

The Helicase HAGE Expressed by Malignant Melanoma-Initiating Cells Is Required for Tumor Cell Proliferation *in Vivo*^{*[5]}

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Background: ABCB5+ MMIC are a population of chemoresistant cancer stem cell-like cells responsible for melanoma initiation, growth, and progression.

Results: HAGE promotes ABCB5+ MMIC-dependent tumorigenesis by enhancing RAS protein expression.

Conclusion: ABCB5+ MMIC require the presence of HAGE for their tumorigenic activity.

Significance: HAGE is expressed only by tumor cells. Hence, targeting HAGE helicase may have broad therapeutic applications.

Malignant melanoma-initiating cells (MMIC) are a subpopulation of cells responsible for melanoma tumor growth and progression. They are defined by the expression of the ATP-binding cassette (ABC) subfamily B member 5 (ABCB5). Here, we identified a critical role for the DEAD-box helicase antigen (HAGE) in ABCB5+ MMIC-dependent tumorigenesis and show that HAGE-specific inactivation inhibits melanoma tumor growth mediated by this tumor-initiating population. Knockdown of HAGE led to a significant decrease in RAS protein expression with a concomitant decrease in activation of the AKT and ERK signaling pathways implicated to play an important role in melanoma progression. To confirm that the reduction in NRAS (Neuroblastoma RAS) expression was dependent on the HAGE helicase activity, we showed that NRAS, effectively silenced by siRNA, could be rescued by reintroduction of HAGE in cells lacking HAGE. Furthermore, we provide a mechanism by which HAGE promotes NRAS unwinding *in vitro*. We also observed using tumor transplantation in Non-obese diabetic/severe combined immunodeficiency mice that the HAGE knockdown in a ABCB5+ melanoma cell line displayed a significant decrease in tumor growth and compared with the control. Our results suggest that the helicase HAGE is required for ABCB5+ MMIC-dependent tumor growth through promoting RAS protein expression and that cancer therapies targeting HAGE helicase may have broad applications for treating malignant melanoma and potentially other cancer types.

The helicase antigen (HAGE), a non-X-linked cancer testis (CT) antigen of 648 amino acids (73 kDa) was originally identified in a rhabdomyosarcoma cell line (1). HAGE, also known as DDX43 or CT13, belongs to the DEAD-box family of ATP-dependent RNA helicases characterized by the presence of nine

conserved motifs grouped into two domains connected by a polylinker SAT region (2). The Q motif, motifs I, II (also called the D-E-A-D-box as a single-letter code for Asp-Glu-Ala-Asp), and III, together with motif IV, bind and hydrolyze ATP molecules. The remaining motifs (V, VI, Ia, and Ib) are thought to interact with the RNA substrate. It has been suggested that RNA helicases browse RNA molecules in a bidirectional fashion using the energy gained from ATP hydrolysis until they encounter ribonucleoprotein-RNA complexes (3). The helicase activity then allows the dissociation of RNA from the ribonucleoprotein to which they have high affinity (4). RNA helicases are often described as the driving forces behind RNA metabolism in processes such as transcription, pre-mRNA splicing, ribosome biogenesis, cytoplasmic transport, translation initiation/elongation, and RNA decay (4–8).

Previous studies have suggested that several RNA helicases, such as DDX1 (9–10), DDX2 (11), DDX6 (12), and DDX53 (13) play an important role in tumor cell development and proliferation in a variety of cancers. In this study, we looked at the role of HAGE in melanoma cancer initiation and growth associated with malignant melanoma-initiating cells (MMIC)³, a subpopulation of chemoresistant cells capable of self-renewal and differentiation and responsible for melanoma tumor initiation, growth, and progression (14). This population of cells express the ATP-binding cassette (ABC) subfamily B member 5 (ABCB5), a protein that renders malignant melanoma drug-resistant and refractory to therapy. As a consequence, this population is thought to be responsible for a highly aggressive type of cancer with a poor prognosis and outcome (15–16). Although the mechanisms by which ABCB5+ MMIC resist systemic therapy have been well studied, their role in promoting tumor growth and progression is still under investigation. Here, we reveal a previously unknown role of the helicase HAGE in ABCB5+ MMIC-dependent tumor growth and pro-

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[5] This article contains supplemental Figs. 1–3 and supplemental data.

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³ The abbreviations used are: MMIC, malignant melanoma-initiating cell; ABC, ATP-binding cassette; ABCB5, ATP-binding cassette subfamily B member 5; IB, immunoblotting; IF, immunofluorescence; HAGE, helicase antigen; NRAS, neuroblastoma RAS; pAKT, phosphorylated AKT; PCNA, proliferating cell nuclear antigen; FOXO, Forkhead box O.

HAGE Promotes ABCB5+ MMIC-dependent Tumor Growth

gression. Using a stem cell proliferation assay, we show that HAGE is required for tumor growth initiated by this population of cells. HAGE knockdown in a melanoma cell line expressing ABCB5 caused a decrease of RAS protein expression, subsequently leading to a significant decrease in the activation of its downstream effectors, AKT and ERK. Silencing of NRAS in the HAGE knockdown melanoma cell line using NRAS-specific siRNA was rescued successfully after reintroduction of HAGE in these cells. We took this further and provide a mechanistic insight by demonstrating the capacity of the helicase HAGE to unwind NRAS RNA complexes using an *in vitro* unwinding assay. Finally, tumor transplantation assays clearly demonstrated the *in vivo* role of HAGE in promoting ABCB5+ MMIC-dependent tumor progression. Collectively, these findings clearly implicate the helicase HAGE in the promotion of tumor growth in melanoma through RAS/AKT and RAS/ERK pathways and shed new light on our understanding of the mechanisms underlying ABCB5+ MMIC-dependent tumorigenesis.

EXPERIMENTAL PROCEDURES

Cell Lines and Growth Conditions—The human melanoma cell lines FM82 and FM55 were a kind gift from Professor D. Schadendorff of the Deutsches Krebsforschungszentrum (Heidelberg, Germany). All cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% FCS and 1% (w/v) L-glutamine (Lonza) and incubated at 37 °C supplied with a 5% CO₂ humid environment.

Stable and Transient Transfection of Cell Lines—A stable knockdown cell line was created using shRNA-bearing plasmids specific for HAGE (SureSilencing shRNA Plasmid for Human DDX43, SABiosciences). FM82 and FM55 cells were seeded at 5×10^4 cells/well into a 24-well plate and grown overnight. 1.2 μ g of SureSilencing shRNA was transfected into cells using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. 48 h later, the cells were seeded into a 96-well plate and grown in media treated with 500 μ g/ml G418 antibiotic to allow selection of plasmid-transfected cells. HAGE expression status was monitored using real-time quantitative PCR, Western blotting, and immunofluorescence. The transient knockdown of NRAS was carried out using an NRAS-specific siRNA molecule (Eurogentec) (sense, 5'-CAGCAGUGAUGAUGGGACUTT-3' and antisense, 5'-AGUCCCAUCAUCACUGCUGTT-3') and Interferin transfection reagent (Polyplus) following the recommendations of the manufacturer. To investigate if the introduction of HAGE into cells results in increased NRAS protein expression, FM82 stable transfectants were firstly cultured and then transfected with NRAS siRNA as described above. 24 h following transfection, a second transfection was carried out using a HAGE-specific cDNA vector, pcDNA3.1-HAGE, to ectopically express HAGE. Transfection was performed using the Lipofectamine 2000 protocol and 12 μ g of the vector to transfect a T25 flask as described by the manufacturer. At 48 hours post-transfection, cells were harvested for protein extraction.

