Polymerase δ-Interacting Protein 2 Promotes Postischemic Neovascularization of the Mouse Hindlimb


Objective—Collateral vessel formation can functionally compensate for obstrusive vascular lesions in patients with atherosclerosis. Neovascularization processes are triggered by fluid shear stress, hypoxia, growth factors, chemokines, proteases, and inflammation, as well as reactive oxygen species, in response to ischemia. Polymerase δ-interacting protein 2 (Poldip2) is a multifunctional protein that regulates focal adhesion turnover and vascular smooth muscle cell migration and modifies extracellular matrix composition. We, therefore, tested the hypothesis that loss of Poldip2 impairs collateral formation.

Approach and Results—The mouse hindlimb ischemia model has been used to understand mechanisms involved in postnatal blood vessel formation. Poldip2+/− mice were subjected to femoral artery excision, and functional and morphological analysis of blood vessel formation was performed after injury. Heterozygous deletion of Poldip2 decreased the blood flow recovery and spontaneous running activity at 21 days after injury. H2O2 production, as well as the activity of matrix metalloproteinases-2 and -9, was reduced in these animals compared with Poldip2+/+ mice. Infiltration of macrophages in the peri-injury muscle was also decreased; however, macrophage phenotype was similar between genotypes. In addition, the formation of capillaries and arterioles was impaired, as was angiogenesis, in agreement with a decrease in proliferation observed in endothelial cells treated with small interfering RNA against Poldip2. Finally, regression of newly formed vessels and apoptosis was more pronounced in Poldip2+/− mice.

Conclusions—Together, these results suggest that Poldip2 promotes ischemia-induced collateral vessel formation via multiple mechanisms that likely involve reactive oxygen species–dependent activation of matrix metalloproteinase activity, as well as enhanced vascular cell growth and survival. (Arterioscler Thromb Vasc Biol. 2014;34:1548-1555.)

Key Words: apoptosis ■ ischemia ■ metalloproteases ■ NADPH oxidase ■ neovascularization

Postnatal vascularization is an inherent and endogenous compensatory mechanism to restore obstructive vascular lesions. Patients with peripheral arterial obstructive disease, mainly caused by atherosclerosis, are at high risk for cardiovascular morbidity and mortality. Promoting vascular regrowth as an adaptive response to limb ischemia to treat peripheral arterial obstructive disease is a major challenge in cardiovascular research. Revascularization occurs via 3 mechanisms: arteriogenesis, angiogenesis, and vasculogenesis.2-6 These processes are triggered by shear stress and hypoxia and occur simultaneously at different levels and involve not only endothelial cell activation and upregulation of cell adhesion molecules but also recruitment of inflammatory cells, expression of growth factors, activation of matrix metalloproteinases (MMPs), as well as proliferation, migration, and apoptosis of vascular cells. The events leading to revascularization are thus complex and multicellular but share some common cellular signaling pathways.

One such mechanism is the production of reactive oxygen species (ROS) and activation of their downstream targets. It has been shown that overexpression of catalase in vascular smooth muscle cells impairs blood flow recovery after femoral artery ligation,7 indicating that H2O2 is necessary for collateral formation. However, excess ROS impairs collateral growth in a model of repetitive ischemia in the heart, suggesting that there is an optimal level of ROS required for neovascularization.8 Several studies have suggested that ROS derived from NADPH (nicotinamide adenine dinucleotide phosphate) oxidases (Nox) are responsible for this requirement.9 Tojo et al10 and Urao et al11 showed that Nox2 in bone marrow–derived cells is essential for ischemia-induced neovascularization. However, others have found that the suppression of hindlimb perfusion in Rac2−/− (Ras-related C3 botulinum toxin substrate2) and Nox2−/− mice does not result from impaired collateral growth.12 Recently, several groups have suggested that the Nox4 homolog also plays a role in ischemia-induced...
neovascularization. Transgenic mice with endothelial-specific Nox4 overexpression showed accelerated recovery of blood flow after hindlimb ischemia and enhanced aortic capillary sprouting. Conversely, knockout of Nox4 led to attenuated angiogenesis in response to femoral artery ligation and after pressure overload–induced cardiac hypertrophy.

