

as a suction pump to draw air past the mouths of the ventrobronchi into the mesobronchus (1). A glass model demonstrated how this geometry gives rise to unidirectional airflow (1).

The mechanism of unidirectional flow in alligator lungs is yet to be determined, but our data support Hazelhoff's model (1), in which key features of the bronchial tree give rise to unidirectional flow. During inspiration, air may jet past the obliquely oriented vestibule of the CVB to enter the larger dorsal bronchial openings and reduce lateral pressure at the CVB orifice to draw air from the CVB into the intrapulmonary bronchus. During exhalation, air in the caudoventral bronchi may jet dorsally (blue arrows in Fig. 1C) to enter the ostia of the dorsobronchi. In this way, a simple arrangement of the bronchi by themselves might give rise to unidirectional airflow. Also, the mechanism of gas exchange in crocodylians is not known; a crosscurrent mechanism has been hypothesized (11), but a countercurrent mechanism cannot be ruled out. Furthermore, the importance of unidirectional airflow for gas exchange efficiency in the alligator lung is not known and cannot be determined from our data, which consist of measurements of airflow.

Previous scenarios for the evolution of unidirectional airflow are that it arose in dinosaurs of coelurosaurian grade (12), convergently in theropods and pterosaurs (13, 15), or not at all in dinosaurs because of a hepatic piston mechanism of breathing (14). Our findings contrast with these previous views in several ways. They demonstrate that the hepatic piston mechanism of breathing, which crocodylians have but birds lack, does not preclude the evolution of unidirectional flow and

that pneumaticity, which crocodylians lack, cannot be used to diagnose unidirectional airflow in fossil taxa, as previously suggested (13, 15). Crocodylians and birds are crown-group Archosauria. Therefore, in contrast to previous views, we suggest that unidirectional flow evolved before the divergence of crurotarsans and dinosaurian archosaurs and was present in the basal archosaurs and their descendants, including phytosaurs, aetosaurs, "rauisuchians," and crocodylomorphs. The crurotarsans and, somewhat later, the dinosaurs supplanted the synapsids as the dominant members of the Triassic terrestrial vertebrate assemblage, with Triassic mammals existing as diminutive mouse-like forms (16, 17). The roles of contingency and competition in the faunal turnover that occurred in the aftermath of the End Permian mass extinction are controversial. The basal archosaurs and archosauromorphs, animals such as *Euparkaria*, appear to have expanded their capacity for vigorous exercise (18) during a period of relative environmental hypoxia (19). In bird lungs, unidirectional airflow coupled with a crosscurrent mechanism of gas exchange facilitates the extraction of oxygen under conditions of hypoxia (20). If such a lung was present at the base of the archosaur radiation, this clade may have been better able than the synapsids to compete for niches that required a capacity for vigorous exercise.

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Materials and Methods

Fig. S1

References

Movies S1 to S3

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G Protein Subunit $G\alpha_{13}$ Binds to Integrin $\alpha_{11b}\beta_3$ and Mediates Integrin "Outside-In" Signaling

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Integrins mediate cell adhesion to the extracellular matrix and transmit signals within the cell that stimulate cell spreading, retraction, migration, and proliferation. The mechanism of integrin outside-in signaling has been unclear. We found that the heterotrimeric guanine nucleotide-binding protein (G protein) $G\alpha_{13}$ directly bound to the integrin β_3 cytoplasmic domain and that $G\alpha_{13}$ -integrin interaction was promoted by ligand binding to the integrin $\alpha_{11b}\beta_3$ and by guanosine triphosphate (GTP) loading of $G\alpha_{13}$. Interference of $G\alpha_{13}$ expression or a myristoylated fragment of $G\alpha_{13}$ that inhibited interaction of $\alpha_{11b}\beta_3$ with $G\alpha_{13}$ diminished activation of protein kinase c-Src and stimulated the small guanosine triphosphatase RhoA, consequently inhibiting cell spreading and accelerating cell retraction. We conclude that integrins are noncanonical $G\alpha_{13}$ -coupled receptors that provide a mechanism for dynamic regulation of RhoA.

