The interaction of $G\alpha_{13}$ with integrin β_1 mediates cell migration by dynamic regulation of RhoA

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ABSTRACT **Heterotrimeric G protein $G\alpha_{13}$ is known to transmit G protein–coupled receptor (GPCR) signals leading to activation of RhoA and plays a role in cell migration. The mechanism underlying the role of $G\alpha_{13}$ in cell migration, however, remains unclear. Recently we found that $G\alpha_{13}$ interacts with the cytoplasmic domain of integrin β_3 subunits in platelets via a conserved ExE motif. Here we show that a similar direct interaction between $G\alpha_{13}$ and the cytoplasmic domain of the integrin β_1 subunit plays a critical role in β_1-dependent cell migration. Point mutation of either glutamic acid in the $G\alpha_{13}$-binding ^{767}EKE motif in β_1 or treatment with a peptide derived from the $G\alpha_{13}$-binding sequence of β_1 abolished $G\alpha_{13}$–β_1 interaction and inhibited β_1 integrin–dependent cell spreading and migration. We further show that the $G\alpha_{13}$-β_1 interaction mediates β_1 integrin–dependent Src activation and transient RhoA inhibition during initial cell adhesion, which is in contrast to the role of $G\alpha_{13}$ in mediating GPCR-dependent RhoA activation. These data indicate that $G\alpha_{13}$ plays dynamic roles in both stimulating RhoA via a GPCR pathway and inhibiting RhoA via an integrin signaling pathway. This dynamic regulation of RhoA activity is critical for cell migration on β_1 integrin ligands.**

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INTRODUCTION

G protein–coupled receptors (GPCRs) transmit signals via the heterotrimeric G proteins and are important, working in concert with β_1 integrins, for directed cell migration (Sakai *et al.*, 1998a,b, 1999). $G\alpha_{13}$ is among the G proteins important in the directed cell migration and chemotaxis in various cell types (Goulimari *et al.*, 2005; Bian *et al.*, 2006; Rieken *et al.*, 2006). It has been believed that this role of $G\alpha_{13}$ is mediated via the $G\alpha_{13}$-dependent activation of RhoGEFs, including p115RhoGEF (ARHGEF1), which subsequently activates the small GTPase RhoA (Kozasa *et al.*, 1998; Suzuki *et al.*, 2003; Radhika *et al.*, 2004; Goulimari *et al.*, 2005; Kreutz *et al.*, 2007). RhoA activates actomyosin–mediated contraction through inhibition of myosin light chain phosphatases (Kimura *et al.*, 1996) and increases actin polymerization through activation of formin (mDia1), events that are important for cell migration (Goulimari *et al.*, 2005).

The integrin family of adhesion receptors mediates cell adhesion and migration on extracellular matrices and is important in many physiological and pathological processes, such as development, wound healing, immunity, thrombosis, and cancer metastasis (Hynes, 2002; Ley *et al.*, 2007; Li *et al.*, 2010; Margadant and Sonnenberg, 2010; Huttenlocher and Horwitz, 2011; Seguin *et al.*, 2015). In particular, the widely expressed β_1 integrins are critical for regulating anchorage-dependent cell survival, proliferation, and migration on extracellular matrices. Integrins transmit signals bidirectionally: high-affinity ligand binding to integrins can be activated by "inside-out" signaling (Ginsberg *et al.*, 2005), whereas ligand binding induces "outside-in" signaling (Shattil, 2005). Outside-in signaling elicits a cascade of intracellular signaling events, resulting in two essential cellular responses to the extracellular matrix proteins: cell spreading (formation of protrusions, filopodia and lamellipodia) and cell retraction, both of which are important in driving cell migration

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Abbreviations used: β_1CD, integrin β_1 cytoplasmic domain; GST, glutathione *S*-transferase; Wt, wild type.

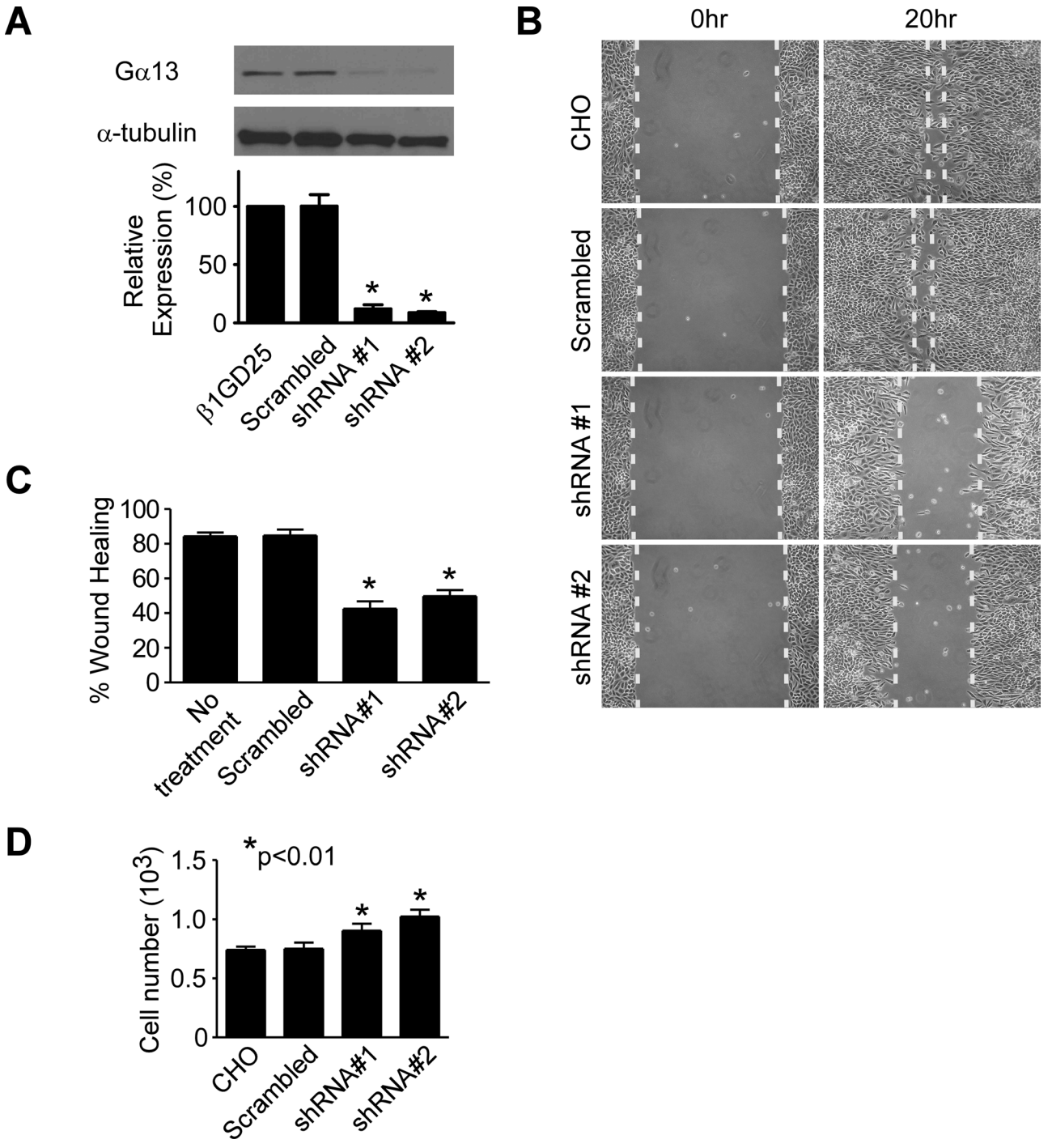


FIGURE 1: The importance of $G\alpha_{13}$ in cell migration. (A) Western blot comparison of $G\alpha_{13}$ expression levels in CHO cells and CHO cells transfected with $G\alpha_{13}$-specific shRNA #1, shRNA #2, or scrambled control shRNA lentivirus. α-Tubulin was used as loading control. Quantitative data are shown as mean ± SD. (B) Phase contrast images of CHO cells before and after 20-h migration following wound scratches. (C) Quantification of data as shown in B; $n = 6$. (D) Total cell counts at 24-h time point (five experiments). Cell count at 0 h is 0.5×10^3.

on extracellular matrices (Huttenlocher and Horwitz, 2011; Shen *et al.*, 2012). It is unclear how integrins signal to coordinate cell spreading and retraction and mediate cell migration. It is also unclear whether there is a connection between the roles of $G\alpha_{13}$ and integrins in cell migration.

Recently we found that the platelet integrin β_3 subunit binds directly to $G\alpha_{13}$, and the $G\alpha_{13}$–β_3 binding plays a critical role in integrin $\alpha_{IIb}\beta_3$–mediated outside-in signaling, cell spreading, and amplification of thrombus formation (Gong *et al.*, 2010; Shen *et al.*, 2013). Of interest, $G\alpha_{13}$ binding to the integrin β_3 subunit requires an ExE motif that is highly conserved among most integrin β subunits, including β_1 (Shen *et al.*, 2013). Thus we hypothesized that $G\alpha_{13}$ might also be important in mediating signaling of β_1 integrins.