We previously demonstrated that polymerase δ-interacting protein 2 (Poldip2) increases Nox4 activity in vascular smooth muscle cells. The Nox4/Poldip2 complex activates RhoA (RAS homolog family member A) in vascular smooth muscle cells, leading to focal adhesion turnover and regulating migration. In a recent study, we showed that Poldip2 knockdown reduces H2O2 production in vivo, leading to increases in extracellular matrix deposition, greater vascular stiffness, and impaired agonist-mediated contraction. Thus, Poldip2 is necessary for vascular integrity and function. However, in addition to regulating Nox4, Poldip2 has several other reported functions, including roles in organizing the mitotic spindle, DNA repair, and cellular adhesion. Many of these processes can potentially contribute to collateralogenesis. Furthermore, Poldip2 has a variably excised N-terminal mitochondrial signal peptide and has been implicated in the mitochondrial fusion that occurs during cell cycle. In this regard, we recently found that loss of Poldip2 impairs cell cycle progression in mouse embryonic fibroblasts, most likely independent of Nox4. Thus, it seems that as a consequence of its multiple binding partners, Poldip2 potentially alters many cellular functions intrinsic to collateral formation. Based on these observations and our previous work implicating Poldip2 in vascular integrity, we hypothesized that loss of Poldip2 would impair collateral formation. Poldip2 heterozygous mice were subjected to femoral artery excision, and functional recovery was assessed by laser Doppler perfusion imaging of blood flow, running test, and histological analysis of blood vessel formation. We found that loss of Poldip2 does impair neovascularization, apparently via reduced endothelial proliferation, excessive regression of newly formed vessels, and inhibition of MMPs.

**Materials and Methods**

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

**Results**

**Blood Flow Recovery in Poldip2+/− Mice After Femoral Artery Ligation**

To examine the effect of heterozygous deletion of Poldip2 on blood flow recovery after hindlimb ischemia, Poldip2+/− and wild-type (WT) mice were submitted to femoral artery ligation and excision, causing hypoperfusion of the lower leg and foot. Functional blood flow recovery was assessed using laser Doppler perfusion imaging at 7-day intervals ≤3 weeks. Laser Doppler perfusion imaging revealed impaired perfusion recovery in Poldip2+/− mice, beginning at 14 days with significant impairment at 21 days after surgery (Figure 1).

**Morphological Assessment of Vascularity After Ischemia**

To determine whether impaired perfusion recovery results from a decrease in vessel number, we assessed capillary density using I-isolectin B4 staining in muscle immediately distal to the site the injury. As expected, WT animals showed an ≈50% increase in capillary density in the ischemic hindlimb at 7 and 14 days after surgery; however, capillary density did not increase in Poldip2+/− animals and was significantly less than that in WT animals at 7 and 14 days (Figure 2A). Capillary formation normalized by day 21 in both groups. The density of mature arterioles was assessed by staining smooth muscle α-actin (Figure 3A). Ischemic hindlimbs from WT mice had a 6-fold increase in α-actin staining compared with the nons ischemic leg. The response in Poldip2−/− mice was much less (1.9±0.4-fold). Of interest, Poldip2+/− animals showed a regression of the vasculature by 50±5% compared with WT at 21 days after ischemia, suggesting that Poldip2 may have dual roles in the formation of new collaterals and maintenance of the structure of new vascular networks. To obtain a more detailed morphological assessment of the collateral development after induction of hindlimb ischemia, legs of WT and Poldip2−/− mice were analyzed by micro–computed tomography 21 days after surgery. Three-dimensional histomorphometric analysis showed that the connectivity ratio of ischemic limbs to nons ischemic limbs was 2.3-fold higher in Poldip2−/− animals compared with WT animals (1.31±0.28 for WT and 3.07±0.10 for Poldip2−/−). No other differences were observed. Taken together, these results strongly suggest that Poldip2+/− mice have impaired neovascularization.

**In Vivo Assessment of Role of Poldip2 in Angiogenesis Processes**

The early decrease in capillary density in Poldip2−/− animals suggests an impairment of angiogenesis. To assess the role of Poldip2 in angiogenic processes in vivo, we used the small intestine submucosa (porcine small intestine submucosa) implant model of angiogenesis. As shown in Figure 2B, Poldip2+/− mice had 45±9% less endothelial invasion into the matrix compared with WT animals (1.31±0.28 for WT and 3.07±0.10 for Poldip2−/−). No other differences were observed.