Integrins mediate cell adhesion and transmit signals within the cell that lead to cell spreading, retraction, migration, and proliferation (1). Thus, integrins have pivotal roles in biological

processes such as development, immunity, cancer, wound healing, hemostasis, and thrombosis. The platelet integrin $\alpha_{11b}\beta_3$ typically displays bidirectional signaling function (2, 3). Signals from within

the cell activate binding of $\alpha_{11b}\beta_3$ to extracellular ligands, which in turn triggers signaling within the cell initiated by the occupied receptor (so-called "outside-in" signaling). A major early consequence of integrin "outside-in" signaling is cell spreading, which requires activation of the protein kinase c-Src and c-Src-mediated inhibition of the small guanosine triphosphatase (GTPase) RhoA (4–7). Subsequent cleavage of the c-Src binding site in β_3 by calpain allows activation of RhoA, which stimulates cell retraction (7, 8). The molecular mechanism coupling ligand-bound $\alpha_{11b}\beta_3$ to these signaling events has been unclear.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) consist of $G\alpha$, $G\beta$, and $G\gamma$ subunits (9). G proteins bind to the intracellular side of G protein-coupled receptors (GPCRs) and transmit signals that are important in many intracellular events (9–11). $G\alpha_{13}$, when activated by GPCRs, interacts with Rho guanine-nucleotide exchange

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factors (RhoGEF) and thus activates RhoA (11–14), facilitating contractility and rounding of discoid platelets (shape change). To determine whether $G\alpha_{13}$ functions in signaling from ligand-occupied integrin, we investigated whether inhibition of $G\alpha_{13}$ expression with small interfering RNA (siRNA) affected $\alpha_{IIb}\beta_3$ -dependent spreading of platelets on fibrinogen, which is an integrin ligand. We isolated mouse bone marrow stem cells and transfected them with lentivirus encoding $G\alpha_{13}$ siRNA. The transfected stem cells were transplanted into irradiated C57/BL6 mice (15). Four to six weeks after transplantation, nearly all platelets isolated from recipient mice were de-

rived from transplanted stem cells, as indicated by the enhanced green fluorescent protein (EGFP) encoded in lentivirus vector (Fig. 1A and fig. S1). Platelets from $G\alpha_{13}$ siRNA-transfected stem cell recipient mice showed >80% decrease in $G\alpha_{13}$ expression (Fig. 1B). When platelets were allowed to adhere to immobilized fibrinogen [$\alpha_{IIb}\beta_3$ binding to immobilized fibrinogen does not require prior “inside-out” signaling activation (16)], platelets depleted of $G\alpha_{13}$ spread poorly as compared with control platelets (Fig. 1A and fig. S2). The inhibitory effect of $G\alpha_{13}$ deficiency is unlikely to be caused by its effect on GPCR-stimulated $G\alpha_{13}$ signaling because (i) washed

resting platelets were used and no GPCR agonists were added, and (ii) prior treatment with 1 mM aspirin [which abolishes thromboxane A_2 (TXA $_2$) generation (17)] did not affect platelet spreading on fibrinogen (fig. S2), making unlikely the endogenous TXA $_2$ -mediated stimulation of $G\alpha_{13}$. Furthermore, $G\alpha_{13}$ siRNA inhibited spreading of Chinese hamster ovary (CHO) cells expressing human $\alpha_{IIb}\beta_3$ (123 cells) (18), which was rescued by an siRNA-resistant $G\alpha_{13}$ (fig. S3). Thus, $G\alpha_{13}$ appears to be important in integrin “outside-in” signaling leading to cell spreading.