In this study, we demonstrate that the interaction of $G\alpha_{13}$ with integrin β_1 plays a critical role not only in β_1 integrin–mediated cell spreading, but also in β_1-dependent cell migration on the

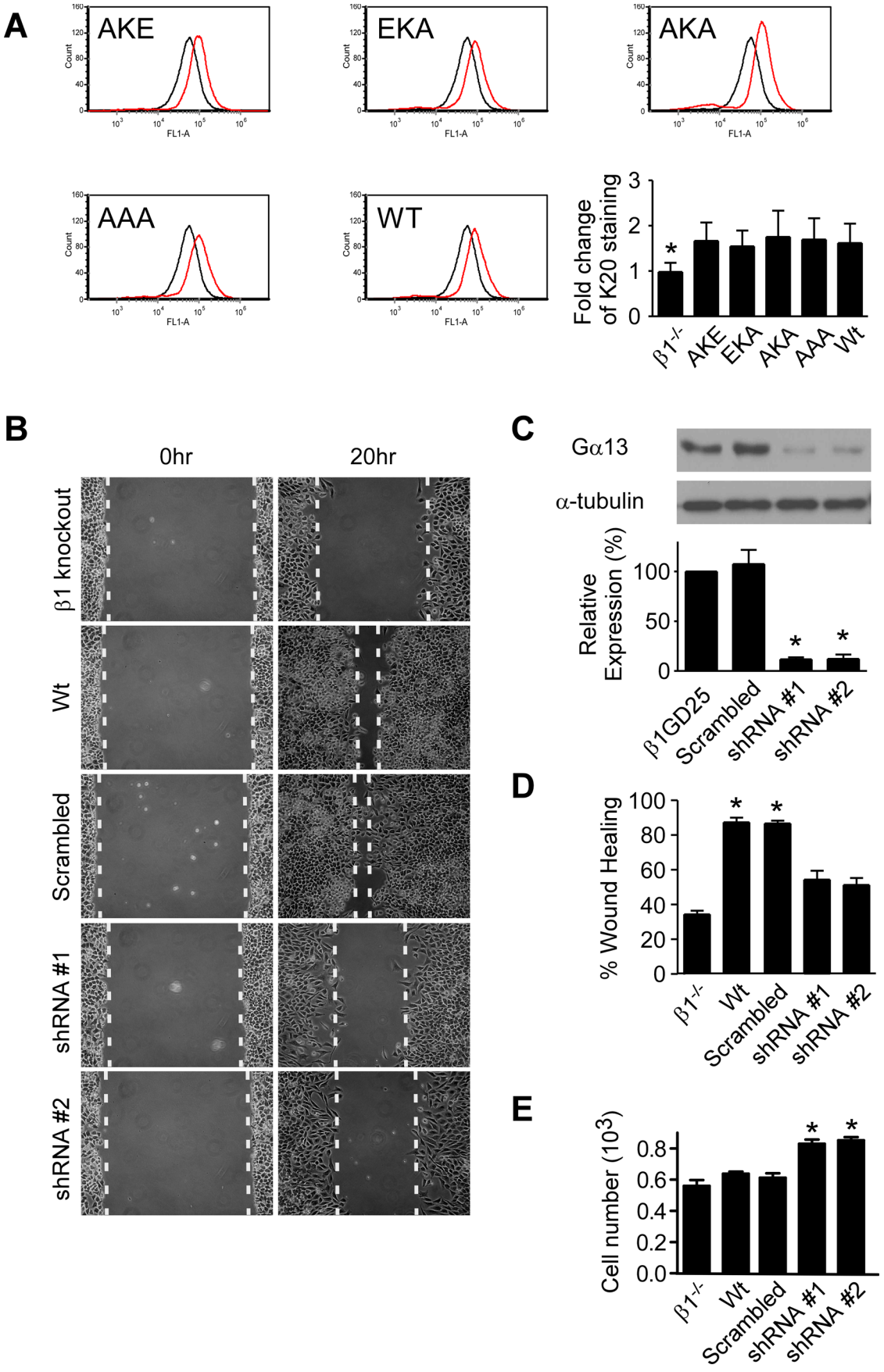


FIGURE 2: The role of $G\alpha_{13}$ in integrin β_1–dependent cell migration. (A) Flow cytometric analysis of β_1 expression on GD25 cell surface after lentiviral transfection of various integrin β_1 mutants. Similar expression levels of these mutants

extracellular matrix. Furthermore, we show that, in contrast to the previously reported role of $G\alpha_{13}$ in the activation of RhoGEF/RhoA pathway (Kozasa *et al.*, 1998; Suzuki *et al.*, 2003), the $G\alpha_{13}$–β_1 interaction mediates transient inhibition of RhoA signaling pathway through activation of Src (Arthur *et al.*, 2000). Thus our studies suggest a new mechanism in which the dynamic regulation of the RhoA signaling pathway by $G\alpha_{13}$ occurs through its binding to RhoGEF GTPases and β_1 integrins. This novel mechanism is critical for β_1 integrin–dependent cell migration.

RESULTS

The importance of $G\alpha_{13}$ in cell migration

To assess the role of $G\alpha_{13}$ in cell migration, we developed two $G\alpha_{13}$-specific short hairpin RNAs (shRNAs) and their corresponding nonspecific scrambled control shRNA using a lentiviral vector. Transfection of the $G\alpha_{13}$-specific shRNA, but not the scrambled control, resulted in ~90% knockdown of $G\alpha_{13}$ in CHO cells (Figure 1A), which express endogenous β_1 and β_5 integrins but not β_3 integrins (Ylanne *et al.*, 1993). We used a scratched wound–healing assay to assess the role of $G\alpha_{13}$ in cell migration. As shown in Figure 1B, 20 h after the scratch, CHO cells and CHO cells transfected with control shRNA had migrated to almost seal the gap. In contrast, $G\alpha_{13}$-knockdown cells were defective in migration (Figure 1, B and C).

The process of scratched wound healing consists of both cell proliferation and cell migration. To exclude the possibility that the phenotypes we observed were attributed to suppression of cell proliferation, we demonstrated that CHO cells with $G\alpha_{13}$ knockdown were not defective in cell proliferation, but instead cell proliferation was slightly increased compared with control (Figure 1D). Thus these data indicate that $G\alpha_{13}$ plays an important role in migration of CHO cells.

The role of $G\alpha_{13}$ in β_1 integrin–dependent cell migration

To determine specifically the role of $G\alpha_{13}$ in β_1 integrin–dependent cell migration, we analyzed cell migration in GD25 cells, an embryonic stem cell–derived fibroblast-like cell line originated from β_1 integrin–knockout mice (Wennerberg *et al.*, 1996), and GD25 cells transfected with wild-type (Wt) or mutant integrin β_1 subunits (Figure 2A).

The $\beta_1^{-/-}$ GD25 cells displayed severely impaired migration in a scratched wound–healing assay after 20 h (Figure 2B). In contrast, Expression of β_1 integrin in GD25 cells corrected the defective migration (Figure 2B). These data are consistent with a previous study showing that β_1 expression in β_1-knockout cell lines enhanced cell migration (Sakai *et al.*, 1998a,b, 1999; Gimond *et al.*, 1999), indicating that GD25 cell migration in the scratched wound–healing analysis is dependent upon integrin β_1. Moreover, knockdown of $G\alpha_{13}$ in the β_1-expressing GD25 cells using either of the two $G\alpha_{13}$ shRNAs caused significant defects in cell migration (Figure 2, B–D), indicating that $G\alpha_{13}$ plays a critical role in β_1-integrin–mediated cell migration. Similar to the aforementioned experiments using CHO cells, $G\alpha_{13}$ knockdown did not inhibit, but instead mildly increased cell proliferation compared with control (Figure 2E), indicating that the defective wound healing in $G\alpha_{13}$-knockdown cells was not due to inhibited cell growth but instead to suppressed cell migration.

The interaction of $G\alpha_{13}$ with β_1 integrins via the ExE motif

We previously showed that $G\alpha_{13}$ directly interacts with integrin β subunits via a conserved ExE motif (Shen *et al.*, 2013). To determine whether $G\alpha_{13}$ interacts with β_1 via the ^{767}EKE sequence, we incubated purified $G\alpha_{13}$ with Sepharose beads conjugated with purified glutathione bead–bound glutathione *S*-transferase–Wt β_1 integrin cytoplasmic domain fusion protein (GST-β_1CD) or with GST-β_1CD carrying mutations that change the ^{767}EKE sequence to AKA or AAA. $G\alpha_{13}$ binds directly to the GST-β_1CD Sepharose beads, whereas $G\alpha_{13}$ failed to bind to the AKA or AAA mutants (Figure 3A). Thus the ExE motif is crucial in the direct binding of $G\alpha_{13}$ to integrin β_1CD. To study whether the interaction between intact β_1 subunit and $G\alpha_{13}$ occurs in cells, we also introduced a set of mutations in the ^{767}EKE sequences changing EKE to AKE, EKA, AKA, or AAA and expressed these mutants in GD25 cells at similar levels (Figure 2A). We then performed coimmunoprecipitation experiments using these cells. There was little integrin β_1 associated with $G\alpha_{13}$ in suspended cells (Figure 3B). However, after cell adhesion on fibronectin for 1 h, the interaction becomes prominent (Figure 3B). This suggests that integrin ligation is required for the binding of $G\alpha_{13}$ to β_1 in these cells. Furthermore, AKE or EKA mutants showed significantly reduced $G\alpha_{13}$–β_1 association, and mutation of the glutamic residues (AKA or AAA) almost totally abolished the interaction (Figure 3B). Thus the β_1 integrin ExE motif is critical for $G\alpha_{13}$–β_1 interaction in cells. Consistent with previous results in β_3 integrins (Shen *et al.*, 2013), AAA mutation in β_1 did not inhibit talin association with β_1 integrin, which was in fact slightly increased (Figure 3C), suggesting that AAA mutation selectively abolished $G\alpha_{13}$–β_1 interaction without inhibiting talin binding.

