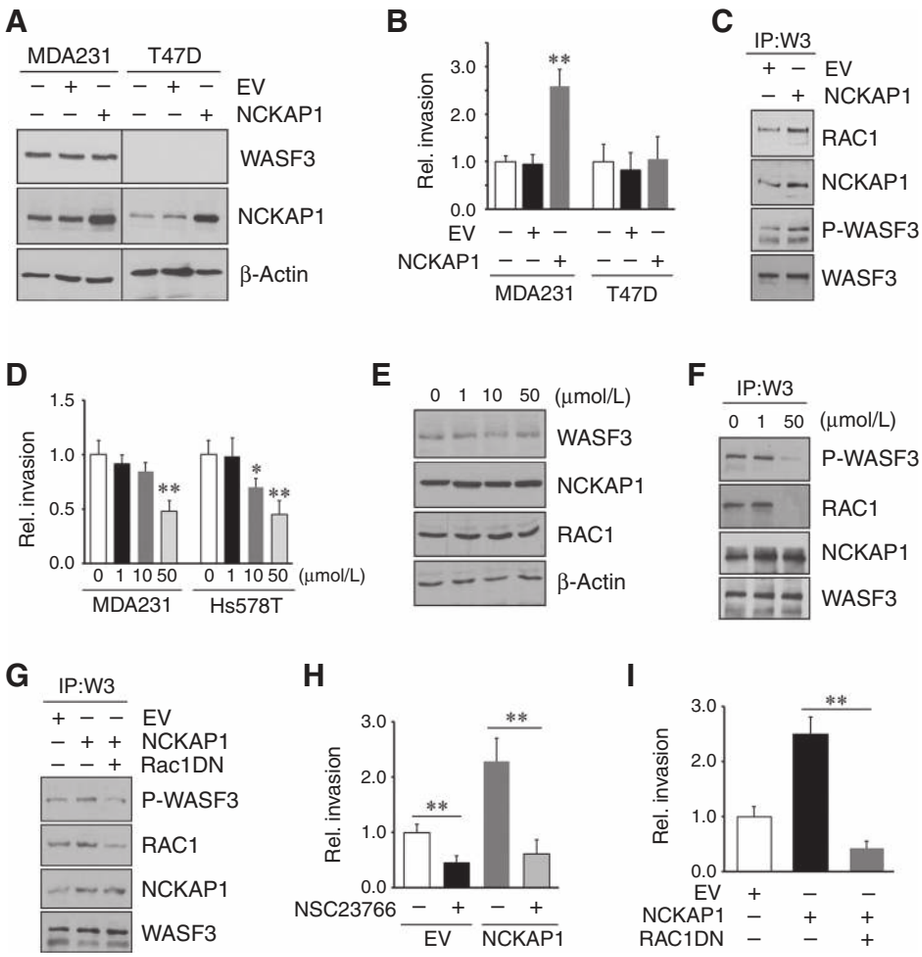

Figure 4.

RAC1 binding to the WASF3 complex is required for NCKAP1-mediated invasion of breast cancer cells. **A,** NCKAP1 overexpression in MDA-MB-231 cells does not affect WASF3 levels, and in T47D cells that do not express WASF3, overexpression of NCKAP1 does not increase WASF3 levels. **B,** Transwell assays demonstrate that overexpressing NCKAP1 in MDA-MB-231 cells significantly increases invasion potential, although T47D cells are unaffected. **C,** IP of WASF3 (W3) from MDA-MB-231 cells shows increased RAC1 levels in the WASF3 complex and increased WASF3 phosphorylation when NCKAP1 is overexpressed. **D** and **E,** treatment of MDA-MB-231 and Hs578T breast cancer cells with the NSC23766 RAC1 inhibitor leads to a dose-dependent reduction in invasion potential (**D**) but does not affect protein levels of either WASF3, NCKAP1, or RAC1 (**E**). **F,** IP of WASF3 (W3) from MDA-MB-231 cells treated with NSC23766 shows that at high (50 μmol/L) concentration, activation of WASF3 is suppressed and RAC1 engagement in the complex is virtually eliminated. When a dominant-negative RAC1 (RAC1DN) is introduced into MDA-MB-231 cells overexpressing NCKAP1, levels of phosphoactivated WASF3 are significantly reduced in concert with reduced RAC1 levels (**G**). **H,** in Transwell assays, NSC23766 leads to a significant reduction in invasion in both MDA-MB-231 parental cells containing the empty vector (EV) and cells overexpressing NCKAP1. **I,** similarly, the RAC1 dominant-negative construct (RAC1DN) significantly suppresses invasion in MDA-MB-231 cells overexpressing NCKAP1. *, $P < 0.05$; **, $P < 0.01$.