Genetic Analysis—FM82 and FM55 control and FM82 and FM55 shRNAs cells were grown to 80% confluence as described above. Total RNA was extracted from cells using RNA-STAT

60 reagent (AMS Biotechnology) as described by the manufacturer. Extracted RNAs were quantified using a Nanodrop spectrophotometer (Thermo Scientific). Muloney murine leukemia virus reverse transcriptase (Promega), oligo(dT) primers (Promega), and 2 μ g of total RNA were subsequently used for cDNA synthesis following the protocol of the manufacturer. Real-time quantitative PCR was then carried out using primers (all MWG Eurofins) specific for HAGE (forward, 5'-GGAGATCGGCCATTGATAGA-3' and reverse, 5'-GGATTGGGGATAGGTCGTTT-3'), NRAS (forward, 5'-AAACCTCAGCCAAGACCAAGA-3' and reverse, 5'-CCCTGAGTCCCATCATCACT-3') and the housekeeping genes TBP1 (forward, 5'-TGCACAGGAGCCAAGAGTGAA-3' and reverse, 5'-CACATCACAGCTCCCCACCA-3') and HPRT1 (forward, 5'-TGACACTGGCAAACAATGCA-3' and reverse, 5'-GGTCCTTTTCAACCAGCAAGCT-3') using a Rotor-Gene 6000 real-time PCR cyclers (Qiagen). Forty cycles were performed, and melt curves were ratified following each analysis. Expression of the genes of interest was normalized using averaged results for the housekeeping genes, and $\Delta\Delta$ CT calculations were performed.

Antibodies—For this study, we used a monoclonal HAGE antibody (1:250 for immunoblotting (IB), 1:50 immunofluorescence (IF), SAB1400618, Sigma), ABCB5 (1:500 for IB, 1:100 for IF, HPA026975, Sigma), β -actin (1:1000, 4967, Cell Signaling Technologies), AKT (1:250 for IB, 9272, Cell Signaling Technologies), p-AKT (Ser-473) (1:250 for IB, 4058, Cell Signaling Technologies), GSK3 β (1:250 for IB, 9315, Cell Signaling Technologies), p-GSK3 β (Ser-9) (1:250 for IB, 9336, Cell Signaling Technologies), NRAS (1:100 for IB, 1:50 for IF, SC-31, Santa Cruz Biotechnology), Kirsten RAS (1:100 for IB, SC-30, Santa Cruz Biotechnology), Harvey RAS (1:100 for IB, SC-29, Santa Cruz Biotechnology), p21^{CIP1} (1:250 for IB, ab80633, Abcam), c-SRC (cellular-SRC) (1:250 for IB, SC-5266, Santa Cruz Biotechnology), GRB2 (1:250 for IB, 3972, Cell Signaling Technologies), SOS1 (1:250 for IB, SC-55528, Santa Cruz Biotechnology), ERK1/2 (1:250 for IB, 4695, Cell Signaling Technologies), p-ERK1/2 (Thr-202/Tyr-204) (1:250 for IB, 9101, Cell Signaling Technologies), p-FOXO1 (Thr-24)/FOXO3a (Thr-32)/FOXO4 (Thr-28) (1:250 for IB, 2599, Cell Signaling Technology), and PCNA (1:100 for IF, ab29, Abcam). HRP-conjugated antibodies were used as secondary antibodies for IB (1:500, Dako). Alexa Fluor 488- and Alexa Fluor 568-conjugated antibodies were used as secondary antibodies for IF (1:500, Invitrogen).

Immunohistochemistry and IF—Paraffin-embedded human melanoma tissue microarrays were purchased from US Biomax. The sections were dewaxed, rehydrated in graded alcohols, rinsed in double distilled H₂O, and then antigen retrieval was performed for 10 min (0.01 M citrate phosphate buffer (pH 6.0)) in a microwave at 1000 Watt. The tumors excised from non-obese diabetic/severe combined immunodeficiency mice were embedded in OCT (optimal cutting temperature) media, fixed in 2-butanol (Sigma) that had been super-cooled in liquid nitrogen, and sectioned using a cryostat CM1900 (Leica). Melanoma cells were fixed in 4% (w/v) paraformaldehyde and then treated as follows. The sections or cancer cells were washed three times in 1 \times PBS for 10 min each, blocked and permeabilized in 10% (w/v)

BSA in 0.1% (v/v) PBS-Tween, incubated overnight with primary antibody (in blocking solution), washed three times for 10 min each with 1× PBS, incubated for 1 h with secondary antibody (in blocking solution), and washed three times with 1× PBS. Sections and melanoma cells were counterstained and mounted with DAPI fluorescent medium (Vector Laboratories) for IF microscopy.

Immunoblotting—For IB, cancer cells were collected, washed with 1× PBS, lysed in 1× solution containing 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, and 10% (v/v) glycerol and loaded on Tris/glycine SDS-polyacrylamide gels. Proteins were separated alongside a molecular weight marker (Bio-Rad). Protein bands were transferred onto Amersham Biosciences Hybond-P PVDF membranes (GE Healthcare). Membranes were blocked with 10% Marvel milk/TBS solution with 0.05% Tween 20 containing sodium orthovanadate and sodium fluoride. Following TBS solution with 0.05% Tween 20 washes, membranes were incubated with primary antibodies (in blocking solution) at 4 °C overnight followed by washing and incubation with secondary antibodies for 1 h at room temperature ahead of visualization with Rapid Step ECL reagent (Calbiochem) and viewed using a charge coupled device camera (Fujifilm). For immuno-dot-blot analysis of signaling phosphoproteins, Proteome Profiler arrays (R&D Systems) specific for the PI3K and MAPK pathways were acquired and used in accordance with the guidelines of the manufacturer using protein lysates extracted from FM82 control and FM82 shRNA1 cells. Blots were visualized in the same manner as described above, and densitometry readings of each dot were carried out using densitometry software (Aida v. 5.2).

