Girdin/GIV regulates transendothelial permeability by controlling VE-cadherin trafficking through the small GTPase, R-Ras

Hitoshi Ichimiya [a], Kengo Maeda [a, *], Atsushi Enomoto [b], Liang Weng [b], Masahide Takahashi [b], Toyoaki Murohara [a]

[a] Department of Cardiology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
[b] Department of Pathology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

ARTICLE INFO

ABSTRACT

Vascular permeability is regulated by intercellular junction organization of endothelial cells, the dysfunction of which is implicated in numerous pathological conditions. Molecular mechanisms of how endothelial cells regulate intercellular junction in response to extracellular signals, however, have so far remained elusive. This study identified that Girdin (also termed GIV), an Akt substrate functioning in post natal angiogenesis, was expressed in a mature endothelial monolayer, where it regulated VE-cadherin trafficking to maintain vascular integrity. Girdin depletion abrogated VEGF-induced VE-cadherin endocytosis and the disassembly of adherens junctions in a monolayer of endothelial cells, thus leading to a significant decrease in the permeability. We also showed that activated R-Ras, a member of the Ras family GTPase, known to be a master regulator of transendothelial permeability, interacts with Girdin, and facilitates the complex formation between Girdin and VE-cadherin in endothelial cells. However, the increased permeability mediated by the loss of R-Ras was rescued by Girdin depletion, thus suggesting that the interaction of Girdin with R-Ras functions in VE-cadherin trafficking pathways distinct from endocytosis. The recycling of VE-cadherin was promoted by the exogenous expression of the active mutant of R-Ras, which was attenuated in the Girdin-depleted endothelial cells. These results show that Girdin regulates transendothelial permeability in synergy with R-Ras and VE-cadherin in an endothelial monolayer.

© 2015 Elsevier Inc. All rights reserved.

1. Introduction

Aberrantly increased vascular permeability is associated with numerous human diseases, such as inflammation, malignant tumors, and diabetic retinopathy [1,2]. Endothelial cells are connected with each other by various junctional proteins, and the integrity of an endothelial monolayer plays a central role in the maintenance of vascular barrier function. Endothelial cells have at least two distinct adhesive junctional regions at intercelluar junctions, which are designated adherens junctions (AJs), and tight junctions (TJs) [3–5].

VE-cadherin is a member of cadherin superfamily, whose expression is exclusively restricted to endothelial cells [6]. *Cis-* and *trans-*homophilic adhesion of VE-cadherin is a crucial factor to construct AJs [1,3]. AJs are dynamic structures that undergo persistent remodeling not only in cells exposed to agents increasing permeability, but also in confluent and resting cells [1,7]. One mechanism for the remodeling of AJs involves continuous trafficking, which includes endocytosis and recycling of VE-cadherin [8–11]. Several lines of evidence indicate that the trafficking of VE-cadherin is regulated by an intracellular signaling pathway [12]. However, detailed mechanisms for the trafficking of VE-cadherin have remained elusive.

We previously discovered an actin binding protein termed Girdin [13], which was also reported as Gα-interacting vesicle associated protein (GIV) [14]. Girdin is a large protein of 220 kDa (KDa) (1870 amino acids) with a unique amino-terminal domain, a long coiled-coil region, and a unique carboxyl-terminal domain [13,15,16]. The amino-terminal domain and coiled-coil region contribute to homophilic oligomerization of Girdin and the carboxyl-terminal domain directly binds to the actin cytoskeleton and the plasma membrane, leading to the hypothesis that Girdin is

a cross-linker of the actin filaments required to remodel the actin meshwork lining the plasma membrane. Girdin is highly expressed in immature endothelial cells, where it plays a pivotal role in VEGF-dependent cell migration, vascular network formation, and post natal angiogenesis that occurs in the retina and brain [17]. The expression of Girdin is not restricted to immature endothelial cells but also found in mature vascular networks composed of capillary and middle-sized vessels in various tissues [17]. However, the function of Girdin in those mature endothelial cells has not been addressed. An insight into the involvement of Girdin in the trans-endothelial permeability has come from our previous studies that showed that Girdin functions as a GAP for dynamine 2 and regulates the endocytosis of E-cadherin and transferrin receptor in MDCK cells [18].

The present study explored the function of Girdin in the regulation of the permeability of a confluent monolayer of endothelial cell. Girdin regulated the remodeling of AJs by controlling the trafficking of VE-cadherin, which was partly mediated by the small GTPase R-Ras that is important in promoting vascular stability and integrity. These observations suggest that Girdin therefore plays an important role in the regulation of vascular permeability and proper functioning of mature vascular networks.

2. Materials and methods

2.1. Plasmid constructs

The construction of adenoviral expression vectors was previously described [17]. Full-length human wild type and mutant R-Ras cDNA were kindly provided by Drs.Negishi and Oinuma (Kyoto University). The cDNAs were amplified by PCR using primers with appropriate restriction enzyme sites, and the resulting cDNA fragments were inserted into the pIRES2-EGFP expression vector (Clontech).

2.2. Cell culture

HUVEC-2 were obtained from BD Biosciences, and cultured in EGM-2-MV medium (Cambrex). Cells from passages 4–7 were used. HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum.

2.3. Antibodies

Rabbit anti-Girdin polyclonal antibody was previously described [13], (IBL, Gumma, Japan). Other antibodies used in this study included anti-Girdin sheep polyclonal antibody (BD Biosciences), anti-R-Ras, anti-VE-cadherin, anti-β-catenin, anti-p120 catenin and anti-clathrin heavy chain antibodies (Cell signaling technology), anti-VE-cadherin monoclonal antibody (clone BV6; Millipore), anti-cadherin5 antibody (BD Biosciences), anti-β-actin antibody (Sigma), and anti-V5 antibody (Invitrogen).

2.4. RNA interference

The siRNA-mediated knockdown was performed as previously described [17]. All siRNAs were purchased from Qiagen.

2.5. Expression of Girdin and R-Ras by the adenovirus system

Adenoviruses bearing Girdin and R-Ras/IRES2-EGFP cDNAs were prepared using the ViraPower Adenovirus Expression System (Invitrogen). HUVECs were infected with viruses two days after plating at a multiplicity of infection of 1:10. The titer of adenoviral stock was determined using QuickTiter™ Adenovirus Titer ELISA Kit (Cell Biolabs Inc., Buffalo, USA).

