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Authors

Cai, Rong
Bade, David
Liu, Xiaochuan
et al.

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Targeted Quantitative Profiling of GTP-Binding Proteins Associated with Metastasis of Melanoma Cells

Rong Cai[‖],
Department of Chemistry, University of California, Riverside, Riverside, California 92521, United States; Suzhou Research Institute, Shandong University, Suzhou, Jiangsu 215123, China

David Bade[‖],
Environmental Toxicology Graduate Program, University of California, Riverside, Riverside, California 92521, United States

Xiaochuan Liu,
Department of Chemistry, University of California, Riverside, Riverside, California 92521, United States

Ming Huang,
Environmental Toxicology Graduate Program, University of California, Riverside, Riverside, California 92521, United States

Tianyu F. Qi,
Environmental Toxicology Graduate Program, University of California, Riverside, Riverside, California 92521, United States

Yinsheng Wang
Department of Chemistry and Environmental Toxicology Graduate Program, University of California, Riverside, Riverside, California 92521, United States;

Abstract

Metastasis is a major obstacle in the therapeutic intervention of melanoma, and several GTP-binding proteins were found to play important roles in regulating cancer metastasis. To assess systematically the regulatory roles of these proteins in melanoma metastasis, we employed a targeted chemoproteomic method, which relies on the application of stable isotope-labeled desthiobiotin-GTP acyl phosphate probes in conjunction with scheduled multiple-reaction monitoring (MRM), for profiling quantitatively the GTP-binding proteins. Following probe

Corresponding Author: Yinsheng Wang – Department of Chemistry and Environmental Toxicology Graduate Program, University of California, Riverside, Riverside, California 92521, United States; Phone: (951) 827-2700; yinsheng.wang@ucr.edu.

[‖]**Author Contributions**: R.C. and D.B. contributed equally to this work.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jproteome.1c00708.
Selected-ion chromatograms for the light and heavy forms of the targeted TGM2 peptide; selected-ion chromatograms for the light and heavy forms of the targeted AK4 peptide; AK4-promoted migration and invasion in the IGR37/IGR39 melanoma cells; wound healing assay for the IGR37/IGR39 melanoma cells; comparison between the results obtained from GTP probe pull-down and SDS-PAGE fractionation followed by MRM analysis; and uncropped western blot images (PDF)
Quantitative profiling of GTP-binding proteins in WM-115/WM-266-4 and IGR39/IGR37 cells (XLSX)

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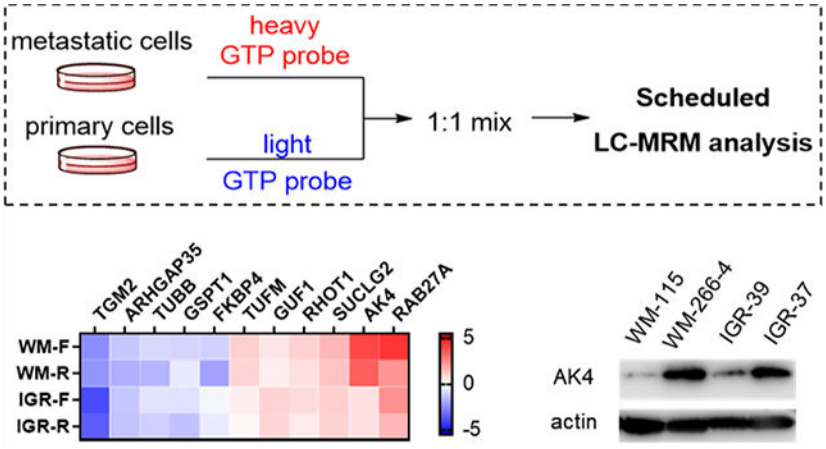
labeling, tryptic digestion, and affinity pull-down of desthiobiotin-conjugated peptides, differences in expression levels of GTP-binding proteins in two matched pairs of primary/metastatic melanoma cell lines were measured using liquid chromatography–MRM analysis. We also showed that among the top upregulated proteins in metastatic melanoma cells, AK4 promotes the migration and invasion of melanoma cells; overexpression of AK4 in primary melanoma cells leads to augmented migration and invasion, and reciprocally, knockdown of AK4 in metastatic melanoma cells results in repressed invasiveness. In summary, we examined the relative expression levels of GTP-binding proteins in two pairs of primary/metastatic melanoma cell lines. Our results confirmed some previously reported regulators of melanoma metastasis and revealed a potential role of AK4 in promoting melanoma metastasis.