³H Incorporation Proliferation Assays—All cells were grown in 24-well plates (Sarstedt) at 5×10^4 cells/well. All necessary treatments were performed in duplicate. Assessment of the effect of HAGE knockdown on cell proliferation was performed on day 1 and day 3 following plating ($n =$ three independent experiments). The day prior to testing, cells were treated with complete RPMI 1640 medium containing ³H and allowed to absorb the isotope during overnight incubation. On the day of testing, medium was removed, and cells were lysed with ddH₂O, leading to the release of incorporated ³H. The contents of the plate were subsequently harvested onto a Unifilter 96-well plate using a Filtermate Harvester (Packard), which was allowed to dry using a drying cabinet (SLS). Each test well was treated with 40 μl of Microscint-O media (PerkinElmer). Plate reading was carried out using a Top-Count NXT microplate scintillation counter (Packard).

Tumor Transplantation Assay—Non-obese diabetic/severe combined immunodeficiency mice were acquired (Harlan Laboratories, UK) and kept in accordance with UK Home Office regulations. Mice were kept in individual ventilated cages supplied with laminar air flow and autoclaved food and water taken *ad libitum*. Mice were separated into two groups and injected subcutaneously into the right flank with 1×10^6 FM82 control or FM82 shRNA cells per 100 μl of serum-free medium. Tumor development was observed twice weekly using caliper measurements. Once one of the tumors reached a diameter of 1 cm, the experiment was stopped, and mice were euthanized prior to

tumors being extracted, snap-frozen in liquid nitrogen-cooled 2-butanol (Sigma), and stored at -80 °C.

Melanoma Spheroid Cell Culture and Differentiation—For the culture of melanospheres, cells from FM82 and FM55 controls and FM82 and FM55 shRNAs were plated at clonal density (10×10^3 cells/ml in 24-well plates) in a DMEM/F12 medium (Invitrogen, 32500) supplemented with N-2 Max Media (R&D Systems, AR009), and daily addition of fibroblast growth factor basic 100 ng/ml and EGF 100 ng/ml (both from Sigma) for a 10-day culture period (17). The differentiation of melanospheres was induced upon FGF basic and EGF withdrawal. The cells were stained by immunofluorescence as described above.

In Vitro Transcription and RNA Labeling—The plasmid pDNR-LIB containing NRAS was purchased from Open Biosystems and used for *in vitro* transcription using the T7 promoter to generate the NRAS transcript (Ambion). NRAS transcripts were labeled using the Pierce RNA 3' end biotinylation kit (Thermo Scientific). The labeling efficiency was determined by dot blotting following the recommendations of the manufacturer. The biotinylated transcripts (100 pmol) were transfected in 6-well plates containing FM82 control and FM82 shRNA cells using Lipofectamine 2000 (Invitrogen). The transcripts were detected by immunofluorescence using Streptavidin Alexa Fluor 488 conjugate (Invitrogen).

Native RNA Gels and Winding/Unwinding Assay—Biotinylated RNA (2 pmol, labeling efficiency higher than 75%, 5'-GACUCAGGGUUGUAUGGGGAUUGCCAUGUGUGGU-GAUGUAA-3') was placed to wind with unlabeled NRAS RNA in a solution containing 3.5 mM equimolar of ATP/MgCl₂ for 5 min at 95 °C followed by 1 h at 37 °C. HAGE recombinant protein was added at two different concentrations (0.6 μg and 1.2 μg) after the RNA winding assay, followed by 1 h of incubation at 37 °C. The reactions were stopped by the addition of 50% (v/v) glycerol, 20 mM EDTA, 2% (w/v) SDS, and 0.025% (w/v) bromophenol blue. Samples were analyzed on 12% (w/v) native acrylamide gels in 1× Tris/Borate/EDTA buffer followed by blot transfer and visualization using the chemiluminescent detection module (Thermo Scientific).

RESULTS

HAGE Promotes the Proliferation of Malignant Melanoma Cells—A number of studies have demonstrated that ATP-dependent RNA helicases belonging to the family of DEAD-box proteins could be involved in the process of tumorigenesis (9–13, 18, 19). Because the role of HAGE in tumor growth has yet to be described, we wanted to determine its role in melanoma growth. We examined HAGE expression in tumor tissue microarrays containing malignant melanoma patient samples by performing immunostaining using a HAGE-specific polyclonal antibody. As a control, we tested normal skin. HAGE expression was observed in 6 of 10 melanoma samples (Fig. 1 and supplemental Fig. 1). At the cellular level, HAGE was demonstrated to be cytoplasmic, with few cells exhibiting a nucleocytoplasmic localization. HAGE expression was confined to cytoplasmic aggregates in the FM82 and FM55 melanoma cell lines (FM82 control and FM55 control) as detected by immunofluorescence, which was markedly decreased when HAGE was stably knocked down using shRNAs (Fig. 2, A and B, and

HAGE Promotes ABCB5+ MMIC-dependent Tumor Growth

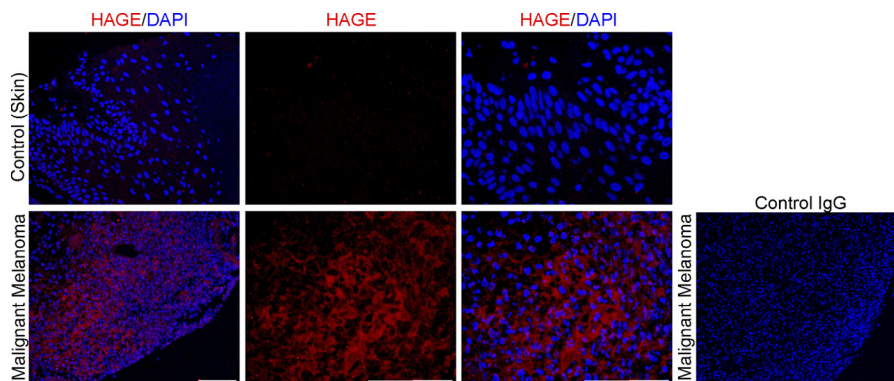


FIGURE 1. **Expression of HAGE protein in malignant melanoma.** Representative image of an immunohistochemistry with antibody to HAGE (red) on malignant melanoma and normal skin sections. The negative control was an IgG isotype from mice. Scale bars = 100 μ m.

supplemental Fig. 2, A and C). To determine the effect of HAGE expression on cell proliferation, we performed a thymidine incorporation assay to measure the proliferation rate of FM82 and FM55 shRNAs cells compared with FM82 and FM55 control cells. A significant reduction in proliferation was observed in the FM82 and FM55 shRNA cell lines after days 1 and 3 (Fig. 2C and supplemental Fig. 2B).

The effect of HAGE expression on tumor growth was investigated using a tumor transplantation assay. FM82 control and FM82 shRNA1 were injected subcutaneously into two groups of immunodeficient mice (non-obese diabetic/severe combined immunodeficiency). Mice injected with FM82 shRNA1 cells developed tumors at a significantly slower rate compared with the FM82 control group (Fig. 2D). The tumors that developed in FM82 control- and FM82 shRNA1-injected mice were excised and histologically prepared for analysis by immunofluorescence using antibodies against HAGE and the proliferation marker PCNA. HAGE and PCNA protein expression was significantly reduced in FM82 shRNA tumors compared with FM82 control tumors (Fig. 2E). Taken together, these results demonstrate that HAGE promotes proliferation and tumor growth of malignant melanoma cells.