The interaction between $G\alpha_{13}$ and β_1 integrins mediates β_1-dependent cell migration

To determine the importance of $G\alpha_{13}$–β_1 interaction in β_1-dependent cell migration, we used the scratched wound–healing assay to examine the migration of GD25 cells expressing Wt β_1 or various β_1 ExE motif mutants. The β_1/$G\alpha_{13}$-dependent cell migration was partially abolished in cells with AKE mutation in integrin β_1 and was totally abolished in cells expressing AKA, AAA, or EKA mutant (Figure 4, A and B). Mutations in the β_1 ExE motif did not affect proliferation of these β_1-expressing cells (Figure 4C). Thus it appears that $G\alpha_{13}$–integrin interaction is responsible for the role of $G\alpha_{13}$ in cell migration, and $G\alpha_{13}$–β_1 interaction is critically important for β_1-dependent cell migration.

To determine the role of $G\alpha_{13}$–integrin interaction in cell migration under different conditions, we also used a transwell migration assay. The integrin β_1 ligand fibronectin was coated on the bottom side of a transwell insert with 8-μm pores, which allow cells on the top side to migrate through the pores to the bottom side of the insert. As shown in Figure 5, transwell migration of GD25 cells requires β_1 integrin expression. $G\alpha_{13}$ knockdown abolished transwell

were achieved by cell sorting with FITC-conjugated anti-β_1 antibody K20. $\beta_1^{-/-}$ GD25 cells were used as negative control, and the quantification of the relative fold changes of K20 staining is shown ($n = 3$). (B) Phase contrast images of β_1(Wt)GD25 cells, with or without $G\alpha_{13}$-specific or scrambled control shRNA lentiviral transfection, before and after 20 h migration following wound scratches. (C) Western blot comparison of $G\alpha_{13}$ expression levels in β_1(Wt)GD25 cells and β_1(Wt)GD25 cells transfected with $G\alpha_{13}$-specific or scrambled control shRNA. α-Tubulin was used as loading control. Quantitative data are shown in the bar graph (mean ± SD, n = 3). (D) Quantification of data as shown in B (mean ± SD, $n = 6$). (E) Total cell count at 24-h time point (five experiments). Cell count at 0 h is 0.5×10^3.

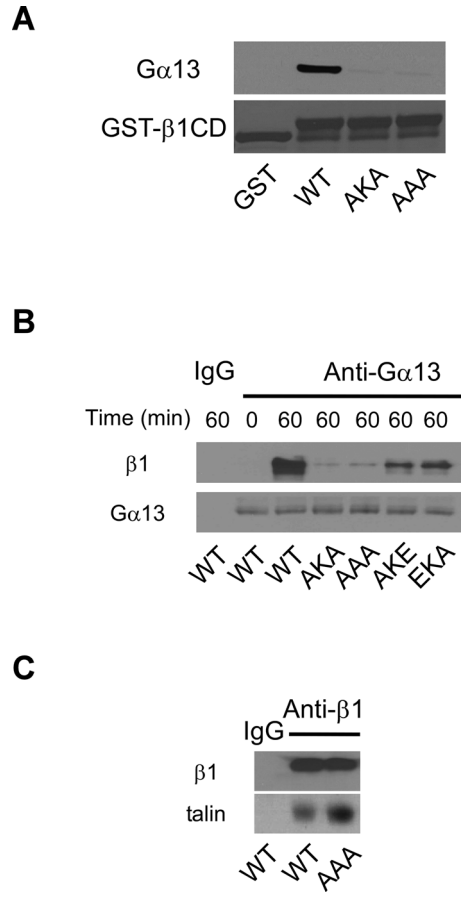


FIGURE 3: The critical role of the β_1 ExE motif in the $G\alpha_{13}$–β_1 interaction. (A) Binding of purified recombinant $G\alpha_{13}$ to glutathione bead–bound glutathione S-transferase (GST), GST-Wt β_1 cytoplasmic domain fusion protein (GST-β_1Wt), GST-(AKA)β_1, and GST-(AAA)β_1 mutants. (B) β_1GD25 cells expressing similar levels of wild-type (Wt) β_1 and various ExE β_1 mutants (AKE, EKA, AKA, or AAA) were allowed to adhere to fibronectin for 1 h. After 1 h, cells were lysed, and cell lysates were immunoprecipitated with anti-$G\alpha_{13}$ antibody or an equal amount of control rabbit IgG. Immunoprecipitates were immunoblotted with anti-$G\alpha_{13}$ and anti-β_1 antibodies. (C) β_1GD25 cells expressing similar levels of Wt and AAA mutant β_1 were allowed to adhere to fibronectin. After 1 h, cells were lysed, and cell lysates were immunoprecipitated with integrin β_1 antibody or IgG. Immunoprecipitates were immunoblotted with anti-talin and anti-β_1 antibodies.

migration almost completely. Similar to the scratched wound–healing assay, the AKE mutant of β_1 only partially supported transwell migration, and the EKA, AKA, and AAA mutants had very little activity (Figure 5, A and B). Furthermore, AAA mutation or $G\alpha_{13}$ knockdown significantly inhibited transwell migration when fibronectin coating was replaced with collagen coating (Figure 5C). On the basis of these results, we conclude that $G\alpha_{13}$–β_1 interaction is required for the β_1-dependent cell migration.

The role of $G\alpha_{13}$–β_1 interaction in mediating β_1 integrin outside-in signaling leading to cell spreading

Next we wanted to determine the mechanism responsible for the importance of $G\alpha_{13}$–integrin interaction in β_1-dependent cell migration. We previously showed that $G\alpha_{13}$ binding to the integrin β_3 is important in $\alpha_{IIb}\beta_3$ outside-in signaling and consequent cell spreading (Shen *et al.*, 2013). Hence we hypothesize that $G\alpha_{13}$-mediated β_1 integrin outside-in signaling and the consequent cell membrane movement (spreading and retraction) are responsible for their functions during cell migration. Thus we investigated how $G\alpha_{13}$ knockdown affects integrin-dependent cell spreading. CHO cells with $G\alpha_{13}$ knockdown were defective in spreading on immobilized integrin ligand fibronectin (Figure 6, A and B), suggesting the importance of $G\alpha_{13}$ in integrin-dependent cell spreading. Previous studies showed that early-phase cell spreading on fibronectin was defective in $\beta_1^{-/-}$ GD25 cells (Wennerberg *et al.*, 1996; Pankov *et al.*, 2003; Green *et al.*, 2009). Our data also show that early-phase cell spreading of GD25 cells on fibronectin is dependent on β_1 integrins. GD25 cells expressing the β_1 ExE motif mutants exhibited diminished early-phase cell spreading to a similar level as observed in β_1-deficient GD25 cells (Figure 6C), suggesting that the $G\alpha_{13}$–integrin interaction is important in β_1-mediated outside-in signaling leading to β_1-dependent cell spreading.

$G\alpha_{13}$–β_1 binding mediates outside-in signaling through activation of c-Src and transient inhibition of RhoA

To determine whether and how $G\alpha_{13}$ binding to β_1 mediates integrin outside-in signaling, we seeded Wt and mutant β_1-expressing GD25 cells onto immobilized fibronectin and measured Src activation (indicated by phosphorylation at Y416) and RhoA activity (indicated by RhoA pull down with GST rhotekin Rho binding domain protein [RBD] (Ren *et al.*, 1999), which specifically binds to active RhoA) at different time points after adhesion. Adhesion of the β_1(Wt)GD25 cells on fibronectin resulted in robust but transient RhoA inactivation (Figure 7, A and B). In contrast, this transient RhoA inactivation was not observed in β_1(AAA)GD25 cells, which showed constant activation of RhoA (Figure 7, A and B). Furthermore, Src was activated in Wt β_1–expressing GD25 cells after adhesion to fibronectin, and Src activation was inhibited in the AAA mutant β_1–expressing GD25 cells (Figure 7, C and D). Because it is known that transient RhoA inhibition after β_1 outside-in signaling is Src dependent (Arthur *et al.*, 2000), our data indicate that $G\alpha_{13}$ binding to the ExE motif of β_1 integrins mediates Src activation and Src-mediated inhibition of RhoA signaling. To explore further the role of the $G\alpha_{13}$-binding $\beta_1{}^{767}$ExE motif in regulating RhoA activity after cell adhesion, GD25 cells adherent to immobilized fibronectin were stained with the GST-rhotekin RBD (Ren *et al.*, 1999; Figure 8). Indeed, Wt but not AAA mutant β_1–expressing cells showed a transient reduction in GST-RBD staining (Figure 8A). The staining of the GST-RDB was specific, as it was abolished by Rho inhibitor C3-transferase, and GST protein alone failed to stain the cells (Figure 8A). Thus these data indicate that transient RhoA inhibition soon after cell adhesion (30 min) depended on $G\alpha_{13}$–β_1 interaction (Figure 8B).