**Role of Poldip2 in Human Umbilical Vein Endothelial Cell Proliferation**

During angiogenesis, endothelial cells are induced to proliferate and migrate out of an existing vessel to form new branches. To determine whether Poldip2 can affect proliferation of endothelial cells, human umbilical vein endothelial cells were transfected with siPoldip2 (small interfering RNA against Poldip2) and proliferation was assessed for 4 consecutive days (Figure 2C). Poldip2 protein expression was reduced after small interfering RNA transfection by 62±3% (Figure 1).
in the online-only Data Supplement). As shown in Figure 2C, the rate of proliferation was significantly decreased in cells with Poldip2 downregulation. However, vascular endothelial growth factor signaling and hypoxia-inducible factor 1α stabilization seem to be not affected by Poldip2 downregulation. No differences were detected between WT and Poldip2+/− mice in vascular endothelial growth factor receptor 2 phosphorylation or accumulation of hypoxia-inducible factor 1α in the ischemic muscle 7 days after surgery (data not shown), suggesting that Poldip2 directly affects endothelial progression through the cell cycle, as we have previously shown in mouse embryonic fibroblasts.

**Apoptosis in the Proximal Muscle of the Ischemic Limb**

The reduced density of arterioles observed in Poldip2+/− mice at 21 days, together with the impaired blood flow recovery, suggests that loss of Poldip2 might lead to inadequate regression of nonfunctional vessels. To test the role of Poldip2 in vessel regression, we measured vascular apoptosis in vivo using TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining. As shown in Figure 3B, apoptosis of vessels surrounding muscle fibers immediately distal to the site of injury was reduced by 82±22% in Poldip2+/− mice compared with WT mice 21 days after surgery. This result suggests that Poldip2 can affect vessel homeostasis.

**Inflammatory Response of Poldip2+/− Mice After Hindlimb Ischemia**

Infiltration of inflammatory cells is also an important early event in collateral vessel formation.

To determine whether inflammatory cell infiltration is impaired in Poldip2+/− mice, histological analysis of the ischemic limbs was performed. Immunostaining for MAC3 (macrophage 3 antigen), a macrophage marker, showed that Poldip2+/− mice had 40±10% less macrophages per section compared with WT mice 7 days after surgery in the proximal muscle of the ischemic limb (Figure 4A). Loss of Poldip2 does not seem to affect macrophage polarization, because WT and Poldip2+/− mice showed similar expression of both M1 and M2 markers (Figure 4B).

**H2O2 Production in the Proximal Muscle of the Ischemic Limb**

Because Poldip2 has been shown to regulate Nox4, which has been implicated in angiogenesis, we measured total H2O2 production in muscle immediately distal to the ligation (Figure 6A). In agreement with previous studies from our group, H2O2 production in Poldip2+/− mice was decreased 44±7% compared with WT mice in the proximal muscle at 21 days after surgery, consistent with reduced Nox4 activity.

**Spontaneous Running Activity of Poldip2+/− Mice After Hindlimb Ischemia**

Finally, to evaluate the extent to which impaired recovery of perfusion and vascular remodeling affected physiological function, we measured motor activity. WT and Poldip2+/− animals
were placed in a voluntary running wheel activity system at 7 and 21 days after surgery, and distance traveled was recorded for 7 days (Figure 6B). Under baseline conditions (day 0, not shown) and 7 days after surgery, WT and Poldip2−/− mice ran similar distances. However, by 21 days after surgery, a time when blood flow recovery and vessel formation are impaired, Poldip2−/− mice ran 25% less than WT mice. These data indicate that impaired neovascularization in Poldip2 mice affects the physiological function of the limb muscles.

Discussion

Neovascularization in response to ischemia is a key adaptive response to preserve functional integrity of tissues; however, therapy to improve vascularization remains elusive.1,27,28 In the present study, we report that Poldip2 downregulation impairs the revascularization process after ischemic insult in the adult mouse femoral artery ligation model. We noted only a partial recovery of perfusion, resulting from reduced capillary density and fewer small caliber vessels. These morphological changes resulted in impaired physiological function as assessed by voluntary running.