2.6. Immunofluorescent studies

HUVECs were plated on gelatin-coated culture slides, and cultured for 2 days to form mature monolayers. The cells were starved in DMEM with 1% BSA for 3 h, and stimulated with 50 ng/ml VEGF-A for indicated times. The cells were fixed with 4% para-formaldehyde, and permeabilized with 0.1% TritonX-100 in PBS. Cells were blocked with donkey serum and stained with indicated primary antibodies overnight at 4 °C, followed by incubation with Alexa Fluor 488, or 594-conjugated secondary antibodies (1:500, Invitrogen) for 1 h at room temperature. Fluorescence was examined using a confocal laser-scanning microscope (Fluoview FV500, Olympus).

2.7. Immunoprecipitation and Western blot analyses

Immunoprecipitation and Western blot analyses were performed with indicated antibodies as previously described [19].

2.8. R-Ras activity assay

The levels of GTP-bound R-Ras in HUVECs were determined using a pull down assay with GST-Raf-RBD (Millipore) as previously described [20].

2.9. FITC-dextran permeability assay

Permeability was assessed by the passage of FITC-dextran (average molecular weight, 40,000, MP Biomedicals LLC, Santa Ana, USA), as previously described [12]. All data were from at least five independent experiments and presented as mean ± S.E.

2.10. Antibody uptake assay

Antibody uptake assay was performed as previously described [21] with some modifications. Confluent HUVECs were starved in DMEM with 1% BSA for 3 h, followed by treatment with 150 μM chloroquine (Sigma) for 30 min. A monoclonal antibody directed against the extracellular domain of VE-cadherin (BV6) was incubated with HUVECs in DMEM with 1% BSA at 4C° for 1 h. Unbound antibody was washed by rinsing cells in ice-cold DMEM with 1% BSA. Then, cells were incubated with 50 ng/ml VEGF-A for 30 min at 37C° in the presence of chloroquine. The cells were washed for 15 min at 4C° in PBS, pH 2.7 containing 25 mM glycine and 1% BSA to remove cell surface bound antibody. The cells were rinsed, fixed, and subjected to immunofluorescence. The cells were treated similarly without chloroquine, and then were incubated in EGM2-MV at 37C° for 20 min after a mild acid wash for the induction of recycling in order to visualize VE-cadherin that was recycled to cell surface.

2.11. Trypsinization assay

Trypsinization assay was performed as described previously [12]. Confluent HUVECs were serum starved in DMEM with 1% BSA for 3 h, followed by treatment with 150 μM chloroquine. Then, cells were incubated in EGM-2-MV at 37C° for 16 h in the presence of chloroquine, and treated with 0.25% trypsin-EDTA. Trypsin was inactivated in serum containing medium. Cells were collected by centrifugation and protein extracts were obtained from cell pellets.

2.12. Statistical analysis

Student's t test was performed for comparisons between two groups. One-way ANOVA followed by post hoc Tukey's test was performed for comparisons among multiple groups. A value of $p < 0.05$ was considered to indicate a statistically significant difference. All data are shown as means ± SEM.

3. Results

3.1. Girdin is involved in the remodeling of AJs

VEGF stimulation induces VE-cadherin to undergo endocytosis, which leads to the disassembly of AJs and enhanced vascular permeability [1]. An immunofluorescence analysis demonstrated that, AJs, in a monolayer of control HUVECs, were disrupted after VEGF stimulation, whereas no apparent disruption of AJs was observed in a monolayer of Girdin-depleted cells (Fig. 1A). We also investigated the effect of Girdin on the interaction between VE-cadherin and catenins. The interaction of VE-cadherin and p120-catenin was attenuated after VEGF stimulation in control HUVECs, but not in Girdin depleted cells (Fig. 1B). The interaction between VE-cadherin and β-catenin was not affected by Girdin depletion. The previous study reported that p120-catenin binding prevented VE-cadherin from endocytosis [22]. Thus, we investigated the effect of Girdin on VE-cadherin endocytosis. Antibody uptake assays revealed that VE-cadherin containing vesicles significantly decreased in Girdin depleted HUVECs, in comparison to control cells (Fig. 1C and D). Control cells contained VE-cadherin containing vesicles, which exhibited partial colocalization with Girdin (Fig. 1C), indicating the involvement of Girdin in the VE-cadherin endocytosis and its intracellular trafficking. The endocytosis of VE-cadherin is regulated by clathrin-dependent endocytosis [8]. Cross-linking experiment was applied to analyze the low affinity interaction of VE-cadherin, clathrin, and its adopter proteins in the previous study [23]. Thus, we also used a cross-linking reagent DSP (dithio-bis-succinimidylpropionate). An immunoprecipitation assay showed that Girdin forms a complex with VE-cadherin as well as clathrin heavy chain (HC) after VEGF stimulation in HUVECs treated with a DSP (Supplemental Fig. 1A).

The involvement of Girdin in VE-cadherin endocytosis was further supported by a trypsinization assay (Fig. 1E). The data showed that the amount of VE-cadherin was decreased by Girdin depletion in trypsin-treated but not control HUVECs, indicating that the amount of VE-cadherin that underwent endocytosis was decreased in Girdin-depleted HUVECs. These data suggest that Girdin is involved in the regulation of AJs through controlling the endocytosis and/or intracellular trafficking of VE-cadherin in HUVECs.

3.2. Girdin is required for VEGF induced transendothelial permeability

The effect of Girdin depletion on the permeability of FITC (fluorescein isothiocyanate)-conjugated dextran was examined in a monolayer of HUVECs (Fig. 1F). VEGF-induced dextran permeability was significantly decreased in a monolayer of Girdin-depleted HUVECs in comparison to control cells. Moreover, the expression of siRNA-resistant Girdin restored the VEGF-induced dextran permeability in Girdin depleted cells (Supplemental Fig. 1B and C). These data indicate that Girdin functions to facilitate vascular permeability after VEGF stimulation by regulating the endocytosis of VE-cadherin.

Akt phosphorylates Girdin at serine-1416 downstream of activated VEGFR2 [17]. Akt1 has an established role in the regulation of vascular permeability and leakage [24]. Thus the possibility that the phosphorylation of Girdin at serine-1416 is involved in the regulation of permeability in HUVECs was examined. Dextran permeability was significantly decreased by the expression of a non-phosphorylated mutant of Girdin (Girdin SA) in which serine-1416 was mutated to alanine (Supplemental Fig. 1D). Another finding from this experiment is that the expression of another mutant of Girdin (Basic-mut), in which six basic residues (lysines and arginines) adjacent to the Akt phosphorylation site were replaced with alanines, also led to the decrease in dextran permeability. Basic-mut of Girdin exhibits decreased Akt-mediated phosphorylation at serine-1416 [25]. Although precise mechanism remains unclear, the result suggests that intact function of Girdin is essential for the regulation of the dextran permeability through the modulation of VE-cadherin endocytosis in HUVECs.

