A Purine Nucleotide Biosynthesis Enzyme Guanosine Monophosphate Reductase is a Suppressor of Melanoma Invasion

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SUMMARY

Melanoma is one of the most aggressive types of human cancers, and the mechanisms underlying melanoma invasive phenotype are not completely understood. Here, we report that expression of guanosine monophosphate reductase (GMPR), an enzyme involved in *de novo* biosynthesis of purine nucleotides, was down-regulated in invasive stages of human melanoma. Loss- and gain-of-function experiments revealed that GMPR down-regulates the amounts of several GTP-bound (active) RHO-GTPases, suppresses the ability of melanoma cells to form invadopodia, degrade extracellular matrix and invade *in vitro* and grow as tumor xenografts *in vivo*. Mechanistically, we demonstrated that GMPR partially depletes intracellular GTP pools. Pharmacological inhibition of *de novo* GTP biosynthesis suppressed, whereas addition of exogenous guanosine increased invasion of melanoma cells as well as cells from other cancer types. Our data identified GMPR as a melanoma invasion suppressor, and established a link between guanosine metabolism and RHO-GTPase-dependent melanoma cell invasion.

INTRODUCTION

Acquisition of the invasive phenotype, a critical event for melanoma metastasis, is initiated in primary cutaneous melanoma. Although various characteristics of primary melanoma such as Breslow thickness determine clinical prognosis, the mechanisms underlying this

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invasive process are not completely understood (Balch et al., 2009, Haass and Smalley, 2009, Leong et al., 2012). One of the major prerequisites for the invasion of malignant cells is the ability to degrade the extra-cellular matrix (ECM) and the underlying basement membrane in order to escape the primary site of growth (Friedl and Alexander, 2011; Lu et al., 2011) Many factors can influence these properties, including formation of invadopodia, specialized subcellular actin-rich structures that recruit proteolytic enzymes to the areas of cell-ECM contact (Caldieri et al., 2009, Ridley, 2011). In many types of cancer, including melanoma, invasion and the ability to form invadopodia have been strongly associated with the activity of small GTPases, in particular those of the RHO-GTPase family (Buccione et al., 2009, Struckhoff et al., 2011).

RHO-GTPases (including most studied members RHOA, RHOC, RAC1, and CDC42) are small 21-KDa proteins that regulate formation of actin structures and processes associated with these structures, including adhesion, migration, and invasion (Takai et al., 2001, Kaibuchi et al., 1999, Ridley, 2006). In their active, GTP-bound, state these small GTPases interact with down-stream effectors to initiate and/or propagate signaling events. Hydrolysis of GTP to GDP renders the GTPases inactive (Takai et al., 2001, Van Aelst and D'Souza-Schorey, 1997). Although small GTPases have an intrinsic GTP hydrolyzing activity, the spontaneous reactions of hydrolysis and subsequent GDP to GTP nucleotide exchange are extremely slow. These processes are regulated by GTPases-activating proteins (GAPs) that enhance intrinsic GTPase activity, guanine nucleotide exchange factors (GEFs) that promote exchange of GDP for GTP (Schmidt and Hall, 2002, Moon and Zheng, 2003) and the guanine nucleotide dissociation inhibitors (GDIs) that maintain GTPase in inactive form in the cytoplasm (Moon and Zheng, 2003). Activities of GAPs, GEFs and GDIs are in turn regulated by multiple signal cascades (Moon and Zheng, 2003, Van Aelst and D'Souza-Schorey, 1997). The question of whether tumor cells possess intrinsic ability to regulate invasion and activity of the above GTPases by manipulating intracellular GTP pools has never been addressed.

Neoplastic cells, including melanoma, are highly dependent on *de novo* biosynthesis of purine and pyrimidine nucleotides (Dang, 2012, Tong et al., 2009) and enzymes involved in these pathways are substantially up-regulated in cancer cells (Liu et al., 2008, Mannava et al., 2008). *De novo* biosynthesis of GMP requires several enzymes including inositol monophosphate dehydrogenase 1 and 2 (IMPDH1 and IMPDH2) that convert inositol monophosphate (IMP) into xanthosine monophosphate (XMP) (Collart and Huberman, 1988), and guanosine monophosphate synthetase (GMPS) that converts XMP into guanosine monophosphate (GMP) (Zalkin, 1985) (Figure 1A). A reverse reaction, catalyzed by guanosine monophosphate reductase (GMPR) (Spector et al., 1979), converts GMP to IMP to fuel back into both the AMP and GMP synthesis pathways (Figure 1A). IMPDH2 has been functionally linked to cell proliferation and carcinogenesis and its levels were suppressed in arrested cells (Jayaram et al., 1999, Mannava et al., 2008, Nagai et al., 1992). The functional role of GMPR in the biology of cancer cells has never been addressed.

The current work presents evidence for a previously unrecognized ability of cancer cells to increase the activity of RHO-GTPases, leading to formation of invadopodia and invasion *via* up-regulation of GTP pools, and identifies GMPR as a potential tumor suppressor that inhibits this regulatory pathway in tumor cells. In addition, using human samples representative of invasive cutaneous and metastatic melanoma we validated our findings in a clinical setting.

RESULTS

Expression levels of GMPR and IMPDH2 are altered in metastatic melanoma cells

To investigate the role of intracellular GTP metabolism in tumor progression of melanocytic cells, we compared protein levels of the enzymes involved in the *de novo* GTP biosynthesis (Figure 1A) in a panel of 3 independently isolated populations of normal human melanocytes (NHM) and 9 arbitrarily chosen human metastatic melanoma cell lines. All populations of melanocytes expressed readily detectable levels of GMPR, in striking contrast with cells from the melanoma panel where GMPR expression was undetectable in all but one melanoma cell line (Figure 1B). A strong suppression of GMPR expression in the majority of metastatic melanoma cell lines was also confirmed at the mRNA level using quantitative reverse-transcription PCR (Figure S1A). Previously, we have demonstrated that levels of IMPDH2 were up-regulated in metastatic melanoma cells (Mannava et al., 2008). We confirmed these findings here as IMPDH2 expression levels were higher in all studied melanoma cells compared to NHM (Figure 1B). GMPS expression levels showed little variation across all examined cell lines and populations (Figure 1B). These data suggest that expression of GMPR and IMPDH2 may be altered in the course of melanoma progression.

GMPR expression is associated with the invasive phenotype in clinical settings

To authenticate the specificity of GMPR and IMPDH2 antibodies used in Figure 1B in an immunohistochemistry (IHC) settings, cell pellets from two populations of NHM and cells from several melanoma lines were formalin-fixed, paraffin-embedded and stained using the above antibodies the same way as patient melanoma specimens (see Extended Experimental Procedures), following by scoring for the intensity of staining. Expression levels of IMPDH2 or GMPR in NHM versus melanoma cells determined by western blotting correlated well with the expression levels identified by IHC staining (Figure S1B).

To validate the expression pattern of GMPR and IMPDH2 observed in cultured cells, we analyzed their protein expression levels by IHC in 249 human melanoma specimens. For the purpose of comparison, all patients were divided into one of three cohorts: primary cutaneous thin melanoma (Breslow thickness 2 mm); primary cutaneous thick melanoma (Breslow thickness > 2 mm), and metastatic melanoma, consisting of 36, 46, and 167 patients respectively (see Extended Experimental Procedures). Melanoma specimens were scored for intensity of staining and percentage of stained cells (see Experimental Procedures). An IHC index was calculated as a product of these parameters and used to semi-categorically assess GMPR and IMPDH2 expression levels (Figure 1C–D). A statistically significant correlation was identified between GMPR expression and Breslow thickness for the primary cutaneous cohorts. The cohort of thin melanomas showed higher GMPR expression than the cohort of thick melanomas (Figure 1C). Importantly and in accordance with the findings in the primary cutaneous cohorts, GMPR expression was significantly lower in metastatic melanomas compared to the thick melanomas cohort (Figure 1C). Unlike GMPR, expression levels of IMPDH2 did not differ between thin and thick melanomas or between the cutaneous and metastatic cohorts (Figure 1C), although IMPDH2 expression was higher in metastatic melanomas compared to thin melanomas. No correlation existed between GMPR or IMPDH2 expression and outcome (metastatic disease) within the thin or thick melanoma cohort (Table S1), suggesting that the above observations are directly related to the invasive process and not to other confounding factors. Additionally, no correlation was found between the GMPR or IMPDH2 expression and patient age for all cohorts, or anatomical location in the primary melanoma cohorts (head and neck, limbs, trunk) (see Table S1). These data demonstrate that expression of GMPR is suppressed in invasive cutaneous and metastatic melanoma.

