Tumorigenesis and Neoplastic Progression

Gab2-Mediated Signaling Promotes Melanoma Metastasis

Basil Horst,* Sofia K. Gruvberger-Saal,[†]
Benjamin D. Hopkins,[†] Lindsey Bordone,[‡]
Ying Yang,[‡] Karen A. Chernoff,[‡] Ijeoma Uzoma,[§]
Volker Schwipper,[¶] Jutta Liebau,^{||}
Norma J. Nowak,** Georg Brunner,^{††}
David Owens,^{**} David L. Rimm,[§]
Ramon Parsons,^{*†} and Julide Tok Celebi[‡]

From the Departments of Pathology* and Dermatology,[‡] and the Institute for Cancer Genetics,[†] Columbia University, New York, New York; the Department of Pathology,[§] Yale University School of Medicine, New Haven, Connecticut; the Departments of Oral and Maxillofacial Surgery[¶] and Cancer Research,^{††} Fachklinik Hornheide, University of Münster, Münster, Germany; the Clinic for Plastic Surgery,^{||} Diakonie-Hospital Kaiserswerth, Duesseldorf, Germany; and the Department of Cancer Prevention and Population Sciences,** Roswell Park Cancer Institute and Department of Biochemistry, University at Buffalo, Buffalo, New York

Metastatic melanoma is a disease with a poor prognosis that currently lacks effective treatments. Critical biological features of metastasis include acquisition of migratory competence, growth factor independence, and invasive potential. In an attempt to identify genes that contribute to melanoma pathogenesis, a genome-wide search using bacterial artificial chromosome array comparative genomic hybridization and single nucleotide polymorphism arrays in a series of 64 metastatic melanoma samples and 20 melanoma cell lines identified increased copy numbers of *Gab2* located on 11q14.1. *Gab2* is an adaptor protein that potentiates the activation of the Ras-Erk and PI3K-Akt pathways and has recently been implicated in human cancer; however, its role in melanoma has not been explored. In this study, we found that *Gab2* was either amplified (~11%) and/or overexpressed (~50%) in melanoma. *Gab2* protein expression correlated with clinical melanoma progression, and higher levels of expression were seen in metastatic melanomas compared with primary melanoma and melanocytic nevi. We found that overexpression of *Gab2* potentiates, whereas silencing of *Gab2* reduces, migration and invasion of melanoma cells. *Gab2* mediated the hy-

peractivation of Akt signaling in the absence of growth factors, whereas inhibition of the PI3K-Akt pathway decreased *Gab2*-mediated tumor cell migration and invasive potential. *Gab2* overexpression resulted in enhanced tumor growth and metastatic potential *in vivo*. These studies demonstrate a previously undefined role for *Gab2* in melanoma tumor progression and metastasis. (Am J Pathol 2009, 174:1524–1533; DOI: 10.2353/ajpath.2009.080543)

Melanoma is the fifth most common cancer in the US, accounting for more than 60,000 new cases each year.¹ If diagnosed in the early stages, melanoma can be cured by surgery; however metastatic disease has a poor prognosis with a median survival of 6 to 9 months.² There is no therapy for thicker or metastatic melanomas proven to have a significant impact on survival. Therefore, gaining mechanistic insight into melanoma metastasis and identifying therapeutic targets are critical for improved patient care.

The *gab* genes, encoding mammalian *Gab1*, *Gab2*, and *Gab3*, represent a family of scaffolding or docking adaptor proteins.³ Gab proteins are recruited to activated receptors by direct or indirect mechanisms, mostly indirectly via Grb2. They contain several functional motifs that mediate interactions with multiple signal relay molecules and can assemble multimeric signaling complexes. On stimulation, *Gab2* (Grb2-associated binding protein 2) undergoes tyrosine phosphorylation, creating a number of docking sites to mediate interactions with SH2 domain-containing proteins such as the tyrosine phos-

Supported by the National Institutes of Health (National Institute of Arthritis and Musculoskeletal and Skin Diseases grants AR050273 and CA133925 to J.T.C.; and National Cancer Institute grant CA016056 to Roswell Park Cancer Institute, Cancer Center Support Grant, and CA114277 to D.L.R.) and the Department of Defense (grant BC061955 to S.K.G.).

Accepted for publication January 9, 2009.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

D.L.R. has stock options in HistoRx.

Address reprint requests to Julide Tok Celebi, M.D., Columbia University, Department of Dermatology, Irving Cancer Research Center, 1130 St. Nicholas Ave., Room 321, New York, NY 10032. E-mail: jt165@columbia.edu.

phatase Shp2 and the p85 subunit of PI3K. The interaction of Gab2 with Shp2 activates Ras-Erk signaling, whereas its association with the p85 subunit of PI3K is crucial in mediating the PI3K-Akt signaling.^{3,4}

Recent studies provide evidence that Gab2 plays a critical role in human cancer. Gab2 is overexpressed in a subset of breast cancers.⁵ Its overexpression increases the proliferative capacity of mammary epithelial cells and alters growth factor dependence.⁶ Gab2 cooperates with receptor tyrosine kinases such as erbB2 or the Src family and promotes an invasive phenotype in breast tumorigenesis.^{6,7} In the Her2/Neu-induced Gab2 knockout mouse model, Gab2-deficient cells exhibit decreased migration and mice show reduced lung metastasis, suggesting its role in promoting mammary tumor metastasis.⁸ Gab2 maps to a region commonly amplified in several human malignancies and is amplified in a subset of Gab2-overexpressing breast tumors. In addition to breast tumorigenesis, Gab2-mediated signaling has been implicated in hematological malignancies. The Grb2-Gab2 pathway is critical for leukemic transformation by Bcr-Abl.⁹ Gab2 can be activated downstream of tyrosine kinases in hematopoietic cells and propagates signals for the transforming activity of those kinases. Although these studies suggest the importance of Gab2-mediated signaling in breast tumorigenesis and leukemia, its role in melanoma is unexplored.