Graphical Abstract



Keywords

GTP-binding protein; melanoma; cancer metastasis; targeted proteomics; chemoproteomics; multiple-reaction monitoring

INTRODUCTION

Melanoma is a principal cause of skin cancer mortality in the United States, accounting for an estimated 106,110 new cases and 7180 deaths in 2021.[1] Early-stage melanoma is typically curable, where a five-year survival rate of 99% was reported with localized melanoma; the prognosis, however, worsens considerably to approximately 27% for cases of distant metastasis.[2] Fortunately, the reported mortality for melanoma in the U.S. decreased dramatically since the FDA approved new therapies, for example, immune checkpoint inhibitor ipilimumab and BRAF inhibitor vemurafenib, for metastatic cancer.[3] Identification and functional characterizations of other proteins modulating melanoma metastasis are of paramount importance, and they may advance therapy and improve overall survival of patients with advanced disease.

GTP-binding proteins serve as molecular switches in cell signaling pathways.[4] Aberrant expression and/or activities of GTP-binding proteins, including small GTPases, are known to

be associated with melanoma metastasis.[5,6] Huang *et al.*[7] reported a targeted proteomic method combining stable isotope labeling by amino acids in cell culture (SILAC), sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) fractionation, and multiple-reaction monitoring (MRM) analysis, to quantify small GTPases in matched pairs of primary/metastatic melanoma cells. Based on liquid chromatography (LC)–mass spectrometry (MS)/MS and bioinformatic analysis, they revealed a potential role of RAB38 in enhancing melanoma metastasis through the upregulation of matrix metalloproteinase 2 (MMP2) and MMP9. Despite these findings, there is a continued need to understand comprehensively the functions of GTPases in melanoma metastasis.

We previously developed isotope-coded desthiobiotin-GTP acyl phosphate probes for the proteome-wide enrichment and quantitative profiling of GTP-binding proteins.[8] In conjunction with an MRM-based proteomic workflow, we were able to quantify variations in GTP-binding proteins in a pair of primary and metastatic colon cancer cell lines. Several up- or downregulated proteins identified with the method were previously shown to play essential roles in regulating colon cancer metastasis.[8] Considering the robustness and efficiency of this method and the importance in discovering novel regulators of melanoma metastasis, we employed this quantitative proteomic method for assessing GTP-binding proteins in two matched pairs of primary/metastatic melanoma cell lines. We found that among the differentially expressed GTP-binding proteins, adenylate kinase 4 (AK4) is markedly upregulated in metastatic melanoma cells, and its elevated expression enhances the migration and invasion of cultured melanoma cells.

EXPERIMENTAL SECTION

Labeling with the Desthiobiotin-GTP Acyl Phosphate Probes

WM-115 and WM-266-4 cells (WM pair) were obtained from ATCC, and IGR39 and IGR37 cells (IGR pair) were generous gifts from Prof. Peter H. Duesberg. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen-Gibco) with 10% fetal bovine serum (FBS) (Invitrogen-Gibco) and penicillin/streptomycin (100 IU/mL) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Unless specifically described, all chemicals were obtained from Sigma-Aldrich. The preparation of isotope-coded desthiobiotin-GTP acyl phosphate probes, cell lysate preparation, and labeling were performed as previously reported.[9] Briefly, approximately 2×10^7 cells were lysed with 1 mL of lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% Triton X-100) containing 1% protease inhibitor cocktail. After removing endogenous nucleotides with NAP-5 columns (Amersham Biosciences), about 1 mg of lysates in 1 mL of lysis buffer was incubated with 5 mM EDTA for 5 min, followed by incubation with 20 μM GTP probe and 20 mM $MgCl_2$ at room temperature for 2 h. In the forward labeling experiments, the primary and metastatic melanoma cells were labeled with light and heavy GTP probes, respectively. The labeling reactions were swapped in the reverse experiments. After the labeling reactions, the unreacted probes were quenched by incubating the mixture with 100 mM glycine at room temperature for 30 min. The labeled lysates from each pair of primary and metastatic melanoma cells were combined at 1:1 ratio (by mass) for further analysis.

Scheduled MRM Analysis

The LC–MS/MS samples were prepared and analyzed by LC–MRM following previously reported procedures,[10] with the details being provided in the Supporting Information. The transition lists for peptides in the library and the raw files for LC–MS/MS analyses for the paired melanoma cells were deposited into PeptideAtlas with the identifier number PASS01639 (http://www.peptideatlas.org/PASS/PASS01639).