GMPR enzymatic activity affects melanoma cell invasion

Invasion is one of the major features distinguishing thick and thin cutaneous melanomas (Balch et al., 2009). Our findings that GMPR levels are decreased in thick melanomas imply that GMPR may negatively regulate invasion. To test this hypothesis, we restored GMPR levels in highly invasive human melanoma cells SK-Mel-103 and SK-Mel-147 *via* lentivirus-based transduction of human GMPR cDNA. By adjusting the lentiviral titer, we were able to achieve physiological levels of exogenous GMPR comparable to the endogenous GMPR levels found in NHM (Figure 2A). Control and GMPR-expressing cells displayed similar proliferative rates, as determined by incorporation of the deoxyuridine analog EdU (Figure 2B). Nonetheless, melanoma cells ectopically expressing GMPR demonstrated a substantial impairment in the ability to invade through Matrigel matrix (Figure 2C).

To investigate whether GMPR enzymatic activity is required for the suppression of melanoma cells invasion, we generated a putatively catalytically-inactive mutant of human GMPR by substituting cysteine with alanine at position 186 (GMPR^{C186A}). This mutation has been previously shown to abolish catalytic activity of GMPR2, a conserved homolog of GMPR, *in vitro* (Li et al., 2006). Overexpression of GMPR^{C186A} at levels comparable to overexpression of wild-type GMPR (Figure 2D) did not affect invasion (Figure 2E), suggesting that enzymatic activity of GMPR is essential for its ability to inhibit invasion of melanoma cells.

Although it has never been shown in cells, by converting GMP to IMP, GMPR may negatively regulate cellular GTP pools (Figure 1A). To determine if this was the case, we measured amounts of ribonucleoside triphosphates in SK-Mel-103 cells expressing vector or GMPR cDNA. We found that GTP levels were reproducibly lower by $\sim$24% in cells expressing GMPR compared to control cells (Figure 2F). Levels of ATP remained virtually unchanged (95.8±0.3% of controls), whereas the amounts of CTP and UTP were slightly elevated in GMPR-expressing cells (by 9.57±0.5% and 17.7±0.3%, respectively).

Recently, Arozarena et al reported that depletion of cyclic GMP (cGMP) *via* up-regulation of cGMP-specific phosphodiesterase PDE5A affects melanoma cell invasion (Arozarena et al., 2011). Therefore, we measured the levels of cGMP in control and GMPR-expressing SK-Mel-103 cells and found that GMPR did not affect cGMP amounts (Figure S2A).

To confirm that depletion of GTP pools is critical for GMPR-dependent inhibition of melanoma cell invasion, control and GMPR-overexpressing cells were tested for invasion and proliferation in media supplemented with 100 μM of exogenous guanosine (a concentration commonly used to reverse intracellular GTP depletion in mammalian cells (Gu et al., 2003, Laliberte et al., 1998)). Addition of guanosine did not increase proliferation rates of GMPR-expressing melanoma cells but substantially increased their invasion (Figure 2G). The invasion of control cells incubated with guanosine was also increased (Figure 2G). Importantly, manipulations of GMPR expression levels or addition of exogenous guanosine, produced similar results in human breast carcinoma cells MDA-MB-231 cells and human colon carcinoma cells HCT116 (Figure 2H, Figure S2B).

All together, these results suggest that GMPR and GTP pools play an important role in invasion of different types of tumor cells.

GMPR affects formation of invadopodia and matrix degradation

Invasion has been in many cases directly linked to their ability of tumor cells to form invadopodia, actin-rich membrane structures that are capable of recruiting proteolytic activities to induce localized degradation of the extracellular matrix (Caldieri et al., 2009,

Nurnberg et al., 2011, Ridley, 2011). To detect active invadopodia, melanoma cells were plated on FITC-conjugated gelatin and active invadopodia were visualized using microscopy by matching immunofluorescent actin puncta with areas of gelatin degradation (Figure 3A) as described earlier (Branch et al., 2012, Alexander et al., 2008). This methodology allowed us to determine the number of cells with active invadopodia, the number of active invadopodia per cell and the area of gelatin degradation per cell (Figure 3B). In agreement with the data described above, ectopic expression of GMPR substantially affected the ability of melanoma cells to form invadopodia and to degrade gelatin, whereas addition of 100 μM guanosine completely alleviated the negative effects of GMPR (Figure 3A). Taken together, these data characterize GMPR as a negative regulator of invadopodia formation and matrix degradation, therefore providing a mechanistic explanation to the hindered invasion potential of melanoma cells overexpressing GMPR.

GMPR affects melanoma cell tumorigenicity

Invasion as a mean for release of space constraints has been shown to contribute to the growth of sub-cutaneous tumor cell xenografts in immuno-compromised mice (Hotary et al., 2003). Therefore, we were interested in testing whether GMPR would affect cell tumorigenicity as well. To this end, SK-Mel-103 and SK-Mel-147 cells transduced with empty lentiviral vector or vector encoding GMPR cDNA were inoculated subcutaneously in both flanks of athymic nude mice (10 mice per condition). We found that ectopic expression of GMPR in SK-Mel-103 and SK-Mel-147 cells significantly increased latency of tumors (calculated as the time period from inoculation date to the appearance of tumors at 50% of inoculated sites) compared to controls: 13 days versus 7 days in SK-Mel-103 cells and 13 days versus 7 days in SK-Mel-147 cells (Figure 3C). GMPR overexpression also suppressed growth of tumor xenografts derived from SK-Mel-103 and SK-Mel-147 cells (Figure 3D). Additionally, fluorescence microscopy showed that tumor xenografts originated from GMPR-over-expressing SK-Mel-103 cells grew in the subcutaneous tissue (panniculus adiposus) without invading the muscular layer (panniculus carnosus) that separates dermis and subcutaneous adipose tissue (Figure S3A). On the other hand, control xenografts showed more aggressive pattern of growth with invasion through the muscular layer into the epidermis. (Figure S3A). To verify that the xenografts retained GMPR expression, GMPR levels were assessed in a separate experiment in transduced SK-Mel-103 cells before inoculation and in individual tumors 11 and 15 days after inoculation. As shown in Figure S3B, GMPR over-expression was retained throughout the course of the experiments. Additionally, we evaluated colony forming potential of vector- and GMPR-expressing cells in semi-solid agarose. Agarose cannot be metabolized by mammalian cells, and therefore the ability of a cell to degrade extracellular matrix should not influence its ability to form colonies in this assay. Indeed, no significant difference in the number or size of colonies was identified between vector- and GMPR-expressing cells (Figure 3E), suggesting that the xenograft latency was not due to a change in anchorage-independent growth. To ascertain that ectopic expression of GMPR was not suppressed in the course of the experiments, GMPR levels were measured in cells cultured for 12 days on a plate or in semi-solid conditions. As shown in Figure S3C, the GMPR levels were retained throughout the experimental time-frame.

Suppression of GMPR promotes invasion and tumorigenicity of melanoma cells

Among tested melanoma cells lines, only SK-Mel-28 cells expressed measurable amounts of GMPR (Figure 1B, Figure S1A). These cells demonstrated proliferation rates similar to those of SK-Mel-103 and SK-Mel-147 cells (Figure 4A), however their invasion index was several fold lower (Figure 4B). Thus, SK-Mel-28 cells represent a convenient model to study the effects of GMPR depletion on melanoma cell invasion. SK-Mel-28 cells were transduced in parallel with two GMPR shRNAs or with control shRNA (Figure 4C). The

transduced populations were tested for proliferation (Figure 4D), Matrigel-invasion (Figure 4E) and combined gelatin degradation assay (Figure 4F,G). In good agreement with the above observations, we found that depletion of GMPR resulted in the increase of all studied parameters of cell invasion (Figure 4E–G). Moreover, supplementation of cultured media of control SK-Mel-28 cells with guanosine led to elevation in the intracellular GTP levels (in agreement with previous studies (Hines et al., 2010, Nouri et al., 2011)) (Figure S4A), and increased invasion similarly to GMPR-depleted cells (Figure 4E–G).

To test whether GMPR depletion would increase tumor formation, SK-Mel-28 cells transduced with control- or two GMPR-shRNAs were subjected to the tumorigenicity assays described in the previous section. We found that GMPR depletion substantially decreased tumor latency of SK-Mel-28-derived tumors, identified as the time period from inoculation date to the appearance of tumors at 25% of inoculated sites (9 and 7 days for cells expressing GMPR shRNA 1 and 2, respectively versus 13 days for control shRNA cells) (Figure 5A). Moreover, suppression of GMPR levels detected before tumor cell inoculation was retained in the tumor xenografts at the time of their appearance (Figure S5A). On the contrary, GMPR depletion did not affect ability of SK-Mel-28 cell to form colonies in semi-solid agarose (Figure 5B) as the amounts of GMPR were depleted throughout the course of the experiments (Figure S5B).