The effect of an inhibitor peptide based on the $G\alpha_{13}$-binding sequence in β_1

The foregoing results indicate that integrin β_1–$G\alpha_{13}$ binding plays an important role in β_1 integrin–mediated activation of Src and transient inhibition of RhoA and in β_1-dependent cell migration. Thus we hypothesize that an inhibitor of β_1–$G\alpha_{13}$ interaction should inhibit β_1-dependent integrin outside-in signaling and cell migration. To test this hypothesis, we synthesized a myristoylated, cell-permeable peptide containing the $G\alpha_{13}$-binding ExE motif, mβ_1P_6 (Myr-FEKEKM). Preincubation of this peptide with β_1(Wt)GD25 cells abolished the interaction of $G\alpha_{13}$ to integrin β_1 in the coimmunoprecipitation assay (Figure 9A). In contrast, the scrambled control peptide mβ_1P_6Scr (Myr-EKMFEK) had no effect. These data indicate that mβ_1P_6 is effective in inhibiting $G\alpha_{13}$–β_1 interaction (Figure 9A).

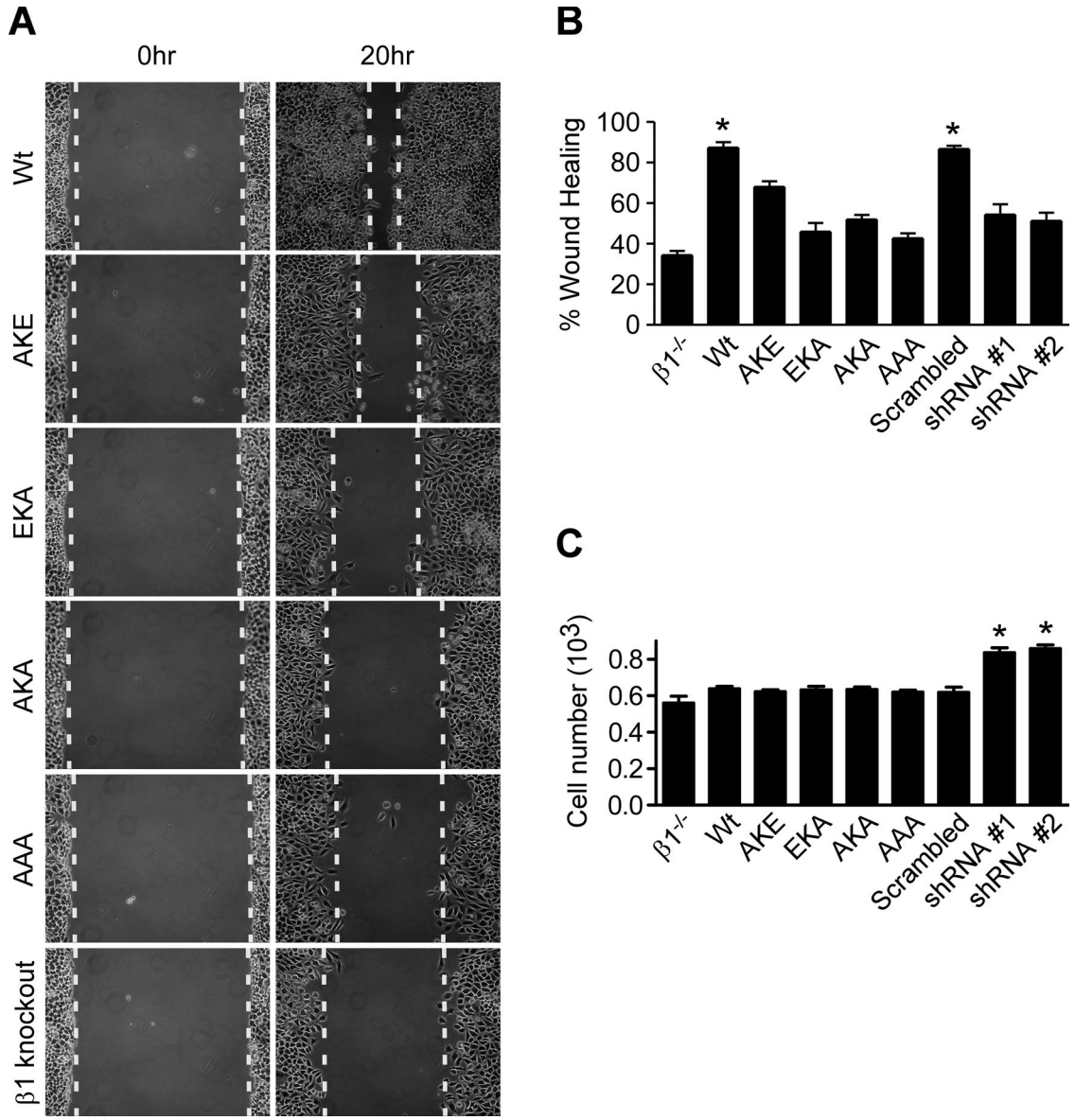


FIGURE 4: The interaction between $G\alpha_{13}$ and β_1 integrins mediates β_1-dependent migration. (A) Phase contrast images of β_1GD25 cells expressing similar levels of wild-type (Wt) β1 or various ExE β_1 mutants (AKE, EKA, AKA, or AAA), before and after 20 h migration following wound scratches. (B) Quantification of migration data in A (mean ± SD, $n = 6$). (C) Total cell count at 24-h time point (five experiments). Cell count at 0 h is 0.5×10^3.

Preincubation of $m\beta_1P_6$ but not the scrambled peptide also significantly inhibited transwell migration in β_1(Wt)GD25 cells (Figure 9, B and C). Furthermore, c-Src activity and transient RhoA inactivation were both inhibited by this inhibitor (Figure 9, D–G), mirroring the results of ExE mutation (Figure 7). Thus this inhibitory peptide of β_1–$G\alpha_{13}$ interaction was an effective blocker of integrin β_1–mediated outside-in signaling and cell migration.

DISCUSSION

In this study, we demonstrated that $G\alpha_{13}$ directly binds to the ExE motif of integrin β_1 cytoplasmic domain, and this binding is required for β_1 outside-in signaling and cell migration on the β_1 integrin ligand fibronectin. Of importance, we showed that $G\alpha_{13}$–integrin interaction mediates transient RhoA inhibition, which is in contrast to the well-known role of $G\alpha_{13}$ in mediating RhoA activation by binding to RhoGEFs (Goulimari *et al.*, 2005; Chen *et al.*, 2012). Thus our data suggest that $G\alpha_{13}$-mediated dynamic regulation of RhoA activity is a novel mechanism responsible for the role $G\alpha_{13}$ during β_1 integrin–dependent cell migration.

The importance of integrin β_1 in cell migration has been reported in various cell types (White *et al.*, 2004; Liu *et al.*, 2010). In addition, evidence has also been given for a role for $G\alpha_{13}$ in directed cell migration (Radhika *et al.*, 2004; Shan *et al.*, 2006; Tan *et al.*, 2006). However, it was not previously appreciated why and how β_1 integrins and $G\alpha_{13}$ play important roles in mediating cell migration and whether there is a connection. Here we show that integrin β_1–dependent cell migration requires direct binding of $G\alpha_{13}$ to the cytoplasmic domain of β_1, establishing a direct connection between these two important molecules in cell migration. Recently we showed that $G\alpha_{13}$ interacts with the platelet