HAGE Promotes the Phosphorylation of AKT and ERK in Melanoma Cells—To determine whether HAGE is involved in tumor growth via regulation of downstream kinases involved in promoting cell proliferation, FM82 control and FM82 shRNA1 cell lysates were diluted and incubated with nitrocellulose membranes spotted with phospho-kinase antibodies. Densitometry analysis of the different spots revealed a significant decrease of intensity of phospho-AKT and phospho-ERK spots when incubated with FM82 shRNA1 cell lysates compared with controls (Fig. 3, A and B). Immunoblotting using phospho-AKT (Ser-473) and phospho-ERK1/2 (Thr-202/Tyr-204) antibodies confirmed that both p-AKT and p-ERK levels were significantly decreased in FM82 and FM55 shRNAs cells lacking HAGE (Fig. 3, C and E, and supplemental Fig. 2C). This result suggests that HAGE promotes predominantly AKT and ERK phosphorylation. The total level of AKT protein appeared to increase following HAGE silencing, whereas ERK1/2 total protein levels were slightly decreased in FM82 shRNAs, suggesting that HAGE may play a role in ERK1/2 protein expression (Fig. 3, C and E).

Phosphorylated AKT and ERK promote proliferation and survival by inducing the phosphorylation of FOXO transcription factors and GSK3 β , thereby inactivating their function in regulating apoptosis and proliferation (20–23) (Fig. 3B). Immunoblotting experiments using antibodies against the phosphorylated forms of FOXO transcription factors (p-FOXO1 (Thr-24)/p-FOXO3a (Thr-32)/p-FOXO4 (Thr-28)) and phospho-GSK3 β (Ser-9) revealed a decrease in phosphorylation of these factors upon HAGE stable knockdown (Fig. 3C). Furthermore, the decreased phosphorylation of FOXO factors correlated with an increase of p21^{CIP1} protein expression (Fig. 3C), a known transcriptional target of FOXO transcription factors. Together these results suggest that HAGE expression promotes the phosphorylation of AKT and ERK proteins, with concomitant effects on their downstream targets.

HAGE Regulates AKT and ERK Phosphorylation through NRAS—AKT and ERK are well known downstream targets of the proto-oncogene RAS (22). RAS activation results in an induction of AKT phosphorylation through the PI3K pathway and ERK phosphorylation through the RAF/MEK pathway (24, 25) (Fig. 3B). To determine whether HAGE-mediated regulation of AKT and ERK phosphorylation was occurring via NRAS, we examined the levels of NRAS expression in the FM82 and FM55 controls and FM82 and FM55 shRNAs cell lines by immunoblotting using NRAS-specific antibody. HAGE silencing resulted in a significant decrease in NRAS protein expression (Fig. 3, D and E, and supplemental Fig. 2C). A decrease of NRAS expression was also observed in the FM82 shRNA1 tumors compared with the controls (Fig. 3F). Furthermore, a decrease of KRAS and HRAS protein expression was also observed in FM82 shRNA compared with the FM82 control (data not shown). These results suggest that HAGE promotes the phosphorylation of AKT and ERK through the regulation of NRAS protein expression.

Receptor tyrosine kinases are upstream activators of the RAS/PI3K/AKT and RAS/RAF/MEK/ERK pathways through signal transduction involving the protein tyrosine kinase SRC, the adapter protein GRB2, and the guanine nucleotide exchange factor SOS1 (26) (Fig. 3B). To exclude the possibility that the decrease in RAS proteins following HAGE silencing might be occurring via a decrease in SRC, GRB2, and/or SOS1,