3.3. Girdin interacts with the active form of R-Ras

A valuable clue to the mechanism for Girdin-mediated regulation of transendothelial permeability was provided by a previous proteomic study that comprehensively identified proteins interacting with activated R-Ras [26]. Proteins interacting with a dominant-active mutant of R-Ras were identified, which included mouse Girdin (mKIAA1212 protein). We tried to address whether the R-Ras/Girdin interaction is involved in the regulation of vascular permeability, since the function of R-Ras is closely associated with vascular permeability and stability [27,28].

Exogenously-expressed Girdin was immunoprecipitated with the constitutively active form of R-Ras (R-Ras 38V), but not its dominant negative form (R-Ras 43N) (Fig. 2A, Supplemental Fig. 2A). Further experiments investigated whether Akt-mediated phosphorylation of Girdin affects its interaction with R-Ras (Supplemental Fig. 2B), implying no apparent involvement of Akt in the formation of R-Ras/Girdin protein complex.

A time-course experiment in HUVECs demonstrated that the activity of endogenous R-Ras was increased at 5 min after VEGF stimulation and sustained for at least 20 min (Fig. 2B). Therefore, the interaction between endogenous Girdin and R-Ras was examined in HUVECs. The result showed that endogenous R-Ras was successfully co-immunoprecipitated with Girdin in VEGF-stimulated HUVECs (Fig. 2C). Collectively, these data indicate that Girdin forms a complex with the active form of R-Ras in endothelial cells after VEGF stimulation.

Various constructs encoding the fragments of Girdin fused with the GST (glutathione S-transferase) were generated and pull-down assays were conducted. However, repeated experiments could not determine the domain/region(s) of Girdin that are required for R-Ras binding, which was probably attributable to non-specific binding of each fragment of Girdin to R-Ras.

3.4. R-Ras regulates subcellular localization of Girdin

Immunocytochemistry was used to investigate the effect of R-Ras depletion on Girdin subcellular localization in HUVECs. Girdin was primarily colocalized with the peripheral actin, accumulating at the cell–cell contact sites in a static monolayer of control HUVECs (Supplemental Fig. 2C, upper panels), which was abrogated in R-Ras-depleted cells (Supplemental Fig. 2C, lower panels). These data indicate that R-Ras regulates subcellular localization of Girdin, which may be a mechanism for the regulation of transendothelial permeability and VE-cadherin trafficking by the R-Ras/Girdin complex.
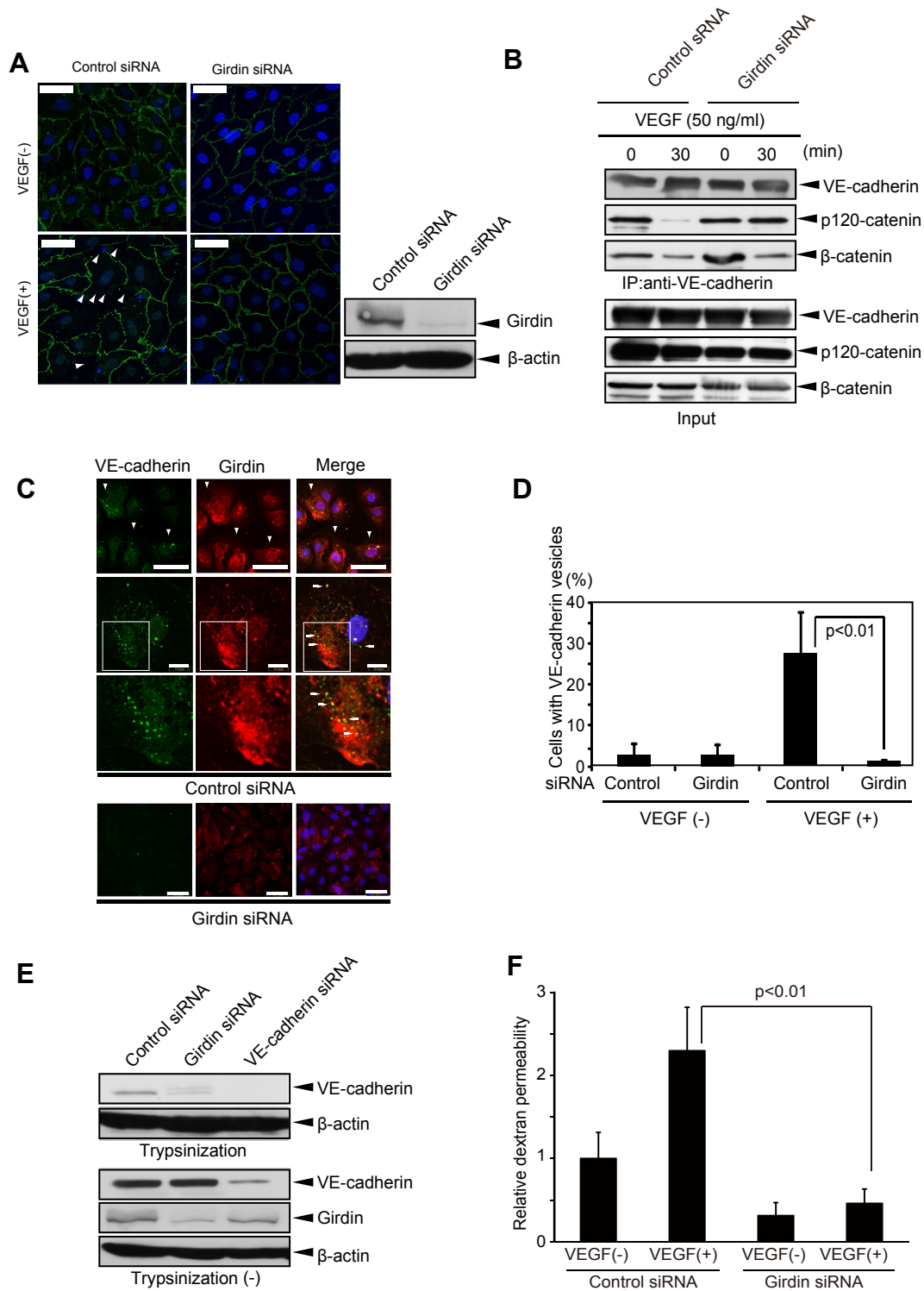
Fig. 1. Girdin is involved in the endocytosis of VE-cadherin and transendothelial permeability of HUVECs monolayer. (A) Immunofluorescence of VE-cadherin: Arrowheads indicate disrupted AJs in control cells. Bar, 50 μm. Shown in the right panel is the data of a Western blot analysis that shows effective depletion of endogenous Girdin in HUVECs. (B) Samples obtained from HUVECs were subjected to Immunoblotting. (C) Antibody uptake assay: Arrowheads indicate cells containing internalized VE-cadherin. Arrows indicate the colocalization of Girdin and internalized VE-cadherin. Bar, 50 μm in the panels in the first and forth lines, 10 μm in the panels in the second line. The panels in the third line indicate the magnification of images in the insets in the panels in the second line. (D) The number of HUVECs containing VE-cadherin vesicles were counted and quantified. Two hundred cells were evaluated in each group. Data represent the mean ± S.E. from three independent experiments. (E) Protein extracts were obtained from HUVECs with or without trypsinization as described in Materials and methods. The VE-cadherin antibody recognizes the extracellular domain of VE-cadherin. (F) FITC-dextran permeability assay of control or Girdin siRNA treated HUVECs.
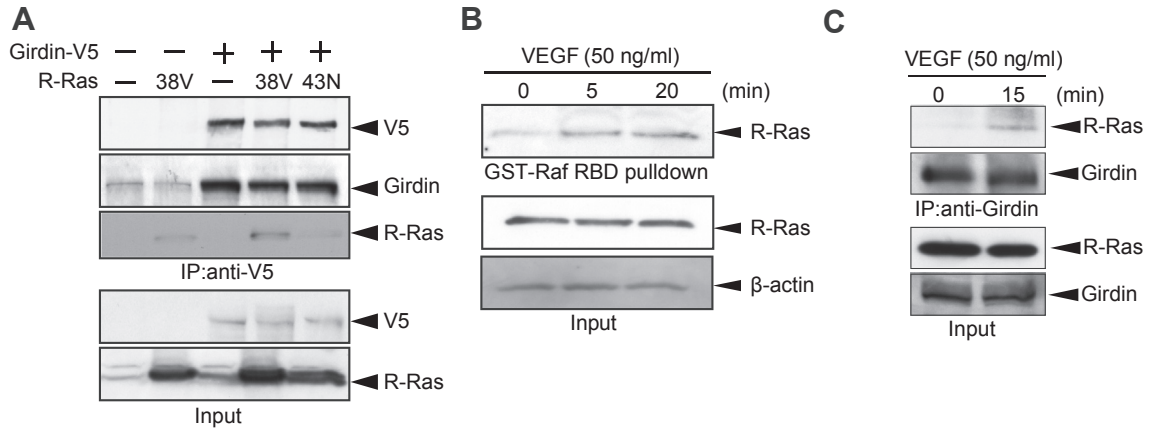
Fig. 2. Girdin interacts with the active form of R-Ras in HUVECs. (A) HEK293 cells were transfected with indicated cDNAs followed by immunoprecipitation assays using anti-V5 antibody. Each immunoprecipitate was analyzed by a Western blot analysis. (B) GTP-bound R-Ras was detected with a GST-Raf RBD pull down assay. (C) Endgeneous R-Ras co-precipitated with Girdin was detected by Western blotting.