In this study, we identified increased copy numbers of *Gab2* in metastatic melanomas through a genome-wide search using bacterial artificial chromosome (BAC) array comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays that led us to analyze its role in melanoma. We found that metastatic melanomas express significantly higher levels of Gab2 than primary melanomas and melanocytic nevi, identifying Gab2 as a molecular marker for neoplastic progression. Moreover, we show that Gab2 promotes tumor cell migration and invasion by activating Akt signaling, and enhances tumor growth and metastasis *in vivo*, suggesting a role for Gab2-mediated signaling in promoting metastatic capability in melanoma.

Materials and Methods

Tumor Specimens and Cell Lines

Sixty-four metastatic melanoma frozen tissue samples were obtained from the tissue banks of the Department of Pathology at Columbia University and the Department of Cancer Genetics at University of Münster, Fachklinik Hornheide, Germany. Tumors were examined histopathologically and those with at least 90% neoplastic tissue were selected. In some samples where tumor cells were admixed with a prominent lymphocytic infiltrate, cells were microdissected using the PixCell II laser capture microdissection system (Arcturus, Mountain View, CA). Twenty-three cell lines derived from primary and metastatic melanomas were obtained from the American Type Culture Collection (Rockville, MD) and the Wistar Institute (Philadelphia, PA) and cultured according to their instruc-

tions. A normal human melanocyte cell line was generated from neonatal foreskin as described¹⁰ and maintained in melanocyte growth medium-4 with supplements and growth factors (Lonza Inc., Allendale, NJ). The protocol was approved by Columbia University's Institutional Review Board.

BAC Array CGH

Arrays with ~19,000 RPCI-11 BAC clones providing an average resolution of 150 kb, were used.¹¹ BAC array CGH was performed as described previously.¹² Briefly, 2 μ g of reference and test sample genomic DNA were fluorescently labeled using the BioArray CGH labeling system (Enzo Life Sciences, Inc., New York, NY). The DNA was denatured in the presence of random primer at 99°C for 10 minutes, cooled to 4°C, labeled by adding dNTP-cyanine 3 mix (or dNTP-cyanine 5) and *Klenow*, and incubated overnight at 37°C. Hybridization to the 19K arrays was performed at 55°C for 16 hours using a GeneTAC hybridization station (Genomic Solutions, Inc., Ann Arbor, MI). The hybridized slides were scanned with a Genepix 4200A scanner (Molecular Devices, Inc., Sunnyvale, CA) and image analysis was performed with ImaGene software (Biodiscovery, Inc., El Segundo, CA). Log₂ ratios were computed and normalized using subgrid loess. Clones were ordered by chromosomal position according to the University of California-Santa Cruz human genome sequence build 36.1 (<http://genome.ucsc.edu>).

SNP Array

SNPs were genotyped and the copy numbers were estimated using 250K arrays (Affymetrix, Santa Clara, CA). Genomic DNA was cleaved with the restriction enzyme, *StyI*, ligated with linkers, followed by polymerase chain reaction (PCR) amplification. The PCR products were purified and digested with *DnaI* to a size ranging from 250 to 2000 bp. Fragmented PCR products were then labeled with biotin and hybridized to the array. Arrays were washed on the Affymetrix fluidics stations. The bound DNA was then fluorescently labeled using streptavidin-phycoerythrin conjugates and scanned using the Gene Chip Scanner 3000 (Affymetrix). DChip software was used for SNP array data analysis as described previously.¹³ Data were normalized to baseline array with median signal intensity at the probe intensity level using the invariant set normalization method. A model-based (PM-MM) method was used to obtain the signal values for each SNP in each array. To infer the DNA copy number from raw signal data, we used the Hidden-Markov model based on the assumption of diploidy for normal samples. Mapping information of SNP locations and cytogenetic band were based on curation of Affymetrix and University of California-Santa Cruz. A cutoff of >2.8 copies in more than three consecutive SNPs were defined as amplification.

Quantitative Real-Time PCR

cDNA was synthesized using the Superscript first strand synthesis kit as per instructions of the manufacturer (In-

vitrogen, Carlsbad, CA). One μg total RNA, 10 mmol/L dNTP mix, and 50 $\mu\text{mol/L}$ oligo(dT)₂₀ were incubated at 65°C for 5 minutes, placed on ice for 1 minute, and cDNA synthesis mix was added. The reaction was incubated at 50°C for 50 minutes and terminated at 85°C for 5 minutes. Real-time PCR was performed with 100 ng of input RNA per reaction containing 1 $\times$ TaqMan universal PCR Master mix and the appropriate TaqMan probe on a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA). *Gab2* (Hs00373045) and β -*Actin* (4326315E) TaqMan probes were purchased from Applied Biosystems. All samples were prepared in triplicates on each plate and at least three plates were analyzed. Relative mRNA levels were determined using the comparative C_T method.

