Author's response to reviews

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Gβγ subunits inhibit Epac-induced melanoma cell migration

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ABSTRACT

Background: Recently we reported that activation of Epac1, an Exchange protein activated by cAMP, increases melanoma cell migration via Ca$^{2+}$ release from the endoplasmic reticulum (ER). G-protein $\beta\gamma$ subunits (G$\beta\gamma$) are known to act as an independent signaling molecule upon activation of G-protein coupled receptor. However, the role of G$\beta\gamma$ in cell migration and Ca$^{2+}$ signaling in melanoma has not been well studied. Here we report that there is crosstalk of Ca$^{2+}$ signaling between G$\beta\gamma$ and Epac in melanoma, which plays a role in regulation of cell migration.

Methods: SK-Mel-2 cells, a human metastatic melanoma cell line, were mainly used in this study. Intracellular Ca$^{2+}$ was measured with Fluo-4AM fluorescent dyes. Cell migration was examined using the Boyden chambers.

Results: The effect of G$\beta\gamma$ on Epac-induced cell migration was first examined. Epac-induced cell migration was inhibited by mSIRK, a G$\beta\gamma$-activating peptide, but not its inactive analog, L9A, in SK-Mel-2 cells. Gp(CH)$_2$pp (Guanosine 5', $\alpha$-β-methylene triphosphate), a constitutively active GTP analogue that activates G$\beta\gamma$, also inhibited Epac-induced cell migration. In addition, co-overexpression of $\beta$1 and $\gamma$2, which is the major combination of G$\beta\gamma$, inhibited Epac1-induced cell migration. By contrast, when the C-terminus of $\beta$ adrenergic receptor kinase ($\beta$ARK-CT), an endogenous inhibitor for G$\beta\gamma$, was overexpressed, mSIRK's inhibitory effect on Epac-induced cell migration was negated, suggesting the specificity of mSIRK for G$\beta\gamma$. We next examined the effect of mSIRK on Epac-induced Ca$^{2+}$ response. When cells were pretreated with mSIRK, but not with L9A, 8-(4-Methoxyphenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-pMeOPT), an Epac-specific agonist, failed to increase Ca$^{2+}$ signal. Co-overexpression of $\beta$1 and $\gamma$2 subunits inhibited 8-pMeOPT-induced Ca$^{2+}$ elevation. Inhibition of G$\beta\gamma$ with $\beta$ARK-CT or guanosine 5'-O-(2-thiodiphosphate) (GDP$\beta$S), a GDP analogue that inactivates G$\beta\gamma$, restored 8-pMeOPT-induced Ca$^{2+}$ elevation even in the presence of mSIRK. These data suggested that G$\beta\gamma$ inhibits Epac-induced Ca$^{2+}$ elevation. Subsequently, the mechanism by which G$\beta\gamma$ inhibits Epac-induced Ca$^{2+}$ elevation was explored. mSIRK activates Ca$^{2+}$ influx from the extracellular space. In addition, W-5, an inhibitor of calmodulin, abolished mSIRK's inhibitory effects on Epac-induced Ca$^{2+}$ elevation, and cell migration. These data suggest that, the mSIRK-induced Ca$^{2+}$ from the extracellular space inhibits the Epac-induced Ca$^{2+}$ release from the ER, resulting suppression of cell migration.

Conclusion: We found the cross talk of Ca$^{2+}$ signaling between G$\beta\gamma$ and Epac, which plays a major role in melanoma cell migration.
INTRODUCTION

Melanoma causes the majority of skin cancer related death, and is prevalent worldwide. The median life span of patients with advanced stage melanoma is less than a year because no therapies are effective once the tumor has spread to vital organs [1]. The tumor metastasis process is conventionally understood as the migration of individual cells that detach from the primary tumor, enter lymphatic vessels or the bloodstream, attach to endothelial cells and undergo transendothelial extravasation, and proliferate in organs [2]. Although numerous efforts have been focused on understanding of melanoma progression, the controlling of melanoma cell migration/metastasis has been unsuccessful.