NCKAP1 with the WASF3 complex could lead to disruption of the WRC and loss of invasion. Currently, no inhibitors target WASF3 function directly, but targeting the WRC may provide a means to suppress invasion. We have previously reported, for example, that targeting a direct protein–protein interaction between CYFIP1 and WASF3 using SPs can effectively suppress invasion in WASF3-overexpressing cancer cells (30). It is possible, therefore, that disrupting the WRC by targeting NCKAP1 might have a similar consequence. Analysis of the crystal structure of the WRC complex, however, showed no direct contact between NCKAP1 and WASF3 (42, 43), even though loss of NCKAP1 leads to reduced WASF3 stability (Fig. 2A). SPs can be designed to mimic α-helical surfaces between proteins to serve as disruptors of protein–protein interactions. In order to disrupt the WRC complex, several α-helices were identified at the interface between CYFIP1 and NCKAP1 (Supplementary Fig. S6) that could potentially serve as disruptors of the WRC and hence suppress invasion. We therefore targeted three regions derived from NCKAP1 that had α-helical interactions with CYFIP1. These PPIs encompassed amino acids 631–642, 933–944, and 1110–1121. SPs, designated **WA**SF3-**N**CKAP1 **T**argets (WANT), were designed against the NCKAP1 surface (Fig. 6A) at these sites. Highly invasive MDA-MB-231 and HS578T breast cancer cells were treated with each of the three WANT peptides independently at a final concentration of 10 μmol/L. When MDA-MB-231 cells were challenged to invade in Transwell chamber assays, no significant effect on invasion

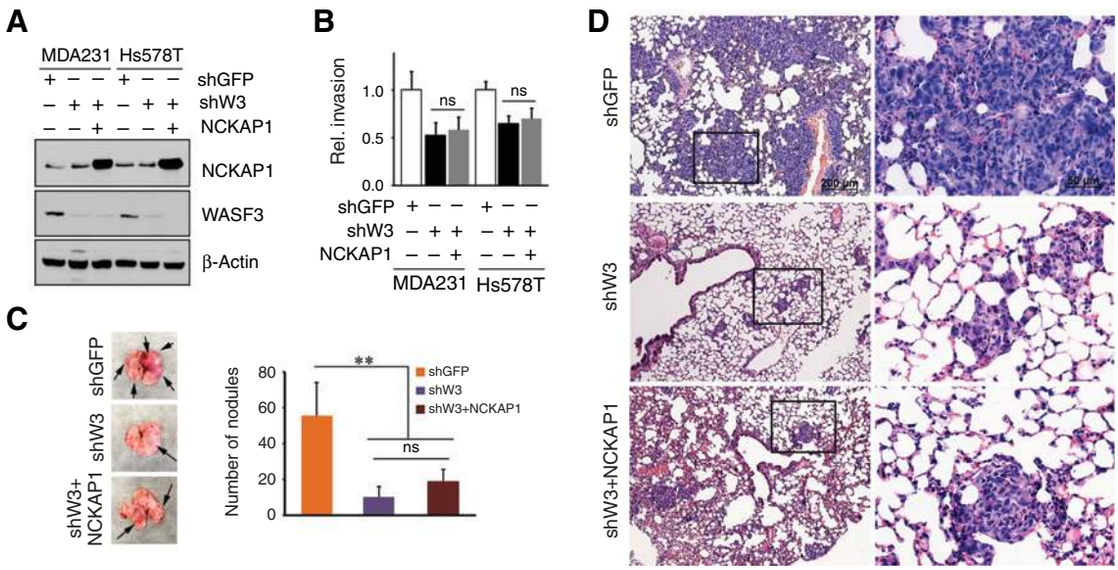

Figure 5.

