The Actin-Binding Protein Girdin and Its Akt-Mediated Phosphorylation Regulate Neointima Formation After Vascular Injury

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**Rationale:** It is well established that the migration and proliferation of vascular smooth muscle cells (VSMCs) have major roles in the vascular remodeling process. Our previous study showed that the Akt substrate Girdin, which is expressed in VSMCs and endothelial cells, is essential for postnatal angiogenesis. However, the function of Girdin and its Akt-mediated phosphorylation in VSMCs and their in vivo roles in vascular remodeling remain to be elucidated.

**Objective:** We investigated the function of Girdin and its Akt-mediated phosphorylation using cultured VSMCs and animal models of vascular remodeling.

**Methods and Results:** The depletion of Girdin by RNA interference disrupted the rearrangement of the actin cytoskeleton in VSMCs, resulting in impaired cell migration. The depletion of Girdin also inhibited VSMC proliferation. Girdin expression was highly upregulated and its serine at position 1416 was phosphorylated in the neointima of carotid arteries after balloon injury in a rat model. The introduction of an adenovirus harboring short hairpin RNA against Girdin attenuated the proliferation of VSMCs and neointima formation without affecting reendothelialization. Furthermore, we found that neointima formation after femoral wire injury was significantly attenuated in Girdin S1416A knock-in mice, in which the Akt phosphorylation site of Girdin was mutated, thus indicating a major role for Girdin phosphorylation in vascular remodeling.

**Conclusions:** These findings indicate that Girdin and its Akt-mediated phosphorylation have major roles in the migration and proliferation of VSMCs and vascular remodeling, making the Akt/Girdin signaling pathway a potential target for the development of new therapeutics for vascular diseases. (Circ Res. 2011;108:1170-1179.)

**Key Words:** Akt ■ Girdin ■ vascular remodeling ■ vascular smooth muscle ■ neointima

Vascular injury caused by angioplasty induces deendothelialization, which promotes the deposition of platelets at the injured site and subsequent recruitment of leukocytes. Growth factors and cytokines released from platelets, leukocytes and vascular smooth muscle cells (VSMCs) stimulate the migration and proliferation of VSMCs, which results in neointima formation and restenosis.1–3 The phosphatidylinositol 3-kinase (PI3K)-Akt pathway is a key regulator of several processes such as cell survival, proliferation and growth downstream of these humoral factors.4–6 Accumulating evidence suggests that the PI3K-Akt pathway and its downstream components also play essential roles in vascular remodeling.7–11 However, the underlying molecular mechanisms are not completely understood.

A search for Akt-binding proteins led to the identification of Girdin (girders of actin filament), also known as Akt phosphorylation enhancer (APE) and Ga-interacting vesicle associated protein (GIV).12–14 Girdin is a large 220-kDa protein with unique amino- and carboxyl-terminal domains flanking a long coiled-coil region. Girdin forms oligomers through its amino-terminal domain and coiled-coil region. The carboxyl-terminal domain contains the actin-binding site and the phosphatidylinositol phosphate-binding motif located near the Akt phosphorylation site (serine 1416). Therefore, Girdin is postulated to crosslink actin filaments and to anchor them to the plasma membrane in quiescent cells. In response to growth factors, Akt phosphorylates Girdin at serine 1416, and the phosphorylated Girdin detaches from the plasma mem-

Original received November 4, 2010; revision received March 4, 2011; accepted March 10, 2011. In February 2011, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.7 days.

From the Departments of Cardiology (H.M., K.M., R.S., H.I., Y.Y.; K.K., T.M.) and Pathology (N.A., M.I.-S., A.E., M.T.), Nagoya University Graduate School of Medicine; and Department of Medical Technology (M.I.-S.), Nagoya University School of Health Science, Japan.

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*Circulation Research* is available at [http://circres.ahajournals.org](http://circres.ahajournals.org) DOI: 10.1161/CIRCRESAHA.110.236174
brane with actin filaments. Subsequently, the phosphorylated Girdin accumulates in the lamellipodia of migrating cells, leading to the rearrangement of the actin cytoskeleton.\textsuperscript{12} To our knowledge, Girdin is the only actin-binding protein that is directly phosphorylated by Akt.

We recently reported that Girdin is expressed in immature endothelial cells to regulate vascular endothelial growth factor-mediated postnatal angiogenesis. Importantly, Akt-mediated phosphorylation of Girdin is required for the migration of endothelial cells.\textsuperscript{12,15} It is of note that other evidence suggests the synergistic action of Girdin and Akt and the involvement of Girdin in multiple signaling pathways. For example, Girdin enhances Akt signaling\textsuperscript{11} and acts as a nonreceptor guanine nucleotide exchange factor to activate the G\textsubscript{q} subunit of heterotrimeric G proteins in resting cells rather than in activated cells.\textsuperscript{16}

Another report suggested the involvement of Girdin in DNA synthesis, although the underlying mechanism has not yet been determined.\textsuperscript{13} Considering the fact that aberrant Akt signaling leads to impaired angiogenesis\textsuperscript{15} and vascular remodeling,\textsuperscript{7} an important but yet unanswered issue is whether Girdin and its Akt-mediated phosphorylation have roles in the pathophysiology of vascular dysfunction, remodeling and diseases.

We previously showed that Girdin is expressed in VSMCs but not endothelial cells in large vessels with a thick smooth muscle layer, such as carotid and femoral arteries.\textsuperscript{15} The roles of Girdin and its phosphorylation in VSMCs have not yet been reported. In the present study, we investigated whether Girdin modulates the vascular remodeling process in vitro and in vivo. We examined the ability of Girdin to induce cell migration, proliferation and survival in human (h)VSMCs. We also evaluated the function of Girdin in neointima formation using an adenoviral vector encoding short hairpin (sh)RNA directed against Girdin, and Girdin S1416A knock-in mice that we generated, in which serine 1416 was replaced with alanine. Our observations indicate that the Akt/Girdin signaling pathway is crucial for the vascular remodeling process.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Generation of Girdin S1416A Knock-In mice**

Conventional gene-targeting techniques were used to generate Girdin S1416A knock-in mice, as detailed in the expanded Online Methods.

**Statistical Analysis**

Data are presented as the means±SEM. A statistical analysis was performed using Student t test. A value of P<0.05 was considered to be significant.