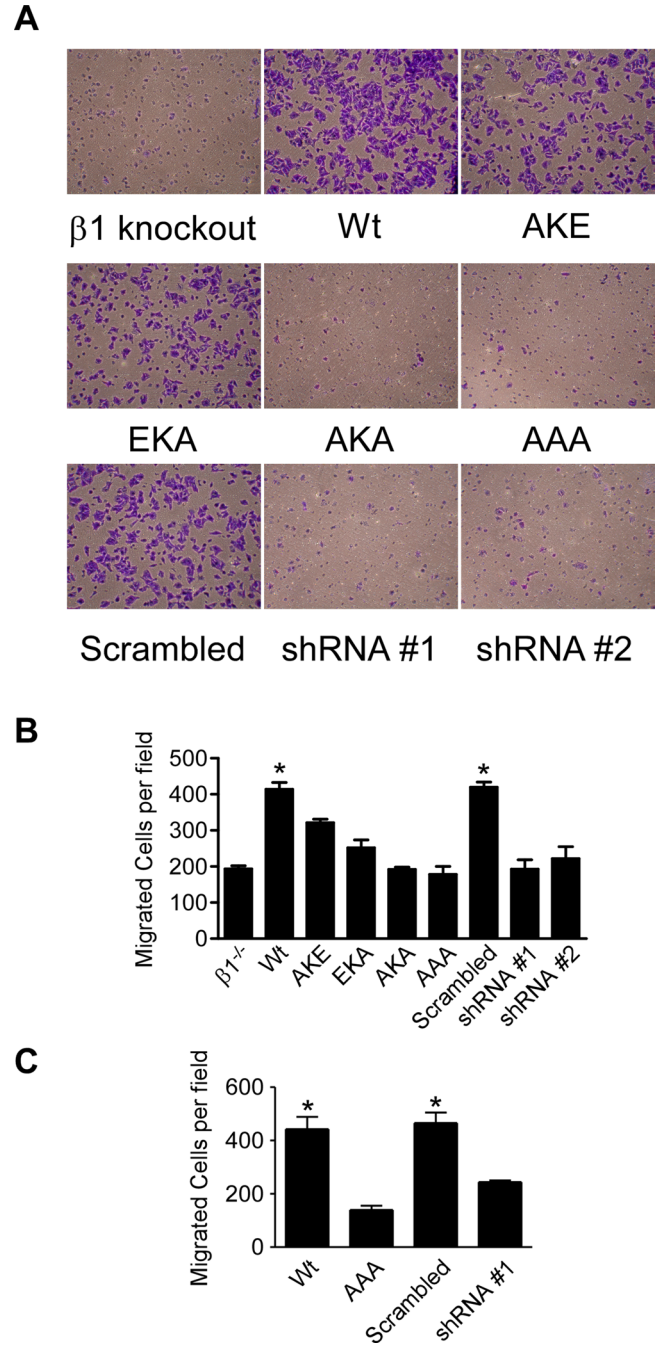


FIGURE 5: The interaction between $G\alpha_{13}$ and β_1 integrins mediates β_1-dependent transwell migration. (A) GD25 cells without or with expression of similar levels of wild-type (Wt) β_1 and various ExE β_1 mutants (AKE, EKA, AKA, or AAA) or β_1(Wt)GD25 cells transfected with scrambled or $G\alpha_{13}$-specific shRNA were compared in a transwell migration assay with fibronectin coated on the bottom side of the insert well. Cells were fixed and stained with crystal violet after 6 h of migration. (B) Quantification of migrated cells in A (mean ± SD, four experiments). (C) Wild-type or AAA mutant β_1GD25 cells or Wt β_1GD25 cells transfected with scrambled or $G\alpha_{13}$-specific shRNA were compared in the transwell migration assay as described in A, with the exception of collagen coating on the bottom side. Cells were fixed and stained with crystal violet after 6 h of migration. Numbers of migrated cells were compared (mean ± SD, three experiments).

integrin $\alpha_{IIb}\beta_3$ and plays an important role in $\alpha_{IIb}\beta_3$ outside-in signaling (Shen *et al.*, 2013). Migration is not ordinarily considered or studied as an activity of platelets, and it is unclear whether $G\alpha_{13}$–β_3 interaction is a common mechanism of integrin signaling that is shared with β_1 integrin signaling and important in cell migration. Furthermore, there are major differences in outside-in signaling mechanisms between platelet β_3 integrins and β_1 integrins in nucleated cells. In particular, β_3 outside-in signaling requires Src binding to the C-terminal site of β_3, which can be cleaved by calpain (Arias-Salgado *et al.*, 2003; Flevaris *et al.*, 2007). Calpain cleavage of β_3 cytoplasmic domain abolishes c-Src binding and switches β_3 integrin signaling from mediating cell spreading to retraction (Flevaris *et al.*, 2007). However, Src does not appear to bind to β_1 C-terminus (Arias-Salgado *et al.*, 2003), and thus its interaction with β_1 is likely to be regulated in a different way. Furthermore, β_1 signaling was reported to involve complex formation of focal adhesion kinase (FAK) with Src and integrin and FAK-dependent Src activation (Xing *et al.*, 1994; Thomas *et al.*, 1998), which has not been shown in platelet $\alpha_{IIb}\beta_3$. We recently showed that platelet $\alpha_{IIb}\beta_3$-dependent activation of Src requires $G\alpha_{13}$ binding to β_3 (Gong *et al.*, 2010; Shen *et al.*, 2013). Here we further show that $G\alpha_{13}$ directly binds to the ExE motif in β_1. Disruption of $G\alpha_{13}$–β_1 binding through mutations in the ExE motif or use of an ExE motif peptide abolished β_1-dependent Src activation and inhibited β_1-dependent outside-in signaling and cell spreading. These data indicate that, despite the difference between β_1 and β_3 signaling, the ExE motif–containing integrins β_1 and β_3 share similar $G\alpha_{13}$-dependent mechanisms of Src activation and outside-in signaling in platelets and migrating nucleated cells. Of importance, our data for the first time indicate that the interaction between $G\alpha_{13}$ and β_1 ExE motif plays a critical role in integrin-dependent cell migration.

Our data not only indicate the important role for the $G\alpha_{13}$–β_1 interaction in cell migration, but they also suggest a novel mechanism of $G\alpha_{13}$-dependent dual regulation of RhoA activity in migrating cells (Figure 10). Cell migration on β_1 integrin ligands involves coordinated integrin-dependent cell spreading and retraction. The alternate protrusion and retraction occur during cell spreading at the leading edge (Machacek *et al.*, 2009; Tkachenko *et al.*, 2011). Retraction in the rear of a cell pulls the cell forward (Lauffenburger and Horwitz, 1996; Ridley *et al.*, 2003). The Rho-family GTPase RhoA is a major regulator of cell retraction. RhoA activates Rho kinase. Rho kinase inhibits myosin light chain (MLC) phosphatase and increases MLC phosphorylation, resulting in actomyosin-mediated cell retraction (Kimura *et al.*, 1996), which drives inward movement of cell membranes. Thus RhoA activity inhibits cell spreading and stimulates cell retraction (Vega *et al.*, 2011). Consequently, RhoA-dependent retractile signaling needs to be dynamically activated and inhibited in order for cells to migrate. On activation by GPCRs, $G\alpha_{13}$ directly stimulates RhoGEFs and activation of RhoA (Kozasa *et al.*, 1998), which is believed to be the reason that $G\alpha_{13}$ is important in cell migration (Bian *et al.*, 2006; Patel *et al.*, 2014). Here we for the first time demonstrate the other aspect of this dynamic regulation. We show that $G\alpha_{13}$ binding to β_1 ExE motif mediates β_1 integrin–dependent activation of Src and transient inhibition of RhoA in migrating cells. This finding is not only consistent with previous data suggesting that integrin β_1 outside-in signaling transiently inhibits RhoA activity via Src during cell spreading (Arthur and Burridge, 2001), but it also provides a plausible mechanism for

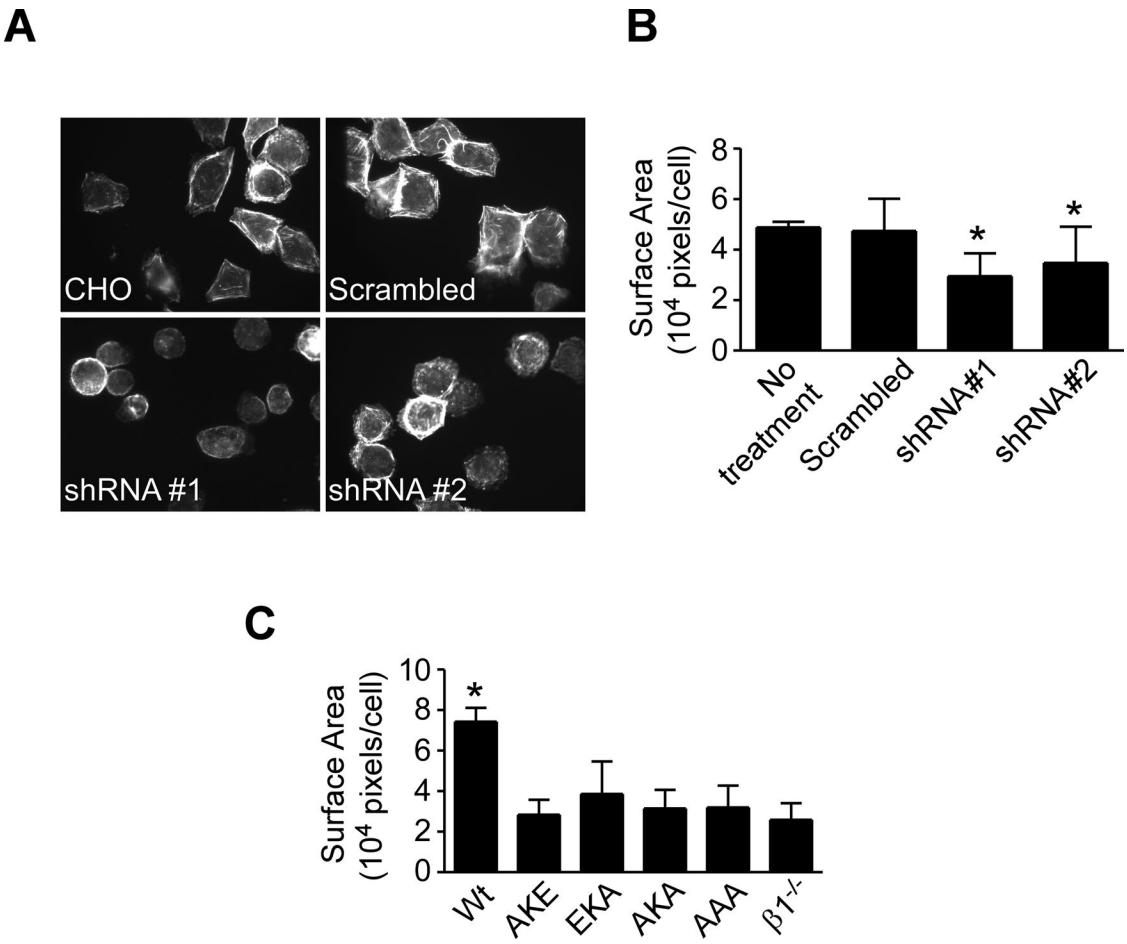


FIGURE 6: The role of $G\alpha_{13}$–β_1 interaction in mediating β_1 integrin outside-in signaling leading to cell spreading. (A) Fluorescence microscopy images of phalloidin-stained CHO cells spreading on fibronectin for 1 h. (B) Quantification of surface areas of individual cells (mean ± SE, $n = 31$, 24, 28, and 29 for CHO and CHO transfected with scrambled or $G\alpha_{13}$-specific shRNAs). (C) Quantification of surface areas of individual GD25 cells spreading on fibronectin for 30 min (mean ± SE). $n = 23$, 25, 26, 24, 28, and 31 for Wt, AKE, EKA, AKA, AAA, and β_1 knockout GD25 cells, respectively.