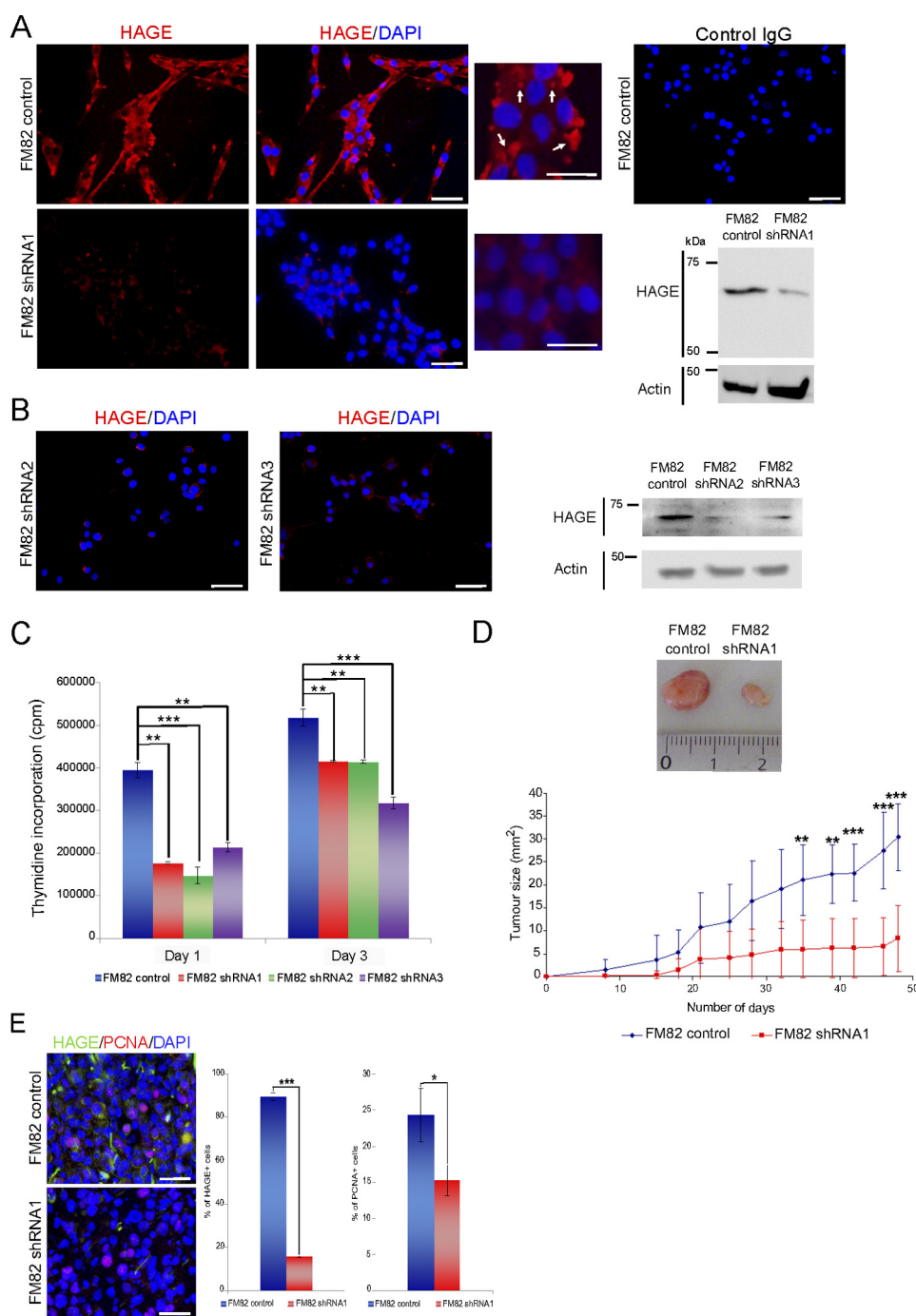


FIGURE 2. HAGE silencing results in a decrease of proliferation *in vitro* and *in vivo*. *A*, immunofluorescence staining showing HAGE expression in the FM82 control and FM82 shRNA1 melanoma cell lines. HAGE aggregates are shown in the cytoplasm by arrows. Scale bars = 20 μ m. Also shown is immunoblotting using an antibody to HAGE in FM82 control and FM82 shRNA1 extracts. β -actin was measured as a loading control. *B*, immunofluorescence staining showing HAGE expression in FM82 shRNA2 and FM82 shRNA3. Scale bars = 20 μ m. Also shown is immunoblotting using an antibody to HAGE in FM82 shRNA2 and FM82 shRNA3 extracts. β -actin was measured as a loading control. *C*, standard 3 H incorporation assays of stable knockdown and control FM82 cells carried out one day and three days post-plating. Mann-Whitney *U* test: **, $p < 0.01$; ***, $p < 0.001$. *D*, measurement of tumor growth in mice injected with either FM82 control cells ($n = 12$) or FM82 shRNA1 cells ($n = 12$). Student's *t* test: **, $p < 0.01$; ***, $p < 0.001$. *E*, immunostaining of FM82 control and FM82 shRNA1 tumors using antibody to HAGE (green) and the proliferative marker PCNA (red). Scale bars = 20 μ m. The graph illustrates the percentage of HAGE- and PCNA-positive cells from 10 random counting microscopic fields. Student's *t* test: *, $p < 0.05$; ***, $p < 0.001$.

immunoblotting using antibodies against these proteins was performed. No significant decrease of SRC, GRB2, and SOS1 was observed in HAGE knockdown (FM82 shRNA1) compared with the control (FM82 control) (Fig. 3D). These findings suggest that the decrease in RAS protein expression following

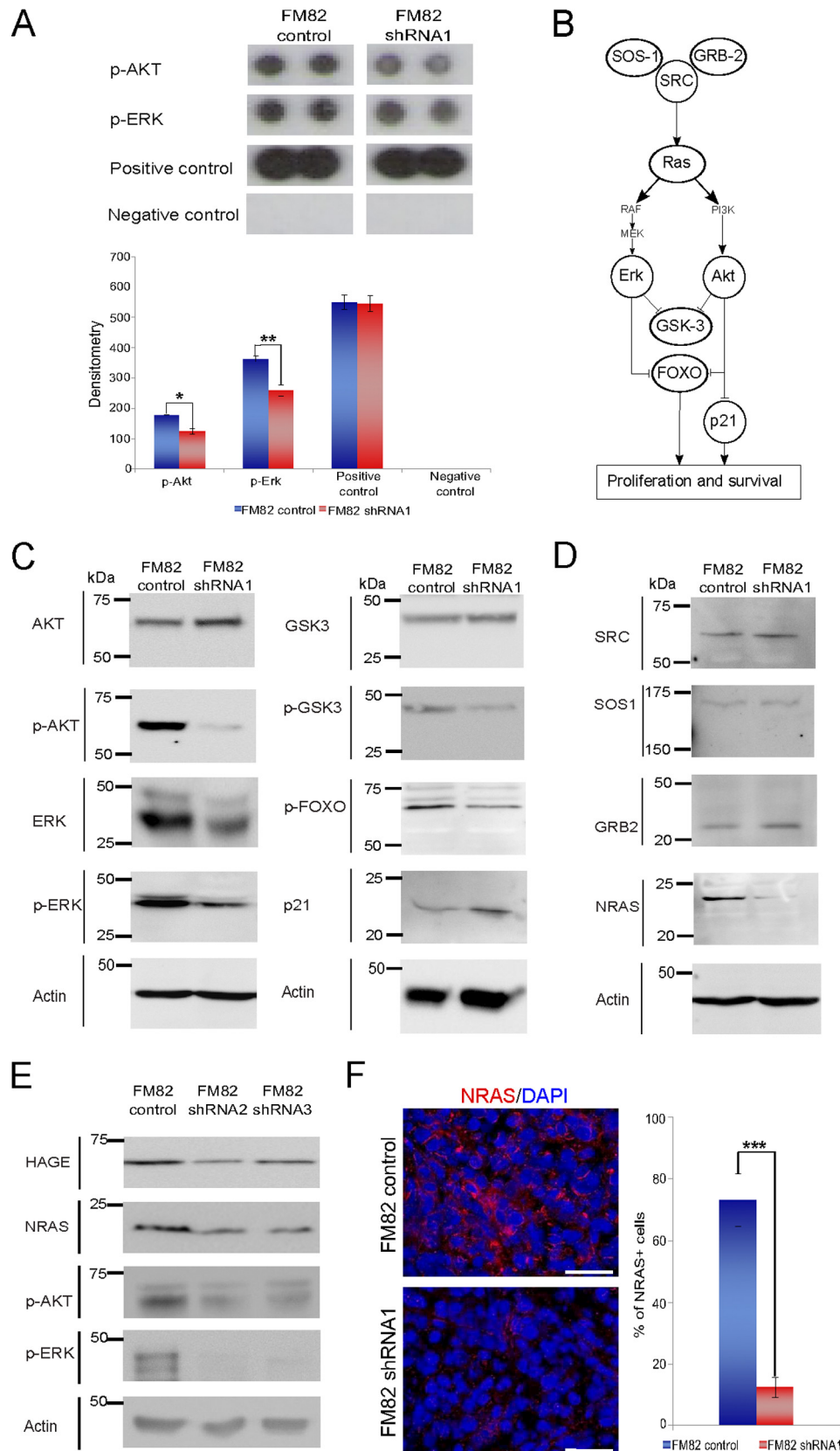
HAGE knockdown is not due to the down-regulation of the upstream activators of RAS.