Received comments

G protein-coupled receptors (GPCRs) belong to a large family of transmembrane receptors. Upon ligand binding, the G-protein $\alpha$ and $\beta\gamma$ subunits ($G_\alpha$ and $G_{\beta\gamma}$, respectively) are dissociated. Each molecule regulates intracellular signal transductions and evokes cellular responses including cell migration [3]. Previous reports suggested a role of $G_{\beta\gamma}$ in cell migration of endothelial cells and breast cancer cells [4-6]; however, the role of $G_{\beta\gamma}$ on cell migration in melanoma is largely unknown. $G_{\beta\gamma}$ is also known to regulate Ca$^{2+}$ homeostasis via regulation of membrane voltage-dependent Ca$^{2+}$ channels in excitable cells [7, 8]. In non-excitable cells, $G_{\beta\gamma}$ activates Ca$^{2+}$ release from the endoplasmic reticulum (ER) [9, 10]. However, the role of $G_{\beta\gamma}$ in Ca$^{2+}$ signaling in cancer cells, including melanoma, remains unknown.

In addition to the traditional target of cAMP, protein kinase A (PKA), a new, PKA-independent signaling pathway has been identified. The exchange protein directly activated by cAMP (Epac), a guanine nucleotide exchange factor [11] has two isoforms, Epac1 and Epac2. Epacs mediate cAMP signaling through activation of a small-molecular-weight G protein, Rap1 [12]. Previous reports demonstrated functions of Epac in cancer cells. Epac mediates cell adhesion in Ovcar3 cells [13], apoptosis [14] and growth arrest [15] in B lymphoma cells, formation of embryonic vasculogenic networks in melanoma cells [16], and proliferation of prostate carcinoma cells [15]. Previously, we have reported that Epac increases melanoma cell migration by modification of heparan sulfate, a major component of the extracellular matrix [17]. More recently, we demonstrated that Epac increases cytosolic Ca$^{2+}$ in melanoma cells, which also led to an increase of cell migration. The major mechanism in Epac-induced Ca$^{2+}$ elevation was activation of inositol triphosphate (IP3) receptor to release Ca$^{2+}$ from the ER [18]. Although calmodulin is a major regulator of IP3 receptors [19-21], the role of calmodulin in melanoma is still unclear.

In the present study, we demonstrated that there is interaction between Ca$^{2+}$ signaling between $G_{\beta\gamma}$ and Epac in melanoma. The interaction evoked by $G_{\beta\gamma}$ inhibited Epac-induced Ca$^{2+}$ elevation and cell migration via additional Ca$^{2+}$ influx from the extracellular space. We thus propose that $G_{\beta\gamma}$-signaling inhibits the Epac-induced cell migration via a Ca$^{2+}$-dependant mechanism in melanoma.
METHODS

Reagents and cell lines: Reagents were purchased from Sigma unless otherwise specified. G-protein βγ activating peptide, myr-SIRKALNILGYPDYD-OH (mSIRK), and its control peptide, myr-SIRKALNIAGYPYDYOH (L9A), 2-aminoethoxydiphenyl borate (2-APB), xestopongin C, ryanodine, N-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) were purchased from EMD Chemicals. Guanosine 5’-α-,β-methylene, triphosphate (Gp(CH)2pp) was purchased from Biomol International. 8-(4-Methoxyphenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-pMeOPT) was purchased from Biolog. Antibodies against Epac1, Gβ1, Gγ2, C-terminal domain of β-adrenergic receptor kinase (βARK-CT) were purchased from Santa Cruz Biotechnology. Anti-α-tubulin antibody was purchased from Abcam. SK-Mel-2 and SK-Mel-24 melanoma cell lines were obtained from the American Type Culture Collection. SK-Mel-187 cell line was kindly provided by Dr. Alan Houghton. C8161 cell line was provided by Dr. Mary JC Hendrix. WM1552C, WM115, WM3248, WM1361A were obtained from Dr. Meenhard Herlyn. SK-Mel-2 and SK-Mel-24 cells were maintained in MEM containing 10% FBS, 1% penicillin-streptomycin. All other melanoma cells were maintained in RPMI containing 10% FBS, 1% penicillin-streptomycin.