initiating Src-dependent transient RhoA inhibition. Thus our data, together with the previously reported role of $G\alpha_{13}$ in activating RhoA, suggest a novel mechanism by which dynamic regulation of RhoA activation by $G\alpha_{13}$ is achieved: $G\alpha_{13}$ binding to ligand-bound integrins induces Src-dependent transient inhibition of RhoA, which is required for cell spreading. On the other hand, $G\alpha_{13}$ binding to RhoGEFs stimulates RhoA and thus drives cell retraction together with RhoA activation induced by late-phase integrin signaling (Dubash *et al.*, 2007). Dynamic regulation of RhoA activation by $G\alpha_{13}$ thus provides a novel mechanism explaining the importance of $G\alpha_{13}$ in driving coordinated cell spreading and retraction, leading to cell migration.

MATERIALS AND METHODS

Reagents

Human integrin β_1 cDNA was cloned into plenti6/V5-DEST vector after digestion with *Eco*RI and *Xho*I. Integrin E-to-A mutants were generated using PCR and cloned into plenti6/V5-DEST vector by *Eco*RI and *Xho*I. GST-β_1CD and recombinant $G\alpha_{13}$ purification was described previously (Shen *et al.*, 2013). Anti-RhoA antibody was from Cytoskeleton (Denver, CO); anti-$G\alpha_{13}$(sc410), anti–c-Src (sc18), anti–integrin β_1 K20 (sc18887), and anti–integrin β_1 JB1B (sc59829) antibodies were from Santa Cruz Biotechnology (Dallas, TX); anti-$G\alpha_{13}$ (26004) was from NewEast Biosciences (King of Prussia, PA); anti–phospho-Src Y^{416} antibody was from Cell Signaling Technology (Danvers, MA); anti–GST tag antibody and Alexa Fluor 555 conjugate were from EMD Millipore (Billerica, MA); Lipofectamine 2000, ViraPower Lentiviral Expression System, and Alexa Fluor 546–conjugated phalloidin were from Invitrogen (Carlsbad, CA); fibronectin was from BD Biosciences (Franklin Lakes, NJ); the Active Rho Pull-Down and Detection Kit was from Pierce, Thermo Scientific (Waltham, MA).

Cell culture

CHO (Gu *et al.*, 1999; Xi *et al.*, 2003), 293FT, and GD25 cells were cultured in DMEM complete (Cellgro) supplemented with 10% fetal bovine serum (FBS; Corning), 2 mM L-glutamine (Corning), 100 U/ml penicillin plus 100 µg/ml streptomycin (Corning), 1 mM sodium pyruvate (Gibco), 0.1 mM nonessential amino acids (Gibco), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Gibco). Cells were serum starved in DMEM containing each of the foregoing components except the FBS for at least 4 h before experiments.

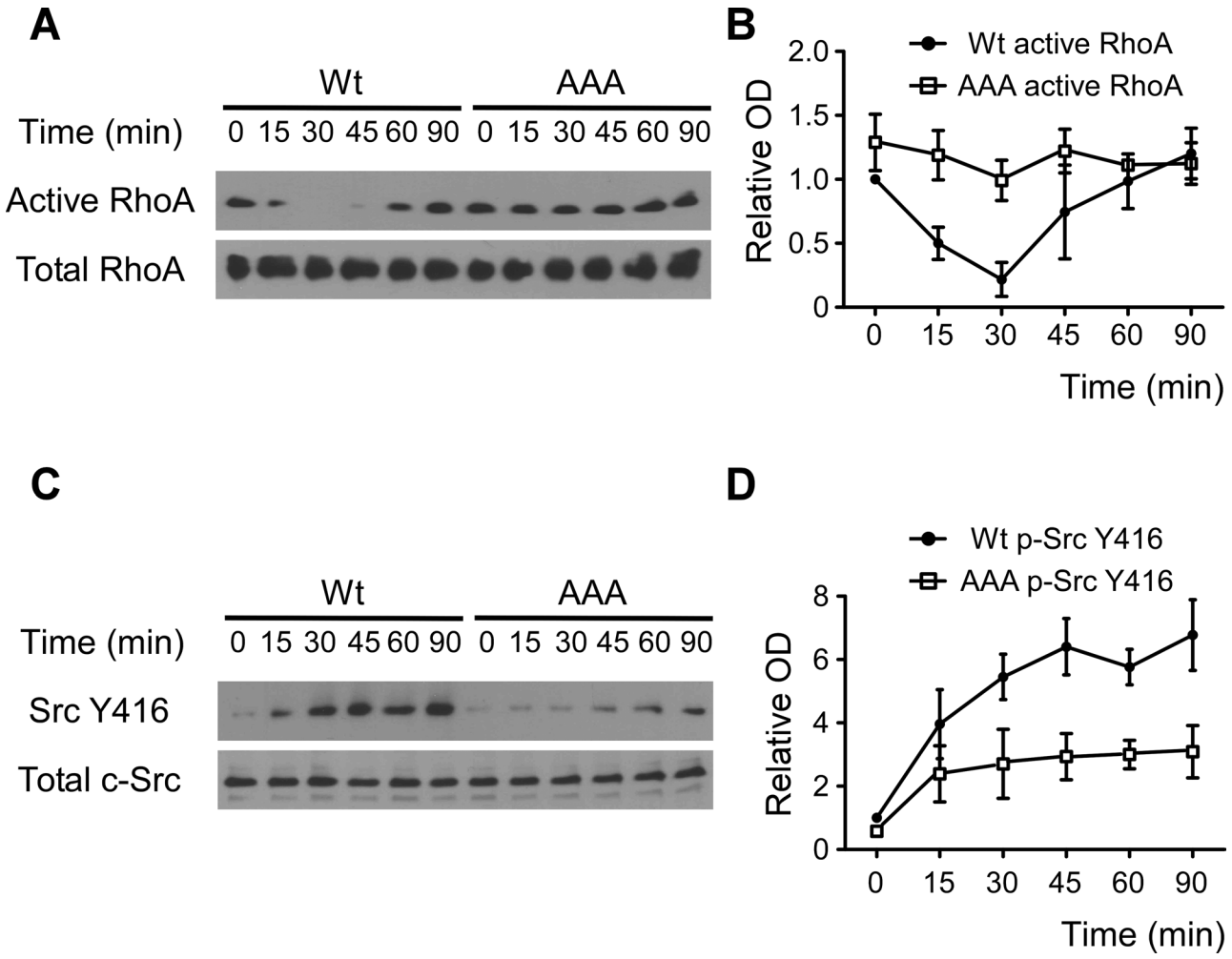


FIGURE 7: $G\alpha_{13}$–β_1 binding mediates activation of c-Src and transient inhibition of RhoA. GD25 cells expressing similar levels of Wt or AAA mutant β_1 were allowed to adhere to immobilized fibronectin, solubilized at various time points, and analyzed for RhoA activation (A) and c-Src Tyr416 phosphorylation (C). (B, D) Quantification of three independent experiments.

Lentiviral infection and integrin β_1 reconstitution

$G\alpha_{13}$ shRNA lentivirus was prepared as described previously (Gong *et al.*, 2010). Briefly, pLL3.7-scrambled shRNA or pLL3.7-$G\alpha_{13}$ shRNA (#1 and #2) were cotransfected into subconfluent 293FT cells with pLP1, pLP2, and pLP/VSVG plasmids (Invitrogen) using Lipofectamine 2000. After 48 h, cell culture supernatant was collected, filtered, and used to infect CHO or GD25 cells. Similarly, expression of integrin β_1 in GD25 cells was achieved by infecting GD25 cells with 293FT supernatant after plenti6-V5-DEST-β_1 transfection using the ViraPower Lentiviral Expression System. Expression of Wt or mutant β_1 integrin on the GD25 cell surface was assayed by flow cytometry using fluorescein isothiocyanate (FITC)–conjugated anti–integrin β_1 antibody K20.