Stable Cell Line Generation

Melanoma cells with stable knockdown or overexpression of the *AK4* gene were generated using a lentiviral approach. The shRNA sequences were selected from Sigma and cloned into pLKO.1-Puro (Addgene). The loop regions for shRNAs were CTCGAG, and the sense strands were 5′-GCCAGTCATTGAATTATACAA-3′ for shAK4-2e, 5′-GCCAGGCTAAGACAGTACAAA-3′ for shAK4-1 and 5′-TCCTAAGGTTAAGTCGCCCTCG-3′ for scrambled control (shCtrl). The coding sequence of the *AK4* gene was PCR-amplified from a cDNA library prepared from WM-266-4 cells and cloned into a pLJM1-EGFP vector (Addgene). The lentiviruses were packaged in HEK293T cells and collected following 48 h of incubation. Primary melanoma cells were infected with lentivirus for AK4 overexpression or an empty vector, and metastatic melanoma cells were infected with lentivirus for AK4 knockdown or shCtrl for 48 h. Successfully infected cells were subjected to puromycin selection (1 μg/mL).

Cell Migration and Invasion Assays

For the migration assay, WM or IGR pairs of melanoma cells were suspended in serum-free media, and 2.5×10^4 (for the WM pair) or 4.0×10^4 (for the IGR pair) cells were added into the upper chamber of the transwell insert (Corning). Complete DMEM supplemented with 10% FBS was added into the lower chamber underneath the insert. After incubation at 37 °C for 24 (WM cells) or 48 h (IGR cells), the inserts were rinsed with PBS, and the unmigrated cells inside the insert were gently removed with a cotton swab. The migrated cells were fixed with 70% ethanol, stained with 0.5% crystal violet, and imaged under an inverted microscope. The WM cells were imaged under bright field, and the IGR cells were imaged using a Texas Red lens. Cells in four randomly selected zones were counted, and the cell numbers were averaged. The invasion assay was performed in parallel with the migration assay under the same conditions, except that the inner surface of the transwell insert was coated with a Matrigel basement membrane matrix (Corning), at a concentration of 200–400 μg/mL, at 37 °C for 1 h prior to seeding of cells.

Wound Healing Assays

Wound healing assay was performed following published procedures.[11] Cells were seeded in 6-well plates at a density of 5×10^5 cells/well. Upon reaching 90% confluency, a scratch was made in the cell monolayer with a 100 μL pipette tip, and the dislodged cells were washed off gently with 1× PBS. The remaining cells were cultured at 37 °C in DMEM without FBS to prevent cell proliferation. Images were then taken under an inverted microscope at 0, 24, and 48 h, to assess the extents of cell migration. The data were then analyzed with Image-Pro v10 (Media Cybernetics).

RESULTS AND DISCUSSION

Quantitative Profiling of GTP-Binding Proteins in Melanoma Cells

We previously reported the application of isotope-labeled desthiobiotin-GTP acyl phosphate probes for the covalent labeling, enrichment, and mass spectrometric analysis of GTP-binding proteins (Figure 1A).[8] These probes were designed to incorporate a desthiobiotin tag to the nucleophilic lysine residues situated at or near the GTP-binding pockets, namely, lysines in the highly conserved Walker A motif (GxxxxGKT/S) present in many GTPases. By introducing an isotope-coded γ-amino-butyryl (GABA) linker between the affinity tag and GTP, targeted proteins could be labeled with a heavy- or a light-labeled probe to facilitate quantitative analysis using LC–MS/MS. Based on shotgun proteomic data, we constructed a Skyline[12] library encompassing 217 probe-labeled peptides of GTP-binding proteins and developed a quantification method based on LC–MRM analysis (Figure 1B).

Lysates of paired primary/metastatic melanoma cells were labeled with light or heavy probes in the forward experiments, respectively, and mixed at equal mass. The labelings were swapped in the reverse experiment. The ensuing protein mixtures were digested with trypsin, and the desthiobiotin-labeled peptides were enriched from the mixture using streptavidin beads for LC–MRM analysis. By employing our previously developed Skyline MRM library, we detected targeted peptides in preselected retention time windows based on iRT calibration. The data were then analyzed using Skyline. Based on the fold changes in the desthiobiotin-labeled peptides of proteins in metastatic over primary cancer cells, probe-binding efficiencies of the corresponding proteins could be quantitatively determined.

Scheduled MRM Analysis of GTP-Binding Proteins in Matched Pairs of Metastatic and Primary Melanoma Cells

Metastasis is one of the primary causes for poor prognosis and mortality in melanoma patients. To assess the roles of differential expression of GTP-binding proteins in melanoma metastasis, we applied our scheduled MRM method on two pairs of matched metastatic and primary melanoma cell lines. WM-115 and WM-266-4 cells were, respectively, developed from primary tumor and skin metastasis of a 55-year-old female patient.[13] IGR39 and IGR37 cells were initiated from primary tumor and lymph node metastasis, respectively, of a 26-year-old male patient.[14]