HAGE Enhances NRAS Protein Expression—The decrease in NRAS protein expression upon HAGE knockdown may be due to a decrease in NRAS gene expression. We therefore per-

HAGE Promotes ABCB5+ MMIC-dependent Tumor Growth

formed a quantitative real-time PCR on RNA isolated from FM82 control and FM82 shRNA1 cells using specific primers for NRAS. No significant difference was observed in NRAS

RNA levels following HAGE knockdown, excluding the possibility that HAGE was directly regulating NRAS transcription (Fig. 4A). RNA helicases of the DEAD-box family unwind RNA



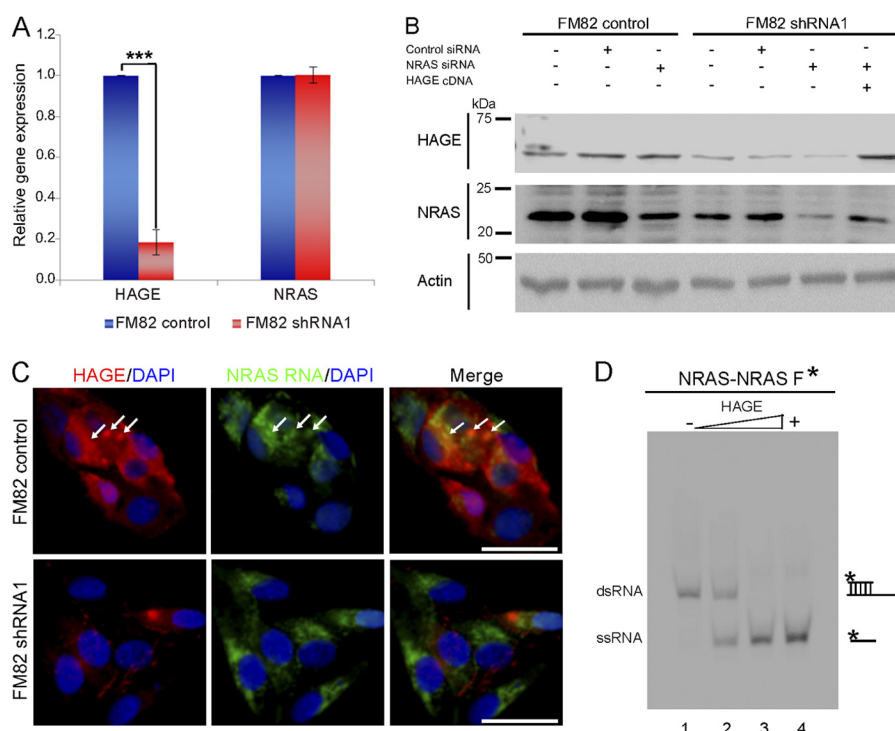


FIGURE 4. HAGE promotes NRAS protein expression. *A*, real-time quantitative PCR of HAGE and NRAS mRNAs isolated from FM82 control and FM82 shRNA1. Mann-Whitney *U* test: ***, $p < 0.001$. *B*, transient silencing of NRAS in HAGE stable knockdown and control FM82 cells followed by rescue of HAGE expression using a HAGE cDNA expression vector. β -actin was measured as a loading control. *C*, immunofluorescence on FM82 control and FM82 shRNA1 cells using antibody to HAGE and streptavidin Alexa Fluor 488-conjugated antibody to biotinylated NRAS mRNA. Partial colocalizations are shown by arrows. Scale bar = 20 μ m. *D*, unwinding of biotinylated NRAS N-terminal complementary RNA sequence-NRAS RNA duplexes in the presence of increasing HAGE protein concentration (lane 1, 0 μ g; lane 2, 0.6 μ g; lane 3, 1.2 μ g). A biotinylated NRAS N-terminal complementary RNA sequence was used as a loading control in lane 4.

duplexes by local strand separation in an ATP-dependent fashion. As a result, RNA helicases function in specific processes, such as ribosome biogenesis, pre-mRNA splicing, and translation (3–8). Recently, the DEAD-box helicase p68 (DDX5) has been shown to be an essential player in the regulation of HRAS expression, implicating RNA helicases in cell proliferation and cancer (18). HAGE may promote NRAS protein expression by unwinding and stabilizing NRAS mRNA, therefore enhancing its translation. To investigate this possibility, siRNA for NRAS mRNA was used to reduce NRAS protein expression in both FM82 control and FM82 shRNA1 cells, as well as in FM82 shRNA1 cells transfected with a HAGE-expressing plasmid. In this experiment, the siRNA would form duplexes with NRAS mRNA, leading to decreased NRAS protein expression. NRAS protein expression was significantly restored when HAGE was ectopically expressed in FM82 shRNA1 compared with the controls (Fig. 4*B*). Interestingly, biotinylated NRAS mRNA introduced in FM82 colocalized partially with HAGE in the cytoplasm and in HAGE aggregates, suggesting that these structures may be sites for RNA unwinding (Fig. 4*C*). To deter-

mine whether the helicase HAGE induces NRAS RNA unwinding, we performed an *in vitro* RNA unwinding assay in the absence or presence of different concentrations of recombinant HAGE protein and NRAS RNA duplexes. Remarkably, HAGE was able to unwind NRAS RNA duplexes in a dose-dependent fashion (Fig. 4*D*). Collectively, these results suggest that HAGE promotes NRAS protein expression by unwinding and stabilizing NRAS mRNA.

HAGE Is Required for ABCB5+ MMIC-dependent Tumor Growth—ABCB5+ MMIC are highly tumorigenic and resistant to chemotherapy. They possess the capacity to self-renew and to generate differentiated cells, properties associated with stem cells and cancer stem cells. To investigate the role of HAGE in ABCB5+ MMIC-dependent tumor growth, we performed dual immunostaining of HAGE and ABCB5+ on tumor tissue microarrays and on the FM82 and FM55 cell lines. We found that HAGE was expressed by ABCB5+ cells and that its absence correlated with a decreased number of ABCB5+ cells (Fig. 5, *A* and *B*, and supplemental Figs. 2, *A* and *C*, and 3*A*). A similar result was observed by immunofluorescence on sections

FIGURE 3. HAGE knockdown leads to a decrease in NRAS protein expression and in phosphorylation of its downstream targets. *A*, comparison of protein lysates extracted from FM82 control and FM82 shRNA1 using Proteome Profiler arrays specific for the PI3K and MAPK pathways. Mann-Whitney *U* test: *, $p < 0.05$; **, $p < 0.01$. Positive controls were not specified by the manufacturer. Negative controls were IgG from goat, mouse, and rabbit. *B*, schematic representation of upstream regulators of RAS proteins and their downstream targets. *C*, immunoblotting using antibodies against AKT, p-AKT, ERK, p-ERK, GSK3 β , p-GSK3 β , p-FOXO, and p21^{CIP1} in shRNA1 cell lysates compared with control cell lysates. β -actin was measured as a loading control. *D*, immunoblotting of FM82 control and FM82 shRNA1 extracts using antibodies to SRC, SOS1, GRB2, and NRAS. β -actin was measured as a loading control. *E*, immunoblotting using antibodies against HAGE, NRAS, p-AKT, and p-ERK in FM82 control, FM82 shRNA2, and FM82 shRNA3. β -actin was measured as a loading control. *F*, representative immunostaining of tumor sections using antibody against NRAS (red). Scale bars = 20 μ m. The graph demonstrates the percentage of NRAS+ cells in FM82 control and FM82 shRNA1 derived from counting 10 random microscopic fields. Student's *t* test: **, $p < 0.01$.

