G-Protein–Coupled Receptor-2–Interacting Protein-1 Is Required for Endothelial Cell Directional Migration and Tumor Angiogenesis via Cortactin-Dependent Lamellipodia Formation

Syamantak Majumder, Mark P. Sowden, Scott A. Gerber, Tamlyn Thomas, Christine K. Christie, Amy Mohan, Guoyong Yin, Edith M. Lord, Bradford C. Berk, Jinjiang Pang

Objective—Recent evidence suggests G-protein–coupled receptor-2–interacting protein-1 (GIT1) overexpression in several human metastatic tumors, including breast, lung, and prostate. Tumor metastasis is associated with an increase in angiogenesis. We have showed previously that GIT1 is required for postnatal angiogenesis during lung development. However, the functional role of GIT1 in pathological angiogenesis during tumor growth is unknown.

Approach and Results—In the present study, we show inhibition of angiogenesis in matrigel implants as well as reduced tumor angiogenesis and melanoma tumor growth in GIT1-knockout mice. We demonstrate that this is a result of impaired directional migration of GIT1-depleted endothelial cells toward a vascular endothelial growth factor gradient. Cortactin-mediated lamellipodia formation in the leading edge is critical for directional migration. We observed a significant reduction in cortactin localization and lamellipodia formation in the leading edge of GIT1-depleted endothelial cells. We specifically identified that the Spa homology domain (aa 250–420) of GIT1 is required for GIT1–cortactin complex localization to the leading edge. The mechanisms involved extracellular signal-regulated kinases 1 and 2–mediated Cortactin-S405 phosphorylation and activation of Rac1/Cdc42. Finally, using gain of function studies, we show that a constitutively active mutant of cortactin restored directional migration of GIT1-depleted cells.

Conclusion—Our data demonstrated that a GIT1–cortactin association through GIT1-Spa homology domain is required for cortactin localization to the leading edge and is essential for endothelial cell directional migration and tumor angiogenesis. (Arterioscler Thromb Vasc Biol. 2014;34:419-426.)

Key Words: cortactin □ endothelial cells □ G-protein–coupled receptor kinase interacting protein-1 □ tumor angiogenesis

Angiogenesis, the formation of new blood vessels from existing ones, is critical for tissue development, tissue repair, as well as many diseases including diabetic retinopathy and tumor growth.1 Directional migration of endothelial cells (EC) toward a gradient of vascular endothelial growth factor (VEGF) determines vascular formation, which is a hallmark of tumor angiogenesis.1-3 This process involves 3 highly coordinated and regulated steps: sensing the stimuli, cytoskeleton rearrangement, and lastly movement of the cell. Lamellipodia are the critical structures for cell directional migration and they are regions of very rapid actin remodeling, which is mainly mediated by the cortical actin remodeling protein, cortactin.4 Cortactin has emerged as a key signaling protein in many cellular processes, including cell adhesion, migration, angiogenesis, and tumor invasion.5,6 Serine 405 phosphorylation by extracellular signal–regulated kinases 1 and 2 (ERK1/2) in the SRC homology 3 domain of cortactin is required for cortical actin cytoskeleton remodeling and cell migration.7,8 The work of Weed et al9 found that active Rac1/Cdc42 induced cortactin-mediated lamellipodia formation. Recent work also revealed the involvement of Cdc42-associated kinase 1 in inducing cortactin activation to promote cortical actin remodeling via Arp2/3-nWiskott -Aldrich syndrome protein complex.10

G-protein–coupled receptor kinase–interacting protein-1 (GIT1) is a multidomain protein involved in diverse cellular processes, including cell adhesion, migration,11 and permeability.12 GIT1, being a scaffold protein, interacts with other proteins, which can affect its cellular localization and activity. The full-length GIT1 protein has an N-terminal ADP-ribosylation factor GTPase-activating protein domain, 3 ankyrin repeats, a Spa2-homology domain (SHD), a synaptic localizing domain and a paxillin-binding site.14 We previously showed that GIT1 is involved in the formation of lamellipodia in cultured endothelial cells through a mechanism that requires Rac1/Cdc42 activation. The Spa homology domain (aa 250–420) of GIT1 is required for GIT1–cortactin association, which is necessary for directional migration.2 In this study, we extended our previous findings by demonstrating that GIT1 is required for cortactin localization and lamellipodia formation, which is critical for directional migration.

Material and Methods

Animals. Wild-type (C57BL/6J) and GIT1-knockout (C57BL/6J) mice were maintained in a barrier facility at the University of Rochester Medical Center. The University of Rochester institutional animal care and use committee approved all animal studies. All methods were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Rochester Medical Center.

Cell Cultures and Matrigel植入. Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Endothelial Cell Growth Medium (Vascular Biology Media, Tewksbury, MA) as previously described.4,10,16 Media was changed every 24 to 48 hours to maintain cell viability. Cells were maintained in 5% CO2 at 37°C. Matrigel implants were cultured as described previously.15

Confocal Imaging. HUVECs grown on coverslips were serum starved for 24 hours and then incubated with or without a non-permeable inhibitor of ROCK (Y-27632) for 24 hours. Y-27632 was obtained from Calbiochem (La Jolla, CA) and was used at a concentration of 1 μM. After this, cells were treated with 10 nM polosin (Calbiochem) for 30 minutes. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes and then permeabilized with 0.2% Triton X-100 for 5 minutes. Cells were permeabilized with 0.2% Triton X-100 for 5 minutes and then incubated with primary antibodies against cortactin (1:200 dilution; Cell Signaling Technology, Danvers, MA) and β-actin (1:1000 dilution; Abcam, Cambridge, MA). Cells were then incubated with secondary antibody against mouse and rabbit (Alexa Fluor 568-goat anti-mouse and Alexa Fluor 568-goat anti-rabbit; Invitrogen, Carlsbad, CA) for 1 hour. Coverslips were mounted on glass slides. Immunofluorescence imaging was performed on a Zeiss LSM710 confocal microscope. Cells were imaged using a 63× Plan Apochromat oil immersion objective, and images were processed using Zeiss LSM Image Browser.