A total of 64 GTP-binding proteins were commonly quantified in both forward and reverse labeling experiments for the two pairs of melanoma cell lines (Figure 2). Among these proteins, several were previously shown to regulate melanoma progression. For example, RAB27A is overexpressed in advanced-stage melanoma patients, and its high expression level is correlated with poorer patient survival.[15] In addition, RAB27A could promote melanoma metastasis by stimulating melanoma cells to secrete pro-invasive exosomes.[15] TGM2, the most downregulated protein in metastatic melanoma cells (Figure 2 and Table S1), is both a GTPase and a transglutaminase and exhibits both anti-apoptotic and proapoptotic effects on cancer cells, which depend on cell types.[16] In this vein, a higher expression level of TGM2 was detected in advanced-stage and highly metastatic melanoma cells.[17] Overexpression of TGM2 promotes the development of chemoresistance, while the

activation of endogenous TGM2 results in rapid apoptosis.[17] A previous study revealed that genetic ablation of *Tgm2* in mice promotes melanoma metastasis;[18] the functions of TGM2 in cancer metastasis, however, remain incompletely understood.

For most of the quantified peptides, the detected intensity ratios in metastatic over primary melanoma cells were reasonably consistent between forward and reverse labeling experiments (Figure 3A,B). We also observed a significant correlation for several differentially regulated proteins between the WM and IGR pairs of cell lines, including AK4, RAB27A, SUCLG2, TGM2, and ARHGAP35 (Figure 3C). To evaluate the quantification accuracy, we examined the differential expressions of AK4 and TGM2 between the primary and metastatic cell lines by employing western blot analysis. The western blot data showed that in both pairs of melanoma cells, TGM2 and AK4 were down- and upregulated, respectively, in the metastatic cell lines (Figure 3D). The results are consistent with our MRM data (Figures S1 and S2), suggesting that our method offers accurate quantifications of GTP-binding proteins.

Huang *et al.*[7] recently reported a targeted quantitative proteomic method for comparing small GTPase expression between paired primary and metastatic melanoma cell lines. To investigate how consistent our data are with those obtained from that study, we compared these two datasets (Figure S5). It turned out that most of the quantified small GTPases showed consistent trends in these datasets. For instance, both studies showed that RAB27A was prominently upregulated in WM-266-4 and IGR37 metastatic cell lines over the matched primary melanoma cell lines, that is, WM-115 and IGR37. These results suggest that both methods offered accurate assessments of differential expression of GTP-binding proteins. It is worth noting that the SDS-PAGE-based fractionation method focuses on small GTPases with molecular weights falling in the range of 15–37 kDa. In the current method, we also included those GTPases with molecular weights exceeding 37 kDa. Thus, the two methods provide complementary coverage of the GTP-binding proteome. Moreover, the probe labeling efficiency of the current method may be modulated by GTP-binding affinity; hence, the method holds potential in revealing the alterations in nucleotide-binding affinities of the small GTPase proteome, which may provide insights into the nucleotide-binding state of small GTPases. In this context, it is of note that a few GTP-binding proteins, that is, RAB28 and SRPRB, showed opposite trends in these two studies, which may reflect a combination of differences in expression levels of the target proteins and their probe binding affinities and labeling efficiencies.

AK4 Promotes the Migration and Invasion of Melanoma Cells

Among the top upregulated proteins in metastatic melanoma cells, AK4 plays an important role in regulating cellular ATP levels.[19] It is potentially involved in the AMPK signaling pathway, which is essential for cellular energy homeostasis.[20] Both ATP and GTP could be used as phosphate donors for AK4 in phosphorylating nucleoside phosphates, whereas only AMP is specifically phosphorylated when GTP is used as the phosphate donor.[21] Xiao *et al.*[22] observed a preference of AK4 in binding with GTP over ATP based on a quantitative proteomic analysis.

There is considerable research showing that AK4 is a prognostic marker in several types of cancer, including ovarian cancer[23] and bladder cancer,[24] where an increased expression of AK4 is usually associated with poorer patient survival. In lung cancer, elevated AK4 expression could promote metastasis by downregulating the transcription factor ATF3, which leads to MMP2 induction.[25] Increased AK4 expression was documented to promote the stabilization of HIF-1α, thereby driving the epithelial-to-mesenchymal transition in lung cancer cells.[26] Additionally, AK4 was found to promote bladder cancer cell proliferation and invasion.[24] Furthermore, Liu *et al.*[27] observed that an elevated AK4 expression confers augmented resistance to tamoxifen in breast cancer cells. Based on our proteomic results, we sought to determine whether AK4 promotes the migratory and invasive capabilities of metastatic melanoma cells by employing transwell migration and invasion assays.