HAGE Promotes ABCB5+ MMIC-dependent Tumor Growth

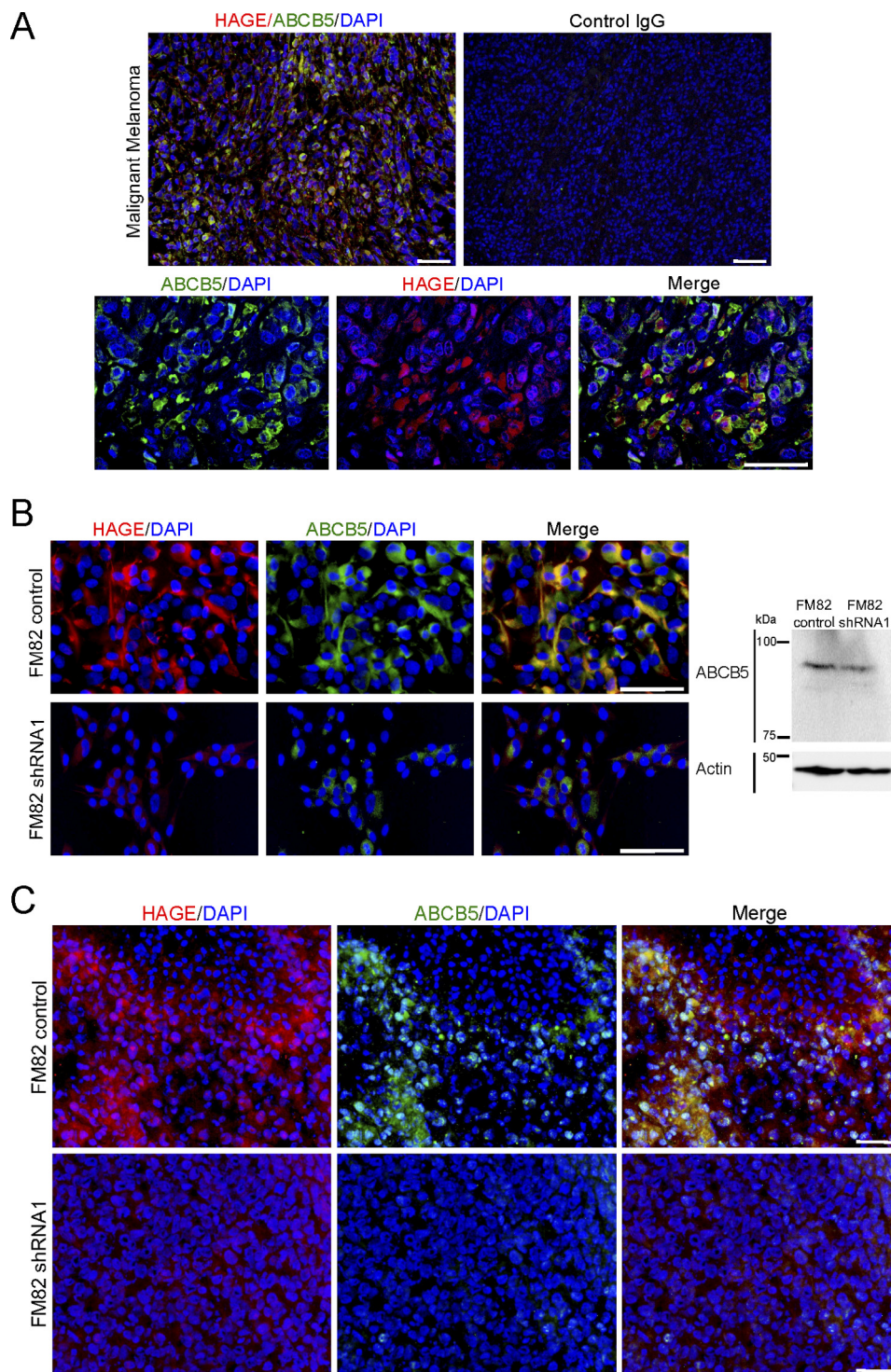


FIGURE 5. Expression of HAGE protein in ABCB5-positive malignant melanoma-initiating cells. *A*, representative image of immunohistochemistry with antibodies to ABCB5 (green) and HAGE (red) in malignant melanoma. The negative control was an IgG isotype from mice. Scale bars = 20 μ m. *B*, immunofluorescence with antibodies to ABCB5 (green) and HAGE (red) in the FM82 control and FM82 shRNA1 melanoma cell line. Scale bars = 20 μ m. Also shown is immunoblotting using an antibody to ABCB5 in FM82 control and FM82 shRNA1 extracts. β -actin was measured as a loading control. *C*, immunostaining of sections from FM82 control and FM82 shRNA1 tumors using antibody against ABCB5 (green) and HAGE (red). Scale bars = 20 μ m.

from FM82 control and FM82 shRNA1 tumors obtained from the tumor transplantation assay described above (Fig. 5C). Using a sphere formation assay, we showed that FM82 and FM55 control cells were able to form melanospheres when plated at clonal cell density. These structures strongly expressed ABCB5 and HAGE and were able to generate

ABCB5+ and ABCB5- cells (Figs. 2D and 6 and supplemental Fig. 3B). Interestingly, FM82 and FM55 generated significantly smaller melanospheres when HAGE was stably knocked down and compared with the controls (Figs. 2D and 5C and supplemental Fig. 3B). Taken together, these results demonstrate that HAGE expression promotes ABCB5+ MMIC-dependent