Invasion and metastasis analysis after NCKAP1 overexpression in WASF3 knockdown cells. When NCKAP1 was overexpressed in WASF3 knockdown MDA-MB-231 and Hs578T cells (**A**), cell invasion was not significantly affected (**B**). Following subcutaneous implantation of MDA-MB-231 cells overexpressing NCKAP1 into NSG mice, the number of nodules on the surface of the lungs after 8 weeks in these animals was not significantly different compared with the WASF3 (shW3) knockdown cells (**C**). Histological analyses showed the same distribution of tumors in the lungs of those mice carrying the NCKAP1-overexpressing cells as seen for the WASF3 knockdown cells (**D**). **, $P < 0.01$; ns, no statistical significance.

potential was observed in the presence of WANT1 and WANT2 (Fig. 6B), albeit with only a single peptide designed against these regions. The lack of activity may be due to the fact that these particular compounds were not able to sufficiently disrupt the targeted protein–protein interface. In contrast, treatment with WANT3 resulted in a highly significant suppression of invasion (Fig. 6B) without affecting cell proliferation (Supplementary Fig. S7A). To investigate the mechanism of WANT3 action, we analyzed WASF3 stability using Western blotting. Compared with treatment with a scrambled peptide control, WANT3 resulted in a significant reduction of WASF3 levels (Fig. 6C) and this coincided with loss of NCKAP1. Flow cytometry analysis demonstrated that, in the two different breast cancer cell lines, uptake of these peptides was rapid, achieving maximal levels after only 10 to 20 minutes (Fig. 6D). Significantly, *in vitro* cell toxicity at 10 μmol/L was minimal (Supplementary Fig. S7), and the ability of WANT3 to suppress WASF3 levels was dose dependent (Supplementary Fig. S8 and Fig. 6E), achieving a maximal effect at 20 μmol/L. Thus, the WANT3 SP serves as a successful tool to suppress invasion.

Because targeting the WASF3–CYFIP1 interface also led to disruption of the WRC and suppression of invasion (30), we investigated the relative ability of targeting the WASF3–CYFIP1 (using the WHAM1 peptide) or CYFIP1–NCKAP1 (using the WANT3 peptide) interaction to disrupt the WRC. Following treatment of MDA-MB-231 cells with WHAM1 or WANT3, it was clear that targeting the CYFIP1–NCKAP1 interface was more efficient in decreasing WASF3 levels compared to targeting the WASF3–CYFIP1 interface (Fig. 6F). WASF3 is activated through its interaction with RAC1 binding to CYFIP1 (44) and so, as expected, because targeting the CYFIP1–NCKAP1 interaction leads to destabilization of the WRC, the engagement of RAC1 in the absence of NCKAP1 is suppressed. The engagement of RAC1 with the WASF3 complex was more significant following treatment with WANT3 than WHAM1 (Fig. 6F). Despite this variance in the level of destabilization of the WRC, Transwell invasion assays demonstrated that both WAHM1 and WANT3 peptides produced a comparable ability to suppress invasion (Fig. 6G). Further, WANT3 could successfully suppress invasion in a concentration-dependent manner (Fig. 6G).

WASF3 also promotes metastasis in prostate and colon cancer (11, 27). To extend these studies to other cancer cell types, we treated highly invasive PC3 prostate and SW620 colon cancer cells with WANT3, where again invasion was suppressed (Supplementary Fig. S9). Thus, targeting the WASF3 complex may have wider efficacy in suppressing metastasis in different cancer subtypes.

The observation that targeting the WRC with SPs leads to suppression of invasion provides preclinical evidence that this target may serve as a means to suppress invasion and potentially metastasis. To evaluate the stability of these SPs, we incubated WANT3 in serum-containing medium for varying lengths of time (1–7 days) at 37°C and then added this medium to MDA-MB-231 cells to evaluate the ability of the preincubated peptide to suppress invasion. As shown in Fig. 6H, WANT3 that had been preincubated for up to 3 days was still able to significantly suppress invasion. Even after 7 days, although not significant, there was a residual effect on suppression of invasion, demonstrating that this peptide has an appreciable half-life in enriched medium. Going forward, WANT3 may serve as a viable candidate for targeted inhibition of the WRC by disrupting the trimeric protein complex and thereby suppress the ability of cancer cells to invade and metastasize.