Fluorescence imaging of intracellular Ca\textsuperscript{2+}: Measurement of intracellular Ca\textsuperscript{2+} release was performed as we previously described [18]. Cells were incubated with HEPES buffer containing 4 µmol/L of Fluo-4AM followed by washing and incubation with HEPES buffered saline containing 1.8 mmol/L of CaCl\textsubscript{2}. An iXon+ 885 charge-coupled device camera (Andor Technology) was used to monitor fluorescence changes. Full images were collected every 4 seconds. The field was illuminated by two wavelengths in rapid succession. Fluo-4 was excited at 488 nm, and data were expressed as normalized changes in background-corrected fluorescence emission (F/F\textsubscript{0}). Data were analyzed using Imaging Workbench (INDEC BioSystems). Representative Ca\textsuperscript{2+} signals averaged from 5 individual cells were shown in the figures.

Western blot analysis: Western blot analysis was performed as we previously described [22, 23]. Briefly, cells were lysed and sonicated in lysis buffer. Equal amounts of protein were subjected to SDS-PAGE. After protein separation by electrophoresis, samples were transferred to Millipore Immobilon-P membrane and immunoblotting with antibodies was performed.

Migration assay: Migration assay was performed using the 24-well Boyden chambers (8 µm pores, BD Biosciences) as we previously described [17]. The cells were plated at a density of 1 x 10\textsuperscript{6} cells/100 µl of medium in the inserts, and incubated for 3 h at 37°C followed by staining using the Diff-Quick kit (Dade Behring). Pictures were taken with a microscope and migrated cells were counted with Image J software using 10 randomly chosen fields.

Overexpression of Epac1 or βARK-CT: Adenoviral overexpression of Epac1, βARK-CT or LacZ was performed as we previously described [18]. βARK-CT adenovirus was kindly provided by Dr. Koch. The recombinant vector was introduced into human embryonic kidney cells (HEK-293) to recover infectious adenovirus. Cells were infected with adenovirus for 24h followed by confirmation of overexpression of target proteins.

Overexpression of Gβ1 and Gγ2 subunits: Plasmid constructs harboring Gβ1 or Gγ2 subunit were
kindly provided by Dr. Simonds [24]. Plasmid transfection was performed as we previously described [22, 23]. Briefly, 750 µl of serum- and antibiotics-free OPTI-MEM was mixed with both 13.5 µl of Lipofectamine2000 and 6.25 µl plasmid DNA. The mixture was then diluted with 5 ml MEM supplement with 5% FBS and gently overlaid onto the cells, followed by incubation for 16 h. The overexpression was confirmed by western blot analyses, and the transfected cells were used for cell migration or cytosolic Ca^{2+} measurement assays.

**Data analysis and statistics:** Statistical comparisons among groups were performed using one factor ANOVA with Bonferroni post hoc test. Statistical significance was set at the 0.05 level.
RESULTS

G\(\beta\gamma\) inhibits Epac-induced cell migration in melanoma.