Results

Inhibition of Angiogenesis in Matrigel Implants. We have previously shown that wild-type mice treated with a non-permeable ROCK inhibitor (Y-27632) have a significant reduction in tumor growth and angiogenesis.15 We now show that GIT1-knockout mice treated with Y-27632 also display a reduction in tumor growth, angiogenesis, and tumor weight. We used a non-permeable ROCK inhibitor (Y-27632) at a concentration of 1 μM for 24 hours to inhibit ROCK activity. Tumor growth and angiogenesis were significantly reduced in GIT1-knockout mice treated with Y-27632 compared with wild-type mice treated with Y-27632 (Figure 1A). Tumor weight was also significantly reduced in GIT1-knockout mice treated with Y-27632 compared with wild-type mice treated with Y-27632 (Figure 1B). We observed a significant reduction in cortactin localization and lamellipodia formation in the leading edge of GIT1-depleted endothelial cells. We specifically identified that the Spa homology domain (aa 250–420) of GIT1 is required for GIT1–cortactin complex localization to the leading edge. The mechanisms involved extracellular signal-regulated kinases 1 and 2–mediated Cortactin-S405 phosphorylation and activation of Rac1/Cdc42. Finally, using gain of function studies, we show that a constitutively active mutant of cortactin restored directional migration of GIT1-depleted cells.

Conclusion—Our data demonstrated that a GIT1–cortactin association through GIT1-Spa homology domain is required for cortactin localization to the leading edge and is essential for endothelial cell directional migration and tumor angiogenesis. (Arterioscler Thromb Vasc Biol. 2014;34:419-426.)

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Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>EC</td>
<td>endothelial cells</td>
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<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinases 1 and 2</td>
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<tr>
<td>GIT1</td>
<td>G-protein–coupled receptor kinase–interacting protein-1</td>
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<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
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<td>MLMEC</td>
<td>mouse lung microvascular endothelial cells</td>
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<tr>
<td>PAK</td>
<td>p21-associated kinase</td>
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<tr>
<td>PIX</td>
<td>PAK-interacting exchange factor</td>
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<td>SHD</td>
<td>Spa homology domain</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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important for ERK1/2 activation in response to multiple stimuli including VEGF and thrombin in EC.15,16 GIT1 is also important in regulating Rac1 and Cdc42 through its p21-associated kinase (PAK)–interacting exchange factor (PIX)–PAK binding in neurons.14,17 Our laboratory recently revealed the crucial role of GIT1 in postnatal angiogenesis during lung vasculature development.12,16 Several data, including Genecard analysis of human tumors, suggested an overexpression of GIT1 in several metastatic tumors, including breast, lung, melanoma, and prostate cancer.18,19 Based on these findings, we hypothesize that GIT1 is essential for tumor angiogenesis by regulating EC directional migration via cortactin-dependent lamellipodia formation through Rac1/Cdc42 and ERK1/2 pathways.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

GIT1 Is Required for Angiogenesis in Matrigel Plugs

Matrigel plug assay is a commonly used angiogenesis model, which resembles pathological angiogenesis during tumor growth.20 Plugs placed onto GIT1-knockout (KO) mice clearly exhibited fewer vessels compared with GIT1-wild type (WT) after 7 days of incubation (Figure 1A). Also, hematoxylin and cosin staining showed fewer EC in the matrigel plugs of GIT1-KO implants compared with GIT1-WT (Figure 1A). Isolectin B4 staining revealed a 45% increase in vessel density in VEGF-treated matrigel plugs of GIT1-WT mice compared with vehicle-treated controls (Figure 1A and 1B). Significantly, we observed a 68% reduction in vessel density in plugs of GIT1-KO mice compared with GIT1-WT controls treated with VEGF (Figure 1B). Together, these data suggest that GIT1 is required for VEGF-mediated angiogenesis in matrigel implants.

Melanoma Tumor Growth and Tumor Angiogenesis Are Reduced in GIT1-KO Mice

Melanoma tumor cells secrete high quantities of VEGF.21 VEGF-induced directional migration of EC is critical for angiogenesis to support tumor growth. Therefore, we chose this tumor model to study the effect of GIT1 on tumor angiogenesis and growth. An equivalent number of tumor cells (B16-F0 cell line, a melanoma spontaneously arising in C57BL/6 mice) which secretes VEGF, were injected intramuscular into GIT1-WT and GIT1-KO mice (2–3 months) and the thigh diameters were measured over time ≤15 days. The tumor volume in GIT1-WT and GIT1-KO mice was similar ≤12 days post injection. However, tumor volume in GIT1-KO was significantly reduced after 13 days compared with WT (Figure 1C; 45% at 15 days). Isolectin B4 staining showed significant reduction (83% and 61%) in vessels of smaller size (0–20 and 20–50 μm, respectively) in GIT1-KO mice (Figure 1D and 1E), whereas no difference was observed in vessels >50 μm between GIT1-WT and GIT1-KO mice (Figure 1D and 1E). Western blots revealed a 54% decrease in platelet endothelial cell adhesion molecule-1 expression in GIT1-KO versus GIT1-WT at 15 days (Figure 1F and 1G). These data demonstrate that GIT1 is an important regulator of tumor angiogenesis and tumor growth.

GIT1 Is Required for EC Directional Migration Toward VEGF Gradient

Angiogenesis requires directional migration of EC toward a stimulus.2 As we observed reduced angiogenesis in matrigel plugs containing VEGF and in a melanoma tumor model in the KO mouse compared with WT, we anticipated that the defect in tumor angiogenesis in GIT1-KO mice could be because of altered directional migration of GIT1-KO EC toward VEGF. Therefore, we performed a Dunn’s chamber experiment using control and GIT1-specific small interfering RNA (siRNA)–transfected human umbilical vein EC (HUVEC). HUVEC treated with control siRNA migrated directionally in response to a VEGF gradient (Figure 2A and 2B; Movies I and II in the online-only Data Supplement). In contrast, cells treated with GIT1 siRNA showed significantly decreased migration toward the gradient (Figure 2A and 2B; Movie III in the online-only Data Supplement). There was also a significant decrease in velocity of GIT1 siRNA–treated cells versus control siRNA–treated cells (6 versus 18 μm/h; Figure 2C). Western blot analysis of GIT1 siRNA–transfected HUVEC revealed a 90% decrease in GIT1 protein compared with control siRNA–transfected control (Figure 2C, inset). These data demonstrate that GIT1 plays a significant role in directional migration of EC toward a VEGF gradient.