Additionally, GTP levels were analyzed in NMH, SK-Mel-103, and SK-Mel-28 cells. Melanoma cells were found to possess increased GTP amounts compared to NHM (33% and 47%, respectively (Figure S4B)). Accordingly, GMPR depletion in NHM resulted in increased GTP levels (9.6% - 10.4%) and elevated motility (Figure S4C–E).

All together, these data demonstrate that depletion of GMPR promotes cellular phenotypes associated with invasion and up-regulates melanoma cell tumorigenicity.

GMPR differentially regulates activity of several RHO-family GTPases

We hypothesized that GMPR suppresses the ability of melanoma cells to form invadopodia, degrade collagen and subsequently invade extracellular matrix *via* inhibition of the activity of one or several RHO-family GTPases, which have been previously reported to play a pivotal role in these processes (Buccione et al., 2009, Struckhoff et al., 2011). We also hypothesized that such inhibition occurs *via* subtle GMPR-induced depletion of intracellular GTP which is required for the activity of these GTPases.

To test these hypotheses, we assessed the activity of RAC1, CDC42, RHOA and RHOC, key regulators of tumor cell invasion, in control and GMPR-expressing SK-Mel-103 cells using a commercially available GTPase pull-down assay. Following precipitation, total GTPase (in the input) and GTP-bound GTPase (in the precipitate) were detected by western blotting with antibodies specific to individual GTPase. As shown in Figure 6A, overexpression of GMPR did not affect total amounts of RAC1, CDC42, RHOA or RHOC, however GMPR-expressing cells contained reproducibly lower levels of GTP-bound RAC1, RHOA and RHOC, but not GTP-bound CDC42 (Figure 6AH).

To independently verify that subtle depletion of intracellular GTP pools may indeed affect the activity of some but not other RHO-GTPases, we utilized mycophenolic acid (MPA), a chemical inhibitor of IMPDH1 and IMPDH2 (Ransom, 1995, Sievers et al., 1997) often used for depletion of GTP pools (Franchetti and Grifantini, 1999, Yalowitz and Jayaram, 2000). By titrating MPA, we were able to determine experimental conditions (0.4 μM of MPA for 24 hours) at which invasion of SK-Mel-103 cells was suppressed without any effects on their proliferation (Figure 6B), i.e. similarly to GMPR overexpression. Importantly, such treatment resulted in depletion of GTP (Figure 6C), comparable to those

achieved by overexpression of GMPR (Figure 2F). Furthermore, like GMPR-overexpressing cells, the amounts of GTP-bound RAC1, RHOA and RHOC but not CDC42 were lower in cells treated with MPA (Figure 6D,H). It is noteworthy, that in each experiment, the amounts of GTP-bound RAC1 were depleted to a higher degree than GTP-bound RHOA or RHOC.

We then asked whether depletion of GMPR would reciprocally lead to increased amounts of GTP-bound small GTPases. To address this question, we performed pull-down experiments in SK-Mel-28 cells expressing control or GMPR-specific shRNA1 or shRNA2. Our results showed that levels of GTP-bound RAC1, RHOA, and RHOC, but not CDC42 were higher in cells depleted of GMPR compared to cells expressing control shRNA (Figure 6E,H). Moreover, addition of exogenous guanosine to uninfected SK-Mel-28 cells also increased the amounts of GTP-bound RAC1, RHOA, and RHOC, but not CDC42, i.e. comparably to GMPR depletion (Figure 6F,H). Furthermore, like GMPR depletion, treatment with guanosine increased the relative amounts of GTP-bound RAC1 more than GTP-bound RHOA or RHOC. To determine whether GMPR acts upstream of studied RHO-GTPases, we tested whether manipulation of GMPR expression in SK-Mel-103 and SK-Mel-28 cells would affect activation status of other important regulators of invadopodia formation and invasion, including NRAS, PI3K, SRC and ERK (Yamaguchi et al., 2011, Murphy and Courtneidge, 2011). Interestingly, the activation status of these pathways was unaffected by changes in GMPR expression (Figure 6G).

Taken together, our data demonstrate that activity of some but not all small GTPases can be regulated *via* GMPR-dependent alterations in intracellular GTP pools.

RAC1^{G12V} supports melanoma cell invasion independently from GMPR or exogenous guanosine

Among all analyzed small GTPases, the ability of RAC1 to bind GTP appeared to be most sensitive to changes in GMPR levels. A constitutively active form of RAC1, RAC1^{G12V} , has been shown to induce invasion in several cell systems (Zhuge and Xu, 2001, Michiels et al., 1995, van Leeuwen et al., 1995) although RAC1 activation had opposite effects in others (Engers et al., 2001, Uhlenbrock et al., 2004, de Toledo et al., 2012). Thus, we were interested in investigating whether sustained activation of RAC1 increases invasion of studied melanoma cells and if so, whether or not RAC1^{G12V}-dependent invasion would be affected by guanylate pools alteration. To this end, SK-Mel-103 cells were transduced with an empty retroviral vector or a vector encoding RAC1^{G12V} cDNA. The resulting cell populations were super-infected with an empty lentiviral vector or a vector expressing GMPR and tested for invasion in the Matrigel assay. Overexpression of RAC1^{G12V} (Figure 7A) increased invasion of SK-Mel-103 cells compared to the "vector" cells (Figure 7B). Super-infection with GMPR cDNA led to a modest decrease in the invasion of RAC1^{G12V}-melanoma cells, whereas invasion of vector-melanoma cells was deeply affected, as expected (Figure 7B). Moreover, unlike control cells, other invasion-associated phenotypes were affected only minimally in RAC1^{G12V}-melanoma cells (Figure S6), and invasion of RAC1^{G12V}-cells was not significantly affected by treatment with 0.4 μM of MPA (Figure 7C).

To complement these studies, we depleted GMPR levels *via* shRNA in vector- or RAC1^{G12V}-expressing SK-Mel-28 cells (Figure 7D). The Matrigel assay demonstrated that invasion of RAC1^{G12V}-expressing cells was not increased by depletion of GMPR (Figure 7E) or by incubation with 100 μM of guanosine (Figure 7F). Therefore, RAC1 contributes to the invasive phenotype of melanoma cells and constitutive activation of this small GTPase supersedes the effects of intracellular GTP levels manipulation.

DISCUSSION

In melanoma, the acquisition of the invasive phenotypes occurs during the early stages of progression and is considered a critical event strongly associated with poor prognosis (Balch et al., 2009). The molecular mechanisms underlying melanoma invasion are still poorly understood. Here, we report the identification of guanosine monophosphate reductase (GMPR) as a negative regulator of the invasive melanoma phenotype. Our data are in agreement with a previous study establishing an expression signature characteristic of "invasive" versus "proliferative" phenotypes of freshly isolated human metastatic melanoma cells (Hoek et al., 2006), where GMPR was among several dozen genes that were under-expressed in melanoma cells with increased invasion.

The function of GMPR in cellular processes has not been well characterized. Through gain- and loss-of-expression experiments, we demonstrated that GMPR negatively regulates phenotypes associated with invasion (formation of invadopodia, localized matrix degradation, invasion through Matrigel). Two independent lines of evidence demonstrate that GMPR suppresses invasion *via* depletion of GTP pools. First, expression of the catalytic-null GMPR^{C186A} mutant did not affect melanoma invasion (Figure 2E). Second, supplementation of culture medium with guanosine restored the GMPR-affected phenotypes (Figure 2G). Accordingly, addition of guanosine increased the endogenous invasive potential of SK-Mel-28, SK-Mel-103 and SK-Mel-147 cells (Figures 2G and 4E), suggesting that elevated guanosine levels in melanoma patients may be associated with a more aggressive invasive phenotype and consequently with less favorable prognosis. The rather modest increase in the invasion of SK-Mel-103 and SK-Mel-147 cells was most likely due to already high basal invasion potential of these cells.

Recently, an elegant study by Arozarena et al, has implicated cyclic-GMP (c-GMP) production in the regulation of invasion of melanoma cells expressing mutant BRAF^{V600E} (Arozarena et al., 2011). We did not detect changes in the intracellular c-GMP amounts in cells ectopically expressing GMPR or treated with mycophenolic acid (Figure S2A), in agreement with a previous report (Kleinschmidt et al., 1977). Additionally, the c-GMP-dependent regulation of invasion was shown to be specific to melanoma cells containing mutant BRAF^{V600E} (Arozarena et al., 2011), whereas manipulation with GMPR levels regulated invasion in cells expressing mutant BRAF^{V600E} (SK-Mel-28), as well as wild type BRAF (SK-Mel-103 and SK-Mel-147). Thus, GMPR appears to control invasion *via* c-GMP-independent mechanisms. Instead, our data demonstrated that GMPR acts *via* finely tuned regulation of RAC1 and, to the lesser extent, RHOA and RHOC (Figure 6 A,D,E,H).