(#Reviewer4-comment1) We have previously demonstrated that Epac increases melanoma cell migration by modification of heparan sulfate [17]. In addition, it has been reported that G\(\beta\gamma\) plays a role in cell migration of endothelial cells and breast cancer cells [4-6], we examined the effects of G\(\beta\gamma\) on melanoma cell migration. We found that mSIRK, a cell-membrane permeable activator of G\(\beta\gamma\) [7], decreased basal cell migration only at the highest dose (50 \(\mu\)M). In contrast, mSIRK inhibited Epac1 overexpression-induced cell migration in a dose-dependent manner (Fig. 1a). These data suggest that the inhibitory effect of G\(\beta\gamma\) is obvious under Epac-activated conditions, but not clear under basal conditions (Fig. 1a). Gp(CH)\(_2\)pp, a constitutively active GTP analogue which dissociates G\(\beta\gamma\) from G\(\alpha\), also inhibited Epac-induced cell migration. In addition, mSIRK inhibited cell migration induced by 8-pMeOPT, an Epac-specific agonist, suggesting that G\(\beta\gamma\) also inhibits cell migration induced by endogenous Epac (Fig. 1b). Further, we examined the specificity of G\(\beta\gamma\) in the inhibition of Epac-induced cell migration. Co-overexpression of G\(\beta\_1\) and G\(\gamma\_2\) subunits (Fig. 1c), which is the major combination of G\(\beta\gamma\) [25], inhibited Epac1-induced cell migration (Fig. 1e). By contrast, overexpression of \(\beta\)ARK-CT (Fig. 1d), an inhibiting-peptide for G\(\beta\gamma\) [26], abolished the mSIRK’s inhibitory effect (Fig. 1e). These data suggest that there is cross talk between G\(\beta\gamma\) and Epac, which affects melanoma cell migration. (#Reviewer2-comment2) Since activation of Epac was achieved by artificial overexpression or the Epac-agonist which does not exist in nature, we tested whether hormonal control of GPCR increases cell migration via Epac. Stimulation of \(\beta\)-adrenergic receptor increased cell migration in melanoma, and it was inhibited by ablation of Epac1 (Fig. S1), suggesting that Epac increases cell migration upon activation of hormone receptors.

G\(\beta\gamma\) inhibits Epac-induced Ca\(^{2+}\) elevation.

We next explored the mechanism by which G\(\beta\gamma\) inhibits Epac-induced cell migration. Since our previous report have shown that Epac1-induced cell migration is mediated by Ca\(^{2+}\) release from the ER via IP3 receptor [18], we examined whether mSIRK affects Epac1-induced cytosolic Ca\(^{2+}\) elevation. When cells were pretreated with mSIRK, but not with L9A, 8-pMeOPT failed to increase Ca\(^{2+}\) signal (Fig. 2a and b). In addition, co-overexpression of G\(\beta\_1\) and G\(\gamma\_2\) also inhibited 8-pMeOPT-induced Ca\(^{2+}\) elevation (Fig. 2c). Further, inhibition of G\(\beta\gamma\) with \(\beta\)ARK-CT, or guanosine 5'-O-(2-thiodiphosphate) (GDP\(\beta\)S), a GDP analogue that inactivates G\(\beta\gamma\), restored 8-pMeOPT-induced Ca\(^{2+}\) elevation (Fig. 2d and e). These data suggest that G\(\beta\gamma\) inhibits Epac-induced Ca\(^{2+}\) release.

G\(\beta\gamma\) activates Ca\(^{2+}\) entry from the extracellular space.