To determine whether $G\alpha_{13}$ serves as an early signaling mechanism that mediates integrin-induced

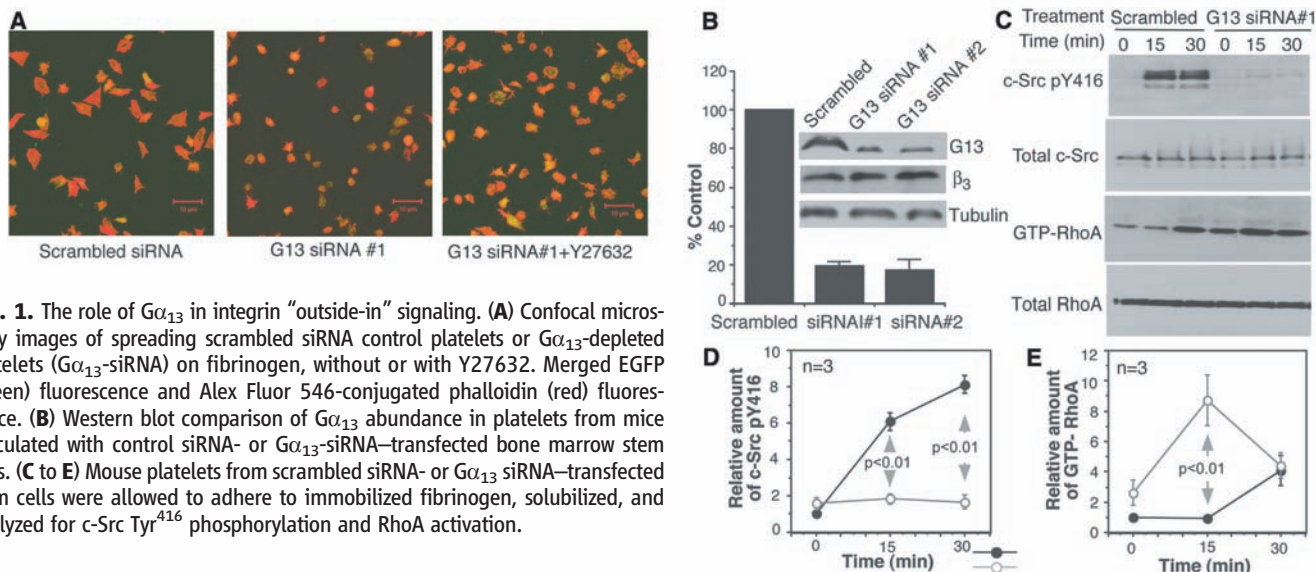


Fig. 1. The role of $G\alpha_{13}$ in integrin “outside-in” signaling. (A) Confocal microscopy images of spreading scrambled siRNA control platelets or $G\alpha_{13}$ -depleted platelets ($G\alpha_{13}$ -siRNA) on fibrinogen, without or with Y27632. Merged EGFP (green) fluorescence and Alex Fluor 546-conjugated phalloidin (red) fluorescence. (B) Western blot comparison of $G\alpha_{13}$ abundance in platelets from mice inoculated with control siRNA- or $G\alpha_{13}$ -siRNA-transfected bone marrow stem cells. (C to E) Mouse platelets from scrambled siRNA- or $G\alpha_{13}$ siRNA-transfected stem cells were allowed to adhere to immobilized fibrinogen, solubilized, and analyzed for c-Src Tyr⁴¹⁶ phosphorylation and RhoA activation.