We first asked whether decreased AK4 expression in metastatic melanoma cells can suppress melanoma cell migration and invasion. We prepared WM-266-4 and IGR37 cell lines with stable knockdown of the *AK4* gene using small-hairpin RNAs (shRNAs). Our results demonstrated that knockdown of AK4 in WM-266-4 and IGR37 cells resulted in significant diminutions in these cells' abilities to migrate and invade, where the knockdown efficiency was assessed against cell lines containing an shCtrl sequence with western blot analysis (Figures 4A–C and S3A–C).

We subsequently modified the WM-115 and IGR39 cell lines to stably overexpress AK4. We compared these cell lines with the corresponding cell lines transfected with an empty vector. By employing the transwell assay, we determined that the invasive capacities of WM-115 and IGR39 cells were substantially increased upon overexpression of AK4 (Figures 4D,E and S3D,E).

To further corroborate the abovementioned results, we employed a wound healing scratch assay to determine if the migratory abilities of melanoma cells are affected by the expression levels of AK4. Our results revealed that relative to the shCtrl cells, stable knockdown of AK4 in WM-266-4 and IGR37 cells conferred attenuation in cell migration at 48 h after the scratch (Figures 5A,B and S4A,B). Reciprocal experiments with WM-115 (Figure 5C,D) and IGR39 (Figure S4C,D) cells showed increases in cell migration following AK4 stable overexpression. Together, these results suggest the vital importance of AK4 in promoting melanoma cell migration and invasion.

CONCLUSIONS

In this study, we employed a high-throughput chemoproteomic method for the quantitative analysis of GTP-binding proteins, where the method involves the use of stable isotope-coded desthiobiotin-GTP acyl phosphate probes and a peptide library encompassing 217 probe-labeled peptides of GTP-binding proteins. We subsequently used this library to design an MRM method for the targeted quantification of these proteins. With this method, we quantified 64 GTP-binding proteins across two pairs of primary and metastatic melanoma cells. From these data, we found several substantially down- or upregulated GTP-binding proteins in the metastatic cell lines compared to their primary counter-parts that were previously shown to be promoters or suppressors for melanoma metastasis. We further

demonstrated, for the first time, that the upregulation of AK4 in these melanoma cells promoted their migration and invasion *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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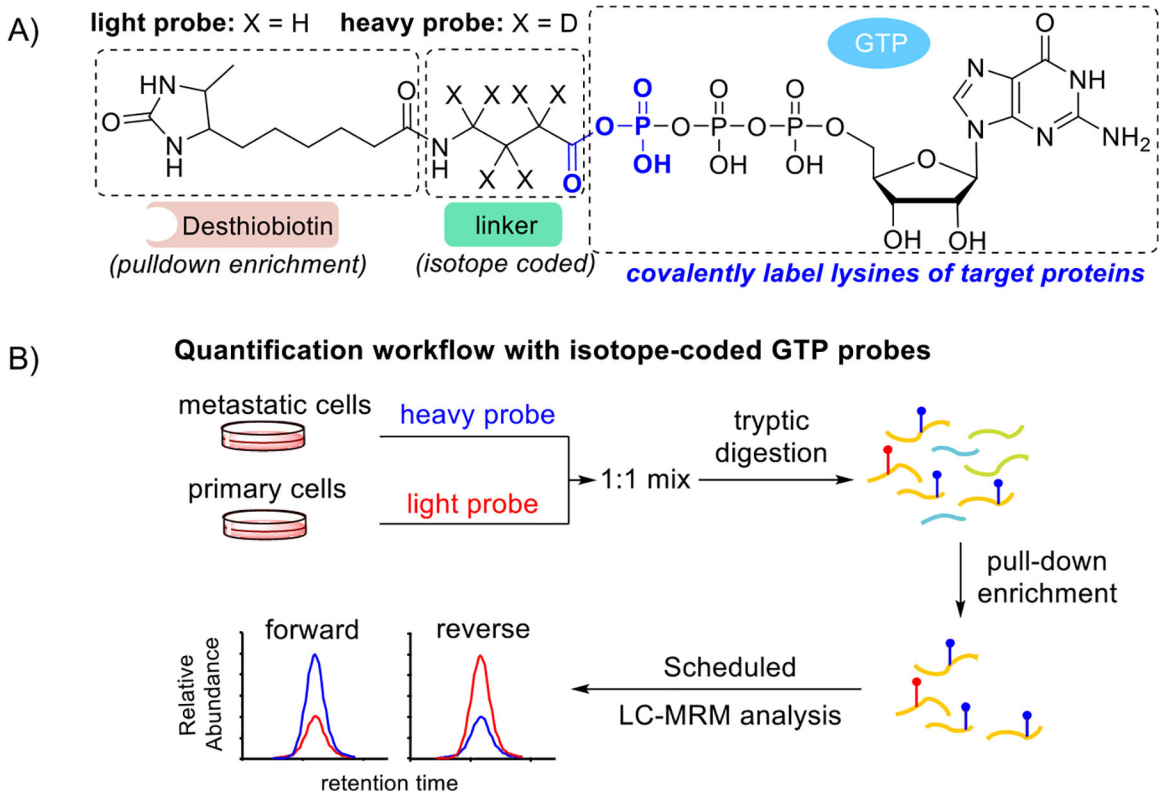

Figure 1.
Design of stable isotope-coded desthiobiotin-GTP probes and the quantification workflow.