**Results**

**Platelet-Derived Growth Factor and Angiotensin II Induced the Phosphorylation of Girdin**

Because platelet-derived growth factor (PDGF) and angiotensin II are potent activators of Akt in VSMCs,\textsuperscript{17–18} we examined whether they can lead to phosphorylation of Girdin at serine 1416, which is a phosphorylation site for Akt, in hVSMCs. The phosphorylation of Girdin was increased by PDGF-BB, and the phosphorylation reached a maximum at 5 minutes after addition of PDGF-BB (Online Figure I, A). Additionally, Angiotensin II caused an increase in the phosphorylation of Akt, and increased the phosphorylation of Girdin at serine 1416 without affecting the overall expression of Girdin (Online Figure I, B). This phosphorylation reached a maximum at 5 minutes after the addition of Angiotensin II. In addition, the treatment with LY294002 or small interfering (si)RNA-mediated Akt knockdown markedly attenuated the phosphorylation of Girdin (Online Figure I, C and D). These data indicate that the phosphorylation of Girdin is increased by both PDGF-BB and Angiotensin II, two important mediators during the process of vascular remodeling, in a PI3K-Akt signaling pathway-dependent manner.

**Depletion of Girdin Has Little Effect on the Activity of Akt and Rho-Kinase**

Considering previous reports suggesting Girdin-mediated regulation of intracellular signaling pathways,\textsuperscript{13,19} we determined the hierarchy of regulation for Girdin and the PI3K-Akt and Rho-Rho-kinase signals in hVSMCs. We assessed the effects of RNA interference-mediated Girdin depletion (knockdown) on these signaling pathways in hVSMCs. The Western blot analyses showed that transfection of Girdin siRNA effectively reduced the expression of Girdin (Online Figure II, A). However, the depletion of Girdin did not affect the phosphorylation of Akt, glycogen synthase kinase-3, or MYPT1 (myosin phosphatase target protein 1) in hVSMCs (Online Figure II, A and B). The phosphorylation of extracellular signal-regulated kinase was also not affected (Online Figure II, A). Although other groups have reported that the depletion of Girdin attenuated the phosphorylation of Akt in several types of immortalized cells,\textsuperscript{13,19} our results revealed that, in hVSMCs, Girdin depletion has little effect on the activity of these signaling pathways.
Girdin Is Essential for Rearrangement of the Actin Cytoskeleton and Migration of hVSMCs

The migration of VSMCs across the internal elastic lamina from the tunica media is a key process in neointima formation after vascular injury. We confirmed that Girdin localizes at actin structures such as stress fibers and lamellipodia in hVSMCs as observed in other cell types (Online Figure III). Therefore, we examined the functions of Girdin on the remodeling of the actin cytoskeleton and the migration of hVSMCs. Serum-starved control and Girdin-depleted hVSMCs showed no apparent morphological differences. However, after stimulation with serum, the stress fibers were disrupted, and the outlines of the cells became irregular in the Girdin-depleted hVSMCs (Figure 1A). In addition, when cells were stimulated with PDGF-BB, the wide extension of lamellipodia at the leading edge was markedly attenuated, and only small membrane protrusions could be seen in the Girdin-depleted cells (Figure 1B). The normalized length of the lamellipodia around Girdin siRNA-transfected cells was significantly reduced (Figure 1C).

These results indicate that Girdin has an important role in actin remodeling at the peripheral lamellipodia in migrating hVSMCs.

Next, the involvement of Girdin in cell migration was examined using wound-healing assays. The number of cells which migrated into the wounded area and the migration velocity were significantly reduced in the Girdin-depleted cells as compared to the control cells (Figure 2A and 2B). Moreover, in Boyden chamber assays to detect directed 3D cell migration toward chemotactic cues, Girdin siRNA-transfected cells displayed a significant delay in cell migration in response to PDGF-BB (Figure 2C). These findings suggest that Girdin is involved in the PDGF-induced migration of hVSMCs. We assessed cell adhesion as shown in Online Figure IV. However, we found no significant difference between the control and Girdin-depleted cells.

Girdin Is Involved in the Proliferation, but Not the Survival, of hVSMCs

To assess the effect of Girdin on hVSMC proliferation, we performed MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] colorimetric assays. On day 5 after the transfection of siRNAs, the proliferation of the Girdin-depleted cells was markedly suppressed (Figure 3A). This suggests that Girdin contributes to hVSMC proliferation. Furthermore, as shown in Online Figure V, Girdin knockdown significantly increased the number of multinucleated cells, which is a characteristic feature of failure in cytokinesis. To test the possibility that the reduced proliferation of Girdin-depleted cells was attributable to increased cell death, we...
examined the effect of Girdin knockdown on apoptosis and cell death using flow cytometric analysis with annexin V and propidium iodide staining. As shown in Figure 3B and 3C, serum starvation and hydrogen peroxide both induced apoptotic cell death. However, the percentage of apoptotic cell death in Girdin-depleted cells was comparable to that of control cells under either normal growing conditions or proapoptotic conditions (Figure 3B and 3C). Girdin knockdown also had little effect on the percentage of dead cells (Figure 3B). These results indicate that Girdin plays a role in the proliferation of hVSMCs without affecting cell survival.

Girdin Is Upregulated and Phosphorylated in the Neointima of Rat Carotid Arteries After Vascular Injury

To examine the in vivo effects of Girdin on vascular remodeling, the expression and phosphorylation of Girdin in serial arterial sections prepared from rat carotid arteries after balloon injury (BI) were assessed by immunohistochemical analysis. As shown in Figure 4A, in the media of the intact artery, the expression and phosphorylation of Girdin was only sporadically detected. The positive staining for Girdin in the tunica adventitia seemed to be a nonspecific reaction, because the Western blot analysis using protein samples from whole intact arteries showed subtle Girdin expression (Figure 4B). As the neointima grew thicker, enhanced expression and phosphorylation of Girdin were observed in the neointima. Both the expression and the phosphorylation of Girdin in the neointima peaked at 14 days after BI, and persisted for at least 28 days. Then, the level dropped to the baseline by 42 days after BI. We confirmed the expression and phosphorylation of Girdin in injured arteries using the Western blot analyses of protein extracts from whole carotid arteries. Only weak expression and phosphorylation of Girdin was detected in the intact artery, and both the expression and phosphorylation were markedly increased over time and became most apparent at day 14 after BI (Figure 4B through 4D).