GIT1 Is Required for Cortactin Localization to the Leading Edge of EC In Vitro

Cortactin-mediated lamellipodia formation in the leading edge is important for directional migration.2 We next performed wound healing assay using mouse lung microvascular EC (MLMEC). There was a 57% decrease in cortactin localization to the leading edge in GIT1-KO MLMEC compared with GIT1-WT MLMEC near the wound (Figure 3A; n=3; P<0.05). To confirm these data, we determined cortactin localization in GIT1 siRNA–treated HUVEC. Control siRNA–treated cells exhibited VEGF-stimulated actin filament reorganization and showed dramatically increased cortactin in lamellipodia like structures that colocalized with actin at the leading edge (Figure IA in the online-only Data Supplement). Both lamellipodia formation and cortactin localization were decreased markedly in HUVEC transfected with GIT1 siRNA; 61% reduction in unstimulated cells and 73% in VEGF-stimulated cells (Figure IA and IB in the online-only Data Supplement).
As actin filament reorganization was also altered significantly (Figure IA in the online-only Data Supplement), the colocalization of cortactin and actin was markedly reduced in GIT1-depleted HUVEC (Figure IA in the online-only Data Supplement). There was a significant 68% decrease in sprout length (Figure IIA–IIC in the online-only Data Supplement) and 84% reduction of cortactin localization in the leading edge of GIT1-KO aortas compared with WT control (Figure IID–IIJ in the online-only Data Supplement).

To confirm that cortactin mislocalization in GIT1-KO EC is directly attributable to the loss of GIT1, we performed a rescue experiment by overexpressing GIT1 in GIT1-KO MLMEC using a GIT1-expressing cytomegalovirus promoter–based lentiviral expression vector in which GIT1 is expressed on an internal ribosome entry site-containing bicistronic mRNA that also expresses enhanced green fluorescent protein. We detected green fluorescent protein expression in ≈60% of GIT1-KO MLMEC infected with either vector control or GIT1-containing lentivirus (Figure 3B). GIT1-WT MLMEC showed high level of cortactin in leading edge, whereas GIT1-KO MLMEC infected with control lentivirus had only 33% of the cortactin in the leading edge. However, overexpression of GIT1 in GIT1-KO MLMEC restored cortactin localization to the leading edge of migrating EC to 94% (n=3; P<0.05). Expression of GIT1 in GIT1-KO MLMEC was confirmed by Western blot (data not shown). These results show that GIT1 is required for cortactin localization to the leading edge of EC during directional migration.

**SHD of GIT1 Is Required for GIT1–Cortactin Association and Membrane Localization to Induce EC Migration**

To characterize the mechanism by which GIT1 regulates cortactin localization in EC, we used an in vitro wound healing assay using HUVEC. VEGF (10 ng/mL, for 6 hours) stimulated a 2.2-fold increase in colocalization of GIT1 and cortactin at the leading edge (Figure IIIA and IIIB in the online-only Data Supplement). Overexpression of GIT1 in GIT1-KO MLMEC showed increased cortactin localization to the leading edge (Figure IIIA and IIIB in the online-only Data Supplement).

**Figure 1.** G-protein–coupled receptor–2–interacting protein (GIT1) is required for tumor angiogenesis. A, Matrigel (250 μL) containing vehicle control or vascular endothelial growth factor (VEGF; 50 ng/mL) was injected subcutaneously on the ventral side of the mouse in the groin area. Plugs were isolated 7 days post injection. Cross sections were stained with either hematoxylin and eosin (H&E) or endothelial cell (EC)–specific marker isoelectin B4 (IB4) to locate the vessels. B, Vessel density was analyzed by counting the number of IB4-stained vessels per field. *Vs control; #vs control treated with VEGF (n=8; * and #P<0.05). C, Melanoma tumor cells were injected into the thigh muscle of GIT1–wild type (WT; n=5) and GIT1–knockout (KO; n=5) mice. Mean thigh diameters were determined and increased tumor volume was calculated. D and E, Tumors from GIT1–WT and GIT1–KO mice were harvested at day 15 after injection. Tumor tissue sections were stained with IB4 and images were acquired in ×10 and ×40 objective. The number of vessels in 3 different diameter groups (>50 [yellow arrow], >20–<50 [green arrow], and ≤20 μm [white arrow]) was counted manually using Image Pro Plus software. *Vs WT (n=5; P<0.05). F and G, Platelet endothelial cell adhesion molecule-1 (PECAM1) expression in tumor samples was detected by Western blot. Quantification of relative expression of PECAM1 normalized to GAPDH. *Vs WT (n=5; P<0.05).

**Figure 2.** G-protein–coupled receptor–2–interacting protein (GIT1) is required for vascular endothelial growth factor (VEGF)–induced directional migration of endothelial cells (EC). A, Human umbilical vein endothelial cell (HUVEC) cultured on fibronectin-coated cover glasses was transfected with either scrambled control (Con) or GIT1–specific small interfering RNA (siRNA) for 24 hours, loaded on Dunn’s chamber with a VEGF (50 ng/mL) gradient and placed into a live cell imaging chamber. Cells were imaged for 24 hours at 10-minute intervals. Videos obtained from live cell imaging experiments were analyzed using Image Pro Plus software. A total of 33 cells from 3 different sets of experiments were tracked from the 2 groups. Trend line showing the average distance traveled by Con and GIT1 siRNA–transfected HUVEC relative to the VEGF gradient. B, Total distance traveled by each cell after 24 hours toward the VEGF gradient from A. *Vs control siRNA (n=3; P<0.05). C, Cell velocity was also measured using Image Pro Plus software. GIT1 depletion by GIT1 siRNA in HUVEC was demonstrated by Western blot. *Vs control siRNA (n=3; P<0.05).
and cortactin association (Figure IIC in the online-only Data Supplement). Furthermore, a 4-fold increase in the level of phosphorylated S405-cortactin in the GIT1–cortactin complex was observed on VEGF stimulation (Figure IIC in the online-only Data Supplement). To identify the cellular compartment of GIT1–cortactin association, we performed cell fractionation and found that GIT1–cortactin association maximally occurs in the membrane of EC (Figure IID in the online-only Data Supplement).