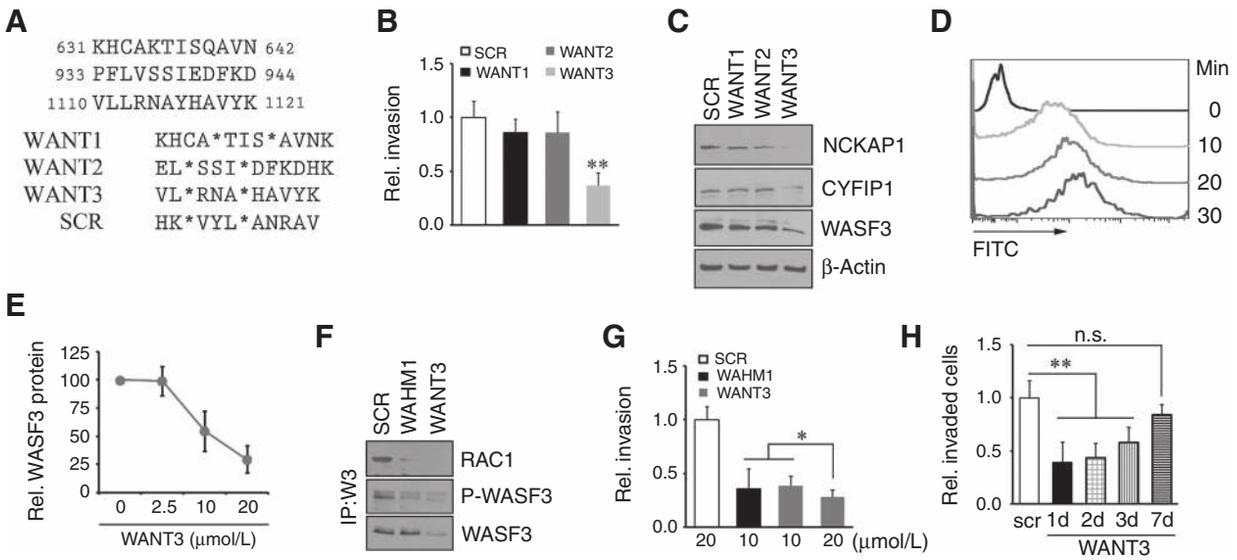

Figure 6.

Targeting the NCKAP1–WASF3 complex using SPs leads to loss of invasion in breast cancer cells. **A,** sequence of amino acid regions 631–642, 933–944, and 1110–1121 in NCKAP1 used to design SPs (top). The three SPs WANT1, 2, and 3 were designed to target interaction surfaces between CYFIP1 and NCKAP1 where (*) represents the position of the non-natural amino acids (bottom). The scrambled peptide was used as a negative control. **B** and **C,** Transwell invasion assays show that only WANT3 significantly suppresses MDA-MB-231 cell invasion (**B**) and suppresses both WASF3 and NCKAP1 protein levels (**C**). **D,** a time course of WANT3–FITC uptake using flow cytometry over the first 30 minutes of exposure shows progressive fluorescein labeling in breast cancer MDA-MB-231 cells. **E,** WANT3 suppresses WASF3 protein levels in a dose-dependent manner. **F,** WANT3 suppresses phosphoactivation of WASF3 more significantly than the WASF3–CYFIP1 peptide mimic WAHM1. **G,** using a high dose of WANT3 (20 μmol/L) leads to a more remarkable reduction in MDA-MD-231 cell invasion compared with low-dose treatment. WANT3 peptides were preincubated in serum-containing medium at 37°C for 1 to 7 days. When this medium was used in invasion assays, significant suppression of invasion in MDA-MB-231 cells was still observed for up to 3 days (**H**).