3.5. Girdin forms a complex with VE-cadherin-containing vesicles dependently of R-Ras

The observations that Girdin localizes at the cell–cell contact sites in the presence of R-Ras and VEGF induces R-Ras activation (Fig. 2 and Supplemental Fig. 2) suggest that the VEGF-R-Ras pathway might regulate the interaction of Girdin with VE-cadherin-containing vesicles at or adjacent to the cell–cell contact sites. Immunoprecipitation assays showed that the interaction between Girdin and VE-cadherin increased overtime and reached a
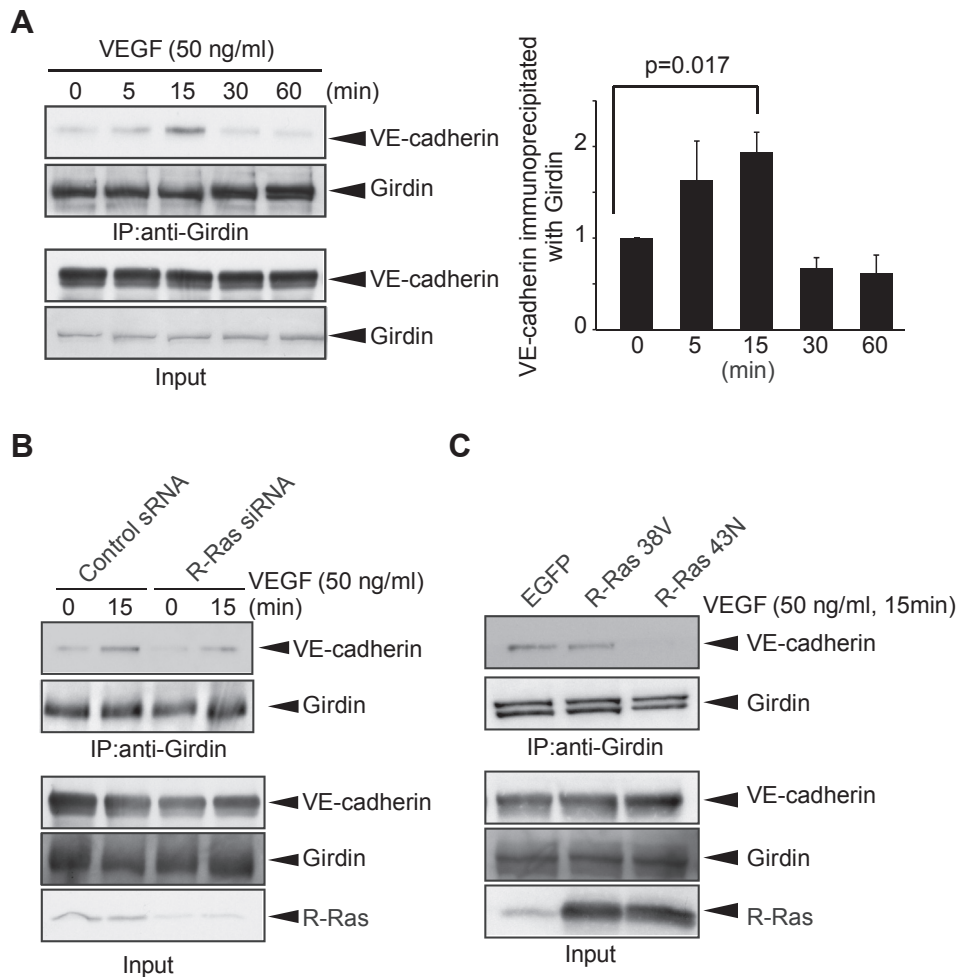
Fig. 3. Girdin forms a complex with VE-cadherin in the presence of R-Ras. (A) Immunoprecipitates with anti-Girdin antibody were subjected to a Western blot analysis using the indicated antibodies. The right panel represents a densitometry analysis of immunoprecipitated VE-cadherin. (B) Confluent HUVECs were transfected with indicated siRNA. Cell lysates were harvested and subjected for immunoprecipitation. (C) Confluent HUVECs were infected with indicated adenoviral vectors. Cell lysates were harvested and subjected to an immunoprecipitation assay.