Girdin Is Essential for Neointima Formation After Balloon Injury In Vivo

To further analyze the involvement of Girdin in vascular remodeling, an adenoviral vector (Ad) encoding nontargeting shRNA (Ad-sh-control) or shRNA directed against Girdin (Ad-sh-Girdin) was transferred into balloon-injured arteries to selectively inhibit the expression of endogenous Girdin. First, we observed that the infection with Ad-sh-Girdin, but not Ad-sh-control, decreased the expression of Girdin in H9c2 cells, a cell line derived from embryonic rat hearts, showing the specificity and efficacy of the engineered adenoviruses (Online Figure VII, A). Next, we confirmed the successful gene transfer to rat carotid arteries with adenoviral vectors (Online Figure VII, B). Then, we introduced either Ad-sh-control or Ad-sh-Girdin into the rat carotid artery immediately after BI. Ad-sh-Girdin effectively suppressed the Girdin expression in the rat carotid artery (Online Figure VII, C). At day 14, thick neointima was observed in the control arteries, whereas the infection with Ad-sh-Girdin markedly prevented neointima formation (Figure 5A). The quantification analysis showed that the intima/media area ratios of Ad-sh-Girdin-infected arteries were significantly reduced at each time point (Figure 5B). In Ad-sh-Girdin-infected arteries, neither the medial area nor the area surrounded by the external elastic lamina differed from those of Ad-sh-control–infected arteries (Online Figure VIII), thus indicating that a smaller intima/media area ratio in Ad-sh-Girdin-infected arteries is attributed to decreased growth of the neointima.

Because an increase in endothelial recovery is correlated with diminished neointima formation,20 we evaluated the effects of Girdin knockdown on reendothelialization after BI with Evans blue dye staining. Regarding the percentage of the total area that was initially denuded
reendothelialized area was approximately 46% in the Ad-sh-control–infected arteries at day 14. Girdin knockdown did not have any significant effect on reendothelialization (Figure 5C and 5D), suggesting that the inhibitory effect of Girdin knockdown on neointima formation was unrelated to reendothelialization.

Girdin Affects Cell Proliferation, but Not Cell Survival, in Injured Arteries

A major step in neointima formation is VSMC accumulation within the intima, which is the sum of cell migration, cell proliferation and apoptotic loss of VSMCs.21–23 We next examined the cell proliferation and apoptosis in balloon-injured arteries using immunostaining for proliferating-cell nuclear antigen (PCNA) and TUNEL staining, respectively (Online Figure IX). In Ad-sh-control–infected arteries, the PCNA labeling index in the neointima reached 40% by day 10. Infection with Ad-sh-Girdin significantly reduced the PCNA labeling indices in the neointima, compared with Ad-sh-control–infected arteries (Figure 6A). The knockdown of Girdin had no apparent effect on PCNA labeling indices in the media (Figure 6B). On the other hand, cell survival in neither the neointima nor media was affected in Ad-sh-Girdin-infected arteries at any time point (Figure 6C and 6D). Collectively, these results suggest the involvement of Girdin in cell proliferation, but not in cell survival, after vascular injury in vivo.

Akt-Mediated Phosphorylation of Girdin at Serine 1416 Is Required for Neointima Formation After Vascular Injury

Finally, we examined the role of the phosphorylation of Girdin in neointima formation by using a mouse femoral artery wire injury model. We previously reported that serine 1416 of Girdin is phosphorylated by Akt, which is required for cell migration induced by growth factors.12 In this study, to examine the role of Girdin phosphorylation in vivo, we generated Girdin S1416A knock-in mice, in which serine 1416 was replaced with alanine by conventional gene targeting (Online Figure X, A). A Western blot analysis using an anti–phosphorylated Girdin antibody revealed that the phosphorylation of Girdin was not observed in the tissues from homozygous mice (Online Figure X, B). These mice survived embryogenesis and did not show apparent gross abnormalities from birth through three months of age, thus suggesting that Girdin phosphorylation may be dispensable for embryonic and postnatal development.

To evaluate the involvement of Girdin phosphorylation in vascular remodeling in adults, P56 mice homozygous for the S1416A mutation and their wild-type littermates (term Girdin<sup>SA/SA</sup> and Girdin<sup>WT/WT</sup>, respectively) were subjected to femoral artery wire injury. In immunohistochemical studies using the injured arteries, the phosphorylation of Girdin could not be detected in the neointima of Girdin<sup>SA/SA</sup> mice (Online
Figure X, C). Injured femoral arteries from Girdin<sup>SA/SA</sup> mice showed less intimal area compared with those of Girdin<sup>WT/WT</sup> mice 21 days after wire injury (Figure 7A). A histological analysis revealed that the intima/media area ratio and intimal area of arteries from Girdin<sup>SA/SA</sup> mice were significantly reduced as compared to those of Girdin<sup>WT/WT</sup> mice (Figure 7B and 7C). The external elastic lamina area and medial area did not differ between the two groups (Online Figure XI). These findings indicate that neointima formation requires not only Girdin expression, but also its phosphorylation at serine 1416. Additionally, the PCNA index in neointima in Girdin<sup>SA/SA</sup> mice was significantly lower than in Girdin<sup>WT/WT</sup> mice (Figure 7D).

When VSMCs were isolated from Girdin<sup>WT/WT</sup> and Girdin<sup>SA/SA</sup> mice, immunocytochemistry revealed that most of the isolated cells were positive for α-SMA, and more multinucleated cells were observed in the VSMCs isolated from Girdin<sup>SA/SA</sup> mice compared with those isolated from the Girdin<sup>WT/WT</sup> mice (Online Figure XII, A and B). We then examined the proliferation and motility of these cells. As a consequence, the proliferation and motility of Girdin<sup>SA/SA</sup> group were significantly impaired compared with the Girdin<sup>WT/WT</sup> group (Online Figure XII, C and D). These data suggest that the attenuated neointima formation observed in Girdin<sup>SA/SA</sup> mice can be attributed to defects in the migration and proliferation of VSMCs.