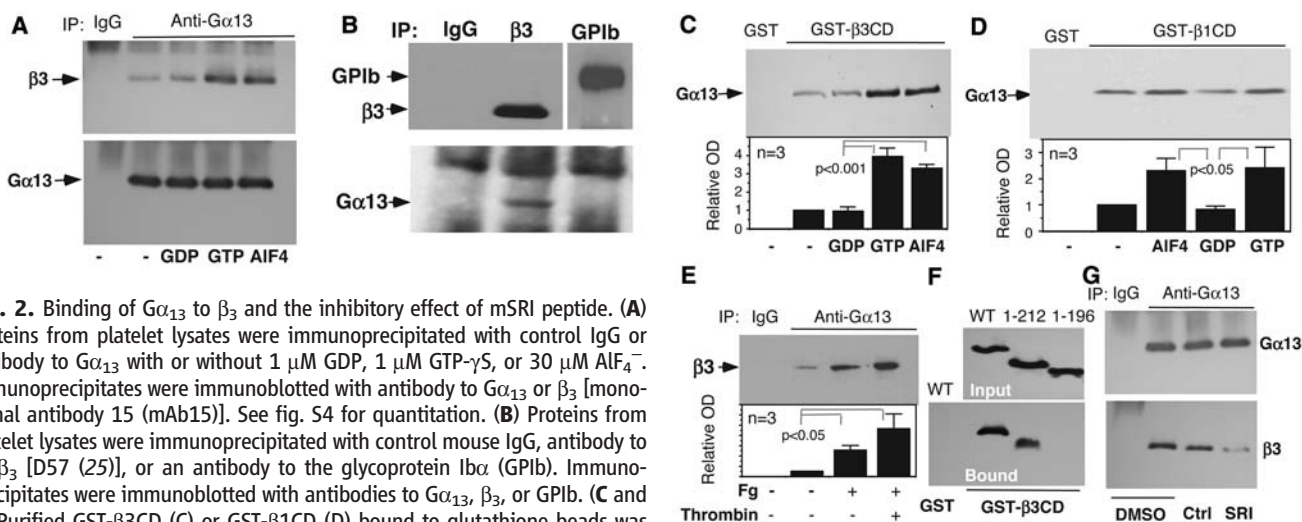
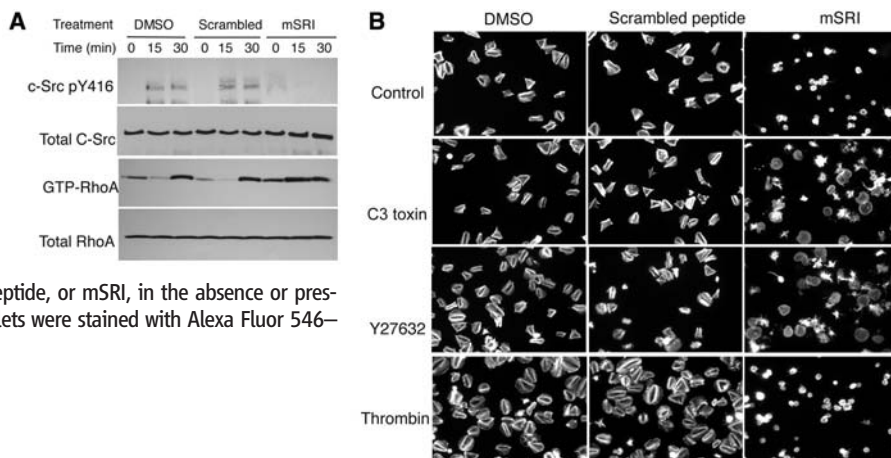


Fig. 2. Binding of $G\alpha_{13}$ to β_3 and the inhibitory effect of mSRI peptide. (A) Proteins from platelet lysates were immunoprecipitated with control IgG or antibody to $G\alpha_{13}$ with or without 1 μ M GDP, 1 μ M GTP- γ S, or 30 μ M AIF $_4^-$. Immunoprecipitates were immunoblotted with antibody to $G\alpha_{13}$ or β_3 [monoclonal antibody 15 (mAb15)]. See fig. S4 for quantitation. (B) Proteins from platelet lysates were immunoprecipitated with control mouse IgG, antibody to $\alpha_{IIb}\beta_3$ [D57 (25)], or an antibody to the glycoprotein Ib α (GPIb). Immunoprecipitates were immunoblotted with antibodies to $G\alpha_{13}$, β_3 , or GPIb. (C and D) Purified GST- β_3 CD (C) or GST- β_1 CD (D) bound to glutathione beads was mixed with purified $G\alpha_{13}$ with or without 1 μ M GDP, 1 μ M GTP- γ S, or 30 μ M AIF $_4^-$. Bound proteins were immunoblotted with antibody to $G\alpha_{13}$. Quantitative data are shown as mean \pm SD and P value (t test). (E) Lysates of control platelets or platelets adherent to fibrinogen in the absence or presence of 0.025 U/ml thrombin were immunoprecipitated with antibody to $G\alpha_{13}$ and then immunoblotted with mAb15. Quantitative data are shown as mean \pm SD and P value (t test). (F) Lysates from 293FT cells transfected with Flag-tagged wild-type $G\alpha_{13}$ or indicated truncation mutants (see fig. S5) were precipitated