Fluorescent in Situ Hybridization

RP11-653J20 and RP11-444N24 BAC clones were obtained from Invitrogen. DNA was prepared from BAC clones using standard methods and labeled by nick-translation using spectrum red or spectrum green dUTP fluorochromes. Spectrum red- or spectrum green-labeled centromeric probes were used to enumerate chromosome numbers (Vysis, Downers Grove, IL). Hybridization signals were scored on at least 20 metaphase spreads of cell lines or 200 interphase nuclei from tissue sections on 4,6-diamidino-2-phenylindole (DAPI)-counterstained slides.

Immunofluorescence and Automated Quantitative Analysis (AQUA) of Tissue Microarrays

For protein expression, tissue microarrays containing specimens obtained from melanocytic nevi ($n = 18$), primary melanomas ($n = 15$), and metastases ($n = 15$) were used. Immunofluorescence staining of tissue microarrays was performed as described previously¹⁴ using an anti-Gab2 antibody (Upstate, Charlottesville, VA). The AQUA software linked to the fluorescence microscopy system was used as described previously¹⁴ for quantification of the Gab2 protein within the tumor region of each tissue microarray core.

Immunoblotting and Antibodies

Cells were lysed with the PhosphoSafe extraction reagent (Novagen, San Diego, CA) containing protease and phosphatase inhibitors. Protein quantity was determined by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins were separated by sodium dodecyl sulfate gel electrophoresis under reducing conditions and then transferred to 0.45- μm nitrocellulose membranes by electroblotting. The membranes were blocked with 5% nonfat powdered milk in phosphate-buffered saline and then probed with primary antibodies at a 1:1000 dilution in TBST with 5% milk. The membranes were then washed with TBST and probed with horseradish peroxidase-conjugated secondary antibodies at 1:2000 dilutions in TBST with 5% milk. After washing in TBST, the membranes

were developed with the ECL Western blotting detection system (Pierce Biotech, Rockford, IL). The films were scanned using a Molecular Dynamics personal densitometer SI and the protein band density was quantified using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The following commercial antibodies were used in this study: Gab2 (Upstate); Gab1, phospho-Akt (Ser-473), total Akt, phospho-PDK1, total PDK1 (Cell Signaling, Danvers, MA); and actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Gene Knockdown Using siRNA

Small interfering RNA against Gab2 and control (scrambled) RNAs were purchased from Dharmacon (Lafayette, CO). Cells (1.5×10^5) were transfected with 300 pmol/L of siRNA using oligofectamine reagent in Opti-MEM I reduced serum medium (Invitrogen). Four hours later, the medium was replaced with culture medium supplemented with 10% fetal bovine serum. Forty-eight hours later the cells were harvested for immunoblotting.

Generation of Gab2-Expressing Stable Cell Lines

Primary melanoma cell lines (WM793, WM278, WM1552, and WM3862) expressing either Gab2 or vector were generated using retroviral transduction as described previously.^{15,16} *Gab2* full-length cDNA, obtained from Origene (Rockville, MD), was subcloned into pBABE-puro retroviral vector (Addgene, Cambridge, MA). Phoenix-Eco (ecotropic) packaging cells were transiently transfected with 20 μg of pBABE-puro retroviral vector by the CaPO₄ co-precipitation method. The packaged viruses released by the Phoenix-Eco cells were used for infecting AM12 (amphotropic) packaging cells to generate stable clones of virus-producing cells and to achieve high viral titers.¹⁵ Forty-eight hours after infection, the cells were split into puromycin selection medium (2 $\mu\text{g}/\text{ml}$) and surviving colonies were selected. Expression levels of Gab2 were evaluated by Western blot analysis of AM12 clones. Viral supernatant was then collected from these producer cells throughout a 24- to 48-hour period, filtered through a 0.45- μm filter to remove cell debris, and used for infecting the melanoma cells. Lysates from infected cells were subjected to Western blot analysis using an antibody against Gab2.

Migration and Invasion Assays

Cell migration was assessed by adding 1×10^5 cells into the upper chamber of a motility chamber system containing 8- μm pores (modified Boyden chamber assay; BD Biosciences, San Jose, CA). Invasion assays were performed by seeding 1×10^5 cells into Biocoat Matrigel invasion chambers (BD Biosciences) in serum-free medium, and by adding 15% fetal bovine serum to the lower wells as chemoattractant. For both assays, the cells were incubated for 24 hours, the filters were stained with crys-

Figure 1. Identification of increased copy numbers of 11q13.5-14.1 in melanoma. **A:** Hierarchical clustering of DNA copy number data from melanoma tumor samples and cell lines show subclusters delineating two independent amplicons on 11q13.2 and 11q13.5-14.1, harboring *cyclin D1* and *Gab2*, respectively. All of the 64 tumor samples consisted of metastatic melanomas. Of the 20 melanoma cell lines, 5 were derived from primary (WM39, WM1552, WM793, WM278, and WM35) and 15 from metastatic melanomas. **B:** 11q14.1 DNA copy number data showing focal amplifications with *Gab2* at the center of the amplicon. **C:** A representative case showing *Gab2* locus amplification by fluorescent *in situ* hybridization. **D:** SNP signal intensity colorgram verifying the 11q13.5-14.1 amplicon in melanoma samples. **E:** Gene expression analysis of *Gab2* by qRT-PCR in tumors with increased copy numbers of 11q14.1 shows consistent up-regulation of *Gab2* (means $\pm$ SD, $n = 3$, $P < 0.05$).

tal violet, and the number of cells that penetrated through the filter was counted under a light microscope at $\times 20$ magnification. For each membrane, the mean number of cells in three randomly selected fields was determined. For each assay, three independent experiments were performed in which the bars represent the mean $\pm$ SD.