Since IP3 receptors are inactivated by prior cytosolic Ca\(^{2+}\) elevation [19-21], we hypothesized that Ca\(^{2+}\) elevation induced by G\(\beta\gamma\) inhibits Epac-induced Ca\(^{2+}\) elevation. mSIRK, but not L9A, indeed increased Ca\(^{2+}\) signal in SK-Mel-2 cells (Fig. 3a and b). mSIRK-induced Ca\(^{2+}\) elevation similarly observed in various melanoma cells including WM1552C, a regional growth pattern primary
melanoma cell line (RGP), WM115, WM1361A, WM3284, vertical growth pattern primary melanoma cells, and SK-Mel-24, SK-Mel-187 and C8161, metastatic melanoma cell lines (MM) (data not shown), suggesting mSIRK’s effect on $\text{Ca}^{2+}$ is universal in different types of melanoma. After the elevation of $\text{Ca}^{2+}$ signal by mSIRK, 8-pMeOPT did not show an additional elevation of $\text{Ca}^{2+}$ signal (Fig. 3b). GDP $\beta S$ inhibited mSIRK induced cytosolic $\text{Ca}^{2+}$ elevation, suggesting the specificity of mSIRK for $\text{G}\beta\gamma$ (Fig. 3c). We next examined the source of $\text{G}\beta\gamma$-induced $\text{Ca}^{2+}$. When extracellular $\text{Ca}^{2+}$ was depleted by removal of $\text{Ca}^{2+}$ or by addition of EGTA, a $\text{Ca}^{2+}$-chelating agent, mSIRK did not increase $\text{Ca}^{2+}$ signal (Fig. 3d and e, respectively). By contrast, inhibition of IP3 receptors or ryanodine receptor did not affect mSIRK-induced $\text{Ca}^{2+}$ signal (Fig. 3g and h). Further, depletion of $\text{Ca}^{2+}$ store in the ER with thapsigargin did not inhibit mSIRK-induced $\text{Ca}^{2+}$ elevation (Fig. 3i). Taken together, these data suggest that $\text{G}\beta\gamma$ induces $\text{Ca}^{2+}$ from the extracellular space, but not from intracellular store.

**Calmodulin is involved in mSIRK-mediated inhibition of $\text{Ca}^{2+}$ elevation and cell migration by Epac.**

Since IP3 receptor is known to be inactivated by $\text{Ca}^{2+}$/calmodulin [19-21], we examined whether calmodulin mediates $\text{G}\beta\gamma$’s inhibitory effect on Epac-induced $\text{Ca}^{2+}$ elevation, and also cell migration. W-5, a calmodulin inhibitor, restored Epac-mediated $\text{Ca}^{2+}$ signal, and cell migration even in the presence of mSIRK (Fig. 4a and c, respectively). In order to identify the $\text{Ca}^{2+}$ channel in which $\text{G}\beta\gamma$ activates $\text{Ca}^{2+}$ influx from the extracellular space, we tested $\text{Ca}^{2+}$ inhibitors in mSIRK-induced $\text{Ca}^{2+}$ elevation. We then found that, 4-methyl-4′-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide (YM58483), which is known as an antagonist for $\text{Ca}^{2+}$-activated $\text{Ca}^{2+}$ channels (CRAC) [27] or canonical transient receptor potential (TRP) channels [28], inhibited mSIRK-induced $\text{Ca}^{2+}$ signal (Fig. 4b). (#Reviewer4-comment2) In addition, YM58483 attenuated $\text{G}\beta\gamma$’s inhibitory effect on Epac-induced cell migration (Fig. 4c). Since YM583483 did not change basal cell migration (Fig. 4c), $\text{G}\beta\gamma$-activation is necessary to inhibit Epac-induced cell migration. In contrast, neither verapamil nor nifedipine inhibit mSIRK-induced $\text{Ca}^{2+}$ (data not shown), suggesting that dihydropyridine $\text{Ca}^{2+}$ channels are not involved in $\text{G}\beta\gamma$-induced $\text{Ca}^{2+}$ signal. Put together, $\text{G}\beta\gamma$ inhibits Epac-induced $\text{Ca}^{2+}$ elevation, and cell migration by $\text{Ca}^{2+}$/calmodulin pathway.
DISCUSSION

In the present study, we demonstrated that G\(\beta\gamma\) interferes with the Ca\(^{2+}\) signaling evoked by Epac, leading to an inhibition of Epac-induced cell migration. We found that G\(\beta\gamma\) activates Ca\(^{2+}\) entry from the extracellular space, which inhibits Epac-induced cytosolic Ca\(^{2+}\) elevation, and cell migration. Calmodulin is presumably involved in these G\(\beta\gamma\)'s effects. Since G\(\beta\gamma\) is a common molecule located downstream of various GPCRs, our findings would provide novel insights in terms of Ca\(^{2+}\) signaling, cell migration, and cross talk of intracellular signaling in melanoma (Fig. 5).