To identify the specific domain of GIT1 responsible for association with cortactin, we transfected GIT1-KO MLMEC with GIT1 deletion mutants that lacked predicted binding domains. Extensive colocalization of GIT1 mutant (green) and cortactin (red) in the cell periphery was observed in MLMEC transfected with green fluorescent protein-GIT1 and green fluorescent protein-GIT1 (1–420; Figure IV in the online-only Data Supplement; Figure 4A). In contrast, GIT1 lacking the functional SHD (del250-420) showed a 71% decrease in colocalization at the cell periphery (Figure IV in the online-only Data Supplement; Figure 4A). Coimmunoprecipitation analysis of HUVEC transfected with the mutants demonstrated that full-length GIT1 and GIT1 (1–420) were associated with cortactin, whereas GIT1(del250-420) that lacks the SHD was not (Figure 4B).

Consistent with our previous finding in GIT1-depleted HUVEC,18 GIT1-KO MLMEC showed a 49% reduction in wound healing compared with GIT1-WT MLMEC (Figure 4C and 4D). Lentivirus-mediated overexpression of full-length GIT1 in GIT1-KO MLMEC completely restored EC migration to 100%, whereas GIT1(del250-420) overexpression had no effect (Figure 4C and 4D). These data demonstrate that GIT1-SHD mediates GIT1 and cortactin association, which is necessary for EC migration by localizing cortactin to the leading edge.

**Figure 3.** G-protein–coupled receptor-2–interacting protein (GIT1) localizes cortactin to the leading edge of endothelial cells (EC) in vitro and in vivo. A, Wounds were created in monolayers of GIT1-wild type (WT) and knockout (KO) mouse lung microvascular EC (MLMEC) and healing proceeded for 6 hours. Cells were then fixed and stained for platelet endothelial cell adhesion molecule-1 (PECAM1) and cortactin. Infected cells were identified by green fluorescent protein (GFP) expression (n=3). B, GIT1 was overexpressed in GIT1-KO MLMEC using a lentiviral vector. After 48 hours of infection, cell migration in a wound healing assay was determined. Cells were fixed and stained for cortactin. Infected cells were identified by green fluorescent protein (GFP) expression (n=3).

**手法**

**GIT1 Is Required for VEGF-Induced Phosphorylation of Cortactin-S405 (pS405) by ERK1/2 That Promotes Cortactin Localization to Lamellipodia**

ERK1/2-mediated phosphorylation of cortactin-S405 is required for lamellipodia formation and cell migration.22 In HUVEC stimulated with VEGF, fetal bovine serum, or EGF, there was an 8-fold increase in pS405-cortactin with a peak at 15 minutes (Figure VA and VB in the online-only Data Supplement). In HUVEC transfected with GIT1 siRNA, there was a 66% decrease in ERK1/2 and 70% decrease in S405-cortactin phosphorylation relative to control siRNA in response to VEGF (Figure 5A; Figure VC and VD in the online-only Data Supplement; n=3; P<0.05). There was no significant change in VEGF-induced phosphorylation of cortactin Y421 on GIT1 depletion (required for actin filament reorganization and stress fiber formation) after GIT1 depletion (Figure VE and VF in the online-only Data Supplement).

Furthermore, in GIT1-KO MLMEC there was a 37% and 43% reduction of S405-cortactin and ERK1/2 phosphorylation, respectively, compared with GIT1-WT MLMEC (Figure 4E and 4F). Lentiviral re-expression of GIT1 in KO MLMEC almost completely recovered S405-cortactin (98%) and ERK1/2 phosphorylation (89%) to the level of GIT1-WT control (Figure 4E and 4F). However, overexpression of GIT1 (del250-420) did not restore S405-cortactin and ERK1/2 phosphorylation (Figure 4E and 4F). Together these data support the role of GIT1-SHD in promoting S405-cortactin and ERK1/2 phosphorylation that is required for EC directional migration.

Treatment of HUVEC with the ERK1/2 inhibitor PD98059 caused a decrease in EC migration, a 42% reduction in cortactin at the leading edge of migrating EC and a concomitant reduction in lamellipodia formation (Figure VIA and VII in the online-only Data Supplement). Treatment of HUVEC with PD98059 completely blocked VEGF-induced S405-cortactin phosphorylation, suggesting that ERK1/2 is the key mediator of S405 phosphorylation of cortactin (Figure VII and VIK in the online-only Data Supplement). These data demonstrated that blocking ERK1/2 activation either by GIT1 depletion or PD98059 treatment inhibited S405-cortactin phosphorylation, which is essential for lamellipodia formation.

**VEGF Promotes the Formation of GIT1–pERK1/2–Cortactin Complex in the Leading Edge of EC**

Previously, we showed EGF-dependent increase in GIT1 association with ERK1/2 in focal adhesion.23 To determine colocalization of GIT1, pERK1/2, and cortactin at the leading edge of migrating EC, we performed triple staining in HUVEC. VEGF significantly increased the GIT1–pERK1/2–cortactin colocalization compared with untreated controls (Figure 5B; n=3). Furthermore, in the wound healing assay, there was a significant decrease in colocalization of pERK1/2...
and cortactin in HUVEC treated with GIT1 siRNA versus control siRNA (30 versus 94 pixel number; Figure VIIA and VIIB in the online-only Data Supplement). In addition, coimmunoprecipitation studies demonstrated that pERK1/2, GIT1, and cortactin coprecipitated (10 ng/mL VEGF; 15 minutes; Figure VIIC in the online-only Data Supplement; n=3). These data demonstrated that GIT1, ERK1/2, and cortactin form a complex that is necessary for phosphorylation of cortactin and its localization to the leading edge of migrating EC.