maximum by 15 min in HUVECs stimulated with VEGF, although weak but still detectable interaction was observed in non-stimulated cells (Fig. 3A). Interestingly, the Girdin/VE-cadherin interaction was significantly attenuated by the depletion of R-Ras or the expression of the 43N mutant of R-Ras in VEGF-stimulated HUVECs (Fig. 3B and C). These results suggest that R-Ras regulates the interaction between Girdin and VE-cadherin-containing vesicles upon VEGF stimulation.
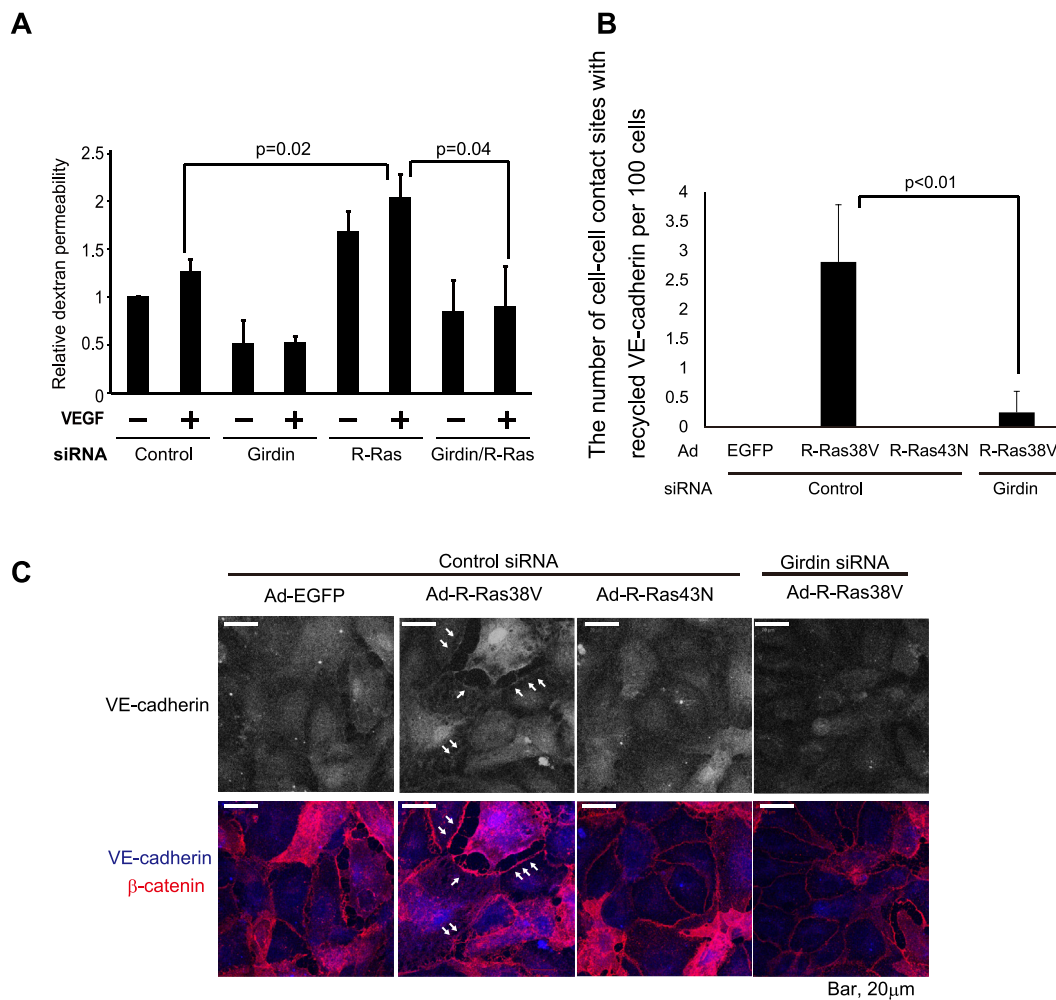
3.6. Girdin regulates the trafficking and transendothelial permeability in synergy with R-Ras

The previous study revealed that the R-Ras activation prevented the endocytosis of VE-cadherin [28]. Thus, we examined the effect of R-Ras and Girdin on transendothelial permeability. The depletion of endogenous R-Ras significantly increased dextran permeability, and the enhanced permeability induced by the R-Ras depletion was negated by the simultaneous depletion of Girdin (Fig. 4A). These data suggest that the interaction of activated R-Ras, Girdin, and VE-cadherin may not simply be necessary for the endocytosis of VE-cadherin, but involved in the recycling of VE-cadherin containing vesicles to the plasma membrane to control the vascular integrity.

Therefore, further experiments were conducted to visualize recycled VE-cadherin by an antibody uptake assay (Fig. 4B and C). The recycling of VE-cadherin was induced by incubation in growth medium after a mild acid wash. The recycled VE-cadherin at cell–cell contact sites was detected in HUVECs transfected with the R-Ras 38V, but not in cells transfected with control (EGFP) or R-Ras 43N. Furthermore, the recycled VE-cadherin at cell–cell contact sites was attenuated by concomitant Girdin depletion suggesting that R-Ras activation promotes the recycling of VE-cadherin-containing vesicles to the plasma membrane in synergy with Girdin, the loss of which leads to the accumulation or degradation of VE-cadherin-containing vesicles in the cytoplasm (Supplemental Fig. 3).