Discussion

Reorganization of the actin cytoskeleton to facilitate cell invasion and metastasis is a complex regulatory process involving many interacting pathways (44–46). One of the key initiating events is the activation of RAC1, which is known to signal actin cytoskeleton reorganization following stimulation with growth factor receptors (34, 47). This facilitates recruitment of WASF family members to the membrane to promote invasion. Here, we demonstrate that RAC1 cannot be recruited to the WASF3 complex in the absence of NCKAP1. High-level expression of NCKAP1 is associated with poorer survival in breast cancer patients. This may be due to the increased stability of WASF3 because, when NCKAP1 levels are increased in MDA-MB-231 cells, invasion potential also increases, and this is associated with both increased RAC1 levels in the WRC complex and increased WASF3 activation. The cascade leading to increased invasion, however, is dependent on WASF3 expression, because noninvasive cells do not respond to increased NCKAP1 expression.

WASF3 is part of a three-member family that share similar structural motifs that define their function in actin cytoskeletal reorganization (20, 48). Knockdown of WASF3 leads to suppression of invasion and metastasis in breast and prostate cancer cells. WASF1 and WASF2 are redundant in this function since they clearly cannot compensate for the suppression of invasion even with sustained expression. We have also shown recently that knockdown of WASF1 and WASF2 in these same breast cancer cells does not lead to suppression of invasion or metastasis (30). While WASF3 regulates lamellipodia formation (5), which is essential for the development of the invasion and metastasis phenotypes, WASF1 appears to regulate dorsal ruffle formation (49) while WASF2 regulates filopodia production (50). It is possible, therefore, that while controlling similar actin dynamics, the specificity of WASF3 in influencing metastasis depends on the mechanisms of its regulation and possibly the proteins it binds to. WASF3, for example, is under the regulation of the STAT3 transcription factor (9, 23) and is activated following cytokine and growth factor stimulation, but WASF1 and WASF2 do not have consensus STAT binding sites in their promoters and do not respond to IL6 stimulation (9). Knockdown of NCKAP1, however, also leads to destabilization of WASF1 and WASF2 protein complexes, but no specific resultant cell phenotypes were evident.

Part of the mechanism proposed for WASF protein function is through recruitment to membrane locations following growth factor stimulation resulting from actin cytoskeleton reorganization through interactions with NCK1 (51, 52). Consistent with this idea, NCK1 is not present in the WASF3 immunocomplex in the absence of serum and WASF3 remains inactive, although addition of serum growth factors activates WASF3 and a sub-pool of protein interacts with NCK1. In contrast, NCKAP1 is associated with WASF3 in both its inactive and active forms, consistent with the idea that its presence is required for protein stability. It is tempting to speculate that NCK1 is an important protein for the recruitment of WASF3 to tyrosine kinase receptor complexes through an interaction with NCKAP1 upon extracellular stimulation. Thus, NCK1–NCKAP1–RAC1 signaling may be critical for WASF3 activation leading to the significant consequence of cell invasion.

The central role of WASF3 in regulating invasion and metastasis (5–12, 23, 25–27, 48), together with its overexpression in high-grade and metastatic tumors (13, 14), provides an ideal target to suppress metastasis. Here, we show that SPs targeting the large interaction interface between two key proteins that maintain the integrity of WASF3 leads to loss of WASF3 and subsequently suppression of invasion, suggesting this complex may serve as a viable target to suppress metastasis. While only one of the three designed peptides demonstrated efficacy, this may simply be due to their ability or inability to effectively disrupt the protein–protein interface. The interface is composed of multiple helical contact points, and each will have differing energetic contributions to the stabilized interface. It is possible that the active compound, WANT3, may additionally have unanticipated off-target effects that may be contributing to the observed phenotypes. This will be explored to fully elucidate the biological mechanism of WANT3. Importantly, because there are currently no inhibitors that directly target WASF3, and the interface lacks pockets required for small molecule targeting, the development of the WANT3 peptide described here, and the WHAM peptides targeting the WASF3–CYFIP1 interaction (30), provide a potential approach to suppress WASF3 function and suppress metastasis. The emerging field of SPs as therapeutic agents is gaining traction through clinical trials currently under way targeting the MDM2/MDMX–p53 protein interaction (53, 54) for cancer patients with tumors expressing wild type p53. Although there are many conventional, unmodified peptides currently in clinical trials, because of their limited ability to penetrate the cell, most still target extracellular proteins. SPs, on the other hand, are constrained in a highly stable helical conformation and address many of the limitations of standard peptides because of (i) their active transport into cells, (ii) their pharmaceutical stability, (iii) low immunogenicity, and (iv) their binding affinity for the target (28). Indeed, we show here that the NCKAP1 peptide mimic WANT3 retains its ability to suppress invasion after incubation in serum for up to 3 days. While the NCKAP1 mimic appears to be more effective in destabilizing the WASF3 complex, CYFIP1 mimics that targeting the WASF3–CYFIP1 interaction (30), suppress WASF3 phosphoactivation. Nonetheless, because we have shown that phosphoactivation is required for the ability of WASF3 to regulate invasion, both peptide mimics suppress invasion equally effectively. This approach validates the WASF3 complex as a viable target for suppression of metastasis and identifies WANT3 as a potential therapeutic lead compound to selectively inhibit invasion and metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Teng, E.J. Kennedy, J. Cowell
Development of methodology: Y. Teng, E.J. Kennedy, J. Cowell
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Teng, H. Qin, A. Bahassan, E.J. Kennedy
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Teng, A. Bahassan, E.J. Kennedy, J. Cowell
Writing, review, and/or revision of the manuscript: Y. Teng, E.J. Kennedy, J. Cowell
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Teng, H. Qin, N.G. Bendzunas, J. Cowell
Study supervision: Y. Teng, J. Cowell