**GIT1 Is Required for VEGF-Induced Activation of Rac1 and Cdc42**

Rac1 and Cdc42 are necessary for the activation of cortactin and the induction of lamellipodia formation.10,24
GIT1-SHD regulates Rac1 and Cdc42 through its PIX–PAK binding domain in neurons,\textsuperscript{14,17} its role in Rac1 and Cdc42 activation in VEGF-stimulated EC is unknown. Rac1 and Cdc42 activation in response to VEGF peaked at 30 minutes in HUVEC (data not shown). In HUVECs treated with GIT1 siRNA, VEGF-induced Rac1 and Cdc42 activation was inhibited by 68% and 80%, respectively, compared with control siRNA–treated cells (Figure 5C and 5D). A previous article by Smith et al\textsuperscript{25} showed that PAK1 activates ERK1/2 in macrophages to regulate lamellipodial stability, which suggests that Rac1/Cdc42 is upstream of ERK1/2. Therefore, we used adenoviral delivery of dominant-negative (DN)-Rac1 and DN-Cdc42 to study their effects on ERK1/2 and cortactin activation. Infection of adeno-DN-Rac1 or adeno-DN-Cdc42 in HUVEC diminished VEGF-induced ERK1/2 activation by 49% and cortactin-S405 phosphorylation by 82% (Figure VIII in the online-only Data Supplement), which suggests that Rac1 and Cdc42 act upstream of ERK1/2. In addition, greater reduction in cortactin phosphorylation compared with ERK1/2 activation implies that Rac1/Cdc42 may also regulate cortactin activation through PAK.\textsuperscript{26} Overall, these data showed that GIT1 promotes the localization of cortactin to lamellipodia through Rac1/Cdc42 and ERK1/2 pathways.

Expression of Constitutively Active Cortactin Rescues GIT1-Depleted Phenotype In Vitro

To confirm that the effect of GIT1 on EC migration is pS405-cortactin dependent, we expressed a constitutively active cortactin, cortactin\textsuperscript{S405D,S418D} (mCortactin), in GIT1-depleted HUVEC. After cotransfection of GIT1 siRNA and mCortactin, we performed a wound healing assay. GIT1-depleted HUVEC showed 54.6±8.0% inhibition of wound healing compared with control HUVEC (Figure 6A–6C). Expression of mCortactin in control siRNA did not stimulate HUVEC migration when compared with pcDNA vector control (Figure 6A–6C). However, mCortactin stimulated a 2.6-fold increase in migration of GIT1-depleted cells (Figure 6A–6C). We also confirmed the presence of mCortactin expressing EC in wound edge (Figure IX in the online-only Data Supplement). These data show that pS405-cortactin is necessary for GIT1-dependent regulation of EC migration.

Discussion

Major findings of the present study are GIT1 is required for VEGF-induced cortactin activation and localization to leading edge to promote lamellipodia formation, which is necessary for EC directional migration and tumor angiogenesis. Three major mechanisms were identified (see model in Figure 6D): (1) GIT1 increases Rac1/Cdc42 activation through PIX–PAK complex to promote cortactin activation and localization to membrane. (2) GIT1 assembles a complex of mitogen-activated protein kinase kinase–1–extracellular signal-regulated kinases 1 and 2 (ERK1/2) that induces Ser405 cortactin phosphorylation. (3) GIT1 forms a complex with cortactin to promote cortactin membrane localization and lamellipodia formation. In addition, we described that GIT1-mediated activation of Rac1/Cdc42 also contributes to ERK1/2-driven activation of cortactin (pathway 4). Importantly, we identified the SHD of GIT1 as the domain essential for these functions.
Recent evidence suggests that GIT1-mediated activation of mitogen-activated protein kinase kinase-1–ERK1/2 induces growth in human liver and colon cancer. Several data, including Genecard analysis of human tumors, suggested increased GIT1 expression in several metastatic tumors, including breast, lung, and prostate. These data all suggest a crucial role of GIT1 in tumor progression. Long established evidence suggested that tumor metastasis is correlated with the extent of vascular structure and increases in angiogenesis that supports the metastatic potential of tumor cells. Our findings reveal that GIT1 is required for tumor angiogenesis to support the growth of tumors. Deletion of GIT1 diminished tumor microvasculature density and prevented tumor growth. We specifically identified that the major reason behind this effect is because of compromised directional migration of GIT1-depleted EC toward a VEGF gradient.

GIT1 promotes the assembly of signaling complexes in focal adhesions where it associates with a complex that includes Pak, Pix, paxillin, and focal adhesion kinase. Moreover, on EGF stimulation, GIT1 colocalized with ERK1/2 in focal adhesions that mediated migration of HeLa cells. However, the mechanisms by which GIT1 regulates lamellipodia formation and directional migration are still unknown. Cortactin plays a key role in lamellipodia formation and directional migration. Our data showed that GIT1 associates with cortactin and localizes it to leading edge to promote lamellipodia formation and directed cell migration. Depletion of GIT1 remarkably impaired cortactin activation and localization. ERK1/2 and Rac1/Cdc42 are the major signaling pathways responsible for cortactin activation. We found significant decreases of both ERK1/2 phosphorylation and Rac1/Cdc42 activation by loss of GIT1. In addition, DN-Rac1/DN-Cdc42 showed greater inhibition on Ser405 cortactin phosphorylation than ERK1/2 phosphorylation, which implies the involvement of other downstream target of Rac1/Cdc42 such as Pak on cortactin activation.

A significant finding of the present study is that the SHD of GIT1 mediates binding and translocation of cortactin to the leading edge. Most importantly, we identified that GIT1-SHD is required for cortactin-S405 phosphorylation, which in turn regulates EC migration. Intriguingly, GIT1 and GIT2 are the only mammalian proteins that contain a SHD. The SHD of GIT1 is responsible for regulating src-mediated phospholipase Cγ activation. ERK1/2 activation in focal adhesions, and the interaction with Pix and Pak complex to regulate small GTPase, such as Rac1 and Cdc42, as well as the regulation of GIT1 binding to focal adhesions. We identified another critical function of GIT1-SHD, which associates with cortactin to promote cortactin activation and localization to the leading edge during angiogenesis. We also found that GIT1-mediated localization of cortactin is important for EC directional migration and angiogenesis in matrigel plugs and tumor.

In recent years, several studies indicated the involvement of GIT1 in cancer including its role in cancer cell migration, cellular transformation, and growth. Our present studies together with previous findings imply that GIT1 overexpression in metastatic tumors could be associated with activation of cortactin, which possibly promote tumor angiogenesis as required during tumor metastasis. Finally, as GIT1-SHD is unique to GIT1 and GIT1 is highly overexpressed in several metastatic tumor, future studies by identifying small molecule inhibitors or peptides to abrogate the functions of GIT1-SHD could enable us to specifically target GIT1 during metastatic tumor progression.