with GST- β_3 CD- or GST-bound glutathione beads. Bead-bound proteins were immunoblotted with antibody to Flag (Bound). Flag-tagged protein amounts in lysates are shown by anti-Flag immunoblot (Input). (G) Protein from platelet lysates treated with 0.1% dimethyl sulfoxide (DMSO), 250 μ M scrambled control peptide (Ctrl), or mSRI were immunoprecipitated with antibody to $G\alpha_{13}$. Immunoprecipitates were immunoblotted with antibody to $G\alpha_{13}$ or β_3 . See fig. S4 for quantitation.

Fig. 3. Effects of mSRI on integrin-induced c-Src phosphorylation, RhoA activity, and platelet spreading. **(A)** Washed human platelets pretreated with DMSO, mSRI, or scrambled control peptide were allowed to adhere to fibrinogen and then solubilized at indicated time points. Proteins from lysates were immunoblotted with antibodies to c-Src phosphorylated at Tyr⁴¹⁶, c-Src, or RhoA. GTP-bound RhoA was measured by association with GST-Rhotekin rho-binding domain (GST-RBD) beads (26). See fig. S4 for quantitative data. **(B)** Spreading of platelets treated with 0.1% DMSO, scrambled control peptide, or mSRI, in the absence or presence of C3 toxin, Y27632, or 0.01 U/ml thrombin. Platelets were stained with Alexa Fluor 546-conjugated phalloidin.



activation of c-Src, we measured phosphorylation of c-Src at Tyr⁴¹⁶ (which indicates activation of c-Src) in control and fibrinogen-bound cells. Depletion of Gα₁₃ in mouse platelets or 123 cells abolished phosphorylation of c-Src Tyr⁴¹⁶ (Fig. 1C and fig. S3), indicating that Gα₁₃ may link integrin α_{IIb}β₃ and c-Src activation. Because c-Src inhibits RhoA (7, 19), we also tested the role of Gα₁₃ in regulating activation of RhoA. RhoA activity was suppressed to baseline 15 min after platelet adhesion and became activated at 30 min (Fig. 1C), which is consistent with transient inhibition of RhoA by c-Src (7). The integrin-dependent delayed activation of RhoA was not inhibited by depletion of Gα₁₃, indicating its independence of the GPCR-Gα₁₃-RhoGEF pathway (Fig. 1C). In contrast, depletion of Gα₁₃ accelerated RhoA activation (Fig. 1C). Thus, Gα₁₃ appears to mediate inhibition of RhoA. The inhibitory effect of Gα₁₃ depletion on platelet spreading was reversed by Rho-kinase inhibitor Y27632 (Fig. 1A), which suggests that Gα₁₃-mediated inhibition of RhoA is important in stimulating platelet spreading. These data are consistent with Gα₁₃ mediating integrin “outside-in” signaling inducing c-Src activation, RhoA inhibition, and cell spreading.