Purified $G\alpha_{13}$ binding to integrin β_1 cytoplasmic domains

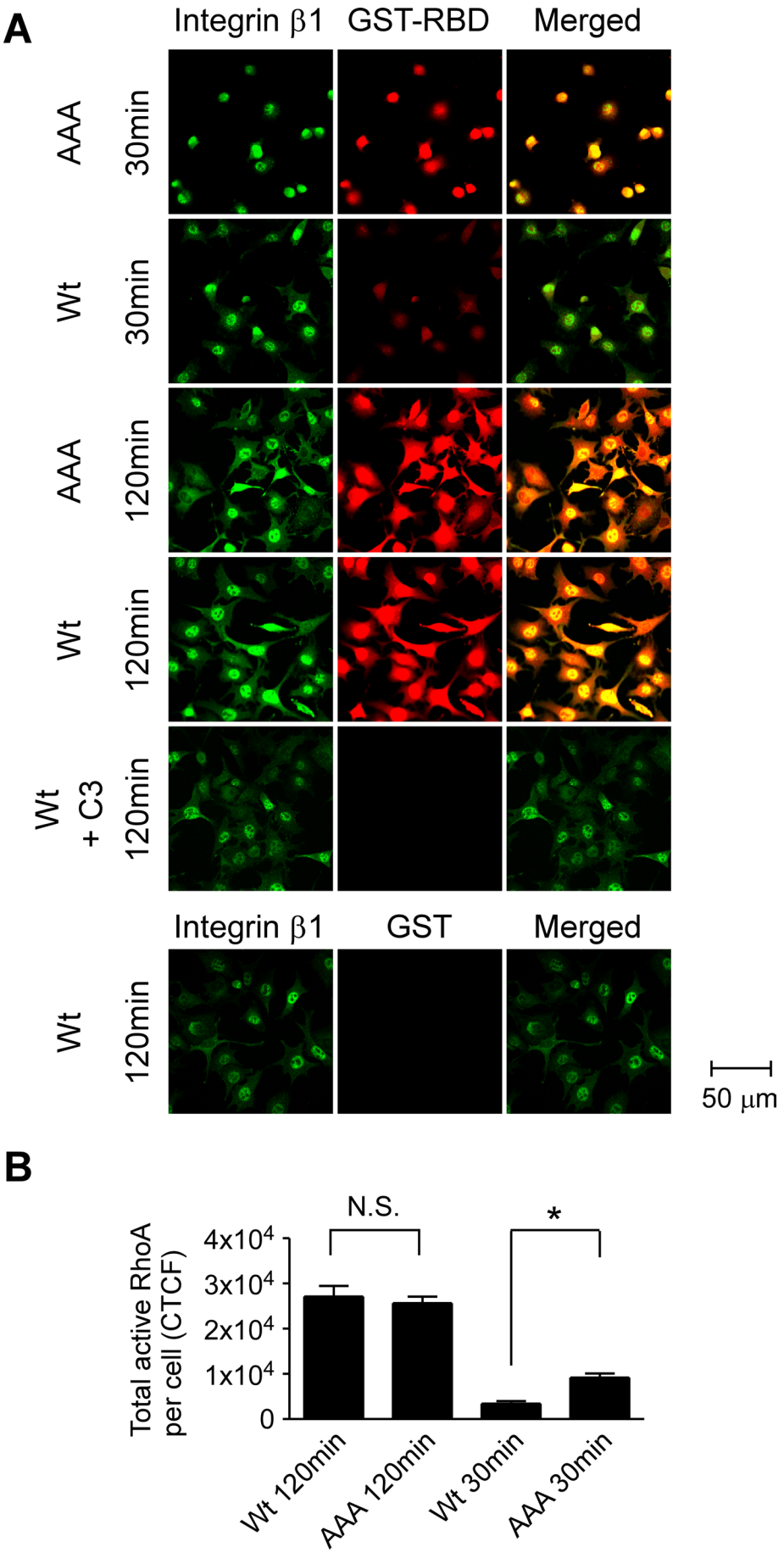
GST-tagged integrin β_1 cytoplasmic domain proteins were incubated with purified recombinant $G\alpha_{13}$ at 4°C overnight in NP40 buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM NaF) with complete protease inhibitor cocktail tablets (1 tablet/5 ml buffer; Roche). After three washes with the NP40 buffer, bound $G\alpha_{13}$ was analyzed by SDS–PAGE and Western blots with antibodies against $G\alpha_{13}$.

Cell-spreading assay

Cultured cells were detached with 0.053 mM EDTA and resuspended in serum-free DMEM (Flevaris *et al.*, 2007). Cells were allowed to spread on 10 μg/ml fibronectin–coated coverslips for different time points, fixed, permeabilized, stained with Alexa Fluor 546–conjugated phalloidin, and viewed with a Leica RMI RB microscope as previously described (O'Brien *et al.*, 2012; Shen *et al.*, 2013). Quantification of the surface area of spreading cells was performed using ImageJ (National Institutes of Health, Bethesda, MD).

Wound-healing assay

Similar to what was previously described (Liang *et al.*, 2007), cells were cultured until subconfluent and serum starved in serum-free DMEM for at least 6 h before the experiment. After starvation, scratches were made using p200 pipet tips, and DMEM complete was supplied to the cells. Cell migration and wound healing were monitored by taking pictures at selected time points using bright-field microscopy.



Transwell migration assay

Fibronectin or collagen (30 μg/ml) was coated on the outer surface of 6.5-mm transwell inserts with 8-μm pore size (Corning) overnight at room temperature. The insert was washed with phosphate-buffered saline (PBS) and blocked with 2% bovine serum albumin in PBS for 30 min at 37°C. Serum-starved cells in DMEM with 0.5% FBS were seeded on the upper chamber of the insert and allowed to migrate for 6 h in a 37°C incubator. After that, cells on the outer surface were fixed with 3.7% paraformaldehyde, stained with crystal violet, and visualized with bright-field microscopy (Huttenlocher *et al.*, 1996).

Coimmunoprecipitation

As previously described (Shen *et al.*, 2013), cells expressing Wt or mutant human integrin β_1 were solubilized in NP40 lysis buffer with complete protease inhibitor cocktail tablets (1 tablet/5 ml buffer; Roche). Lysis debris was cleared after centrifugation at 14,000 × *g* for 10 min. Lysates were then immunoprecipitated with rabbit anti-Gα_{13} immunoglobulin G (IgG) or an equal amount of control rabbit IgG for at least 6 h before protein A/G Sepharose beads were added. After incubation of protein A/G Sepharose beads for 45 min at 4°C, beads were centrifuged down and washed six times with NP40 lysis buffer. Immunoprecipitates were analyzed by immunoblotting using anti-β_1 antibody JB1B or anti-Gα_{13} antibody (26004).

In situ RhoA immunofluorescence assay

GD25 cells were allowed to spread on immobilized fibronectin (10 μg/ml), fixed with 3.7% paraformaldehyde, permeabilized, and incubated with purified GST-RBD proteins as previously described (Flevaris *et al.*, 2007). Then cells were stained with anti–integrin β_1 antibody conjugated with Alexa Fluor 488 and anti–GST tag antibody conjugated with Alexa Fluor 555 and viewed with a Zeiss LSM510 META confocal microscope.

FIGURE 8: In situ RhoA activation analysis of the role Gα_{13} binding to β_1. (A) GD25 cells were allowed to adhere to immobilized fibronectin, fixed, and stained with anti-β_1 antibody (green) and GST-RBD proteins (red). β_1(Wt)GD25 cells were also preincubated with 20 μg/ml C3-transferase and stained with anti-β_1 (green) and GST-RBD (red). Cells stained with anti-β_1 antibody (green) and GST proteins (red) served as additional negative controls. (B) Quantification of the active RhoA in A (mean ± SD, $n = 3$).