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Disclosures

The provisional patent application of G-protein-coupled receptor–2–interacting protein 1 Spa homology domain acting as a therapeutic molecule inhibitors or peptides to abrogate the functions of GIT1 could enable us to specifically target GIT1 during metastatic tumor progression.

References


### Significance

Recent evidence suggests G-protein–coupled receptor–2–interacting protein (GIT1) overexpression in several human metastatic tumors, including breast, lung, and prostate. Tumor metastasis is associated with increases in angiogenesis. The present study is significant by showing that GIT1 is required for tumor angiogenesis and tumor growth via cortactin-dependent directional migration of endothelial cells. There are 3 major mechanisms: (1) GIT1 increases Rac1/Cdc42 activation through p21-associated kinase-interacting exchange factor–p21-associated kinase complex to promote cortactin activation and localization to membrane. (2) GIT1 assembles a complex of MEK1–extracellular signal–regulated kinases 1 and 2 that induces Ser405 cortactin phosphorylation. (3) GIT1 forms a complex with cortactin to promote cortactin membrane localization and lamellipodia formation. Most importantly, we identified the Spa homology domain of GIT1 as the domain essential for this function. Because the GIT1-Spa homology domain is unique in the mammalian genome, understanding the structural nature of its interaction with multiple signal mediators may enable specific targeting of individual pathways necessary for angiogenesis.
G-Protein–Coupled Receptor-2–Interacting Protein-1 Is Required for Endothelial Cell Directional Migration and Tumor Angiogenesis via Cortactin-Dependent Lamellipodia Formation

Syamantak Majumder, Mark P. Sowden, Scott A. Gerber, Tamlyn Thomas, Christine K. Christie, Amy Mohan, Guoyong Yin, Edith M. Lord, Bradford C. Berk and Jinjiang Pang

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SUPPLEMENTAL MATERIALS

GIT1 mediated Cortactin localization to the Leading Edge Is Required For Endothelial Cell Directional Migration and Tumor Angiogenesis

Syamantak Majumder, Mark P Sowden, Scott A. Gerber, Tamlyn Thomas, Christine K Christie, Amy Mohan, Guoyong Yin, Edith M. Lord, Bradford C Berk, Jinjiang Pang

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Supplemental Figure I. GIT1 is required for VEGF induced cortactin localization to the leading edge. (A) Scratch wounds were created as described in the methods in monolayer of control or GIT1-specific siRNA transfected HUVEC. After 6 hours cells were treated with VEGF (10 ng/mL) for 15 minutes and stained for cortactin (green) and F-actin (red). Arrowheads show the presence of cortactin in the leading edge of EC in wound edge. (B) Level of cortactin per unit area in leading edge of EC was measured using Adobe Photoshop CS5. * versus control without VEGF, # versus control with VEGF (n=3, * and # P<0.05).
Supplemental Figure II. GIT1 is necessary to localize cortactin to the leading edge of sprouting EC during ex vivo angiogenesis. (A,B) Aorta rings from GIT1-WT and GIT1-KO mice were isolated and cultured in matrigel coated cover glasses. After 6 days of incubation, aorta rings were fixed and stained for PECAM1. Images show the presence of endothelial sprouts from aorta rings. (C) Images of the sprouts were analyzed using Angioquant software. Sprouts from the aorta ring of GIT1-KO mice were smaller compared to GIT1-WT controls. * versus WT (n=3, *P<0.05). (D-I) Sprouts were co-stained with PECAM1 and cortactin to localize cortactin. (J) Images were analyzed to quantify the level of cortactin in leading edge of EC sprouts of GIT1-WT and KO mice using Adobe Photoshop CS5. There was an 86% decrease in cortactin in the leading edge in GIT1-KO compared to WT. Absence of GIT1 in aorta was confirmed by western blot. * versus WT (n=3, *P<0.05).
**Supplemental Figure III. VEGF induced GIT1-Cortactin association.** (A) Scratch wounds were created as described in the methods. After 6 hours, cells were treated with VEGF (10 ng/mL) for 15 minutes and then fixed and stained for GIT1 (green), cortactin (red) and cell nuclei (DAPI, blue). Cells were imaged with Confocal imaging system with Z-scanning. (B) Level of co-localization was analyzed using Image Pro Plus software. * versus control (n=3, *P<0.05). (C) HUVEC were starved with serum free media for 2 hours and then stimulated with VEGF (10 ng/mL) for 15 minutes. Total cell lysates were co-immunoprecipitated with GIT1 antibody and probed for cortactin and pS405-Cortactin. (n=3) (D) VEGF treated HUVEC were processed using cell fractionation protocol as described in methods. Both membrane and cytosolic fractions were immunoprecipitated with GIT1 antibody. Cortactin and GIT1 were detected in both IP and the total fractionation samples. GAPDH and PECAM1 were used to assess the quality of the fractionation. (n=2)
Supplemental Figure IV. SHD of GIT1 is important for GIT1-cortactin association. GFP tagged WT-GIT1 and two deletion mutants (1-420)GIT1 and (ΔSHD)GIT1 were expressed in GIT1-KO MLMEC. Cells were fixed and stained for cortactin (red). Arrowheads show co-localization of GFP tagged GIT1 and mutants with cortactin. (n=3)
Supplemental Figure V. GIT1 is important for VEGF induced Ser405 phosphorylation but not Tyr421 phosphorylation of cortactin. (A) HUVEC cultured in 6 well plates to 90% confluence were starved for 2 hours in serum free media and stimulated with VEGF (10ng/ml) for different times and used to measure pSer405-cortactin by western blot. (n=3) (B) Ability of VEGF to induce phosphorylation of Ser405 residue of cortactin was compared with two known inducers (Epidermal Growth Factor (EGF) and FBS). (n=3) (C-D) Blots from Figure 5A were analyzed using Image J software. Results are expressed as mean±SD of 3 independent experiments. * versus control (n=3, *P<0.05). (E-F) We measured the Ser405 and Tyr421, two regulatory phosphorylation sites of cortactin in GIT1 depleted HUVEC treated with VEGF (10ng/ml). *P<0.05 versus control; #P<0.05 versus VEGF alone treated set (n=3, * and # P<0.05).
Supplemental Figure VI. Blocking ERK1/2 activation inhibited cortactin Ser405 phosphorylation, membrane localization and lamellipodia formation. (A-H) Scratch wounds were created in monolayers of HUVEC and pretreated with PD98059 (10µM) for 30 minutes. Cells were then stimulated with VEGF for 15 minutes and then fixed and stained for cortactin (red) and F-actin (red). Arrowheads show the co-localization (yellow) of cortactin and F-actin in cell periphery. (I) Level of cortactin localization in the cell periphery specifically in the leading edge was analyzed using Image Pro Plus software. * versus DMSO (n=3, *P<0.05). (J) HUVEC were pretreated with PD98059 (10µM) for 30 minutes and stimulated with VEGF for 15 minutes. Ser405 cortactin and ERK1/2 phosphorylation was measured in treated cells. (K) Blots were analyzed using Image J software and plotted after normalization. Results are expressed as mean±SD of 3 independent experiments. *P<0.05 versus control; #P<0.05 versus VEGF alone treated set (n=3, * and # P<0.05).
Supplemental Figure VII. GIT1 depletion blocked pERK1/2 and cortactin association and localization in the leading edge. (A) Scratch wounds were created in monolayer of control and GIT1 siRNA transfected HUVEC. After 6 hours cells were fixed and stained for cortactin (green) and pERK1/2 (red). Arrowheads show the co-localization (yellow) of cortactin and pERK1/2. (B) Level of co-localization in the cell periphery specifically in the leading edge, was analyzed using Image Pro Plus software. * versus control siRNA (n=3, *P<0.05). (C) HUVEC were stimulated with VEGF (10 ng/mL) for 15 minutes. Cell lysates were co-immunoprecipitated with cortactin antibody and probed for GIT1 and pERK1/2. GAPDH was used as a loading control. (n=3)
Supplemental Figure VIII. Overexpression of dominant negative (Dn) Cdc42 and Rac1 inhibited VEGF induced cortactin and ERK1/2 activation. (A-F) HUVEC at 70% confluence were infected with adenoviral vectors containing either DN Cdc42 (A-C) or DN Rac1 (D-F). Cells were stimulated with VEGF (10ng/mL) for 15minutes and western blot analysis was performed of the total cell lysates. Black arrowheads indicate the expression of DN-Cdc42 (A) and DN Rac1 (D). (n=2)
Supplemental Figure IX. Flag tagged mCortactin expression in wound edge cells. HUVEC were transfected with Flag-tagged mCortactin expressing plasmid. After 36 hours scratch wounds were created in the monolayer and incubated for 6 hours. Cells were fixed and stained using anti-Flag antibody (green) and counterstained with phalloidin-red (F-actin) and Hoechest-blue (nucleus). HUVEC expressing mCortactin were observed in the wound edge. (n=3)
Materials and Methods