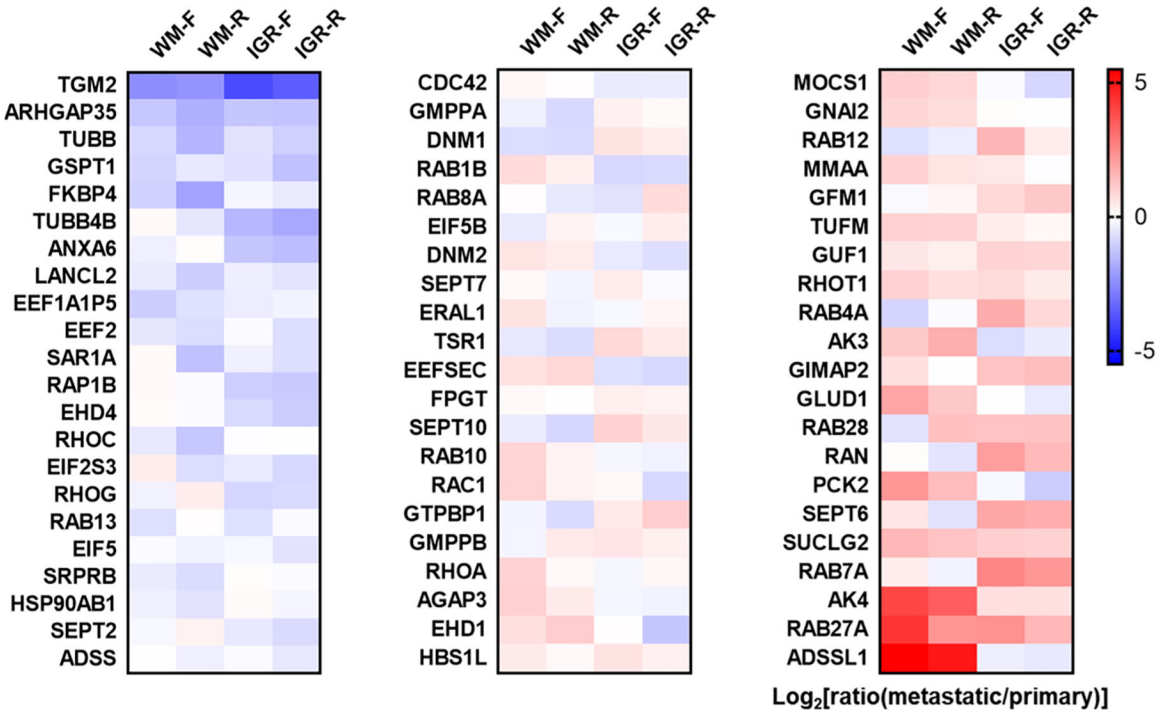

Figure 2.

Heatmap showing the quantification results for GTP-binding proteins in all four experiments. The values for WM-F and WM-R are results obtained for the WM pair of cell lines from the forward and reverse probe labeling experiments, respectively. The values for IGR-F and IGR-R represent the corresponding quantification results for the IGR pair of cell lines.