Collectively, our results suggest that the Akt/Girdin signaling pathway plays a crucial role in neointima formation after vascular injury.

**Discussion**

In the present study, we demonstrated that Girdin regulates the remodeling of the actin cytoskeleton, migration and proliferation of hVSMCs. Transduction of shRNA directed against Girdin into the rat artery suppressed neointima formation after balloon injury. This was accompanied by decreased cell migration and proliferation of VSMCs. In addition, the neointima formation after wire injury was significantly suppressed in Girdin<sup>SA/SA</sup> mice, indicating that Akt-mediated phosphorylation is essential for vascular remodeling (Figure 8).

The PI3K-Akt signaling pathway has essential roles in neointima formation after vascular injury by regulating VSMC migration, proliferation and survival. Previous studies have demonstrated that administration of wortmannin, a PI3K inhibitor, decreased VSMC replication in the tunica media, whereas transfection of a dominant-negative Akt mutant attenuated neointima formation by inhibiting cell proliferation. In accord with this, several Akt substrates have been reported to have pivotal roles in neointima formation after vascular injury. However, despite intensive research...
Girdin is phosphorylated in hVSMCs, as well as LY294002, and siRNA-mediated Akt knockdown effectively attenuated Girdin phosphorylation in VSMCs. We previously reported that Akt phosphorylation is attenuated by PQK, consistent with our previous report in endothelial cells, though other groups have reported that Akt phosphorylation is attenuated by depletion of Girdin. One way to explain this discrepancy is that the process may be dependent on the cell type used. The other authors obtained their results using several types of immortalized cell lines, whereas we used primary cells. Therefore, it may be plausible that the involvement of...
Girdin in Akt phosphorylation depends on the cell type. Further studies are needed to understand the synergy between Girdin and Akt.

Our data demonstrated that depletion of Girdin abrogated neointima formation even at an early stage after BI, when cell replication was maintained at a lower level. Because the intimal VSMCs at early stages are considered to be derived mainly from medial VSMC migration into the intima, it seems plausible that the attenuation of neointima formation by Girdin knockdown would be due, at least in part, to decreased early VSMC migration. In support of this possibility, we demonstrated an attenuated cell migration in Girdin-depleted hVSMCs and VSMCs isolated from GirdinSA/SA mice.

Interestingly, our results showed that Girdin is involved in VSMC proliferation, both in vitro and in vivo. Our previous reports showed that Girdin had little effect on the proliferation of endothelial cells and breast cancer cells, whereas Anai et al showed attenuated DNA synthesis in Girdin-depleted HepG2 cells. We found that Girdin knockdown or its S1416A mutation decreased the number of PCNA-positive cells in the neointima, thus supporting their data, and demonstrating for the first time that Girdin is involved in cell proliferation in vivo. Our results suggested that the impaired proliferation of VSMCs is due, at least in part, to cell cycle delay caused by the defect in cytokinesis. It remains unknown, however, why the effect of Girdin on proliferation depends on cell types. This issue should therefore be determined in future studies.

Reendothelialization is an important aspect of the response to vascular injury. It is traditionally believed that endothelial cells migrate and proliferate from intact
neighboring arterial segments, resulting in the reendothelialization of the injured segment. Although we previously reported that the depletion of Girdin prevents the migration of cultured human umbilical vein endothelial cells, the results of the present study demonstrated that the depletion of Girdin did not affect the reendothelialization in a balloon-injured rat carotid. Considering that the expression of Girdin is not observed in endothelial cells in arteries with thick smooth muscle layers, the migration of endothelial cells may be independent of Girdin during the process of reendothelialization. In fact, we observed the Girdin and α-SMA negative cells lining the vessel lumen. Alternatively, in light of these data and our previous findings, it is likely that the expression of Girdin is spatially and temporally regulated in the endothelial cells of rat carotid arteries. Therefore, another possibility is that the Ad-sh-Girdin infection was limited to the injured segment, which would still allow the noninfected endothelial cells to express Girdin protein transiently during their migration from an adjacent intact area, leading to successful reendothelialization.

In conclusion, Girdin is essential for the rearrangement of the actin cytoskeleton, as well as for the migration and proliferation of hVSMCs, which is vital for neointima formation after vascular injury. Our findings support the notion that the Akt/Girdin signaling pathway functions as a crucial regulator of vascular remodeling. Therefore, Girdin may be an important therapeutic target for vascular proliferative diseases such as restenosis and atherosclerosis.

Sources of Funding
This work was supported by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to K.M.) and a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to M.T.).

Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**
- Akt signaling pathway is known to have a pivotal role in neointima formation after vascular injury.
- The actin-binding protein Girdin is a novel Akt substrate that regulates cell motility.

**What New Information Does This Article Contribute?**
- Girdin is essential for motility and proliferation of vascular smooth muscle cells (VSMCs).
- Girdin expression and phosphorylation are required for neointima formation after vascular injury.
- The Akt/Girdin signaling pathway is crucial for vascular remodeling process.

The Akt signaling pathway plays a pivotal role in neointima formation after vascular injury. However, the mechanism by which Akt regulates VSMC motility is poorly understood. Girdin, an actin-binding protein, is a novel Akt substrate that regulates cell motility. In this study, we investigated whether Girdin is involved in neointima formation. We found that Girdin is essential for the migration and proliferation of VSMCs. We also demonstrate that Girdin knockdown attenuates neointima formation after vascular injury without affecting re-endothelialization and cell viability. Furthermore, our results with a knock-in transgenic mouse model show that Akt-mediated phosphorylation of Girdin is important for neointima formation in adulthood, but not for embryonic vasculogenesis. Our data indicate that the Akt/Girdin signaling pathway is crucial for neointima formation after vascular injury, and that Girdin may be an important therapeutic target for vascular proliferative diseases such as restenosis and atherosclerosis.
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_Circ Res._ 2011;108:1170-1179; originally published online March 17, 2011;
doi: 10.1161/CIRCRESAHA.110.236174

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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SUPPLEMENTAL MATERIAL

Detailed Methods

Cell culture and treatments
hVSMCs were purchased from Kurabo (Osaka, Japan) and cultured with Hu-Media SG2 (Kurabo) containing 5% fetal bovine serum (FBS), antibiotics (50μg/mL gentamicin, 50ng/mL amphotericin B), human basic fibroblast growth factor (2ng/mL), human epidermal growth factor (0.5ng/mL) and insulin (5μg/mL). hVSMCs at passage 4 to 7 were grown to 70% to 80% confluence and used in the experiments. In Boyden chamber assays and scratch wound assays, cells were made quiescent by culturing them in 1% FBS media (Hu-Media SD2, Kurabo) containing antibiotics (50μg/mL gentamicin, 50ng/mL amphotericin B) and heparin (30μg/mL) for 48 hours before experiments. H9c2 cells were purchased from the ATCC (Manassas, VA, USA) and cultured with Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) containing 10% FBS.