The integrin α_{IIb}β₃ was coimmunoprecipitated by antibody to Gα₁₃, but not control immunoglobulin G (IgG), from platelet lysates (Fig. 2A). Conversely, an antibody to β₃ immunoprecipitated Gα₁₃ with β₃ (Fig. 2B). Coimmunoprecipitation of β₃ with Gα₁₃ was enhanced by guanosine triphosphate γS (GTP-γS) or AlF₄⁻ (Fig. 2A and fig. S4). Thus, β₃ is present in a complex with Gα₁₃, preferably the active GTP-bound Gα₁₃. To determine whether Gα₁₃ directly binds to the integrin cytoplasmic domain, we incubated purified recombinant Gα₁₃ (20) with agarose beads conjugated with glutathione S-transferase (GST) or a GST-β₃ cytoplasmic domain fusion protein (GST-β₃CD). Purified Gα₁₃ bound to GST-β₃CD, but not to GST (Fig. 2C). Purified Gα₁₃ also bound to the β₁ integrin cytoplasmic domain fused with GST (GST-β₁CD) (Fig. 2D). The binding of Gα₁₃ to GST-β₃CD and GST-β₁CD was detected with GDP-loaded Gα₁₃,

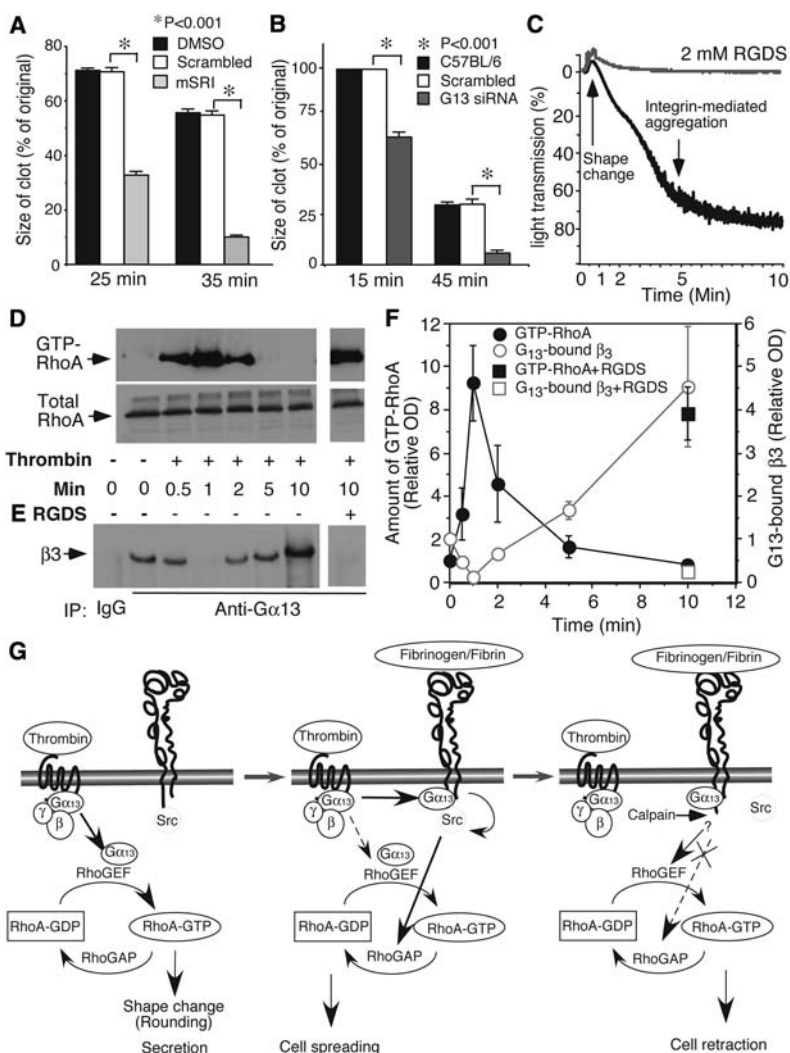


Fig. 4. The role of Gα₁₃ in clot retraction and dynamic RhoA regulation. **(A)** Effect of 250 μM mSRI peptide on clot retraction of human platelet-rich plasma compared with DMSO and scrambled peptide. Clot sizes were quantified using Image J (mean ± SD, n = 3, t test). **(B)** Comparison of clot retraction (mean ± SD, n = 3, t test) mediated by control siRNA platelets and Gα₁₃-depleted platelets. **(C to F)** Platelets were stimulated with thrombin with or without 2 mM RGDS and monitored for turbidity changes of platelet suspension caused by shape change and aggregation (C). The platelets were then solubilized at indicated time points and analyzed for amount of β₃ coimmunoprecipitated with Gα₁₃ (D) and amount of GTP-RhoA bound to GST-RBD beads (E) by immunoblot. (F) Quantitative data (mean ± SD) from three experiments. **(G)** A schematic illustrating Gα₁₃-dependent dynamic regulation of RhoA and crosstalk between GPCR and integrin signaling.

but enhanced by GTP- γ S and AlF_4^- (Fig. 2, C and D), indicating that the cytoplasmic domains of β_3 and β_1 can directly interact with $\text{G}\alpha_{13}$ and that GTP enhances the interaction. The $\text{G}\alpha_{13}$ - β_3 interaction was enhanced in platelets adherent to fibrinogen, and by thrombin, which stimulates GTP binding to $\text{G}\alpha_{13}$ via GPCR (Fig. 2E). Hence, the interaction is regulated by both integrin occupancy and GPCR signaling.