4. Discussion

The present study demonstrates that Girdin regulates transendothelial permeability by facilitating VE-cadherin trafficking at least partly in concert with R-Ras. VEGF stimulation activates R-Ras, which binds to Girdin and mediates the interaction of Girdin with VE-cadherin on intracellular vesicles such as endosomes around the cell–cell contact sites. Our data and the previous study on R-Ras

Fig. 4. R-Ras regulates the trafficking of VE-cadherin and transendothelial permeability in HUVECs. (A) HUVECs transfected with the indicated siRNA were subjected for an FITC-dextran permeability assay. (B) Monolayers of HUVECs were subjected to antibody uptake assay. Cells were incubated in EGM2-MV at 37C° for 5 min after a mild acid wash for the induction of VE-cadherin recycling. The number of cell–cell contact sites containing recycled VE-cadherin was counted and 200 cell–cell contact sites were evaluated in each group. Data are shown as the number of the cell–cell contact sites containing recycled VE-cadherin per 100 cells. Data represent the mean ± S.E. from three independent experiments. (C) Representative images described in (B). In the lower panels, β-catenin was co-stained to visualize a cell–cell contact site. Arrows indicate recycled. Bar, 20 μm.

function in the membrane trafficking system [29] suggest that R-Ras/Girdin complex promotes the recycling of VE-cadherin-containing vesicles to the plasma membrane. The current data also indicate the role of Girdin in endocytosis of VE-cadherin leading to the disassembly of AJs and the increase of trans-endothelial permeability. However, it remains unknown precisely how R-Ras binding to Girdin modulates its function, how Akt-mediated phosphorylation of Girdin is involved in VE-cadherin endocytosis, and how R-Ras/Girdin complex regulates the recycling of VE-cadherin containing vesicles.

We recently reported that Girdin functioned as a GAP for dynamin 2 and regulated clathrin dependent endocytosis in a cargo specific manner [18]. The study revealed that Girdin regulated the endocytosis of E-cadherin in MDCK cells where R-Ras was not expressed. The present study further elucidated the biological significance of Girdin as a regulator of clathrin dependent endocytosis. The finding that R-Ras, in concert with Girdin, regulates the intracellular trafficking of VE-cadherin and transendothelial permeability is consistent with a recent report that R-Ras regulates the integrity of tumor vessels [28]. The study revealed that exogenous expression of R-Ras 38V attenuated the internalization of VE-cadherin accompanied with the reduction of serine 665 phosphorylation of VE-cadherin, which resulted in the enhanced endothelial barrier function. Although it is unclear whether Girdin is involved in the phosphorylation of serine 665 of VE-cadherin at present, the present study identified Girdin as a protein that interacts with the active form of R-Ras, suggesting that Girdin, as an effector for R-Ras, may be vital in the regulation of vascular integrity and post-natal angiogenesis. It would be a challenge to examine how R-Ras utilize its various effectors to differently regulate AJs and cell-extracellular matrix adhesion in endothelial cells.

Finally, it is important to note that R-Ras and Girdin may be candidate proteins useful for the effective treatment for pathological conditions with vascular hyperpermeability. A recent study revealed that the dysregulation of VE-cadherin and disruption of AJs are important initial events in the pathogenesis of the atheroma formed in hyperlipidemia model mice [30]. Therefore, the development of antagonists for R-Ras and Girdin may lead to the development of useful therapeutic modalities for a variety of vascular diseases including atherosclerosis.

Source of funding

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.012.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.012.

References

[1] E. Dejana, E. Tournier-Lasserve, B.M. Weinstein, The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications, Dev. Cell 16 (2009) 209–221.

[2] R. Chibber, B.M. Ben-Mahmud, S. Chibber, E.M. Kohner, Leukocytes in diabetic retinopathy, Curr. Diabetes Rev. 3 (2007) 3–14.

[3] Y. Komarova, A.B. Malik, Regulation of endothelial permeability via paracellular and transcellular transport pathways, Annu Rev. Physiol. 72 (2010) 463–493.

[4] L. Gonzalez-Mariscal, R. Tapia, D. Chamorro, Crosstalk of tight junction components with signaling pathways, Biochim. Biophys. Acta 1778 (2008) 729–756.

[5] E. Dejana, F. Orsenigo, C. Molendini, P. Baluk, D.M. McDonald, Organization and signaling of endothelial cell-to-cell junctions in various regions of the blood and lymphatic vascular trees, Cell Tissue Res. 335 (2009) 17–25.

[6] F. Breviario, L. Caveda, M. Corada, I. Martin-Padura, P. Navarro, J. Golay, M. Introna, D. Gulino, M.G. Lampugnani, E. Dejana, Functional properties of human vascular endothelial cadherin (7B4/cadherin-5), an endothelium-specific cadherin, Arterioscler. Thromb. Vasc. Biol. 15 (1995) 1229–1239.

[7] S. Fukuhra, A. Sakurai, A. Yamagishi, K. Sako, N. Mochizuki, Vascular endothelial cadherin-mediated cell-cell adhesion regulated by a small GTPase, Rap1, J. Biochem. Mol. Biol. 39 (2006) 132–139.

[8] E. Delva, A.P. Kowalczyk, Regulation of cadherin trafficking, Traffic 10 (2009) 259–267.

[9] D.M. Bryant, M.C. Kerr, L.A. Hammond, S.R. Joseph, K.E. Mostov, R.D. Teasdale, J.L. Stow, EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin, J. Cell Sci. 120 (2007) 1818–1828.

[10] J.G. Lock, J.L. Stow, Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin, Mol. Biol. Cell 16 (2005) 1744–1755.

[11] D.M. Bryant, J.L. Stow, The ins and outs of E-cadherin trafficking, Trends Cell Biol. 14 (2004) 427–434.

[12] J. Gavard, J.S. Gutkind, VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin, Nat. Cell Biol. 8 (2006) 1223–1234.

[13] A. Enomoto, H. Murakami, N. Asai, N. Morone, T. Watanabe, K. Kawai, Y. Murakumo, J. Usukura, K. Kaibuchi, M. Takahashi, Akt/PKB regulates actin organization and cell motility via Girdin/APE, Dev. Cell 9 (2005) 389–402.

[14] H. Le-Niculescu, I. Niesman, T. Fischer, L. DeVries, M.G. Farquhar, Identification and characterization of GIV, a novel Galpha i/s-interacting protein found on COPI, endoplasmic reticulum-Golgi transport vesicles, J. Biol. Chem. 280 (2005) 22012–22020.

[15] L. Weng, A. Enomoto, M. Ishida-Takagishi, N. Asai, M. Takahashi, Girding for migratory cues: roles of the Akt substrate Girdin in cancer progression and angiogenesis, Cancer Sci. 101 (2010) 836–842.