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References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
2. Steeg PS. Angiogenesis inhibitors: motivators of metastasis? Nat Med 2003;9:822–3.
3. Nguyen DX, Massagué J. Genetic determinants of cancer metastasis. Nat Rev Genet 2007;8:341–52.
4. Hurst DR, Welch DR. Metastasis suppressor genes at the interface between the environment and tumor cell growth. Int Rev Cell Mol Biol 2011;286:107–80.
5. Sossey-Alaoui K, Li X, Ranalli TA, Cowell JK. WAVE3-mediated cell migration and lamellipodia formation are regulated downstream of phosphatidylinositol 3-kinase. J Biol Chem 2005;280:21748–55.
6. Sossey-Alaoui K, Li X, Cowell JK. c-Abl-mediated phosphorylation of WAVE3 is required for lamellipodia formation and cell migration. J Biol Chem 2007;282:26257–65.
7. Teng Y, Liu M, Cowell JK. Functional interrelationship between the WASF3 and KISS1 metastasis-associated genes in breast cancer cells. Int J Cancer 2011;129:2825–35.
8. Teng Y, Ngoka L, Mei Y, Lesoon L, Cowell JK. HSP90 and HSP70 are essential for stabilization and activation of the WASF3 metastasis promoting protein. J Biol Chem 2012;287:10051–9.
9. Teng Y, Ghoshal P, Ngoka L, Mei Y, Cowell JK. Critical role of the WASF3 gene in JAK2/STAT3 regulation of cancer cell invasion. Carcinogenesis 2013;34:1994–9.
10. Sossey-Alaoui K, Safina A, Li X, Vaughan MM, Hicks DG, Bakin AV, et al. Down-regulation of WAVE3, a metastasis promoter gene, inhibits invasion and metastasis of breast cancer cells. Am J Pathol 2007;170:2112–21.
11. Teng Y, Ren MQ, Cheney R, Sharma S, Cowell JK. Inactivation of the WASF3 gene in prostate cancer cells leads to suppression of tumorigenicity and metastases. Br J Cancer 2010;103:1066–75.
12. Teng Y, Xie X, Walker S, White DT, Mumm JS, Cowell JK. Evaluating human cancer cell metastasis in zebrafish. BMC Cancer 2013;13:453.
13. Prat A, Parker JS, Karginova O, Fan C, Livasy C, Herschkowitz JI, et al. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. Breast Cancer Res 2010;12:R68.
14. Kulkarni S, Augoff K, Rivera L, McCue B, Khoury T, Groman A, et al. Increased expression levels of WAVE3 are associated with the progression and metastasis of triple negative breast cancer. PLoS One 2012;7:e42895.
15. Wu J, Wang GC, Chen XJ, Xue ZR. Expression of WASF3 in patients with non-small cell lung cancer: correlation with clinicopathological features and prognosis. Oncol Lett 2014;8:1169–74.
16. Yue Z, Feng W, Xiangke L, Liuxing W, Qingxia F, Jianbo G. WAVE3 promotes epithelial–mesenchymal transition of gastric cancer through upregulation of Snail. Cancer Gene Ther 2014;21:499–506.
17. Ji Y, Li B, Zhu Z, Guo X, He W, Fan Z, et al. Overexpression of WAVE3 promotes tumor invasiveness and confers an unfavorable prognosis in human hepatocellular carcinoma. Biomed Pharmacother 2015;69:409–15.