Antibodies, small interfering RNA and reagents
Anti-Cortactin (Mouse) was purchased from EMD Millipore, Billerica, MA, USA; anti-pS405-cortactin from Protea Bio., Inc., Morgantown, WV, USA; anti-GIT1 (Goat), anti-GAPDH, anti-ERK1, anti-ERK2, anti-GFP and anti-actin from Santa Cruz Biotech., Inc., Santa Cruz, CA, USA; anti-GIT1 (Rabbit) and anti-Flag from Sigma-Aldrich, St. Louis, MO, USA; anti-pY421-cortactin and anti-pERK1/2 from Cell Signaling Tec., Inc., Danvers, MA, USA; anti-tubulin from Invitrogen Life Tech., Grand Island, NY, USA; rat anti-mouse platelet EC adhesion molecule 1 (PECAM-1) from BD Transduction Laboratories, San Jose, CA, USA. Alexa Fluor 488– or 546 or 680 or 800– conjugated secondary antibodies were purchased from Molecular Probes Life Tech., Grand Island, NY, USA. Alexa Fluor 680– or 800– conjugated goat anti–rabbit, anti-mouse and donkey anti-goat antibodies were purchased from Invitrogen Life Tech., Grand Island, NY, USA. HRP conjugated secondary antibodies were purchased from GE Health Care UK Ltd., Buckinghamshire, UK. PAK1-PBD bound to Agarose beads was purchased from Cell Biolabs., Inc., San Diego, CA, USA. Prevalidated human-specific GIT1 small interfering RNA (AAGCTGCAAGAAGAAGCTAC) was from Applied Biosystems, Life Tech., Grand Island, NY, USA and control siRNA was from Dharmacon RNA Technologies, Thermo Scientific, Waltham, MA, USA. VEGF was purchased from Roche Applied Science, Indianapolis, IN, USA.

Plasmid and Viral construct
GFP tagged mutants of GIT1 have been described previously\(^1\). The pseudo-phosphorylated (Cortactin\(^{S405D,418D}\)) construct of cortactin was from Dr. Alan S. Mak\(^2\). Adenovirus construct for Dominant negative (DN)-Rac1 and Cdc42 were used as previously described by our lab\(^3\). pLV-CMV-IRES-GFP is an HIV-1 based lentiviral expression vector that allows simultaneous expression of GIT1 or GIT1 delSHD cDNAs and EGFP from the CMV promoter. Infectious viral particles were generated by cotransfection of the transgene, with plasmids expressing viral gag/pol genes (psPAX2) and VSV-G coat protein (pMD2.G) into HEK293T cells using Fugene6 (Promega). 48-72 hours post-transfection viral containing supernatants were collected, filtered through 0.45µm cellulose acetate filters and stored in aliquots at -80°C. For viral concentration, supernatants were spun at 25,000rpm in a SW28 rotor for 2 hours at 4°C and the viral pellet resuspended in 200µl of HBSS.

Cell culture and transfection
HUVEC were obtained from collagenase-digested umbilical veins and collected in M200 medium supplemented with low serum growth supplement (Cascade Biologic), 5% fetal calf serum (GIBCO), 50 U/mL penicillin, and 50 µg/mL streptomycin. MLMEC were isolated from GIT1-WT and GIT1-KO mice lungs as previously described\(^4\). MLMEC were grown in DMEM with high glucose and supplemented with 20% Fetal Bovine serum, 1X non-essential amino acids, 50 U/mL penicillin, and 50 µg/mL streptomycin, 50 µg/mL Gentamicin, 4 µg/mL Amphotericine B, 1X Sodium Pyruvate, 100 µg/mL Heparin and EC growth factor (ECGF). For transient expression experiments, 80% confluent cells were transfected with 0.5 µg per 6 well of DNA and Lipofectamine 2000
(Invitrogen) for 3 hours. For siRNA depletion of GIT1, HUVEC were transiently transfected with 100nM GIT1 or control siRNA by Lipofectamine 2000. The cells were harvested 48 hours after siRNA transfection.