[16] P. Jiang, A. Enomoto, M. Jijiwa, T. Kato, T. Hasegawa, M. Ishida, T. Sato, N. Asai, Y. Murakumo, M. Takahashi, An actin-binding protein Girdin regulates the motility of breast cancer cells, Cancer Res. 68 (2008) 1310–1318.

[17] T. Kitamura, N. Asai, A. Enomoto, K. Maeda, T. Kato, M. Ishida, P. Jiang, T. Watanabe, J. Usukura, T. Kondo, F. Costantini, T. Murohara, M. Takahashi, Regulation of VEGF-mediated angiogenesis by the Akt/PKB substrate Girdin, Nat. Cell Biol. 10 (2008) 329–337.

[18] L. Weng, A. Enomoto, H. Miyoshi, K. Takahashi, N. Asai, N. Morone, P. Jiang, J. An, T. Kato, K. Kuroda, T. Watanabe, M. Asai, M. Ishida-Takagishi, Y. Murakumo, H. Nakashima, K. Kaibuchi, M. Takahashi, Regulation of cargo-selective endocytosis by dynamin 2 GTPase-activating protein girdin, EMBO J. 33 (2014) 2098–2112.

[19] H. Miyake, K. Maeda, N. Asai, R. Shibata, H. Ichimiya, M. Isotani-Sakakibara, Y. Yamamura, K. Kato, A. Enomoto, M. Takahashi, T. Murohara, The actin-binding protein Girdin and its Akt-mediated phosphorylation regulate neointima formation after vascular injury, Circ. Res. 108 (2011) 1170–1179.

[20] A.L. Cole, G. Subbanagounder, S. Mukhopadhyay, J.A. Berliner, D.K. Vora, Oxidized phospholipid-induced endothelial cell/monocyte interaction is mediated by a cAMP-dependent R-Ras/PI3-kinase pathway, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1384–1390.

[21] K. Xiao, D.F. Allison, K.M. Buckley, M.D. Kottke, P.A. Vincent, V. Faundez, A.P. Kowalczyk, Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells, J. Cell Biol. 163 (2003) 535–545.

[22] B.A. Nanes, C. Chiasson-MacKenzie, A.M. Lowery, N. Ishiyama, V. Faundez, M. Ikura, P.A. Vincent, A.P. Kowalczyk, p120-catenin binding masks an endocytic signal conserved in classical cadherins, J. Cell Biol. 199 (2012) 365–380.

[23] C.M. Chiasson, K.B. Wittich, P.A. Vincent, V. Faundez, A.P. Kowalczyk, p120-catenin inhibits VE-cadherin internalization through a Rho-independent mechanism, Mol. Biol. Cell 20 (2009) 1970–1980.

[24] A. Di Lorenzo, C. Fernandez-Hernando, G. Cirino, W.C. Sessa, Akt1 is critical for acute inflammation and histamine-mediated vascular leakage, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 14552–14557.

[25] Y. Wang, N. Kaneko, N. Asai, A. Enomoto, M. Isotani-Sakakibara, T. Kato, M. Asai, Y. Murakumo, H. Ota, T. Hikita, T. Namba, K. Kuroda, K. Kaibuchi, G.L. Ming, H. Song, K. Sawamoto, M. Takahashi, Girdin is an intrinsic regulator of neuroblast chain migration in the rostral migratory stream of the postnatal brain, J. Neurosci. 31 (2011) 8109–8122.

[26] L.E. Goldfinger, C. Ptak, E.D. Jeffery, J. Shabanowitz, J. Han, J.R. Haling, N.E. Sherman, J.W. Fox, D.F. Hunt, M.H. Ginsberg, An experimentally derived database of candidate Ras-interacting proteins, J. Proteome Res. 6 (2007) 1806–1811.

[27] M. Komatsu, E. Ruoslahti, R-Ras is a global regulator of vascular regeneration that suppresses intimal hyperplasia and tumor angiogenesis, Nat. Med. 11 (2005) 1346–1350.

[28] J. Sawada, T. Urakami, F. Li, A. Urakami, W. Zhu, M. Fukuda, D.Y. Li, E. Ruoslahti, M. Komatsu, Small GTPase R-Ras regulates integrity and functionality of tumor blood vessels, Cancer Cell 22 (2012) 235–249.

[29] A. Takaya, T. Kamio, M. Masuda, N. Mochizuki, H. Sawa, M. Sato, K. Nagashima, A. Mizutani, A. Matsuno, E. Kiyokawa, M. Matsuda, R-Ras regulates exocytosis by Rgl2/Rlf-mediated activation of RalA on endosomes, Mol. Biol. Cell 18 (2007) 1850–1860.

[30] T. Miyazaki, Y. Taketomi, M. Takimoto, X.F. Lei, S. Arita, J.R. Kim-Kaneyama, S. Arata, H. Ohata, H. Ota, M. Murakami, A. Miyazaki, m-Calpain induction in vascular endothelial cells on human and mouse atheromas and its roles in VE-cadherin disorganization and atherosclerosis, Circulation 6 (2011) 2522–2532.

Supplemental Information

Supplemental Methods

Intracelluar DSP cross-linking

Intracelluar cross-linking using DSP [dithiobis (succinimidylpropionate)] was performed as previously described [1-2].   Briefly, confluent HUVECs were starved for 3 hours in DMEM with 1% BSA, and incubated with 50 ng/ml VEGF-A for 15 minutes. Then, cells were placed on ice, rinsed twice with PBS, and incubated with 1 mM DSP (Thermo Scientific) for 2 hours at 4C°. The reaction was quenched by treatment with 50 mM Tris, pH7.4 in PBS for 15 minutes at room temperature. The cells were lysed and subjected for immunoprecipitation.

Supplementary Figure Legends

Supplemental Figure 1

(A) Confluent HUVECs were stimulated with VEGF-A (50 ng/ml, 15 minutes) followed by treatment with DSP. Cell lysates were harvested and subjected to immunoprecipitation assay. **(B)** HUVECs transfected with Girdin siRNA were infected with adenovirus harboring EGFP or siRNA resistant Girdin, followed by FITC-dextran permeability assay. **(C)** Lysates from HUVECs used for the assay described in B were analyzed by a Western blot analysis using the indicated antibodies. **(D)** Monolayers of HUVECs were treated with siRNA against Girdin, following add-back of siRNA-resistant Girdin using adenoviral vectors containing wild type (WT) or the indicated mutants of Girdin. The FITC-dextran permeability assay was performed as described in Materials and Methods.