Antibodies
The anti-Girdin and anti-phospho Girdin (Ser1416) polyclonal antibodies were previously described1-2. The rabbit anti-Girdin polyclonal antibody was developed against the 19 carboxyl-terminal amino acids of Girdin and affinity–purified with the immunized peptide. The rabbit anti-P-Girdin antibody was raised by immunizing rabbits with a keyhole-limpet hemocyanin-conjugated phospho-peptide corresponding to amino acids 1408–1420 (CDINRERQkpSLTLT) of Girdin. Antiserum was purified as a bound fraction of the phosphopeptide-conjugated column. Other antibodies used in this study include the anti-Akt polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA), anti-phospho Akt (Ser473) polyclonal antibody (Cell Signaling Technology), anti-GSK-3 monoclonal antibody (Upstate, Lake Placid, NY, USA), anti-phospho GSK-3 polyclonal antibody (Cell Signaling Technology), anti-phospho MYPT1 (Thr850) polyclonal antibody (Millipore, Billerica, MA, USA), anti- ERK polyclonal antibody (Cell Signaling Technology), anti-phospho ERK polyclonal antibody (Cell Signaling Technology), anti-α smooth muscle actin (α-SMA) monoclonal antibody (Sigma), anti-β-actin monoclonal antibody (Sigma), anti-PCNA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GFP polyclonal antibody (MBL, Nagoya, Japan). The polyclonal anti-MYPT1 antibody was generated as previously described3.

Immunoprecipitation and Western blot analyses
hVSMCs were lysed in buffer containing 1% NP-40, 20mmol/L Tris-HCl (pH 7.4), 150mmol/L
NaCl, 1mmol/L ethylenediaminetetraacetic acid, 0.5mmol/L sodium orthovanadate, 20mmol/L β-glycerophosphate, and 1mmol/L phenylmethylsulfonyl fluoride supplemented with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA) and Phosphatase Inhibitor Cocktail 1 (Sigma). Protein concentrations were determined utilizing the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Lysate samples from hVSMCs were incubated for 16 hours at 4°C with 10μg of anti-Girdin antibody bound to 30μL of immobilized protein A beads (Sigma). Beads were washed three times with cold lysis buffer and suspended in Laemmli sodium dodecyl sulfate (SDS) sample dilution buffer [125mmol/L Tris-HCl(pH 6.8), 2% SDS, 10μg/mL bromophenol blue, 80mmol/L dithiothreitol]. For the Western blot analyses, the samples were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes, blocked in 3% egg albumin in phosphate-buffered saline (PBS) and 0.05% Tween 20, incubated with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark). The signal was detected using ECL Western Blotting Detection Reagents (Amersham, Little Chalfont, UK). For quantification, the images were analyzed using the NIH Image software.

RNA interference
The siRNA-mediated knockdown of Girdin was performed as previously described1-2. The targeted sequence was as follows (only the sense sequence is shown): 5'-AAGAAGGCTTAGGCAGGAATT-3' (nucleotides 780–800). As for the knockdown of Akt, we employed an siRNA targeting both Akt1 and Akt2. The targeted sequence was as follows: 5'-CCTGCCCTTCTACAACCAGGA-3'. The 21 nucleotide synthetic duplexes were prepared by Qiagen (Valencia, CA, USA). The hVSMCs were transfected with the siRNA or a 21 nucleotide irrelevant RNA (Qiagen) as a control, by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

For shRNA-mediated knockdown of Girdin, an adenovirus harboring a shRNA for Girdin was generated as previously described2. A set of single-stranded oligonucleotides encoding the Girdin target and its complement was synthesized as follows (only the sense sequence is shown): 5’-GAAGGAGAGGCAACTGGAT-3’ (nucleotides 4166–4184). The oligonucleotides, directed against sequences common to both human and rat Girdin RNAs, were selected and provided by Dragon Genomics (Takara, Yokkaichi, Japan). The shRNA adenoviral vector was transfected into 293 A cells using Lipofectamine 2000. Approximately 12 days after transfection, the adenovirus-containing 293A cells were harvested and lysed to prepare a crude viral stock. The resultant viral stock was amplified and subsequently purified with the ViraBind Adenovirus Purification Kit (Cell Biolabs, San Diego, CA, USA). The titer of adenovirus was determined by limiting dilution assay using the QuickTiter Adenovirus Titer ELISA Kit (Cell Biolabs).
Immunofluorescence staining

Immunofluorescence staining was performed as previously described\(^1\). In brief, hVSMCs were plated on fibronectin (1mg/cm\(^2\); BD Biosciences, Sunnyvale, CA, USA)-coated culture slides (BD Biosciences). To observe stress fibers and lamellipodia, hVSMCs were treated with DMEM containing 3% bovine serum albumin for 24 hours, followed by stimulation with DMEM containing 10% FBS or PDGF (20ng/mL), respectively. Next, the cells were fixed with methanol and 4% paraformaldehyde, blocked with goat serum and stained with an anti-\(\beta\)-actin antibody (1:500) overnight at 4\(^\circ\)C, followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1:500, Invitrogen, Carlsbad, CA, USA) for 1 hour. Fluorescence was examined by using confocal laser-scanning microscopy (Fluoview FV500, Olympus, Tokyo, Japan).

In rat carotid arteries, transverse sections (5\(\mu\)m) were blocked with goat serum for 1 hour and subsequently incubated with a mouse monoclonal anti-\(\alpha\)-SMA antibody and rabbit polyclonal anti-Girdin antibody overnight at 4\(^\circ\)C. Thereafter, the sections were incubated with goat anti-mouse IgG antibody conjugated with Alexa Fluor 594 (Invitrogen) and goat anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (Invitrogen) for 1 hour. Hoechst 33258 (Invitrogen) was used for nuclear counterstaining.