We previously demonstrated that the expression of Epac1 is increased in metastatic melanoma than in primary melanoma in human melanoma samples. In addition, overexpression of Epac1 enhanced melanoma metastasis in mice [17]. These previous findings indicate that Epac1 accelerates melanoma metastasis, and thus a molecule that can inhibit Epac1 is a potential drug for suppressing melanoma metastasis. The major mechanism by which Epac1 enhances melanoma metastasis is, at least in part, an increase of cell migration via the Ca\(^{2+}\)-dependent mechanism [18]. We demonstrated that Epac activates the PLC/IP3 pathway, leading to the activation of IP3 receptor in the ER. Subsequently, Ca\(^{2+}\) released from the ER modifies actin assembly, and as a result, increases cell migration. (#Reviewer2-comment1) In our preliminary study, Epac-induced migration was inhibited by stimulation of different types of GPCRs, i.e., \(\beta\) adrenergic receptor, lysophosphatidic acid receptor and endothelin receptor. Since types of G\(\alpha\) subunit are different in these GPCRs whereas G\(\beta\gamma\) is common in these receptors, we hypothesized that G\(\beta\gamma\) mediates the inhibition in Epac-induced cell migration.

Although it is well known that G\(\beta\gamma\) regulates membrane Ca\(^{2+}\) channels in excitable cells, little attention has been paid to its role in non-excitable cells including cancer cells. A report demonstrated that G\(\beta\gamma\) activates IP3 receptor via phospholipase C [29]; however, this is not the case in melanomas because antagonists of IP3 receptors did not inhibit mSIRK-induced Ca\(^{2+}\) elevation (Fig. 3 f and g). Instead, we found that G\(\beta\gamma\) induces Ca\(^{2+}\) from the extracellular space in all melanoma cell lines which we have tested in this study. Moreover, the prior Ca\(^{2+}\) elevation by G\(\beta\gamma\) inhibited Epac-induced Ca\(^{2+}\) release, suggesting the crosstalk of Ca\(^{2+}\) signaling between G\(\beta\gamma\) and Epac. We also found that calmodulin, an ubiquitously expressed calcium binding protein, presumably mediates this G\(\beta\gamma\)'s effect (Fig. 4a). Since the activity of IP3 receptor is reduced by cytosolic Ca\(^{2+}\) elevation via calmodulin [19-21], and Epac activates IP3 receptor, it is tempting to speculate that calmodulin is activated by G\(\beta\gamma\)-induced Ca\(^{2+}\), leading to inactivation of IP3 receptor.

A question remains unclear what Ca\(^{2+}\) channel(s) is(are) activated by G\(\beta\gamma\). We demonstrated that YM58483 inhibits mSIRK-induced Ca\(^{2+}\) elevation. YM58483 is known to inhibit CRAC and TRP channels [28], suggesting involvement(s) of these receptors in G\(\beta\gamma\)-induced Ca\(^{2+}\) entry. Further vigorous experiments including knockdown of CRAC or TRP channels are necessary to elucidate the mechanism of G\(\beta\gamma\)-mediated Ca\(^{2+}\) influx. In addition, it is necessary to identify which G-protein coupled receptor(s) is(are) related to G\(\beta\gamma\)-induced Ca\(^{2+}\) entry, and to further investigate whether knocking down of a specific subtype(s) of G\(\beta\) or G\(\gamma\) subunit reduces melanoma cell
migration, and metastasis. (#Reviewer3-comment4) Meanwhile, mSIRK inhibited Epac-induced cell migration in a dose dependent manner whereas it did not reduce basal cell migration with the exception of the higher concentration (50 µM) (Fig. 1a), suggesting that mSIRK’s inhibition is obvious only when Epac is activated. This is in accordance with our previous report showing that inhibition of IP3 receptor reduces melanoma cell migration only under Epac-activated conditions [18].