18. Fernando HS, Sanders AJ, Kynaston HG, Jiang WG. WAVE3 is associated with invasiveness in prostate cancer cells. Urol Oncol 2010;28:320–7.
19. Shen L, Wang P, Yang J, Li X. MicroRNA-217 regulates WASF3 expression and suppresses tumor growth and metastasis in osteosarcoma. PLoS One 2014;9:e109138.
20. Sossey-Alaoui K, Head K, Nowak N, Cowell JK. Characterization of the genomic organization and expression profile of the human and mouse WAVE gene family. Mamm Genome 2003;14:314–22.
21. Takenawa T, Miki H. WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. J Cell Sci 2001;114:1801–9.
22. Eden S, Rohatgi R, Podtelejnikov AV, Mann M, Kirschner MW. Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. Nature 2002;418:790–3.
23. Teng Y, Ross JL, Cowell JK. The involvement of JAK-STAT3 in cell motility, invasion, and metastasis. JAKSTAT 2014;3:e28086.
24. Ogden A, Rida PC, Aneja R. Heading off with the herd: how cancer cells might maneuver supernumerary centrosomes for directional migration. Cancer Metastasis Rev 2013;32:269–87.
25. Teng Y, Mei Y, Hawthorn L, Cowell JK. WASF3 regulates miR-200 inactivation by ZEB1 through suppression of KISS1 leading to increased invasiveness in breast cancer cells. Oncogene 2014;33:203–11.
26. Ghoshal P, Teng Y, Lesoon LA, Cowell JK. HIF1A induces expression of the WASF3 metastasis-associated gene under hypoxic conditions. Int J Cancer 2012;131:E905–15.
27. Teng Y, Ren X, Li H, Shull A, Kim J, Cowell JK. Mitochondrial ATAD3A combines with GRP78 to regulate the WASF3 metastasis-promoting protein. Oncogene 2016;35:333–43.
28. Verdine GL, Hilinski GJ. Stapled peptides for intracellular drug targets. Methods Enzymol 2012;503:3–33.
29. Walensky LD, Bird GH. Hydrocarbon-stapled peptides: principles, practice, and progress. J Med Chem 2014;57:6275–88.
30. Teng Y, Bahassan A, Dong D, Hanold LE, Ren X, Kennedy EJ, et al. Targeting the WASF3–CYFIP1 complex using stapled peptides suppresses cancer cell invasion. Cancer Res 2016;76:965–73.
31. Dong C, Nichols CD, Guo J, Huang W, Lambert NA, Wu G. A triple arg motif mediates α(2B)-adrenergic receptor interaction with Sec 24C/D and export. Traffic 2012;13:857–68.
32. Zhang X, Wang G, Dupré DJ, Feng Y, Robitaille M, Lazartigues E, et al. Rab1 GTPase and dimerization in the cell surface expression of angiotensin II type 2 receptor. J Pharmacol Exp Ther 2009;330:109–17.
33. Li W, Hu P, Skolnik EY, Ullrich A, Schlessinger J. The SH2 and SH3 domain-containing Nck protein is oncogenic and a common target for phosphorylation by different surface receptors. Mol Cell Biol 1992; 12:5824–33.
34. Rivera GM, Antoku S, Gelkop S, Shin NY, Hanks SK, Pawson T, et al. Requirement of Nck adaptors for actin dynamics and cell migration stimulated by platelet-derived growth factor B. Proc Natl Acad Sci U S A 2006;103:9536–41.
35. Lebensohn AM, Kirschner MW. Activation of the WAVE complex by coincident signals controls actin assembly. Mol Cell 2009;36: 512–24.
36. Zhang G, Chen X, Qiu F, Zhu F, Lei W, Nie J. A novel interaction between the SH2 domain of signaling adaptor protein Nck-1 and the upstream regulator of the Rho family GTPase Rac1 engulfment and cell motility 1 (ELMO1) promotes Rac1 activation and cell motility. J Biol Chem 2014: 289:23112–22.