In Vivo Tumorigenicity and Metastasis Assays

Tumor growth was evaluated by subcutaneous injection of 1×10^6 vector or *Gab2*-expressing stable cell lines into the flanks of 7-week-old female severe combined immunodeficient (SCID) mice ($n = 4$ in each group). Tumor length and width were measured using calipers. Mice were sacrificed when tumor surface area reached 1.5 cm^2 and tumors were harvested and examined histologically. For experimental metastasis assays, vector or *Gab2*-expressing stable cell lines were injected into SCID mice via the tail veins (1×10^6 cells per mouse, $n = 12$ in each group). Mice were sacrificed 3 weeks after inoculation, the lungs were harvested and examined histologi-

cally. Animal experiments were performed under a protocol approved by the Columbia University's Institutional Committee for Care and Use of Animals.

Statistical Analysis

Statistical analyses were performed with a *t*-test in which $P < 0.05$ was considered as statistically significant. Differences between the groups in the experimental metastasis assays were analyzed using Fisher's exact test.

Results

Increased DNA Copy Numbers of *Gab2* in Metastatic Melanoma

To identify genes critical for melanoma pathogenesis, we used BAC array CGH and SNP arrays to search for genome-wide copy number alterations in 64 metastatic melanoma samples and 20 melanoma cell lines. We

Figure 2. Gab2 is overexpressed in metastatic melanoma. Total cell lysates were isolated and Gab2 levels were measured by Western blot analysis. β -Actin was used as a loading control. Quantitation of Gab2 expression was determined by densitometry and was normalized with β -actin. The *BRAF* and *NRAS* mutation status of the cell lines is indicated. All melanoma cell lines with a *BRAF* mutation harbor the *BRAF*^{N600E} mutation. The WM1361 cell line has the *NRAS*^{Q61R} mutation. Gab2 is expressed at very low levels, if any, in a normal human melanocyte cell line and primary melanoma cells, whereas it is expressed at high levels in metastatic cell lines.

found several distinct amplicons on chromosome 11q. Among them, increased DNA copy numbers of the *cyclin D1* locus on 11q13.2, previously described for melanoma, was present in 5 of the 84 (6%) samples (1 of 64 tumors, 5 of 20 cell lines). More importantly, we noted a distinct amplicon, independent of *cyclin D1*, in 9 of the 84 (11%) samples (7 of 64 tumors, 2 of 20 cell lines), in which consistent focal amplifications with *Gab2* gene at the center of the amplicon were observed (Figure 1, A, B, and D). These data were further confirmed by fluorescence *in situ* hybridization analysis (Figure 1C). Six of the nine cases were available for evaluation of Gab2 mRNA expression by qRT-PCR, which showed consistent up-regulation in all six cases compared with a human melanocyte cell line and nonamplified tumor samples (Figure 1E). Identification of increased DNA copy numbers of *Gab2* among metastatic melanomas along with gene amplification in a subset of tumors resulted in further analysis of *Gab2* in melanoma.

Gab2 Is Overexpressed in Metastatic Melanoma

We next examined the expression profile of Gab2 in a normal human melanocyte cell line and a panel of melanoma cell lines by Western blot analysis. Gab2 protein was

expressed at low levels in the melanocyte cell line and in primary melanoma cells whereas high-level Gab2 expression was observed in metastatic cell lines (Figure 2). To confirm the above results and to investigate the expression profile of Gab2 during clinical melanoma progression, we extended our analysis by studying an independent series of melanocytic tumor samples, including melanocytic nevi, primary and metastatic melanomas. For these studies, we used the automated quantitative analysis (AQUA) system, which allows for rapid analysis of immunofluorescence on tissue microarrays, reduces the human variability of scoring by eye, and results in a continuous range of protein expression rather than ordinal (0, +1, +2, and +3) scores. It also allows for quantification of the protein of interest within user-defined subcellular compartments within the tumor region of each tissue microarray spot. Specifically, we performed AQUA analysis of Gab2 protein expression on 18 melanocytic nevi, 15 primary and 15 metastatic melanoma samples each represented by two tissue microarray spots. Results showed that Gab2 protein is expressed at significantly higher levels in metastatic melanomas compared with melanocytic nevi ($P = 0.0044$) and primary melanomas ($P = 0.0137$), identifying Gab2 as a molecular marker of neoplastic progression (Figure 3).

Figure 3. Gab2 expression correlates with clinical tumor progression. **A:** Quantitative analysis of Gab2 protein expression (AQUA-based analysis) in a series of nevi, primary melanomas, and metastatic melanomas is shown. Although the variance is large, there are significantly higher expression levels of nonnuclear Gab2 in metastatic lesions compared with both primary melanomas and nevi. The score for each case is the average of two observations from two unique tissue spots of a single block tissue microarray. Data shown in this figure is from 18 nevi and 15 primary tumors and 15 metastatic tumors. **B:** Representative examples of primary and metastatic melanoma immunostaining of Gab2 are shown.