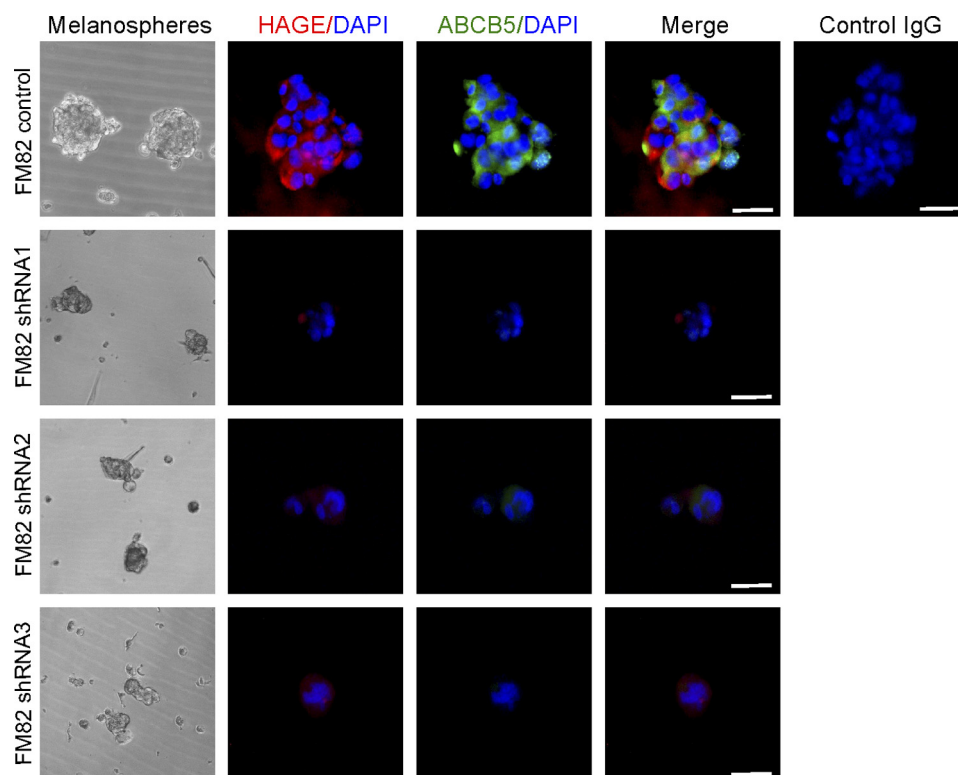


FIGURE 6. **Sphere-forming assay with HAGE stable knockdown and control FM82 cells.** The spheres were stained by immunofluorescence with antibodies to ABCB5 (green) and HAGE (red). The negative controls were IgG isotypes from both mouse and rabbit. Scale bars = 50 μ m.

tumor growth through oncogenic activation of the NRAS/ERK/AKT pathway and that HAGE is implicated in tumor initiation and progression in melanoma cancer.

DISCUSSION

The DEAD-box RNA helicases are ubiquitous, highly conserved enzymes that participate in nearly all aspects of RNA metabolism (4–8). Their role in tumor growth has been reported previously for some members of this family. For instance, p68 (DDX5), p72 (DDX17), and Cancer Associated Antigen (CAGE) (DDX53) have been implicated in promoting cell proliferation and survival in cancer cell lines from different tissue origins (18, 27–31). Members including Cancer Associated Antigen (CAGE) and HAGE (DDX43) were found to be highly expressed in different cancer tissues and cancer cell lines, suggesting their possible role in tumorigenesis (13, 28, 32–34). In this study, we investigated whether the helicase HAGE (DDX43) could promote ABCB5+ MMIC-dependent tumor growth.

We found that HAGE was expressed *in vitro* and *in vivo* by ABCB5+ MMIC, a population of cancer cells resistant to chemotherapy (35). This population possesses properties associated phenotypically with stem cells and cancer stem cells. They are able to self-renew and generate differentiated cells. HAGE knockdown in the FM82 and FM55 melanoma cell line resulted in a significant decrease in proliferation of ABCB5+ cells, measured *in vitro* by the capacity of these cells to form melanospheres. Interestingly, a decrease of ABCB5+ cells was observed in FM82 shRNA tumors, suggesting that HAGE plays a role in ABCB5+ cells survival within the tumors. Mechanis-

tically, HAGE was found to enhance cell proliferation by promoting the expression of NRAS protein. The small GTP-binding proteins NRAS, HRAS, and KRAS belong to a highly conserved family of oncoproteins involved in cellular signaling transduction that regulate cellular processes such as cell growth, differentiation, and apoptosis (24, 36–38). The RAS genes have been shown to be affected by point mutations that occur at codons 12, 13, or 61, which render the protein product unresponsive to GTPase-activating proteins that serve to inactivate RAS (24, 39). Such mutations result in the constitutive activations of RAS proteins and deregulation of downstream cellular cascades, leading to tumorigenesis (24, 40). Knockdown of HAGE led to a significant decrease in RAS protein expression in the FM82 melanoma cell line (supplemental Fig. 2C).

RAS oncoproteins mediate their functions by activating, among others, the PI3K/AKT and RAF/MEK/ERK pathways through a series of phosphorylation cascades, resulting in AKT and ERK phosphorylation, subsequently leading to their activation (24, 36, 41). The phosphorylated forms of AKT and ERK were consistently and markedly decreased upon HAGE knockdown, suggesting a role for HAGE in activating those signaling pathways by regulating NRAS protein expression. Furthermore, we demonstrated that upon HAGE knockdown there was a decrease in phosphorylation of downstream targets of AKT and ERK, consistent with the observed decrease of phosphorylation of both AKT and ERK.

FM82, the melanoma cell line used for this study, carries an active mutant form of BRAF (BRAF^{V600E}) (42). This raised the

HAGE Promotes ABCB5+ MMIC-dependent Tumor Growth

question of whether BRAF^{V600E} is responsible for the increased proliferation observed with the FM82 cell line. This is probably partly true, as FM82 cells also harbor a wild-type form of BRAF (BRAF^{WT}) expressed to the same level as the mutant form (42). Because mutant BRAF is unresponsive to RAS activity, this means that the wild-type form of BRAF in FM82 could possibly be available for activation by RAS signaling and capable of functioning separately from the mutant form (BRAF^{V600E}). HAGE silencing resulted in a decrease of ERK phosphorylation that appears to be independent from ERK activation by BRAF^{V600E}. Furthermore, similar results were obtained using another melanoma cell line (FM55).

To understand the mechanism by which the DEAD-box RNA helicase HAGE (DDX43) promotes NRAS protein expression, we thought about using a specific siRNA to alter NRAS protein expression in a model where HAGE expression was silenced in a stable manner (FM82 shRNA), naturally expressed (FM82 control), or ectopically expressed in FM82 shRNA. siRNAs are RNA molecules that form duplexes with target mRNAs preventing their protein expression. Therefore, we used these molecules as a system to mimic RNA duplex formation. We found that in the presence of HAGE, NRAS protein expression was enhanced significantly, suggesting the possibility that HAGE may play a role in unwinding NRAS mRNAs/siRNA duplexes, thus promoting NRAS protein expression. This possibility was tested using the *in vitro* unwinding assay, and we showed that HAGE may play a role in the unwinding of NRAS mRNA secondary structures to enhance NRAS translation. It is important to note that NRAS RNA expression was not affected by HAGE knockdown, excluding a transcriptional regulation of NRAS by HAGE.

Collectively, these findings provide evidence for a previously unknown role of HAGE in ABCB5+ MMIC-dependent tumor growth. We clearly demonstrated that HAGE promotes tumor cell proliferation by regulating RAS protein expression, which in turn leads to the activation of downstream signaling pathways implicated in cell proliferation and survival. Finally, as HAGE is expressed only by tumor cells, these results suggest that cancer therapies targeting HAGE helicase may have broad applications for treating ABCB5+ malignant melanoma.

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