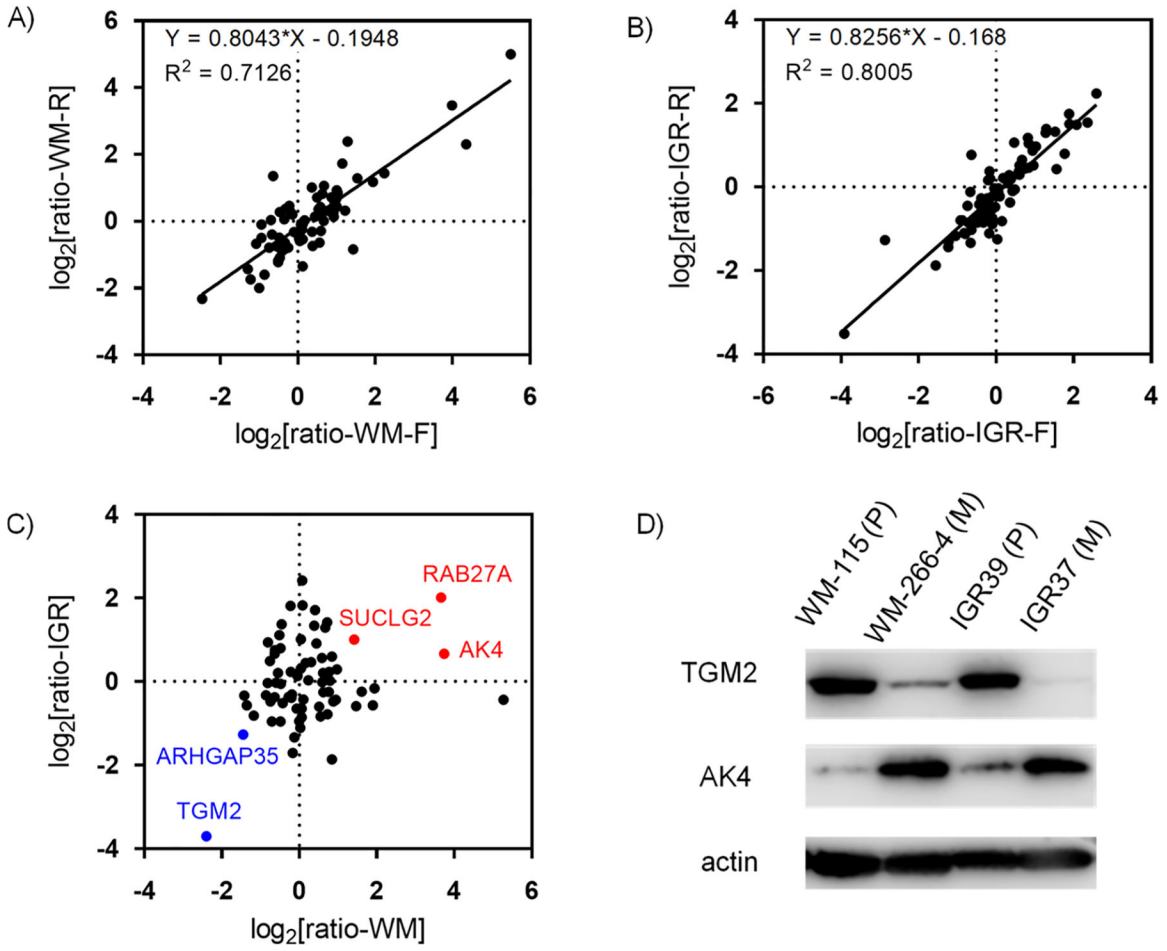

Figure 3.

Analysis and validation of the MRM quantification results of GTP-binding proteins. (A) Comparison between the forward and reverse labeling experiments of the WM pair of cell lines; (B) comparison between the forward and reverse labeling experiments of the IGR pair of cell lines; (C) comparison between the average ratios for WM and IGR pairs of cell lines; proteins that are substantially up- and downregulated in both lines of metastatic melanoma cells are colored in red and blue, respectively; and (D) western blot for the validation of expression levels of selected GTP-binding proteins with western blot; “P” and “M” designate primary and metastatic cell lines, respectively.