At the same time, GMPR expression did not affect the amounts of GTP-bound CDC42, another member of the same RHO family of small GTPases and NRAS, a small GTPase that does not belong to RHO-family (Figure 6A,E). These findings are not surprising since differential reaction to the same stimulus has been reported for different members of RHO-GTPases (Hallett et al., 2003, Noren et al., 2001). For instance, antimycin A-induced depletion of ATP and GTP pools in porcine proximal tubule cells, resulted in differential suppression of the activity of RHOA, RAC1, and CDC42 (Hallett et al., 2003)

An antagonistic relationship between RAC1 and RHOA has been documented in several cell systems (Alberts et al., 2005, Nimnual et al., 2003, Wildenberg et al., 2006, Wang et al., 2003), however in our experiments both GTPases were affected by GTP depletion. One possible explanation for this discrepancy is that GTP depletion affects RHOA and RAC1 to an extent that supersedes the inverse relationship between these proteins. Alternatively, since in a few instances it has been reported a concordant regulation of RAC1 and RHOA (Clerk et al., 2001, Zhu et al., 2013), this may be the case in our system as well.

What are the mechanisms by which GMPR controls activity of RAC1, RHOA or RHOC in studied cells? On one hand, we failed to detect GMPR-dependent changes in the activation status of major regulators of invasion that act upstream of these GTPases, including NRAS, AKT, ERK or Src-family proteins. On the other hand, subtle depletion of total cellular GTP pools by GMPR or MPA still leaves the remaining GTP pools at concentrations that are substantially higher the GTP affinity constants of studied GTPases (Zhang and Zheng, 1998) arguing against GMPR-dependent interference with GTP loading of RHO-GTPases. However ATP and GTP may not be homogeneously distributed in the cell. Indeed, using luminescence-based sensors, several recent studies demonstrated temporal and spatial variation in ATP levels in response to various stimuli. (Manfredi et al., 2002, Imamura et al., 2009, Kennedy et al., 1999) Additionally, it has been reported that ectopic expression of a salvage ATP biosynthesis enzyme adenylate kinase 1 (AK1) increased cell motility and spreading to the highest degree when it was tagged to the focal contacts compared to membrane or cytosol (van Horssen et al., 2009). Unlike ATP, presently, there are no sensors for the direct measurement of GTP levels in a live cell. However, since GTP, like ATP, is one of the most common allosteric modulators of protein functions, it is conceivable that GTP distribution in the cell follows similar pattern. Therefore, GTP depletion may be evenly distributed throughout the gradient of GTP amounts and affect GTP binding of a GTPase at the areas where localized GTP concentration is already low. Alternatively, the degree of GTP depletion may vary in different compartments of a cell and thus, may be more prominent in localized areas (i.e. at the invadopodia sites) where it leads to inhibition of GTP loading. Finally, we cannot rule out the existence of yet unidentified mechanisms of GMPR-dependent regulation of RHO-GTPase activity.

In summary, we have identified GMPR as a novel suppressor of melanoma invasion which is down-regulated already at early invasive stages of melanoma progression and whose activity inhibits melanoma cell invasion by depleting intracellular GTP pools. Moreover, our data provide experimental evidences that melanoma cells utilize small alterations of intracellular GTP pools as an important regulatory mechanism of the activity of several small RHO-GTPases involved in cell invasion.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents

Melanoma cell lines were obtained from Memorial Sloan Kettering Cancer Center and cultured in DMEM supplemented with 10% fetal calf serum, 2mM glutamine and penicillin-streptomycin antibiotics. Populations of normal human melanocytes were purchased from Invitrogen (Carlsbad, CA) and maintained in Medium 254 (Invitrogen) supplemented with Human Melanocyte Growth Supplement (Invitrogen). Mycophenolic acid, guanosine, and Hoechst were purchased from Sigma-Aldrich (St. Louis, MO).

Immunoblotting

Membranes were developed with alkaline phosphatase-conjugated secondary antibodies and signals were visualized using the Alpha-Innotech FluorChem HD2 imaging system (Alpha Innotech Corporation, San Leandro, CA) and quantified using ImageQuant software (GE Healthcare Life Sciences). For a list of antibodies used in this study see the Extended Experimental Procedures.

Immunohistochemistry

Formalin fixed and paraffin-embedded human melanocytic cells, cutaneous and metastatic melanoma tissues were processed at the Pathology Core Facility (Roswell Park Cancer Institute). Positive and negative control slides were supplied by the Pathology Core Facility

and were included with every immunochemistry run. For IMPDH2 antibodies (Atlas Antibodies, HPA001400) and GMPR antibodies (Sigma-Aldrich HPA021476), the Novocastra (Newcastle,UK) PowerVision kit was used for visualization, followed by Fast Red (Thermo Scientific, Wilmington, DE). The slides were manually counterstained with hematoxylin. Human tissue specimens were scored for intensity of staining by a board-certified pathologist. Samples of cultured melanocytic cells were scored only for intensity. For more information see the Extended Experimental Procedures.

Statistical Analysis

Student's t-test was used to assess the significance of differences in data obtained in cell-based experiments or tumor xenograft growth assay. The data obtained in tumor latency assay were analyzed using the Log-rank test. Xenograft invasion was analyzed with Exact Fisher's test.

Plasmids and Infection

Lentiviral and retroviral infection protocols were described previously (Mannava et al., 2008). All infected cells were briefly selected for resistance to respective selectable markers and subjected to the assays. For a list of plasmid used in this study see the Extended Experimental Procedures.

Proliferation Assay

Melanoma cells were plated in 96-well plates at ∼30% confluence one day before the assay. Cells were tested for EdU-incorporation using the Click-iT®EdU Cell Proliferation Assays (Invitrogen) following the manufacturer's instructions. The proportion of EdU-positive cells was determined by counting at least 100 cells under a fluorescent microscope.

Matrigel™-based Invasion Assay

Invasion assay was performed using the BioCoat Matrigel invasion chambers (BD Bioscience, San Diego, CA) according to the manufacturer's instructions with some modifications. For details see the Extended Experimental Procedures.

Combined Gelatin Degradation Assay

Pre-washed coverslips (see Extended Experimental Procedures) were coated with warm Oregon Green® 488-conjugated gelatin (Invitrogen) at 0.2mg/ml in 2% sucrose/PBS, followed by cross-linking with ice-cold glutaraldehyde 0.5% in PBS, incubation with sodium borohydride (5mg/ml), and sterilization with 70% ethanol. Coverslips were quenched with serum-free media for 1 hour at 37°C. Melanoma cells (7.5×10^4) were seeded on the coverslips and after 16-hour incubation at 37°C they were fixed in 4% paraformaldehyde in PBS and stained with rhodamine-conjugated phalloidin (Invitrogen). Coverslips were mounted onto glass slides with aqua-mount media (Polysciences, Warrington, PA). Invadopodia were identified as β-actin puncta co-localizing with areas of gelatin degradation. The area of gelatin degradation was quantified using imageJ (NIH) software.

GTP-bound GTPase Pull-Down Assay

The assay was performed using RHOA/RHOC/RAC1/CDC42 Activation Assay Combo kit (Cell Biolabs) according to the manufacturer's recommendations. For details see the Extended Experimental Procedures. Samples were resolved on polyacrylamide gels along with total lysates as control and visualized as described above.

Quantitative Real Time PCR

Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was prepared using cDNA reverse transcription kit (Invitrogen). Quantitative reverse transcription PCR was performed on 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) using TaqMan Universal Master Mix II (Applied Biosystems) and probes specific for human β-actin and human *GMPR* genes. PCR data were analyzed using sequence detection software 2.4 (Applied Biosystems).