To map the β_3 binding site in $\text{G}\alpha_{13}$, we incubated cell lysates containing Flag-tagged wild type or truncation mutants of $\text{G}\alpha_{13}$ (fig. S5) with GST- β_3 CD beads. GST- β_3 CD associated with wild-type $\text{G}\alpha_{13}$ and the $\text{G}\alpha_{13}$ 1 to 212 fragment containing α -helical region and switch region I (SRI), but not with the $\text{G}\alpha_{13}$ fragment containing residues 1 to 196 lacking SRI (Fig. 2F). Thus, SRI appears to be critical for β_3 binding. To further determine the importance of SRI, $\text{G}\alpha_{13}$ - β_3 binding was assessed in the presence of a myristoylated synthetic peptide, Myr-LLARRPTKGIHEY (mSRI), corresponding to the SRI sequence of $\text{G}\alpha_{13}$ (197 to 209) (21, 22). The mSRI peptide, but not a myristoylated scrambled peptide, inhibited $\text{G}\alpha_{13}$ binding to β_3 (Fig. 2G), indicating that mSRI is an effective inhibitor of β_3 - $\text{G}\alpha_{13}$ interaction. Therefore, we further examined whether mSRI might inhibit integrin signaling. Treatment of platelets with mSRI inhibited integrin-dependent phosphorylation of c-Src Tyr⁴¹⁶ and accelerated RhoA activation (Fig. 3A). The effect of mSRI is unlikely to result from its inhibitory effect on the binding of RhoGEFs to $\text{G}\alpha_{13}$ SRI because $\text{G}\alpha_{13}$ binding to RhoGEFs stimulates RhoA activation, which should be inhibited rather than promoted by mSRI (22). Thus, these data suggest that β_3 - $\text{G}\alpha_{13}$ interaction mediates activation of c-Src and inhibition of RhoA. Furthermore, mSRI inhibited integrin-mediated platelet spreading (Fig. 3B), and this inhibitory effect was reversed by C3 toxin (which catalyzes the ADP ribosylation of RhoA) or Y27632, confirming the importance of $\text{G}\alpha_{13}$ -dependent inhibition of RhoA in platelet spreading. Thrombin promotes platelet spreading, which requires cdc42/Rac pathways (23). However, thrombin-promoted platelet spreading was also abolished by mSRI (Fig. 3B), indicating the importance of $\text{G}\alpha_{13}$ - β_3 interaction. Thus, $\text{G}\alpha_{13}$ -integrin interaction appears to be a mechanism that mediates integrin signaling to c-Src and RhoA, thus regulating cell spreading.