**Dunn’s Chamber cell migration assay and time-lapse microscopy**
Chemotaxis of HUVEC exposed to a VEGF gradient was measured using a Dunn’s Chamber. HUVEC were plated on fibronectin-coated cover-glasses at a density of ~5000 cells/cover-glass. Next day, cells were placed into the Dunn’s chamber and a VEGF gradient was created by loading serum free M200 containing 50 ng/mL VEGF into the outer chamber. Real-time imaging was performed on a confocal microscope (FluoView 1000) equipped with a 10× objective, FV10-ASW acquisition software (Olympus), and an environmental chamber that maintained 37°C and 5% CO₂ throughout the experiment.

**In vitro scratch wound healing assay**
Wound healing assays were performed as described previously.

**Immunofluorescence and Confocal Microscopy**
HUVEC cultured on coverslips were fixed with 2% paraformaldehyde in PBS for 12 minutes and permeabilized with 0.2% TritonX-100 for 2 minutes followed by blocking with 3% BSA in PBS for 1 hour before staining. Rhodamine phalloidin (1 µM; Invitrogen) was used to visualize F-actin. Alexa Fluro 405/488/540 conjugated secondary antibodies (Molecular Probe) against mice/rabbit/goat were used at 1:500 for 1 h at 37°C. Images were acquired with a confocal microscope (Olympus) with an Apochromat oil 40×/1.40 NA objective lens (Olympus) using a camera (DP71 camera, Olympus) and Fluoview software. Image analysis was performed using Adobe Photoshop CS5 or Image Pro Plus or Image J software.

**Subcellular fractionation**
Subcellular fractionation was performed as previously described. HUVEC were stimulated with VEGF (10ng/mL) for 15 minutes and were lysed in cell fractionation buffer (250 mmol/L Sucrose, 20 mmol/L HEPES, 10mM KCL, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1:1000 protease cocktail Inhibitor (Sigma) and lysates passed 20 times through a 25G needle and then centrifuged at 3000g for 10 minutes. The supernatant was transferred and centrifuged at 8000g for 15 minutes to separate the mitochondria (pellet) and the cytosolic fraction. The final cytosolic fraction was centrifuged at 100000 g for 1 hour. The membrane fraction (pellet) and the cytosolic fraction (supernatant) were collected. Membrane and cytosolic fractions (150µg of total protein) were used for co-immunoprecipitation studies using GIT1 antibody.

**Rac1/Cdc42 activation assays**
The GTPase activity of Rac1 and Cdc42 was measured as previously described. After knocking down GIT1 in HUVEC using GIT1 specific siRNA, cells were serum starved for 2 hours and stimulated with VEGF (10 ng/mL) for 30 minutes. Equal amounts of protein (400 µg) were incubated with 20 µg PAK1-PBD bound to agarose beads (Cell
Bio. Labs.) for 60 min at 4°C. Active Rac1 and Cdc42 were precipitated with GST-PBD. The amount of active Rac1, and Cdc42 was then analyzed by western blotting.

**Western blot analysis and co-immunoprecipitation**
Western blot Analysis and co-immunoprecipitation analysis were performed as described earlier.

**Generation of GIT1-KO mice**
GIT1 knockout mice (C57BL/6 background) were generated as described previously. All animal experiments were conducted in accordance with experimental protocols approved by the Institutional Animal Care and Use Committee at the University of Rochester.

**Ex vivo aorta ring assay and immunostaining**
Thoracic aortas excised from 8 to 12-week old GIT1-WT and GIT1-KO mice and were cut into 1mm long cross sections. Rings were placed on Matrigel-coated wells with Medium199 (Invitrogen) supplemented with L-Glutamine, penicillin and streptomycin, heparin (10 U/mL), 1% FBS and VEGF (50 ng/mL). After 6 days of incubation, aorta rings were fixed with 4% paraformaldehyde followed be permeabilization with 0.4% Triton X100 for 10 minutes and blocked with 5% normal goat serum for 1 hour. Next, the tissues were probed with PECAM1 antibody (BD Pharmingen) and cortactin antibody (Millipore) for overnight at 4°C. Tissues were incubated with corresponding secondary antibodies and mounted on glass slides with vectashield (Vector Laboratories). The tissues were imaged by confocal microscopy and the sprouting analysis was performed using Angioquant Software.

**In vivo Matrigel Plug Angiogenesis Assay**
Matrigel (250µL) containing vehicle or VEGF (50ng/mL) were injected subcutaneously on the ventral side of the mouse in the groin area close to the dorsal midline; control on left center of back and Matrigel/VEGF on right center of back. Each mouse obtained both vehicle and VEGF containing matrigel injections. Seven days post injections, animals were sacrificed and the matrigel plugs were isolated with skin and images acquired.

**Melanoma Tumor Model**
Tumor cells (B16-F0 cell line, a melanoma spontaneously arising in C57BL/6 mice, $2 \times 10^5$ in 100 µl HBSS (Sigma-Aldrich, St. Louis, MO) were injected i.m. into the thigh muscle of GIT1-WT and GIT1-KO mice. Mean thigh diameters were measured as described previously. Mice were sacrificed at day 15 after injection.

**Immunohistochemistry of tissue sections**
Matrigel Plugs were fixed with 10% formalin and processed for paraffin embedded tissue sectioning. Sections obtained from the implants were stained with H&E, probed with IB4 to locate the vessel. Melanoma tumor tissues were embedded in OCT in -80°C and frozen sections from the OCT embedded tissues were obtained using the cryostat. Tissue sections were processed as stated earlier.
Statistical analysis
Values calculated from at least three independent experiments were compared by a Student’s t test, and P < 0.05 was considered statistically significant. Results are expressed as mean±SD of 3 independent experiments.

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