Cell migration assays

Directional cell migration of hVSMCs was stimulated in a monolayer using an in vitro scratch wound assay as previously described\(^2\). The hVSMCs were seeded on fibronectin-coated 35mm glass bottom dishes and transfected with either control or Girdin siRNA. The confluent cells were scratched with a 200-\(\mu\)L disposable plastic pipette tip and allowed to migrate toward the wound. Wounded cells were incubated with PDBF-BB (10ng/mL, Sigma) and 0.1% FBS in DMEM (Sigma) at 37\(^\circ\)C and digital images were taken with the microscope (BZ-8100, Keyence, Osaka, Japan).

The Boyden chamber assay was performed as previously described\(^4\). Chemotaxis of hVSMCs was assessed using 48-well modified Boyden chambers (Neuro Probe, Gaithersburg, MD, USA) and polycarbonate membranes (8 \(\mu\)m pores, Neuro probe) coated with fibronectin. hVSMCs (2.0\(\times\)10\(^4\) cells/well) were added to the upper chamber, and DMEM supplemented with PDGF-BB (10ng/mL) was added to the lower chamber. Cells were allowed to migrate through the pores of the membrane for 6 hours at 37\(^\circ\)C. After the removal of cells on the upper surface of the membrane by gentle scraping, the cells on the lower surface of the membrane were fixed with methanol and subsequently were subjected to May-Grunwald-Giemsa staining. The cells were counted using light microscopy (IX-71, Olympus). The experiments were performed at least in quadruplicate in each group.
Adhesion assay

hVSMCs transfected with either the control or Girdin siRNA were detached by trypsin digestion, after which the cells were harvested and resuspended in culture medium. After gentle mixing, the cells were seeded onto fibronectin-coated plates for 1 hour. Nonadherent cells were removed by gentle washing. Cells were fixed and stained with an anti-β-actin antibody, followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody. Hoechst 33258 was used for nuclear counterstaining.

MTS assays

Mitogenic activity was measured by reduction of MTS (Cell Titer96, Promega, Madison, WI), as previously described\(^2\). The hVSMCs (2.0×10\(^4\) cells/well) transfected with either control or Girdin siRNA were plated in 24-well plates and allowed to proliferate for 1, 3 and 5 days with Hu-Media SG2, followed by incubation with MTS reagent for 2 hours. Thereafter, aliquots were pipetted into 96-well plates. The absorbance of each well was measured at 490nm using a microplate reader (Model 680, Bio Rad, Hercules, CA, USA). The absorbance of control siRNA-transfected cells at day 1 was defined as 1. The experiments were performed at least in quadruplicate in each group.

Plasma membrane staining

To count the number of multinucleated cells, the plasma membranes of hVSMCs were stained with CellMask Orange Plasma Membrane Stain (Invitrogen) according to the manufacturer’s protocol. Cell nuclei were labeled with Hoechst 33258.

Flow cytometric analysis

To assess cells for the presence of apoptosis, flow cytometry was performed using FACSCalibur (BD Bioscience) as described previously\(^5\). hVSMCs transfected with either control or Girdin siRNA were treated with DMEM containing 3% bovine serum albumin for 48 hours or hydrogen peroxide (500\(\mu\)mol/L) for 3 hours. The cells were incubated for 15 minutes with Annexin V-Fluos (Roche) and PI (Sigma) labeling solution according to the manufacturer’s protocol. Simultaneous dual parameter analysis of annexin V and PI was used to estimate the percentage of apoptotic cells, defined as annexin V-positive and PI-negative. Dead cells were defined as annexin V-positive.

Rat balloon injury model and infection with adenovirus

Male Sprague-Dawley rats weighing 400 to 450g were obtained from SLC (Nagoya, Japan), and animal protocols were approved by the Animal Care and Use Committee of Nagoya University
Graduate School of Medicine. Balloon injury of the right carotid artery was performed as described previously. Rats were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg). Rat carotid arteries were denuded of the endothelium with a 2F Fogarty balloon embolectomy catheter (Edwards Lifescience, Irvine, CA, USA) which was introduced into the right common carotid artery through the external carotid artery. The balloon was inflated, and the catheter was retracted. This was repeated three times, with the catheter turned 120° each time while it was retracted. Immediately after balloon injury, gene transfer into the injured arterial wall was performed with the adenoviral vectors (1.0 × 10⁹ plaque-forming units/mL, 100 µL) by introducing viral solution into the lumen and incubating it for 20 minutes without blood flow. Then the viral solution was removed, and blood flow was restored. Five rats were studied in each group for each time point.

**Generation of Girdin S1416A knock-in mice**

Conventional gene-targeting techniques were used to generate Girdin S1416A knock-in mice as depicted in Online Figure V, A. The 5' 2.4 kb pairs and 3' 6.3 kb fragments of the mouse girdin gene (ccdc88a), which contains exons 24 and 25 (5' arm) and 26 - 28 (3' arm), respectively, were isolated by polymerase chain reaction (PCR) and cloned into the target vector. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) to introduce the S1416A mutation. The final targeting construct contains the S1416A mutation-carrying exon 25, with a LoxP-flanked PGK-neo resistance gene, which were cloned downstream of exon 25. The diphtheria toxin-A (DTa) chain gene was cloned adjacent to the 5'-flanking homologous arm for negative selection against random insertion. The linearized targeting vector was electroporated into CSL3 embryonic stem cells (a gift from Dr. Chyuan-Sheng Lin), and homologous recombinants were selected by growth in G418. Targeted embryonic stem cell clones identified by a Southern blot analysis using an external PCR probe were injected into C57BL/6 blastocysts to generate chimeric mice. Chimeras that were consistently germline transmitting were mated with β-actin promoter/Cre transgenic mice to excise the neo resistance gene and evaluate the possible presence of interference. The presence of the mutations was verified by genomic DNA sequencing. Heterozygous 129Sv background mice were crossed with each other to generate homozygous mice, or were mated with C57BL/6 mice to generate mice on a C57BL/6 genetic background. We confirmed that the phenotypes of the Girdin mutant mice do not depend on the genetic backgrounds (data not shown). All animal protocols were approved by the Animal Care and Use Committee of Nagoya University Graduate School of Medicine.