Nucleotide Quantification

Cells were harvested by trypsinization, extracted with 0.4 N perchloric acid and neutralized. NTPs were separated and quantified using a strong anion exchange column (Whatman, Hillshore, OR) with a gradient HPLC system (Waters Milford, MA) equipped with a photodiode array detector and controlled by Millennium 2010 software. Nucleotides were eluted with 0.005M ammonium phosphate, pH 2.8, for five minutes followed by a linear gradient to 0.75M ammonium phosphate, pH 3.7, over 60 minutes. Nucleotides were identified based on their UV absorbance spectrum and quantified at either 254 or 281 μM by comparison to the absorbance of a known amount of authentic standard.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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HIGHLIGHTS

- GMPR is down-regulated in invasive stages of cutaneous and metastatic melanoma

- GMPR inhibits invasion and tumorigenicity of melanoma cells

- GMPR suppresses activity of several RHO-GTPases

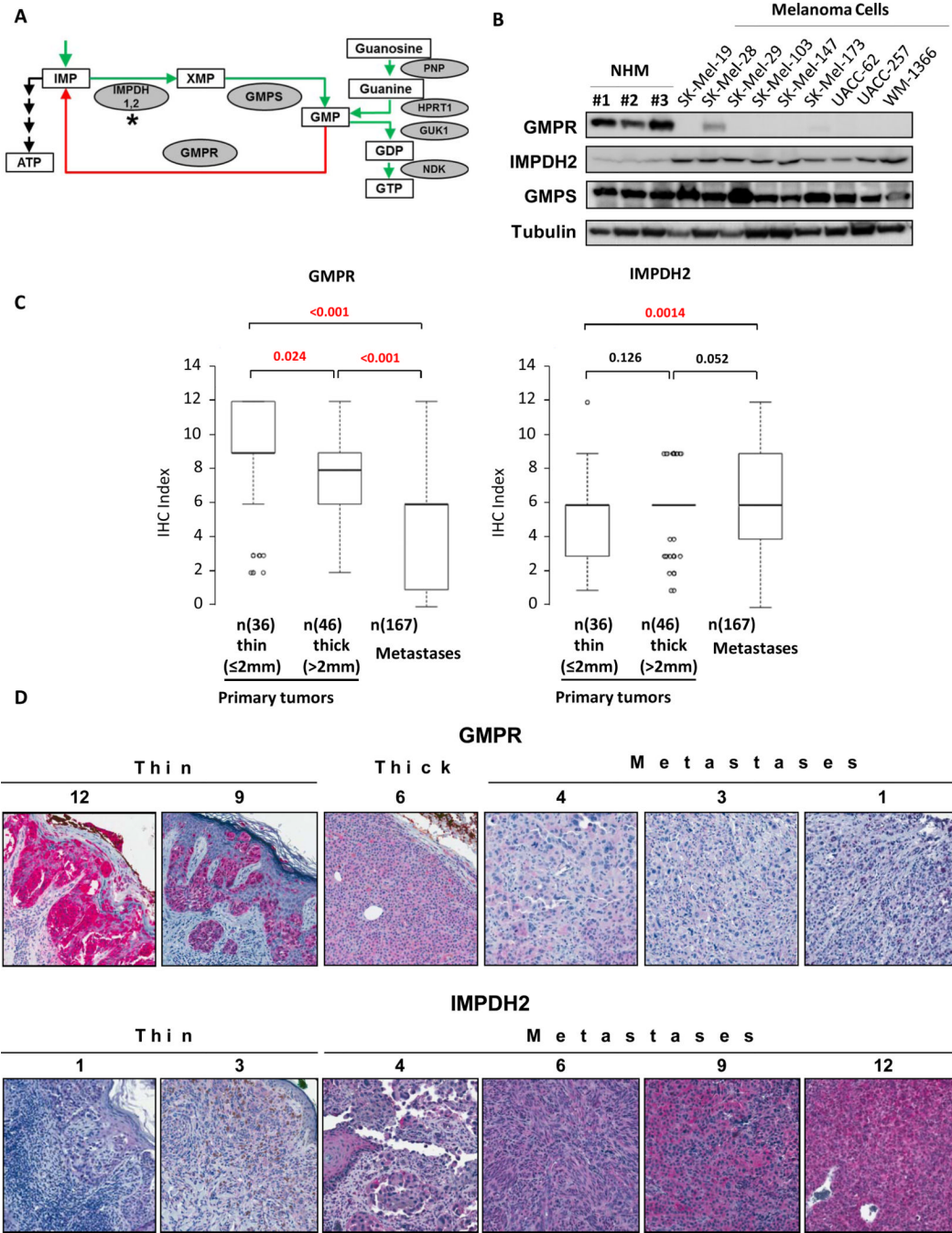- Other enzymes of GTP biosynthesis regulate tumor cell invasion

Figure 1. GMPR is Down-regulated at Invasive Stages of Melanoma

(**A**) Schematic representation of the *de novo* purine biosynthesis and salvage pathway. Enzymes and their products are shown by ovals and open boxes, respectively. * IMPDH 1/2 are rate-limiting enzymes and MPA targets. (**B**) Total cellular extracts from independently isolated populations of normal human melanocytes (NHM) and indicated melanoma cell lines were probed in western blotting with indicated antibodies. (**C**) Expression of GMPR and IMPDH2 in thin, thick primary melanomas and melanoma metastases. The box plots represent the distribution of the IHC index. The median, first quartile, and third quartile are shown in the "box" with outlying samples represented by points. The dashed lines represent

the interquartile range (IQR) times 1.5 added to the first and third quartiles. The number of patient samples (n) is indicated for each cohort. **(D)** Representative IHC images for GMPR and IMPDH2 from the data presented in *C*. See also Figure S1.
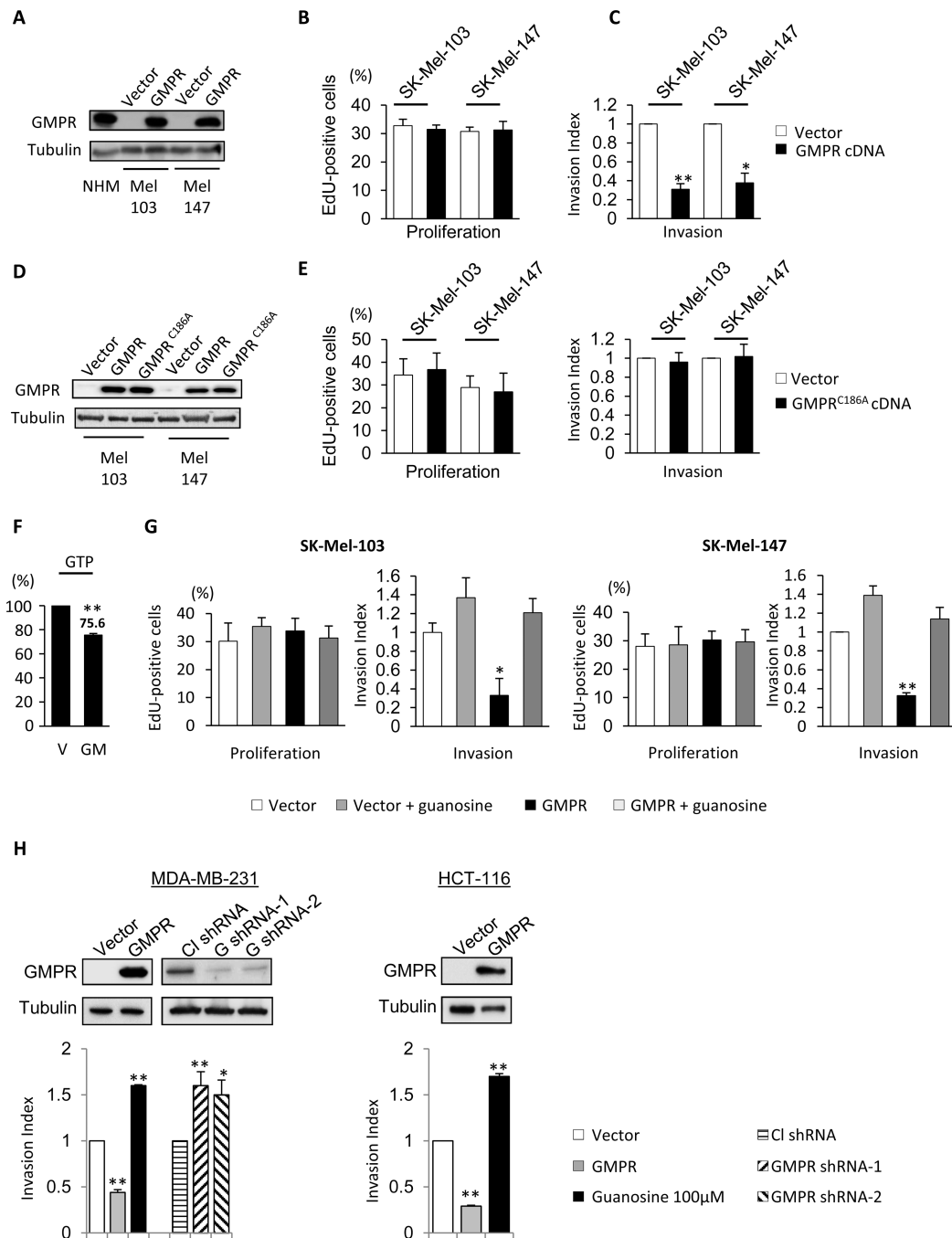
Figure 2. GMPR Enzymatic Activity is Required for Suppression of Invasion
(A) Indicated melanoma cells were transduced with empty lentiviral vector or vector expressing human GMPR cDNA. 4 days after transduction, total protein extracts from transduced cells and from normal human melanocytes were probed in western blotting with indicated antibodies. Cells were also tested for proliferation **(B)** or invasion **(C)**. **(D)** Melanoma cells were transduced with empty lentiviral vector, vector expressing wild-type GMPR or putative catalytic-null mutant of GMPR (GMPR^{C186A}). 4 days after transduction, total protein extracts from infected cells were probed in western blotting with indicated antibodies and assayed for proliferation or invasion **(E)**. **(F)** Intracellular GTP amounts were