Figure 4. Gab2 promotes tumor cell migration and invasion *in vitro*. **A** and **B**: Western blot analysis, migration and Matrigel invasion assays of control (scrambled) or *Gab2* knockdown with siRNA of metastatic melanoma cell lines (Mewo, A2058, and Ht144 cells) are shown. *Gab2* knockdown resulted in decreased migration (means $\pm$ SD, $n = 3$). *Gab2* silencing resulted in a significant reduction of the invasive ability of the cells (means $\pm$ SD, $n = 3$, $*P < 0.05$). **C** and **D**: Western blot analysis of forced *Gab2* expression in primary melanoma cell lines (WM793, WM278, WM1552, and WM3862) as well as migration and Matrigel invasion assay results are shown. Forced expression of *Gab2* in primary melanoma cells resulted in a significant increase of melanoma cell migration and invasion (means $\pm$ SD, $n = 3$, $*P < 0.05$). **E**: Representative examples of vector and *Gab2*-expressing primary melanoma cells (WM793) invading through the Matrigel are shown.

Gab2 Promotes Migration and Invasion of Melanoma Cells

To investigate the role of *Gab2* in tumor progression, we used siRNA technology to down-regulate *Gab2* in three metastatic melanoma cell lines that overexpress *Gab2*; Mewo, Ht-144, and A2058. *Gab2* silencing did not affect *Gab1* expression (Figure 4A). As shown in Figure 4B, *Gab2* knockdown decreased the migratory potential of tumor cells and led to a significant decrease in their invasive capacity ($P < 0.05$), suggesting that continuous *Gab2* expression is required for *in vitro* invasion of metastatic melanoma cells. We next determined whether *Gab2* overexpression could enhance migration and invasiveness of primary melanoma cells. For these experiments, we used WM793, WM278, WM1552, and WM3862 cells that are derived from primary melanomas and have low or undetectable levels of *Gab2*. All of these cell lines except WM3862 cells harbor the *BRAF*^{V600E} mutation. Forced expression of *Gab2* in these cells led to a signif-

icant enhancement of migration and invasion ($P < 0.05$) (Figure 4, C–E). Taken together, these studies show that *Gab2* is capable of enhancing motility and invasive capabilities of primary melanoma cells.

Gab2 Accelerates Tumorigenic Potential and Induces Metastasis in Vivo

To study the effects of *Gab2* on tumor growth *in vivo*, we injected WM3862 primary melanoma cells with forced *Gab2* expression or vector alone subcutaneously into SCID mice. The mice were sacrificed when tumor surface area reached 1.5 cm². All mice injected with *Gab2*-overexpressing cells reached the tumor burden threshold between days 16 to 21, whereas mice injected with vector alone reached this threshold between days 26 to 46. The survival curves in Figure 5A indicate that tumor growth was significantly increased in response to forced *Gab2* expression suggesting a critical role for *Gab2* in promoting tumor

Figure 5. Gab2 enhances tumor growth and metastatic capability of melanoma cells *in vivo*. **A:** *In vivo* tumor growth of the WM3862 primary melanoma cell line expressing either vector or Gab2 was determined. Mice were injected with 1×10^6 cells expressing either Gab2 or vector alone subcutaneously. Tumors were measured with a caliper in two dimensions and mice were sacrificed when tumor surface area reached 1.5 cm^2 . Kaplan-Meier curves show that mice injected with Gab2-overexpressing cells reached tumor burden threshold faster than the vector group ($*P < 0.05$). All mice injected with Gab2-overexpressing cells reached the tumor burden threshold between days 16 to 21, whereas mice injected with vector alone reached this threshold between days 26 to 46. **B:** Representative tumor sections stained with H&E and Gab2 (by immunohistochemistry) are shown. Note that Gab2 overexpression alters melanoma cell morphology from epitheloid to spindle in subcutaneous tumors. **C:** *In vivo* metastasis of WM3862 cell line expressing either vector or Gab2 was evaluated. Mice were injected with vector or Gab2-overexpressing WM3862 cells via the tail veins. Seven of eleven (63%) mice in the Gab2-overexpressing group developed lung metastasis whereas none in the vector group had lung tumors ($*P < 0.05$). **D:** Representative examples of lung sections stained with H&E and Gab2 are shown. Note that Gab2-expressing tumor cells form nodules within the lung and invade into the lung parenchyma.

growth (Figure 5, A and B; $P < 0.05$). Intriguingly, WM3862 cells expressing vector alone led to pigmented tumors in SCID mice, whereas WM3862 cells overexpressing Gab2 were amelanotic (see Supplemental Figure 1 at <http://ajp.amjpathol.org>). We then tested whether Gab2 activation confers a metastatic behavior in Gab2-overexpressing WM3862 cells in an experimental metastasis assay. Vector or Gab2-overexpressing cell lines were injected into SCID mice via the tail veins and animals were assessed for lung metastases 3 weeks after inoculation. One mouse in the Gab2-overexpressing group died prematurely and could not be evaluated. We found tumor cells invading the lung parenchyma in 7 of 11 (63%) mice

injected with Gab2-overexpressing WM3862 cells, whereas none of the mice injected with WM3862 cells expressing vector alone showed any evidence of lung metastases on multiple sections (Figure 5, C and D; $P < 0.05$). These results suggest that Gab2 increases the intrinsic ability of melanoma cells to metastasize *in vivo*.