**Mouse wire injury model**
The mouse wire injury was performed in Girdin$^{WT/WT}$ or Girdin$^{SA/SA}$ mice by inserting a straight spring wire (0.38mm in diameter, No.C-SF-15-15, Cook, Bloomington, IN, USA) in the left femoral artery at the age of 8 weeks as described previously$^7$. The wire was left in place for 1 minute to denude and dilate the artery (Girdin$^{WT/WT}$, n=6, Girdin$^{SA/SA}$, n=3).

**Histological assessment**
After the rats and mice were sacrificed using an overdose of sodium pentobarbital and perfusion-fixed at 100mmHg with 4% paraformaldehyde, the arteries were excised and embedded in paraffin. Sections (5μm) from the middle of segments were stained with hematoxylin-eosin. The cross sectional area of the blood vessel layers, including the intimal and medial areas, were measured with a computerized digital image analysis system (BZ-8100, Keyence) and averaged in 3 independent sections.

**Immunohistochemistry**
Immunohistochemistry was performed as described previously$^8$. Transverse sections (5μm) of the arteries were incubated with the indicated antibodies overnight at 4°C. After they were washed with PBS, the sections were incubated with horseradish peroxidase-conjugated secondary antibodies for 30 minutes. Staining was visualized with 3, 3’-diaminobenzidine and hematoxylin was used as a counterstain.

**Analysis of reendothelialization**
An analysis of reendothelialization in injured arteries was performed as described previously$^6$. A planimetric analysis of reendothelialization after balloon injury was performed with a computerized digital image analysis system (AxioVision, Carl Zeiss, Munich, Germany). For this procedure, 30 minutes before rats were euthanized, they received an intravenous injection of 0.5% Evans blue dye (20mg/kg, Sigma) to identify areas of non-endothelialized artery with a blue stain. After perfusion-fixation with 100% methanol, the injured arteries were excised, incised longitudinally, and then photographed with a microscope (SteREO Lumar. V12, Carl Zeiss). The initially injured area was defined as the total surface of the harvested arterial segment. The total harvested segment corresponded to the total length of the injured segment; in each case, this length was similarly defined proximally by the carotid bifurcation and distally by the edge of the omohyoid muscle. The reendothelialized area was defined as the area not stained with Evans blue dye. The extent of reendothelialization (% reendothelialization) was expressed as a percentage of the initially injured area.

**Detection of cell proliferation and apoptosis in vivo**
PCNA-positive or TUNEL-positive nuclei were evaluated as previously described\(^9\). To detect proliferation, transverse sections (5\(\mu\)m) of the rat carotid arteries and mouse femoral arteries were blocked with PBS containing 10% goat serum for 1 hour and subsequently incubated with a mouse monoclonal anti-PCNA antibody (1:50) overnight at 4\(^\circ\)C. Thereafter, the sections were incubated with goat anti-mouse IgG antibody conjugated with Alexa Fluor 488 (1:500, Invitrogen) for 1 hour. To detect apoptosis, we used MEBSTAIN Apoptosis Kit Direct (MBL, Nagoya, Japan) according to manufacturer’s protocol. In each experiment, TO-PRO-3 (Invitrogen) was used for nuclear counterstaining. PCNA-positive and TUNEL-positive nuclei were counted using a fluorescence microscopy (Axio Observer. Z1, Carl Zeiss). The quantitative analysis was performed in 3 independent sections for each rat (n=5). The numbers of PCNA-positive and TUNEL-positive cells were expressed as PCNA and TUNEL indices (PCNA-positive and TUNEL-positive cells/total cells \times 100), respectively.

**Isolation of VSMCs from murine aortas**
Smooth muscle cells were isolated from mice as previously described\(^{10}\) with minor modifications. In brief, thoracic aortas were removed from Girdin\(^{WT/WT}\) or Girdin\(^{SA/SA}\) mice at 10-12 weeks of age. After incubation in Hanks' balanced salt solutions (HBSS, Invitrogen) containing 0.15% collagenase type II (Worthington, Lakewood, NJ, USA) and 0.025% elastase (Sigma) for 10 min at 37 \(^\circ\)C, the adventitia and endothelial cells were carefully removed. After additional enzymatic digestion for 2 hours, cells were collected by centrifugation and cultured in DMEM containing 10% FBS. The VSMCs at passage 3 to 5 were grown to 70% to 80% confluence and used in the experiments.

**Statistical analysis**
Data are presented as the means \pm standard error of the mean (SEM). A statistical analysis was performed using Student's \(t\)-test. A value of \(P<0.05\) was considered to be significant.
Supplemental References


Online Figure I. Girdin is phosphorylated at serine 1416 in hVSMCs following PDGF and Angiotensin II stimulation. A and B, hVSMCs were stimulated with PDGF-BB (10ng/mL) (A) or Angiotensin II (100nmol/L) (B) for the indicated times. Immunoprecipitates with the anti-Girdin antibody were subjected to the Western blot analyses using the indicated antibodies. C, The hVSMCs were treated with or without the PI3K inhibitor, LY294002 (50µmol/L), and then stimulated with PDGF-BB (20ng/mL) for 10 minutes. Immunoprecipitates with the anti-Girdin antibody were then subjected to the Western blot analyses using the indicated antibodies. D, The hVSMCs transfected with either control or Akt siRNA were stimulated with PDGF-BB (20ng/mL) for 10 minutes. Immunoprecipitates with the anti-Girdin antibody were subjected to the Western blot analyses using the indicated antibodies.
Online Figure II. Depletion of Girdin has little effect on the activity of Akt and Rho-kinase. A and B, The hVSMCs transfected with either control or Girdin siRNA were stimulated with PDGF-BB (20ng/mL) (A) or lysophosphatidic acid (LPA, 10µmol/L) (B) for the indicated times. Total cell lysates were subjected to the Western blot analyses using the indicated antibodies.
Online Figure III. Girdin colocalizes with the actin structures. 