measured by HPLC in SK-Mel-103 cells expressing empty vector or GMPR. Normalized amounts of the nucleotides are shown. **(G)** Cells described in *A* were treated or not with 100 μM of guanosine for 24 hours followed by assessment of invasion indices and incorporation of EdU. **(H)** Human breast carcinoma MDA-MB-231 and human colon carcinoma HCT116 cell expressing or not the indicated constructs and treated or not with guanosine were assayed for invasion as described above. Cells expressing indicated constructs were tested in immunoblot for GMPR expression. All panels show representative experiments. Data represent mean ± S.E.M. *$p<0.05$; **$p<0.001$ by Student *t*-Test. See also Figure S2.

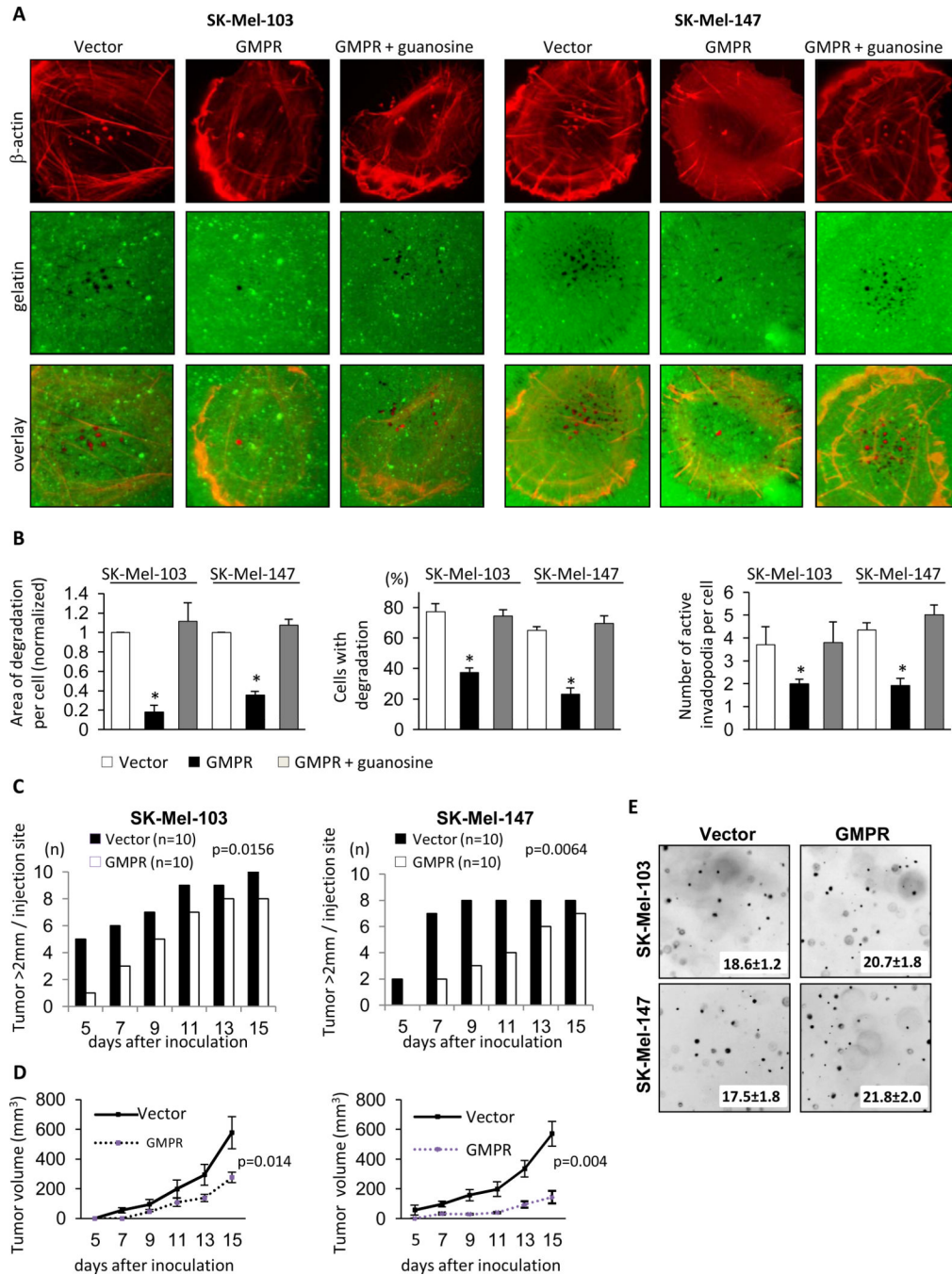**Figure 3. GMPR Affects Invasion-associated Phenotypes of Melanoma Cells**

(A) Melanoma cells were transduced with the indicated constructs. 4 days after transduction, cells were plated on FITC- gelatin for 16 hours, fixed and stained with rhodamine-conjugated phalloidin. Where indicated, cells were grown in media supplemented with 100 μM guanosine for 24 hours prior to and after plating on the gelatin. **(B)** Area of degradation was determined by Image J software. The number of cells with gelatin degradation was determined by counting at least 50 cells. Active invadopodia were counted as phalloidin-positive puncta overlapping with the area of gelatin degradation. **(C)** Indicated melanoma cells expressing vector or GMPR were inoculated in both flanks of nude mice (n= number of

inoculation sites). Tumors were recorded when they reached at least 2 mm in one dimension. **(D)** Tumor xenografts formed by melanoma cells described in *C* were measured at indicated time points. The results are expressed as mean volume ± SEM. **(E)** Cells described in *C* were grown in semi-solid agarose for 12 days. All panels describe representative experiments. Data represent mean ± S.E.M., "+/–" represents S.D. *$p<0.05$ by Student *t*-Test. See also Figure S3.
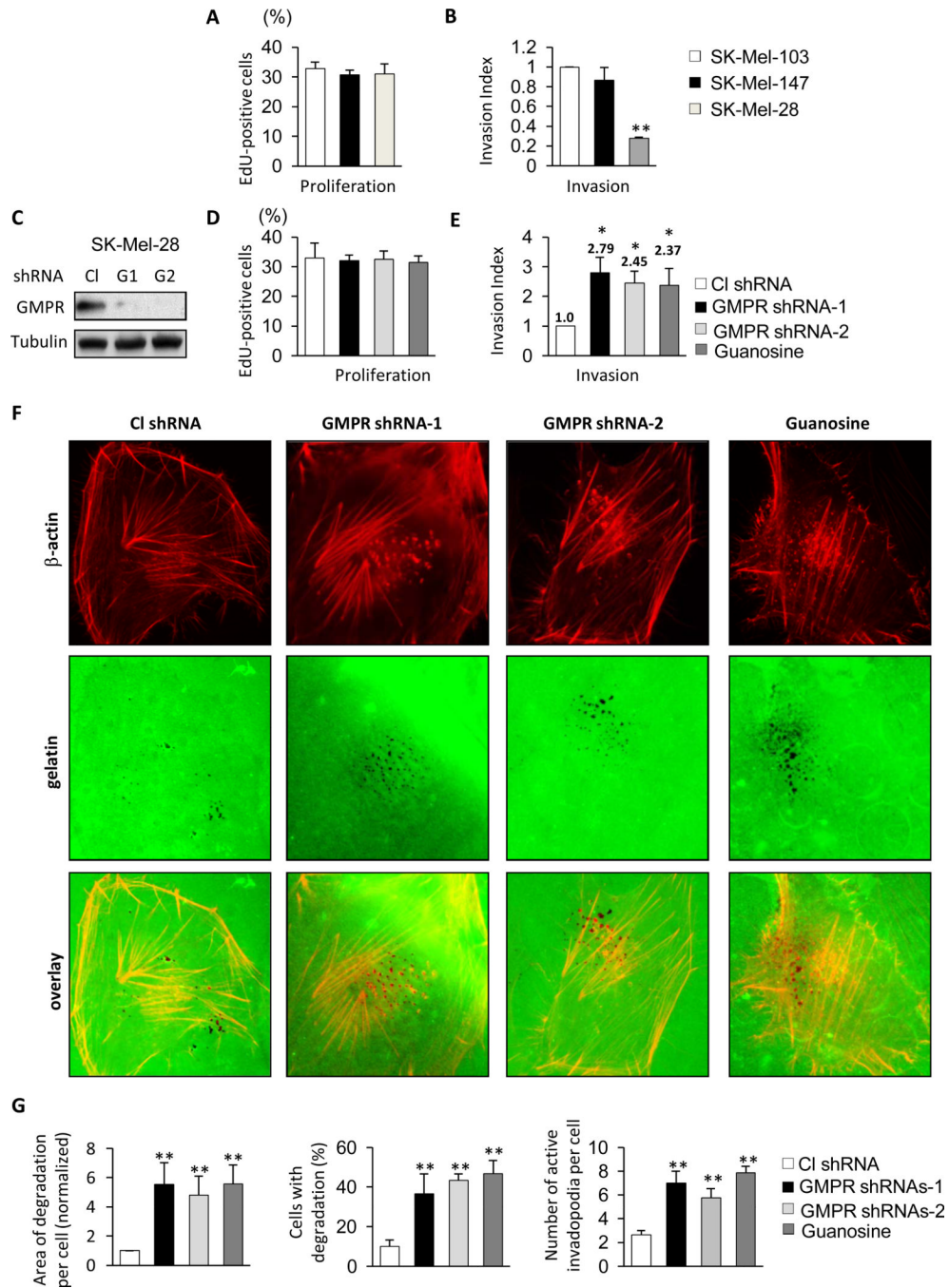
Figure 4. Suppression of GMPR Promotes Invasion of Melanoma Cells