To further determine whether $\text{G}\alpha_{13}$ mediates inhibition of integrin-induced RhoA-dependent contractile signaling, we investigated the effects of mSRI and depletion of $\text{G}\alpha_{13}$ on platelet-dependent clot retraction (shrinking and consolidation of a blood clot requires integrin-dependent retraction of platelets from within) (7, 8). Clot retraction was accelerated by mSRI and depletion of $\text{G}\alpha_{13}$ (Fig. 4, A and B, and fig. S6), indicating that $\text{G}\alpha_{13}$ negatively regulates RhoA-dependent platelet retraction and coordinates cell spreading and retraction. The coordinated cell spreading-retraction process is also important in wound healing, cell migration, and proliferation (24).

The function of $\text{G}\alpha_{13}$ in mediating the integrin-dependent inhibition of RhoA contrasts with the traditional role of $\text{G}\alpha_{13}$, which is to mediate GPCR-induced activation of RhoA. However, GPCR-mediated activation of RhoA is transient, peaking at 1 min after exposure of platelets to thrombin, indicating the presence of a negative regulatory signal (Fig. 4, D and F). Furthermore, thrombin-stimulated activation of RhoA occurs during platelet shape change before substantial ligand binding to integrins (Fig. 4, C, D, and F). In contrast, after thrombin stimulation, β_3 binding to $\text{G}\alpha_{13}$ was diminished at 1 min when $\text{G}\alpha_{13}$ -dependent activation of RhoA occurs, but increased after the occurrence of integrin-dependent platelet aggregation (Fig. 4, E and F). Thrombin-stimulated binding of $\text{G}\alpha_{13}$ to $\alpha_{\text{IIb}}\beta_3$ and simultaneous RhoA inhibition both require ligand occupancy of $\alpha_{\text{IIb}}\beta_3$ and are inhibited by the integrin inhibitor Arg-Gly-Asp-Ser (RGDS) (Fig. 4, D to F). Thus, our study demonstrates not only a function of integrin $\alpha_{\text{IIb}}\beta_3$ as a noncanonical $\text{G}\alpha_{13}$ -coupled receptor but also a new concept of $\text{G}\alpha_{13}$ -dependent dynamic regulation of RhoA, in which $\text{G}\alpha_{13}$ mediates initial GPCR-induced RhoA activation and subsequent integrin-dependent RhoA inhibition (Fig. 4G). These findings are important for our understanding of how cells spread, retract, migrate, and proliferate, which is fundamental to development, cancer, immunity, wound healing, hemostasis, and thrombosis.

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Supporting Online Material

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Materials and Methods

Figs. S1 to S6

References

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Functional and Evolutionary Insights from the Genomes of Three Parasitoid *Nasonia* Species

The *Nasonia* Genome Working Group*†

We report here genome sequences and comparative analyses of three closely related parasitoid wasps: *Nasonia vitripennis*, *N. giraulti*, and *N. longicornis*. Parasitoids are important regulators of arthropod populations, including major agricultural pests and disease vectors, and *Nasonia* is an emerging genetic model, particularly for evolutionary and developmental genetics. Key findings include the identification of a functional DNA methylation tool kit; hymenopteran-specific genes including diverse venoms; lateral gene transfers among Pox viruses, *Wolbachia*, and *Nasonia*; and the rapid evolution of genes involved in nuclear-mitochondrial interactions that are implicated in speciation. Newly developed genome resources advance *Nasonia* for genetic research, accelerate mapping and cloning of quantitative trait loci, and will ultimately provide tools and knowledge for further increasing the utility of parasitoids as pest insect-control agents.

Parasitoid wasps are insects whose larvae parasitize various life stages of other arthropods (for example, insects, ticks, and

mites). Female wasps sting, inject venom, and lay eggs on or in the host, where the developing offspring consume and eventually kill it. Parasitoids