A, The hVSMCs were stimulated with serum to induce stress fiber formation. Cells were stained with an anti-Girdin antibody and an anti-β-actin antibody. Note that Girdin localizes on the actin stress fibers. Bar, 20 µm.

B, The hVSMCs were stimulated with PDGF-BB (20 ng/mL) for 10 min and stained with the anti-Girdin antibody and the anti-β-actin antibody. The arrows denote lamellipodia at the leading edge. Note that Girdin localizes at the leading edge of hVSMCs. Bar, 20 µm.
Online Figure IV. Girdin has little effect on cell-extracellular matrix adhesion. A, The hVSMCs transfected with either the control or Girdin siRNA were seeded onto fibronectin-coated plates for 1 hour. Cells were then stained with an anti-β-actin antibody. Cell nuclei were labeled with Hoechst 33258. Bar, 50 µm. B, The number of hVSMCs per field is shown. The number of cells per field in the control siRNA-transfected cells was defined as 1. C, The surface area of cells with a spread-out morphology was quantified. N.S. not significant.
Online Figure V. Girdin knockdown increases the number of multinucleated cells. A, The plasma membrane of hVSMCs transfected with either the control or Girdin siRNA were stained with CellMask Orange Plasma Membrane Stain (Invitrogen) at 3 and 5 days after siRNA transfection. Cell nuclei were labeled with Hoechst 33258. The arrow denotes a multinucleated cell. Bar, 50 µm. B, Quantification of the percentage of multinucleated cells in total hVSMCs. The results are presented as the means ± SEM of three independent experiments. The asterisk indicates statistical significance (P<0.05).
Online Figure VI. Girdin localizes in \(\alpha\)-SMA-positive cells in the neointima. A and B, Rat carotid arteries at 7 (A) or 14 (B) days after BI were subjected to immunofluorescence staining using the anti-Girdin antibody (green) and anti-\(\alpha\)-SMA antibody (red). Cell nuclei were labeled with Hoechst 33258. The boxes are magnified in the lower panels. The arrows denote both \(\alpha\)-SMA and Girdin-negative cells.
Online Figure VII. Construction of an adenovirus harboring a shRNA directed against Girdin. A, The results of a Western blot analysis showing the effective knockdown of Girdin in the rat embryonic heart-derived cardiomyoblast cell line, H9c2, which was transduced with Ad-sh-Girdin. B, Representative immunohistostaining for green fluorescent protein (GFP) showing the expression of enhanced GFP (EGFP) in rat carotid arteries 4 days after BI with or without transduction of the adenoviral vector encoding EGFP (Ad-EGFP). Bar, 50 µm. We administered $1.0 \times 10^8$ plaque-forming units per 100 µL of Ad-EGFP, and successful gene transfer of EGFP was observed in the media of rat carotid arteries. Therefore, we used this dose of adenovirus for shRNA gene transfer in the present study. C, The Western blot analyses were performed to examine the efficacy of Ad-sh-Girdin in rat whole carotid arteries after BI and infection with adenovirus.
Online Figure VIII. Girdin is essential for neointima formation after balloon injury in vivo. A through D, Quantification of the intimal area (A), medial area (B), lumen area (C) and EEL area (D) at days 7, 10 and 14 after rat carotid artery balloon injury (n = 5 for each group). The asterisks indicate statistical significance (P<0.05). N.S., not significant.
Online Figure IX. Girdin affects cell proliferation, but not cell survival, in injured arteries. A, Carotid arteries were subjected to immunofluorescence staining using an anti-PCNA antibody (green) 10 days after BI. B, Carotid arteries were subjected to TUNEL staining (green) 10 days after BI. N, neointima. M, media. DIC, differential interference contrast. Bar, 50 µm.
Online Figure X. Generation of Girdin S1416A mutant knock-in mice. A, A schematic diagram of the targeted S1416 allele. The girdin gene consists of 33 coding exons, and exons 23 - 29 are shown here. Homologous recombination of the gene-targeting vector at the girdin locus (wild-type allele; top) was designed to insert a PGK-neo cassette into intron 25 and introduce the S1416A mutation that is encoded by exon 25. The structure of the targeted girdin allele (bottom) is shown. The position of the PCR-amplified genomic DNA probe used to screen embryonic stem cell colonies by a Southern blotting analysis is shown in the red box. Restriction enzyme sites for the Southern blotting analysis and predicted sizes of bands are also shown. As the phenotypes of the knock-in mutant mice with or without the neo gene were identical, all data from mutant mice in this paper were prepared in the mutant mice without the neo gene. SA, S1416A. DTa, diphtheria toxin-A. B, The Western blot analyses of lysates extracted from the brains of wild-type, heterozygous and homozygous mice (denoted Wt, He and Ho respectively). C, Murine femoral arteries were injured with a straight spring wire. The mice were sacrificed at 21 days after wire injury. Their femoral arteries were subjected to immunohistochemical analysis using the phospho-Girdin (Ser1416) antibody. Bar, 50µm.
Online Figure XI. Akt-mediated phosphorylation of Girdin at serine 1416 is required for neointima formation after vascular injury. A through C, Quantification of the medial area (A), lumen area (B) and EEL area (C) at day 21 after femoral artery wire injury in Girdin$^{SA/SA}$ mice (n=3) and Girdin$^{WT/WT}$ littermates (n=6). N.S., not significant.
Online Figure XII. Phosphorylation of Girdin at serine 1416 has an important role in VSMC migration and proliferation. 

A, VSMCs isolated from Girdin\textsuperscript{WT/WT} or Girdin\textsuperscript{SA/SA} mice were seeded onto fibronectin-coated plates. Three days after, cells were stained with an anti-α-SMA antibody. Cell nuclei were labeled with Hoechst 33258. The arrows denote multinucleated cells. Bar, 100\,\mu m.

B, Quantification of the percentage of multinucleated cells in total VSMCs.

C, The cell proliferation assay as determined using the MTS colorimetric assay. The absorbance of Girdin\textsuperscript{WT/WT} mouse-derived VSMCs at day 1 was defined as 1. OD, optical density.

D, The Boyden chamber assay was performed, and the number of migrated cells is shown. The number of Girdin\textsuperscript{WT/WT} mouse-derived VSMCs that had migrated per field in the absence of PDGF-BB was defined as 1. The asterisks indicate statistical significance (P<0.05).