(A–B) SK-Mel-28, SK-Mel-103 and SK-Mel-147 cells were tested for proliferation and invasion. (C) Total protein extracts of SK-Mel-28 cells expressing control shRNA (Cl), GMPR shRNA1 (G1) or GMPR shRNA2 (G2) were probed in western blotting with indicated antibodies. Incorporation of EdU (D) and invasion index (E) were determined for indicated cells treated or not with 100 μM of guanosine ("Guanosine"). (F) Cells described in *A* were plated on FITC- gelatin for 16 hours, fixed and stained with rhodamine-conjugated phalloidin. Where indicated, cells were grown in media supplemented with 100 μM guanosine for 24 hours prior to and after plating on the gelatin. (G) Area of degradation

was determined by Image J software. Number of cells with gelatin degradation was determined by counting at least 50 cells. Active invadopodia were counted as phalloidin-positive puncta overlapping with the area of gelatin degradation. All panels describe representative experiments. Data represent mean ± S.E.M., *$p<0.05$; *$p<0.001$ by Student *t*-Test. See also Figure S4.
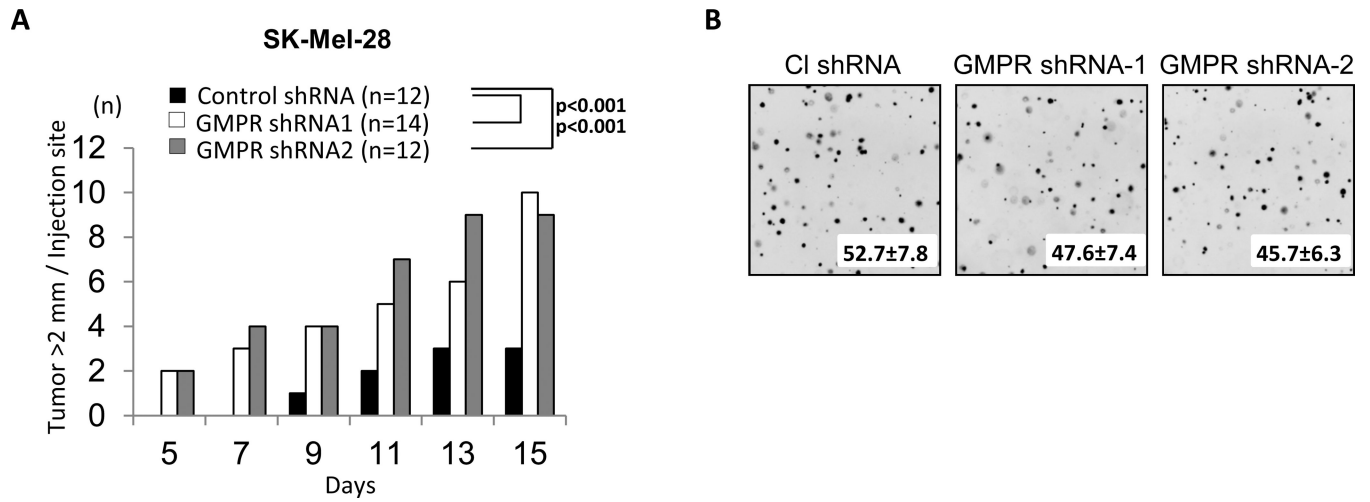
A

SK-Mel-28

B

Figure 5. Suppression of GMPR Increases Melanoma Cell Tumorigenicty
(A) SK-Mel-28 cells expressing indicated shRNAs were inoculated into flanks of nude mice (n= number of inoculation sites). Tumors were recorded when they reached at least 2 mm in one dimension. **(B)** Cells described in *A* were grown in semi-solid agarose for 12 days . All panels describe representative experiments. "+/–" represents S.D. *$p<0.05$ by Student *t*-Test. See also Figure S5.
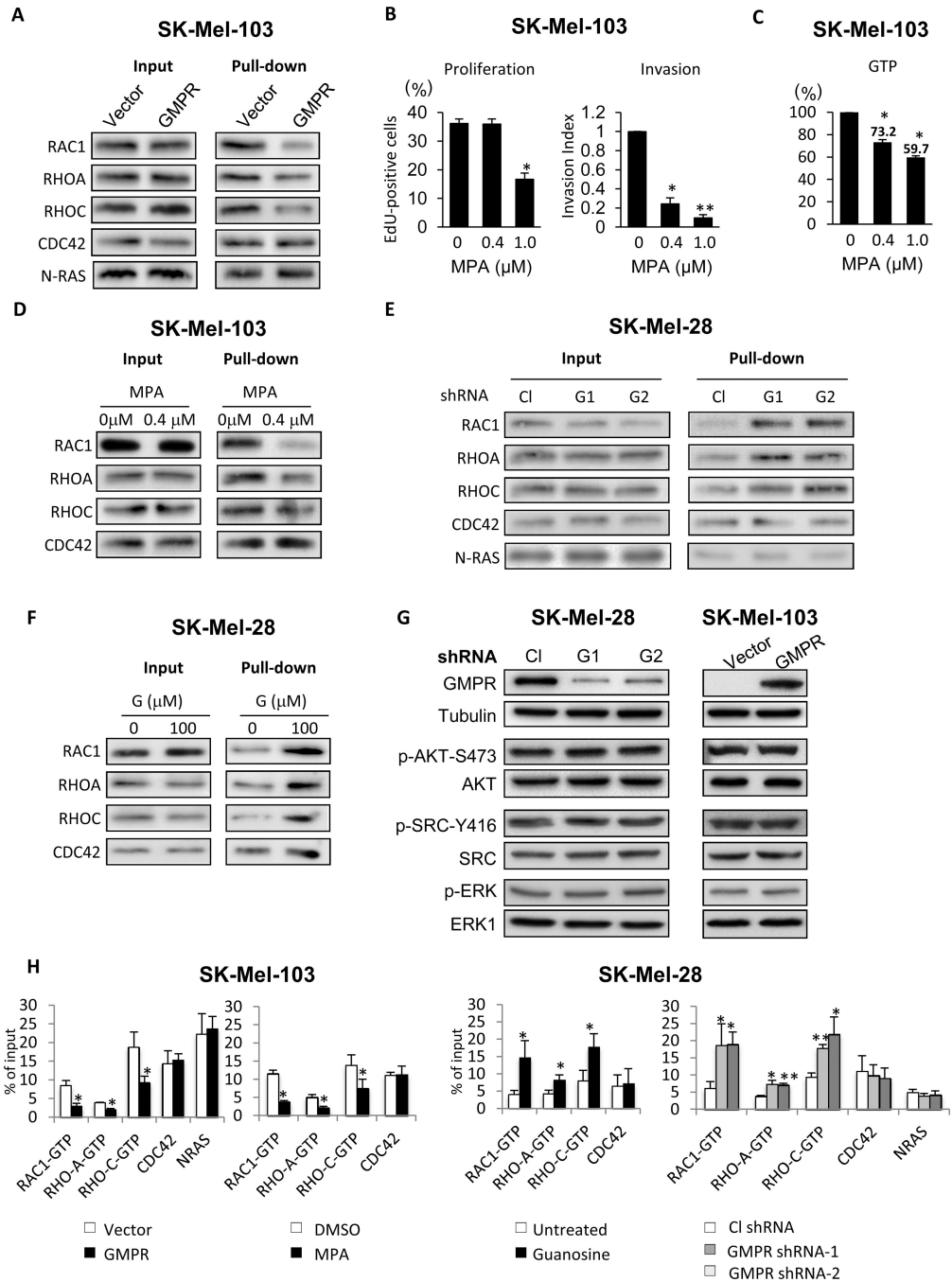
Figure 6. GMPR Regulates Activity of RHO-GTPases