PI3K-Akt Signaling Is Crucial for Gab2-Mediated Metastatic Phenotype

To gain mechanistic insight into signaling pathways that may contribute to Gab2-mediated invasive phenotype in

Figure 6. Gab2 promotes tumor cell migration and invasion via Akt activation. **A:** Gab2 silencing in the Mewo metastatic melanoma cell line using siRNA technology shows decreased Akt phosphorylation. **B:** Western blot analysis showing vector or Gab2 forced expression into primary melanoma cell lines WM793, WM278, WM1552, and WM3862 after serum starvation for 24 hours. Gab2 overexpression results in Akt and PDK1 phosphorylation in melanoma cells. **C:** Treatment of Gab2-overexpressing primary melanoma cells with the PI3K inhibitor, LY294002 (25 μ mol/L), for 24 hours results in a significant decrease in tumor cell migration and invasion potential (means $\pm$ SD, $n = 3$, $*P < 0.05$).

melanoma cells, we examined AKT activation. Silencing of *Gab2* in a Gab2-overexpressing metastatic melanoma cell line, Mewo, resulted in a marked decrease in Akt phosphorylation (Figure 6A). Furthermore, in a reciprocal experiment, forced expression of Gab2 into four primary melanoma cell lines with low endogenous Gab2 levels (WM793, WM278, WM1552, WM 3862; Figure 2) increased Akt activation in the absence of exogenous growth factors (Figure 6B), indicating that Gab2 activation leads to stimulation of Akt in melanoma cells. Because PDK1 is a downstream effector of PI3K that is recruited to the plasma membrane by phosphatidylinositol 3-phosphate (PIP₃) and phosphorylates the activation loop of Akt and is critical for regulating cell migration, we examined its activation in cells with ectopic Gab2 expression. All melanoma cells with Gab2 overexpression also showed increased PDK1 phosphorylation (Figure 6B), suggesting that Gab2 activation in melanoma cells may be resulting in Akt phosphorylation by signaling through PDK1.

To explore the possibility that Gab2 mediates the invasive phenotype via PI3K-Akt signaling, we examined the effect of the PI3K inhibitor, LY294002, on *in vitro* cell migration and invasion. Inhibition of PI3K-Akt signaling in Gab2-overexpressing melanoma cells decreased migration and invasive capability significantly ($P < 0.05$, Figure 6C). Collectively, these data suggest that Gab2-mediated signaling results in Akt stimulation and promotes an invasive and metastatic phenotype in melanoma cells via PI3K/PDK1/Akt signaling.

Discussion

Metastasis is a multistep process that requires the cell's ability to migrate and invade. In melanoma, both Erk and

Akt activation are implicated in tumor cell migration and invasion. *BRAF*^{V600E} and MEK activity are required for melanoma cell invasion *in vitro*.¹⁷ Silencing of MMP-1, which is activated by Erk signaling, decreases metastatic capability of melanoma cells by inhibiting tumor cell collagenase activity and tumor-induced angiogenesis.¹⁸ Mutant *BRAF* in melanoma cells disrupts actin cytoskeletal organization and focal adhesion dynamics through suppression of Rho/ROCK/LIM kinase/cofilin pathway.¹⁹ Blocking PI3K signaling in melanoma cells suppresses cell migration with concomitant increase in E-cadherin.²⁰ Combined targeting of both Ras-Erk and PI3K-Akt signaling pathways decreases invasiveness of melanoma cells suggesting a promising treatment strategy.²¹ In this study, we show that Gab2 confers an invasive phenotype and drives progression of primary melanoma cells and enhances their metastatic capability via activation of the PI3K-Akt pathway. Gab2 expression correlates with clinical tumor progression in which higher levels of Gab2 are seen in metastatic melanomas. Collectively, our data highlight the role of a previously unexplored molecule in melanoma, Gab2, in tumor progression and metastasis.

Alterations of both Ras-Erk and PI3K-Akt pathways are frequent in melanoma and occur in concert.²² Gain-of-function mutations in *NRAS* or *BRAF* lead to Erk activation.²³ *NRAS* is mutated in 10 to 15% and *BRAF* is mutated in 50 to 70% of melanomas, and are mutually exclusive.²⁴ Co-expression of *NRAS*^{Q61R} and *BRAF*^{V600E} results in a senescent phenotype in melanoma cells, thus explaining the epistatic relationship because of selection against double-mutant cells.²⁵ PTEN function is lost in between 5 to 20% of late-stage melanomas leading to PI3K-Akt pathway activation²⁶ and Akt is overexpressed in up to 60% of melanomas.²⁷ *NRAS* mutations and *PTEN*

inactivation occurs reciprocally in melanoma, which is thought to be attributable to activated *NRAS* being able to stimulate both Erk and Akt signaling pathways, and when present, not requiring *PTEN* deregulation. Ras transforms melanocytes more efficiently than BRAF possibly because of the PI3K component.²⁸ Gab2 can stimulate both Erk and Akt signaling through its interactions with Shp2 and p85 subunit of PI3K, respectively. In this study, the finding that Gab2 overexpression co-existed with *BRAF*^{V600E} in several melanoma cell lines (Ht-144, Mel-1, A2058) and that Gab2 overexpression in primary melanoma cell lines with *BRAF*^{V600E} resulted in increased invasiveness by activating Akt signaling, suggests that Gab2 may be cooperating with oncogenic BRAF to for the acquisition of an invasive and metastatic phenotype in these cells. The relationship between increased DNA copy numbers of *Gab2* and Gab2 overexpression in the context of *NRAS* and *BRAF* mutations and PTEN inactivation remains to be determined.