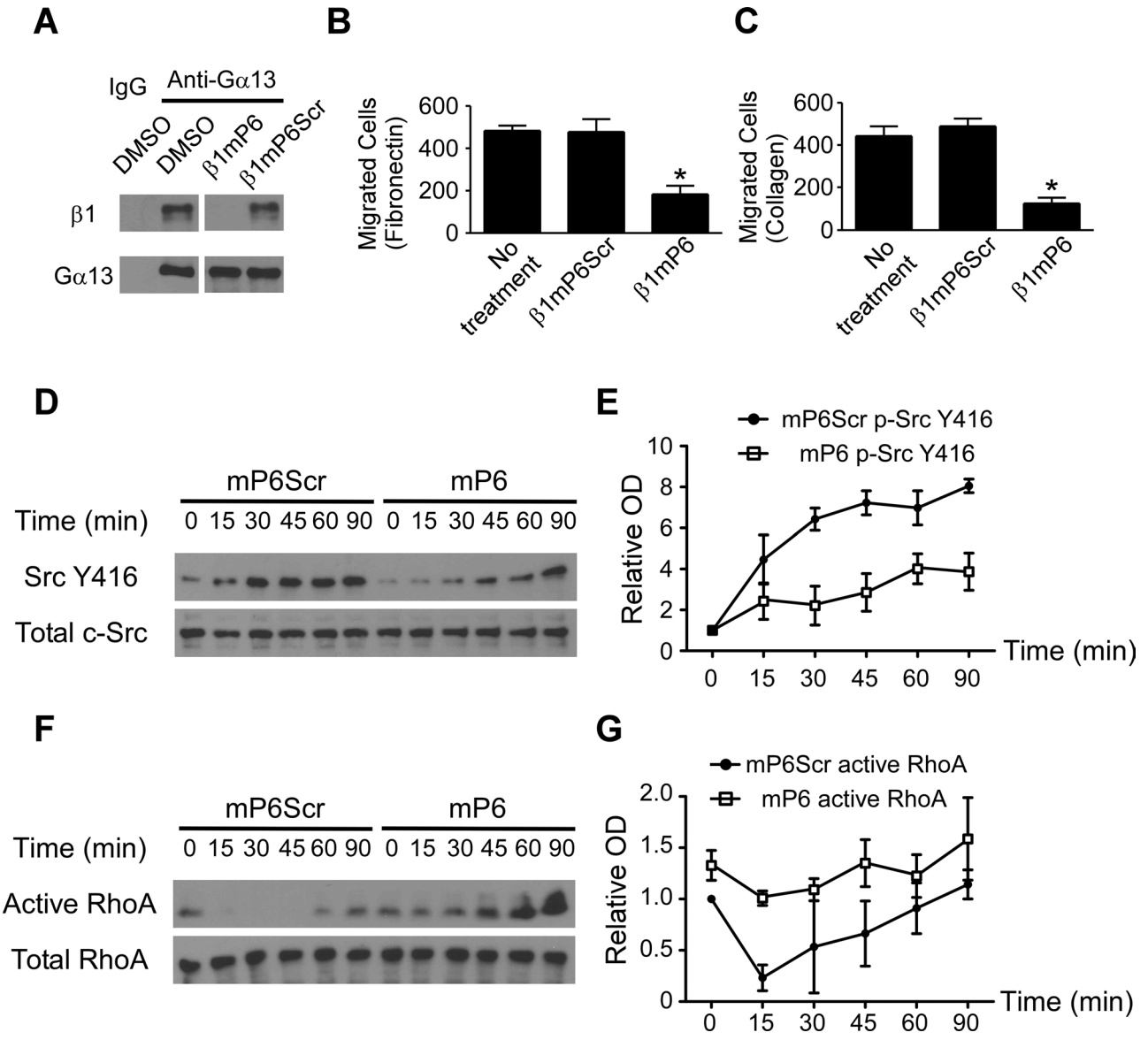


FIGURE 9: The inhibitory effects of a myristoylated peptide $m\beta_1P_6$ on $G\alpha_{13}$–β_1 interaction, cell migration, c-Src activation, and transient RhoA inactivation. (A) β_1(Wt)GD25 cells treated with 100 μM β_1mP_6 or scrambled control peptide β_1mP_6Src were allowed to adhere to fibronectin for 1 h. Cells were lysed, and cell lysates were immunoprecipitated with anti-$G\alpha_{13}$ antibody or an equal amount of control rabbit IgG. Immunoprecipitates were immunoblotted with anti-$G\alpha_{13}$ and anti-β_1 antibodies. (B) Transwell migrated β_1(Wt)GD25 cells with coated fibronectin 6 h after migration. Cells were treated with 100 μM β_1mP_6 or scrambled control peptide β_1mP_6Src before migration and stained with crystal violet after fixation. Number of migrated cells was compared (mean ± SD, $n = 3$). (C) Quantification of migrated cells using coated collagen (mean ± SD, $n = 3$). (D–F) β_1(Wt)GD25 cells treated with 100 μM β_1mP_6 or scrambled control peptide β_1mP_6Src were allowed to adhere to immobilized fibronectin, solubilized at various time points, and analyzed for RhoA activation (D, E) and c-Src Tyr416 phosphorylation (F, G). (D, F) typical gels. (E, G) Quantification of the Western blots (mean ± SD, $n = 3$).

Images were analyzed using ImageJ, and the quantification of the total fluorescence per cell, which is associated with RhoA activity, was performed using the corrected total cell fluorescence.

Src phosphorylation and RhoA activity assay

Cells in modified Tyrode's buffer or adherent on immobilized fibronectin were solubilized in cold NP40 lysis buffer at 4°C, and debris-cleared lysates were immunoblotted for phospho-Src Y418, total c-Src, or total RhoA. To measure the RhoA activity, debris-cleared lysates were incubated for 1 h with purified GST-RBD beads (Pierce, Thermo Scientific), washed, and then immunoblotted with an anti-RhoA monoclonal antibody (Ren *et al.*, 1999).

Peptide inhibitors

Myristoylated peptides $m\beta_1P_6$ (Myr-FEKEKM) and $m\beta_1P_6$Scr (Myr-EKMFEK) were synthesized and purified at the Research Resource Center at the University of Illinois at Chicago. The peptides were prepared in dimethyl sulfoxide for use in biochemistry experiments, dissolved in DMEM complete, and filtered for transwell migration experiments.

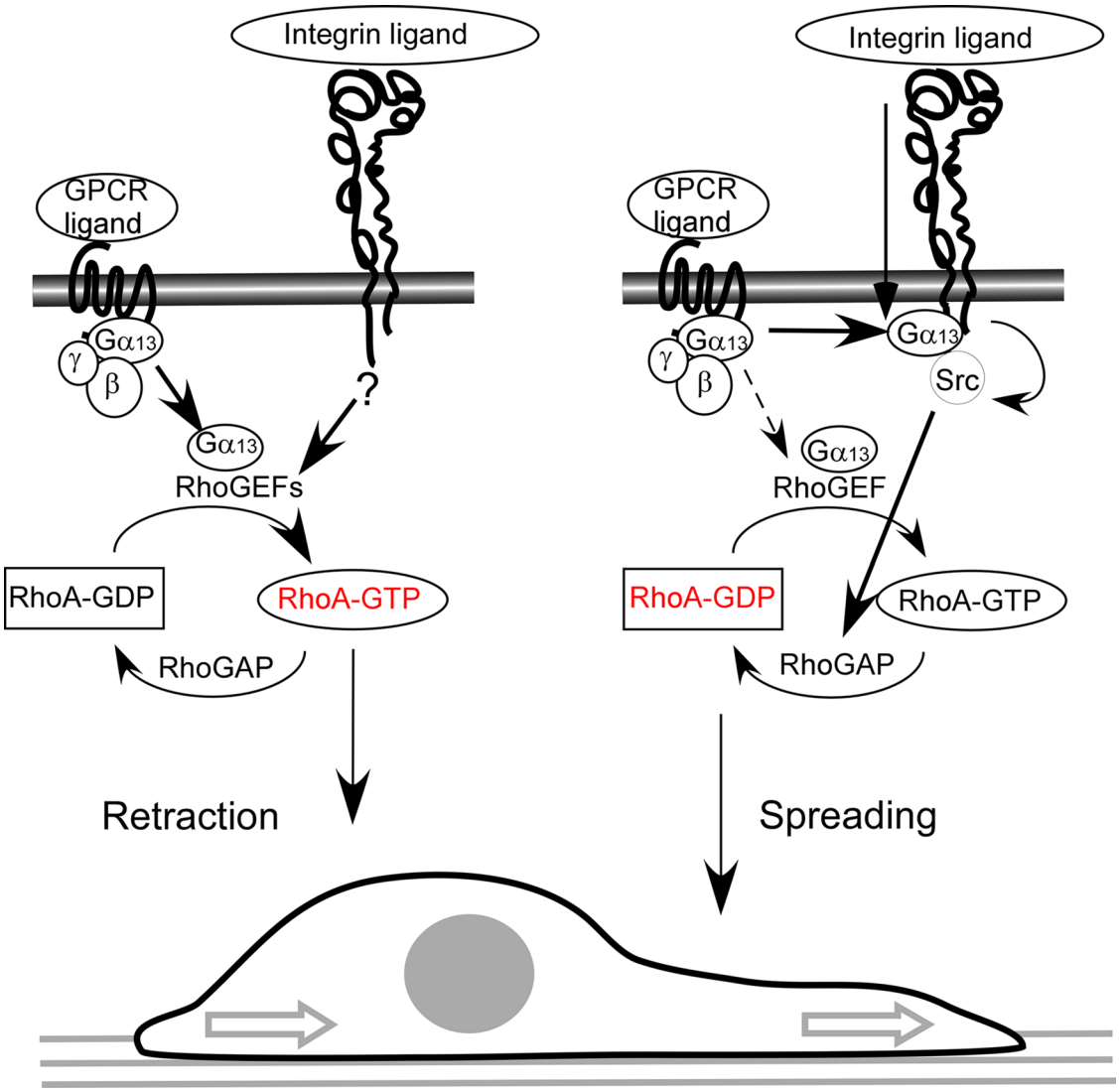


FIGURE 10: A new model for the $G\alpha_{13}$-dependent dynamic regulation of RhoA and cell migration. GPCR-dependent activation of $G\alpha_{13}$ stimulates the activation of RhoGEFs, leading to Rho activation, which has been suggested to be important for cell retraction in response to GPCR stimuli. Integrin ligation induces the interaction between the cytoplasmic domains of β subunits and the activated $G\alpha_{13}$, which mediates Src-dependent transient inhibition of RhoA and activates the Rac1 and PI3K pathways. These events lead to spreading of cells (lamellipodia and filopodia) toward the direction of migration. Late-phase integrin signaling results in reactivation of RhoA and cell retraction, driving the cell movement toward the direction of migration. Thus $G\alpha_{13}$-dependent dynamic regulation of RhoA results in coordinated cell spreading and retraction.

Statistics

Student's *t* tests and one-way analysis of variance were used for comparison; Analyses were performed with GraphPad Prism 4 software. Unless otherwise specified, an asterisk indicates $p < 0.005$.

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