37. Nakao S, Platek A, Hirano S, Takeichi M. Contact-dependent promotion of cell migration by the OL-protocadherin–Nap1 interaction. J Cell Biol 2008;182:395–410.
38. Le J, Mallery EL, Zhang C, Brankle S, Szymanski DB. Arabidopsis BRICK1/HSPC300 is an essential WAVE-complex subunit that selectively stabilizes the Arp2/3 activator SCAR2. Curr Biol 2006;16:895–901.
39. Steffen A, Rottner K, Ehinger J, Innocenti M, Scita G, Wehland J, et al. Sra-1 and Nap1 link Rac1 to actin assembly driving lamellipodia formation. EMBO J 2004;23:749–59.
40. Győrffy B, Lanczky A, Eklund AC, Denkert C, Budczies J, Li Q, et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res Treat 2010;123:725–31.
41. Gao Y, Dickerson JB, Guo F, Zheng J, Zheng Y. Rational design and characterization of a Rac1 GTPase-specific small molecule inhibitor. Proc Natl Acad Sci U S A 2004;101:7618–23.
42. Chen B, Brinkmann K, Chen Z, Pak CW, Liao Y, Shi S, et al. The WAVE regulatory complex links diverse receptors to the actin cytoskeleton. Cell 2014;156:195–207.
43. Chen Z, Borek D, Padrick SB, Gomez TS, Metlagel Z, Ismail AM, et al. Structure and control of the actin regulatory WAVE complex. Nature 2010;468:533–8.
44. Insall RH, Machesky LM. Actin dynamics at the leading edge: from simple machinery to complex networks. Dev Cell 2009;17:310–22.
45. Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev 2009;28:15–33.
46. Krause M, Gautreau A. Steering cell migration: lamellipodium dynamics and the regulation of directional persistence. Nat Rev Mol Cell Biol 2014;15:577–90.
47. Akin O, Zipursky SL. The shape of things to come. Cell 2014;156:13–4.
48. Teng Y, Pi W, Wang Y, Cowell JK. WASF3 provides the conduit to facilitate invasion and metastasis in breast cancer cells through HER2/HER3 signaling. Oncogene 2016;doi: 10.1038/onc.2015.527.
49. Goh WI, Lim KB, Sudhaharan T, Sem KP, Bu W, Chou AM, et al. mDia1 and WAVE2 proteins interact directly with IRSp53 in filopodia and are involved in filopodium formation. J Biol Chem 2012;287:4702–14.
50. Suetsugu S, Yamazaki D, Kurisu S, Takenawa T. Differential roles of WAVE1 and WAVE2 in dorsal and peripheral ruffle formation for fibroblast cell migration. Dev Cell 2003;5:595–609.
51. Dart AE, Donnelly SK, Holden DW, Way M, Caron E. Nck and Cdc42 cooperate to recruit N-WASP to promote FcγR-mediated phagocytosis. J Cell Sci 2012;125:2825–30.
52. Pils S, Kopp K, Peterson L, Delgado Tascón J, Nyffenegger-Jann NJ, Hauck CR. The adaptor molecule Nck localizes the WAVE complex to promote actin polymerization during CEACAM3-mediated phagocytosis of bacteria. PLoS One 2012;7:e32808.
53. Chang YS, Graves B, Guerlavais V, Tovar C, Packman K, To KH, et al. Stapled α-helical peptide drug development: a potent dual inhibitor of MDM2 and MDMX for p53-dependent cancer therapy. Proc Natl Acad Sci U S A 2013;110:E3445–54.
54. Chu Q, Moellering RE, Hilinski GJ, Kim YW, Grossmann TN, Yeh JT, et al. Towards understanding cell penetration by stapled peptides. Med Chem Commun 2015;6:111–9.