(**A**) GTP-bound RAC, CDC42, RHOA, RHOC and NRAS were pulled down from total cell lysates of SK-Mel-103 melanoma cells expressing human GMPR or empty vector. GTP-bound (active) and total RAC1, CDC42, RHOA, RHOC and NRAS were detected by immuno-blotting. (**B**) SK-Mel-103 melanoma cells were treated with the indicated amounts of MPA for 24 hours and tested for proliferation and invasion. Shown are the percent of EdU positive cells and relative invasion index of indicated cells. (**C**) GTP amounts were measured by HPLC in SK-Mel-103 cells treated or not with 0.4 μM or 1.0 μM of MPA. Normalized amounts of GTP are shown. (**D**) Pull-down assays of GTP-bound RAC1,

CDC42, RHOA and RHOC in SK-Mel-103 melanoma cells treated or not with 0.4 μM of MPA for 24 hours. **(E)** SK-Mel-28 cells expressing control shRNA (Cl), GMPR shRNA1 (G1) or GMPR shRNA2 (G2) and **(F)** SK-Mel-28 cells growing in media supplemented or not with guanosine ("G") for 24 hours were subjected to pull-down assays as described above. For all panels representative images are provided **(G)** Whole cell extracts from the indicated cell lines and treatments were probed with indicated antibodies. **(H)** Data representing mean ± S.E.M of quantification of GTP-bound GTPase to total GTPase from three independent experiments as described in A, D, E, and F. *$p<0.05$; **$p<0.001$ by Student *t*-Test.
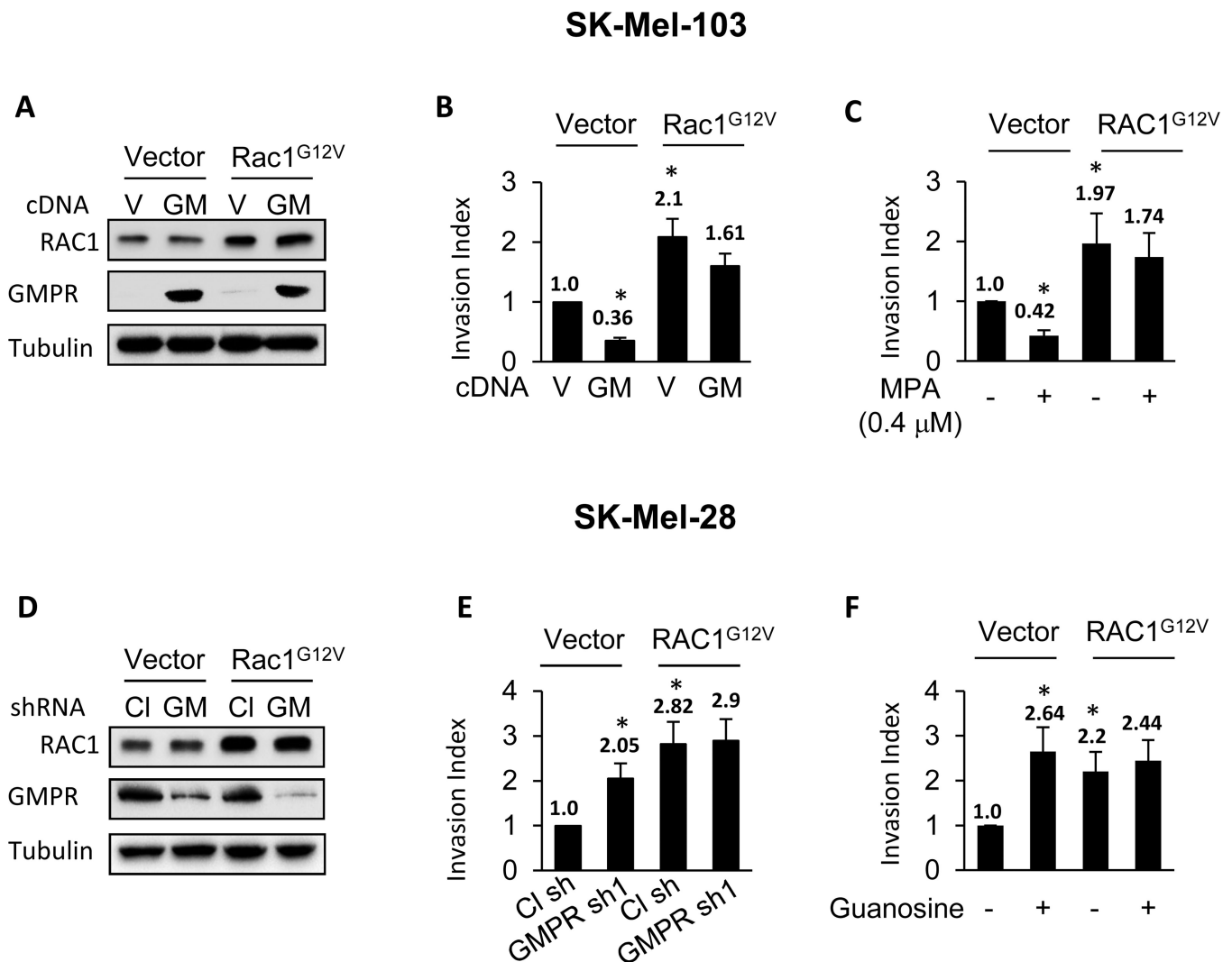
SK-Mel-103

A

cDNA: Vector (V GM) Rac1^{G12V} (V GM)

RAC1

GMPR

Tubulin

B

Invasion Index

Vector: V 1.0, GM 0.36*
Rac1^{G12V}: V 2.1*, GM 1.61

cDNA V GM V GM

C

Invasion Index

Vector: - 1.0, + 0.42*
RAC1^{G12V}: - 1.97*, + 1.74

MPA (0.4 μM): - + - +

SK-Mel-28

D

shRNA: Vector (Cl GM) Rac1^{G12V} (Cl GM)

RAC1

GMPR

Tubulin

E

Invasion Index

Vector: Cl sh 1.0, GMPR sh1 2.05*
RAC1^{G12V}: Cl sh 2.82*, GMPR sh1 2.9

F

Invasion Index

Vector: - 1.0, + 2.64*
RAC1^{G12V}: - 2.2*, + 2.44

Guanosine: - + - +

Figure 7. RAC1^{G12V} Suppresses GMPR phenotype
(**A**) SK-Mel-103 melanoma cells were transduced with an empty retroviral vector or a vector expressing human RAC1^{G12V} cDNA followed by super-infection with empty lentiviral vector ("V") or human GMPR cDNA ("G"). 4 days after the second transduction, total protein extracts from transduced cells were probed in western blotting with indicated antibodies. (**B**) Cells described in *A* were tested for invasion. (**C**) Cells described in *A* were treated or not with 0.4 μM of MPA followed by invasion assay. (**D**) SK-Mel-28 cells were transduced with empty lentiviral vector or vector expressing human RAC1^{G12V} cDNA followed by superinfection with control shRNA ("Cl sh") or with GMPR shRNA1 ("GMPR sh1"). 4 days after the second infection, total protein extracts from transduced cells were probed in western blotting with indicated antibodies. (**E**) Cells described in *D* were tested for invasion. (**F**) SK-Mel-28 cells expressing vector or RAC1^{G12V} were treated with the indicated amounts of guanosine for 24 hours and tested for invasion. *$p < 0.05$ by Student *t* - Test, compared to control. Data represent mean ± S.E.M. All panels describe representative experiments. See also Figure S6.