Conclusions: In summary, the current study demonstrated the potential new cross talk pathway in melanoma in terms of Ca\textsuperscript{2+} homeostasis and cell migration. We demonstrated that there is interaction in cytosolic Ca\textsuperscript{2+} between G\textsubscript{βγ} and Epac in melanoma. The interaction evoked by G\textsubscript{βγ} inhibited Epac-induced Ca\textsuperscript{2+} elevation and cell migration via additional Ca\textsuperscript{2+} influx from extracellular space. We thus propose that G\textsubscript{βγ}-signaling inhibits the Epac-induced cell migration via a Ca\textsuperscript{2+}-dependent mechanism in melanoma. Further studies are needed whether the effects of G\textsubscript{βγ} are universal in other non-excitable cells including other types of cancer.

Abbreviations used: exchange protein activated by cAMP: Epac1; endoplasmic reticulum: ER; inositol 1,4,5-trisphosphate: IP3; G-protein βγ subunits: G\textsubscript{βγ}; guanosine 5′-O-(2-thiodiphosphate): GDP\textsubscript{βS}; guanosine 5′, α-β-methylene triphosphate: Gp(CH)\textsubscript{βpp}; protein kinase A: PKA; myr-SIRKALNILGYPDYD-OH: mSIRK; myr-SIRKALNIAGYPDYD-OH: L9A; 2-aminoethoxydiphenyl borate: 2-APB; N-(6-aminohexyl)-1-naphthalenesulfonamide: W-5; 8-(4-Methoxyphenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate: 8-pMeOPT; C-terminal domain of β-adrenergic receptor kinase: βARK-CT; 4-methyl-4′-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide: YM58483; Ca\textsuperscript{2+}-activated Ca\textsuperscript{2+} channels: CRAC; transient receptor potential cation channels: TRPC;

Competing Interests: We declare no financial and non-financial competing interests existing in relation to this manuscript.

Authors' contributions:
EB designed the study, performed experiments and wrote the manuscript. MU and L-H X performed calcium experiments and participated in the design of the intracellular calcium experiments. MN participated in the design of the study. KI designed the study, performed experiments and wrote the manuscript. All authors read and approved the final manuscript.

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FIGURE LEGEND

Fig. 1
**Gβγ inhibits Epac-induced cell migration.**
a) SK-Mel-2 cells were infected with adenovirus harboring LacZ or Epac1 followed by the migration assay in the presence of mSIRK or Gp(CH2)pp (10 µM). mSIRK inhibited Epac1-induced melanoma cell migration. *, p<0.05 vs. LacZ control, #, p<0.05 vs. Epac1 control. n=4 except 5 and 10 µM of mSIRK (n=2). b) Migration assay was performed in SK-Mel-2 cells in the presence or absence of 8-pMeOPT (200 µM), L9A (20 µM) or mSIRK (20 µM). mSIRK, but not L9A, inhibited 8-pMeOPT-induced cell migration. n=4. c and d) Following the termination of adenovirus harboring LacZ or Epac1, SK-Mel-2 cells were subjected to co-overexpression of Gβ1 and Gγ2 subunits or βARK-CT. Western blot analyses showed increased expression of the target proteins. e) Migration assay was performed in SK-Mel-2 cells. Co-overexpression of Gβ1 and Gγ2 inhibited Epac1-induced cell migration. Overexpression of βARK-CT restored Epac1-induced cell migration even in the presence of mSIRK (20 µM).