Supplemental Figure 2

(A) Lysates prepared from HUVECs infected with adenovirus harboring indicated cDNA were subjected to immunoprecipitation assay using anti-V5 antibody, followed by a Western blot analysis.

(B) Monolayers of HUVECs were infected with adenovirus harboring R-Ras 38V cDNA and treated with indicated siRNA. Alternatively, cells were treated with DMSO or LY294002 (10 μM) for 1 hour. The cell lysates were harvested and subjected to immunoprecipitation with anti-Girdin antibody. **(C)** Serum-starved HUVECs were subjected to immunocytochemistry. Bar, 50 μm in the left three panels, and 20 μm in the right panels. Arrowheads indicate Girdin accumulating in cell-cell contact sites.

Supplemental Figure 3

Schematic illustration of a working model of how Girdin regulates AJs disassembly with R-Ras. **(A)** VEGF stimulation induces Girdin, but not R-Ras, to mediate clathrin-dependent endocytosis of VE-cadherin. Subsequently, R-Ras activated by GEF proteins downstream of VEGFR2 mediates the binding of Girdin with VE-cadherin, leading to the promotion of intracellular trafficking (recycling) of VE-cadherin-containing vesicles. **(B–C)** Schematic illustration of the effects of the depletion of Girdin and R-Ras and the expression of the dominant negative (DN) mutant of R-Ras on VE-cadherin trafficking shown in this study.
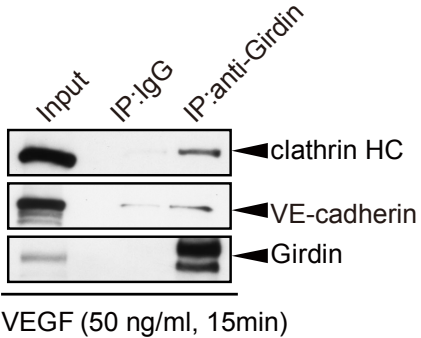
Supplemental References

[1] C.M. Chiasson, K.B. Wittich, P.A. Vincent, V. Faundez, A.P. Kowalczyk, p120-catenin inhibits VE-cadherin internalization through a Rho-independent mechanism, Mol Biol Cell, 20 (2009) 1970-1980.

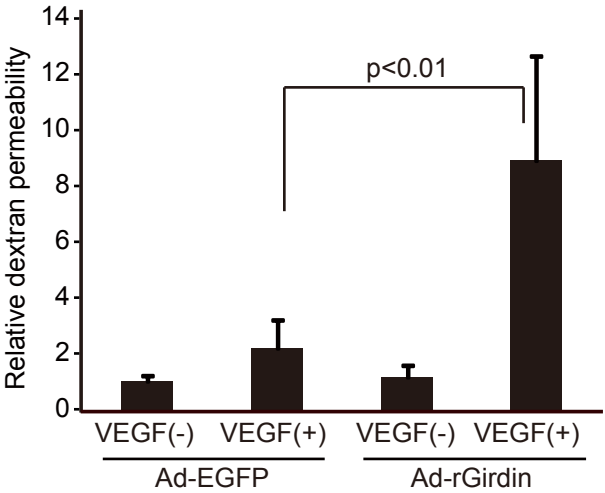[2] B. Craige, G. Salazar, V. Faundez, Phosphatidylinositol-4-kinase type II alpha contains an AP-3-

sorting motif and a kinase domain that are both required for endosome traffic, Mol Biol Cell, 19 (2008) 1415-1426.
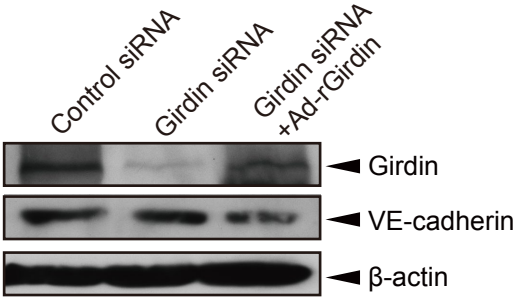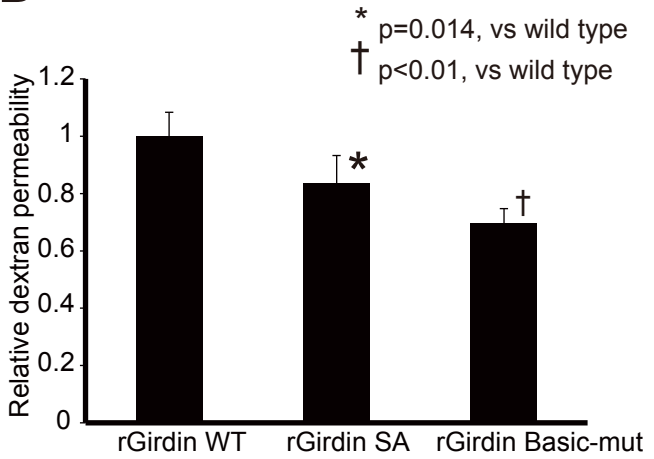
Supplemental Figure 1

A

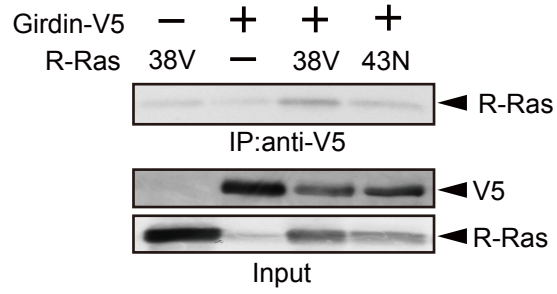VEGF (50 ng/ml, 15min)

B

C

D

* p=0.014, vs wild type
† p<0.01, vs wild type

Supplemental Figure 2

A

Girdin-V5   −   +   +   +

R-Ras   38V   −   38V   43N

◄ R-Ras

IP:anti-V5

◄ V5

◄ R-Ras

Input

B

Ad R-Ras 38V

siRNA   control   Akt

◄ Girdin

◄ R-Ras

IP:anti-Girdin

◄ Girdin

◄ Akt

◄ R-Ras

Input

Ad R-Ras 38V

DMSO   LY294002

◄ Girdin

◄ R-Ras

IP:anti-Girdin

◄ Girdin

◄ pAkt

◄ Akt

◄ R-Ras

Input

C

	β-actin	Girdin	Merge	High magnification

Control siRNA

R-Ras siRNA

Supplemental Figure 3