11q13-14 amplification is observed in several types of malignancy including melanoma,²⁹ breast,^{30,31} and ovarian cancer.³² The amplification of this region was originally thought to involve a single amplicon spanning many megabases; however, fine mapping of the region has identified four independent core regions.³⁰ Core 3 harbors *cyclin D1*, the overexpression of which is known to contribute to carcinogenesis of various tissue types including melanoma.^{30,33} The amplification frequency of the *cyclin D1* locus in primary melanoma is ~11% and is more frequent in acral melanoma subtype.³³ Down-regulation of *cyclin D1* expression in melanoma cell lines reduces cell proliferation and induces apoptosis *in vitro*, and thus *cyclin D1* has been suggested as an oncogene in melanoma driving the 11q13 amplicon.³³ Recently, *Gab2* has been identified as a candidate for core 1 that contributes to breast cancer.⁶ Studies provide evidence that amplification of *Gab2* is a candidate mechanism for Gab2 overexpression in breast tumors with 11q13-14.1 amplification.⁶ In this study, we show that increased copy numbers of *Gab2* is seen in 11% of melanoma tumor samples and is independent of *cyclin D1* locus, suggesting that *Gab2* region is not amplified as a passenger to *cyclin D1* in melanoma. Our results show that *Gab2* is amplified in a subset of Gab2-overexpressing melanomas.

Acknowledgments

We thank Paul Quinn, Devin McQuaid, and Sameena Azmi for technical support.

References

- Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ: Cancer statistics, 2006. *CA Cancer J Clin* 2006, 56:106–130
- Tsao H, Atkins MB, Sober AJ: Management of cutaneous melanoma. *N Engl J Med* 2004, 351:998–1012
- Liu Y, Rohrschneider LR: The gift of Gab. *FEBS Lett* 2002, 515:1–7
- Gu H, Neel BG: The “Gab” in signal transduction. *Trends Cell Biol* 2003, 13:122–130
- Daly RJ, Gu H, Parmar J, Malaney S, Lyons RJ, Kairouz R, Head DR, Henshall SM, Neel BG, Sutherland RL: The docking protein Gab2 is overexpressed and estrogen regulated in human breast cancer. *Oncogene* 2002, 21:5175–5181
- Bentires-Alj M, Gil SG, Chan R, Wang ZC, Wang Y, Imanaka N, Harris LN, Richardson A, Neel BG, Gu H: A role for the scaffolding adapter GAB2 in breast cancer. *Nat Med* 2006, 12:114–121
- Bennett HL, Brummer T, Jeanes A, Yap AS, Daly RJ: Gab2 and Src co-operate in human mammary epithelial cells to promote growth factor independence and disruption of acinar morphogenesis. *Oncogene* 2008, 27:2693–2704
- Ke Y, Wu D, Princen F, Nguyen T, Pang Y, Lesperance J, Muller WJ, Oshima RG, Feng GS: Role of Gab2 in mammary tumorigenesis and metastasis. *Oncogene* 2007, 26:4951–4960
- Sattler M, Mohi MG, Pride YB, Quinnan LR, Malouf NA, Podar K, Gesbert F, Iwasaki H, Li S, Van Etten RA, Gu H, Griffin JD, Neel BG: Critical role for Gab2 in transformation by BCR/ABL. *Cancer Cell* 2002, 1:479–492
- Valyi-Nagy IT, Hirka G, Jensen PJ, Shih IM, Juhasz I, Herlyn M: Undifferentiated keratinocytes control growth, morphology, and antigen expression of normal melanocytes through cell-cell contact. *Lab Invest* 1993, 69:152–159
- Osoegawa K, Mammoser AG, Wu C, Frengen E, Zeng C, Catanese JJ, de Jong PJ: A bacterial artificial chromosome library for sequencing the complete human genome. *Genome Res* 2001, 11:483–496
- Nowak NJ, Miecznikowski J, Moore S, Gaile D, Bobadilla D, Smith DD, Kernstine K, Forman SJ, Mhawech-Fauceglia P, Reid M, Stoler D, Loree T, Rigual N, Sullivan M, Weiss LM, Hicks D, Slovak ML: Challenges in array CGH for the analysis of cancer samples. *Genet Med* 2007, 9:585–595
- Zhao X, Weir BA, LaFramboise T, Lin M, Beroukhir R, Garraway L, Beheshti J, Lee JC, Naoki K, Richards WG, Sugarbaker D, Chen F, Rubin MA, Janne PA, Girard L, Minna J, Christiani D, Li C, Sellers WR, Meyerson M: Homozygous deletions and chromosome amplifications in human lung carcinomas revealed by single nucleotide polymorphism array analysis. *Cancer Res* 2005, 65:5561–5570
- Camp RL, Chung GG, Rimm DL: Automated subcellular localization and quantification of protein expression in tissue microarrays. *Nat Med* 2002, 8:1323–1327
- Levy L, Broad S, Zhu AJ, Carroll JM, Khazaal I, Peault B, Watt FM: Optimised retroviral infection of human epidermal keratinocytes: long-term expression of transduced integrin gene following grafting on to SCID mice. *Gene Ther* 1998, 5:913–922
- Owens DM, Romero MR, Gardner C, Watt FM: Suprabasal alpha6beta4 integrin expression in epidermis results in enhanced tumorigenesis and disruption of TGFbeta signalling. *J Cell Sci* 2003, 116:3783–3791
- Huntington JT, Shields JM, Der CJ, Wyatt CA, Benbow U, Slingluff Jr CL, Brinckerhoff CE: Overexpression of collagenase 1 (MMP-1) is mediated by the ERK pathway in invasive melanoma cells: role of BRAF mutation and fibroblast growth factor signaling. *J Biol Chem* 2004, 279:33168–33176
- Blackburn JS, Rhodes CH, Coon CI, Brinckerhoff CE: RNA interference inhibition of matrix metalloproteinase-1 prevents melanoma metastasis by reducing tumor collagenase activity and angiogenesis. *Cancer Res* 2007, 67:10849–10858
- Klein RM, Spofford LS, Abel EV, Ortiz A, Aplin AE: B-RAF regulation of Rnd3 participates in actin cytoskeletal and focal adhesion organization. *Mol Biol Cell* 2008, 19:498–508
- Stewart AL, Mhashikar AM, Yang XH, Ekmekcioglu S, Saito Y, Sieger K, Schrock R, Onishi E, Swanson X, Mumm JB, Zumstein L, Watson GJ, Snary D, Roth JA, Grimm EA, Ramesh R, Chada S: PI3 kinase blockade by Ad-PTEN inhibits invasion and induces apoptosis in RGP and metastatic melanoma cells. *Mol Med* 2002, 8:451–461
- Meier F, Busch S, Lasithiotakis K, Kulms D, Garbe C, Maczey E, Herlyn M, Schitteck B: Combined targeting of MAPK and AKT signalling pathways is a promising strategy for melanoma treatment. *Br J Dermatol* 2007, 156:1204–1213
- Vivanco I, Sawyers CL: The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2002, 2:489–501
- Cohen C, Zavala-Pompa A, Sequeira JH, Shoji M, Sexton DG, Cotsonis G, Cerimele F, Govindarajan B, Macaron N, Arbisser JL: Mitogen-activated protein kinase activation is an early event in melanoma progression. *Clin Cancer Res* 2002, 8:3728–3733