Fig. 2
**Gβγ inhibits Epac-induced Ca^{2+} elevation.** SK-Mel-2 cells were subjected to Ca^{2+} signal measurements. Arrows indicate addition of 8-pMeOPT (200 µM). a and b) SK-Mel-2 cells were pre-incubated with L9A (20 µM) or mSIRK (20 µM) for 5 min followed by the addition of 8-pMeOPT. mSIRK, but not L9A, inhibited 8-pMeOPT-induced Ca^{2+} elevation. c) 8-pMeOPT did not increase Ca^{2+} signal in SK-Mel-2 cells with co-overexpression of Gβ1 and Gγ2. d) 8-pMeOPT increased Ca^{2+} signal in SK-Mel-2 cells with overexpression βARK-CT even in the presence of mSIRK. e) When SK-Mel-2 cells were pretreated with the combination of mSIRK and GDPβs, 8-pMeOPT increased Ca^{2+} signal.

Fig. 3
**Gβγ induces Ca^{2+} influx from the extracellular space.** SK-Mel-2 cells were subjected to Ca^{2+} signal measurements. a and b) SK-Mel-2 cells were incubated with L9A (20 µM) or mSIRK (20 µM) followed by 8-pMeOPT stimulation (200 µM). mSIRK, but not L9A, increased Ca^{2+} signal. 8-pMeOPT failed to show an additional increase of Ca^{2+} signal after mSIRK. c, d and e) mSIRK-induced Ca^{2+} signal was inhibited by pretreatment with GDPβS (100 µM) for 5 min (c), by Ca^{2+} removal from the media (d) or by depletion of Ca^{2+} in the extracellular space with EGTA (5 mM) (e). f, g and h) Inhibition of IP3 receptors with 2-APB (1 µM) (f) or xestopongin C (1 µM) (g), and blocking of ryanodine receptor with ryanodine (10 µM) (h), did not inhibit mSIRK-induced Ca^{2+} elevation. i) mSIRK increases Ca^{2+} signal SK-Mel-2 cells after depletion of Ca^{2+} in the ER with thapsigargin (2 µM).

Fig. 4
Calmodulin is involved in Gβγ’s inhibition on Epac-induced Ca\(^{2+}\) elevation, and cell migration.
a) mSIRK (20 \(\mu\)M) and 8-pMeOPT (200 \(\mu\)M) were added in the presence of W-5 (100 \(\mu\)M) in SK-Mel-2 cells. W-5 restored 8-pMeOPT-induced Ca\(^{2+}\) elevation even in the presence of mSIRK. b) YM58483 (5 \(\mu\)M) inhibited mSIRK-induced Ca\(^{2+}\) elevation in SK-Mel-2 cells. c) Cell migration assay was performed in SK-Mel-2 cells in the presence or absence of 8-pMeOPT, mSIRK, W-5 or YM58483. W-5 and YM58483 restored Epac-induced cell migration even in the presence of mSIRK. n=4.

**Fig. 5**

**Ca\(^{2+}\) signal cross talk between Epac and Gβγ**
Activation of GPCR releases two signaling molecules, G\(\alpha\) and Gβγ. G\(\alpha\) activates cAMP production, leading to Ca\(^{2+}\) release from IP3 receptor in the ER via Epac/PLC/IP3 pathway. Ca\(^{2+}\) release from IP3 receptor induces cell migration. On the other hand, Gβγ stimulates Ca\(^{2+}\) influx from the extracellular space, leading to activation of calmodulin and the following inactivation of IP3 receptor. Finally, Gβγ inhibits Epac-induced Ca\(^{2+}\) release from the ER, leading to inhibition of cell migration.

**Fig. S1**

**Ablation of Epac1 inhibits GPCR-induced cell migration in melanoma.**
SK-Mel-2 cells were infected with lentivirus harboring Epac1- or control-shRNA as we previously described [18]. Migration assay was performed in the presence or absence of isoproterenol (ISO) (100 \(\mu\)M), a β-adrenergic receptor agonist. Ablation of Epac1 inhibits both basal and ISO-induced cell migration. n=4.
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