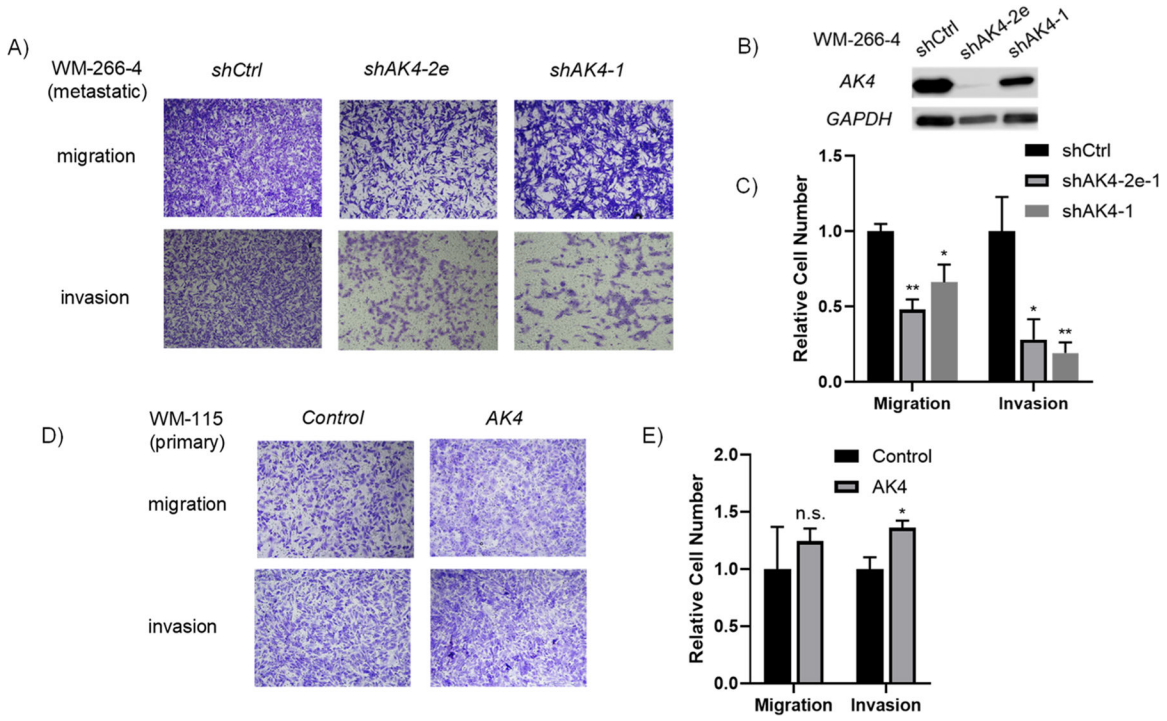

Figure 4.

AK4-promoted migration and invasion of the WM pair of cell lines. Shown are representative images (A) and quantification results (C) of the migration and invasion assay showing the influence of stable AK4 knockdown (with two different shRNAs) compared with shCtrl in WM-266-4 cells; (B) western blot showing the expression level of AK4 in WM-266-4 cells after stable knockdown of AK4; and (D,E) representative images (D) and quantification results (E) from the migration and invasion assay showing the influence of AK4 overexpression on the migratory and invasive capacities of WM-115 cells.

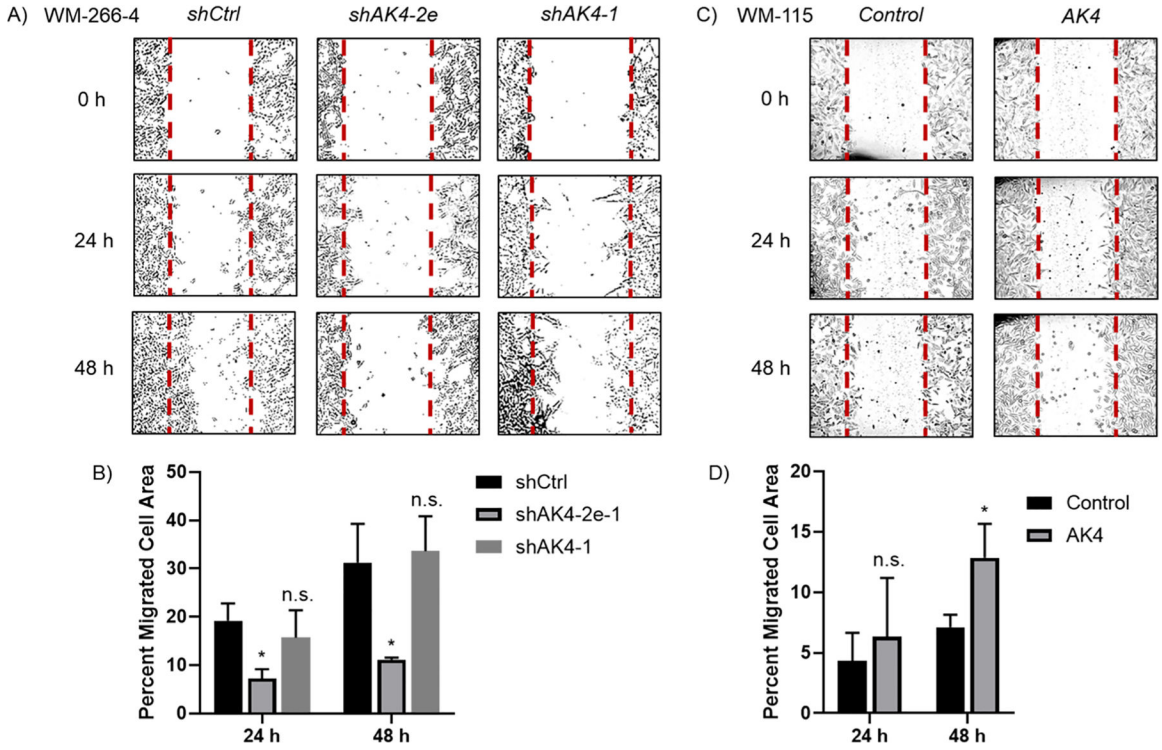

Figure 5.

Wound healing assay for the WM pair of cell lines. (A,B) Representative images (A) and quantification results (B) showing the influence of AK4 knockdown in WM-266-4 cells and (C,D) representative images (C) and quantification results (D) showing the influence of AK4 overexpression on the migration and invasion of WM-115 cells.