24. Gray-Schopfer V, Wellbrock C, Marais R: Melanoma biology and new targeted therapy. *Nature* 2007, 445:851–857
25. Petti C, Molla A, Vegetti C, Ferrone S, Anichini A, Sensi M: Coexpression of NRASQ61R and BRAFV600E in human melanoma cells activates senescence and increases susceptibility to cell-mediated cytotoxicity. *Cancer Res* 2006, 66:6503–6511
26. Wu H, Goel V, Haluska FG: PTEN signaling pathways in melanoma. *Oncogene* 2003, 22:3113–3122
27. Stahl JM, Sharma A, Cheung M, Zimmerman M, Cheng JQ, Bosenberg MW, Kester M, Sandirasegarane L, Robertson GP: Deregulated Akt3 activity promotes development of malignant melanoma. *Cancer Res* 2004, 64:7002–7010
28. Chudnovsky Y, Adams AE, Robbins PB, Lin Q, Khavari PA: Use of human tissue to assess the oncogenic activity of melanoma-associated mutations. *Nat Genet* 2005, 37:745–749
29. Bastian BC, Kashani-Sabet M, Hamm H, Godfrey T, Moore II DH, Bocker EB, LeBoit PE, Pinkel D: Gene amplifications characterize acral melanoma and permit the detection of occult tumor cells in the surrounding skin. *Cancer Res* 2000, 60:1968–1973
30. Ormandy CJ, Musgrove EA, Hui R, Daly RJ, Sutherland RL: Cyclin D1, EMS1 and 11q13 amplification in breast cancer. *Breast Cancer Res Treat* 2003, 78:323–335
31. Bekri S, Adelaide J, Merscher S, Grosgeorge J, Caroli-Bosc F, Perucca-Lostanlen D, Kelley PM, Pebusque MJ, Theillet C, Birnbaum D, Gaudray P: Detailed map of a region commonly amplified at 11q13->q14 in human breast carcinoma. *Cytogenet Cell Genet* 1997, 79:125–131
32. Lambros MB, Fiegler H, Jones A, Gorman P, Roylance RR, Carter NP, Tomlinson IP: Analysis of ovarian cancer cell lines using array-based comparative genomic hybridization. *J Pathol* 2005, 205:29–40
33. Sauter ER, Yeo UC, von Stemm A, Zhu W, Litwin S, Tichansky DS, Pistrutto G, Nesbit M, Pinkel D, Herlyn M, Bastian BC: Cyclin D1 is a candidate oncogene in cutaneous melanoma. *Cancer Res* 2002, 62:3200–3206