Collateral formation and remodeling are complex processes involving recruitment, migration, proliferation, and apoptosis of vascular cells.3–6 One of the most intriguing aspects of our results is the finding that Poldip2+/− mice exhibited impaired collateral formation at early time points and also an enhanced loss of collaterals at later time points. These findings suggest that both formation and regression of newly formed vessels are altered in these animals. The complexity of angiogenesis involves not only growth but also maturation and regression of the blood vessel network. Although regulated regression is an important aspect of neovascularization and formation of intact networks, the exact endogenous antiangiogenic factors and the mechanisms responsible for blood vessel regression after robust vessel sprouting are not well understood.29 In general, 1 week after injury, the density of blood vessels in the wound bed is >3× higher than that of the uninjured tissue. After a peak in vessel density, some newly sprouted vessels that have integrated in the existing perfused network undergo maturation. Vessels that are not perfused and functional are targeted for elimination. The most accepted mechanism for this process is apoptosis of endothelial cells. Analysis of apoptosis in the proximal muscle of the hindlimb ischemia revealed that Poldip2+/− mice had more vascular cell death compared with WT mice (Figure 3B). This vascular rarefaction in Poldip2+/− mice suggests that Poldip2 is required for cell survival, which is compatible with other prosurvival roles of Poldip2 reported in the literature.30 Impaired collateral formation and angiogenesis are also highly dependent on proliferation of both endothelial cells and smooth muscle cells. Depletion of Poldip2 had a profound inhibitory effect on endothelial cell proliferation (Figure 2C), consistent with other work in our laboratory showing that growth of vascular smooth muscle cells and mouse embryonic fibroblasts is also adversely affected by loss of Poldip2.25 This was reflected in impaired angiogenesis (Figure 2A). These results clearly show that Poldip2 has multiple roles in response to ischemia.

Extracellular matrix also has an important role in collateral formation. Matrix proteins provide not only a supportive...
scaffold for cells but also serve as crucial effectors of cellular function by sequestering and releasing growth factors and cytokines, including vascular endothelial growth factor-A, tumor necrosis factor-\(\alpha\), and interleukins. These vital proangiogenic factors both initiate and maintain vascular remodeling. However, degradation of extracellular matrix is required for cells to migrate and form new vessels. For these reasons, MMP activity is a major regulator of vasculogenesis. In the hindlimb ischemia model, MMP9 activity has been shown to increase in the gastrocnemius muscle tissue beginning at 3 days after injury and remains elevated until perfusion is restored. In the present study, we saw elevated but similar MMP activity in WT and Poldip2+/− mice 7 days after injury; however, at 21 days the gelatinase/collagenase activity, as well as the activity of MMP2 and MMP9, was decreased in Poldip2+/− compared with WT mice. This suggests that one mechanism for the impaired vasculogenesis seen in Poldip2+/− mice is a failure to adequately degrade extracellular matrix. This deficiency may also contribute to the increase in extracellular collagen that we previously reported in aortas from these animals.

An important source of MMPs is inflammatory cells such as macrophages and neutrophils that infiltrate vascular tissue. We found that macrophage infiltration in Poldip2+/− mice is diminished compared with that in WT mice at 7 days after surgery. Limited inflammation would result in compromised blood vessel growth, as we observed here, because it has been shown that inflammation is required for collateralogenesis. A reduction in inflammatory cells also means that MMP and growth factor release from the matrix is altered and may not be sufficient to sustain newly formed vessels. Such an explanation would be consistent with the enhanced regression of newly formed vasculature that we observed in Poldip2+/− mice. However, we observed no difference in MMP activity between WT and Poldip2+/− mice at 7 days after induction of ischemia, suggesting that other cell types contribute to the reduced MMP activity observed at later times.

There is a strong link among MMP activation, oxidative stress, and collateral formation. ROS production via Nox enzymes during mechanical stretch enhances MMP2 mRNA expression and pro-MMP2 release. In atherosclerosis, monocytes exhibit increased ROS production via Nox enzymes, which leads to enhanced secretion and activity of MMP9. ROS can have a direct effect on MMP activation via the cysteine switch or by altering the interaction between TIMPs and MMPs, as well as by increasing the expression of certain MMPs. We observed a difference in MMP activity but no change in expression, suggesting that 1 of the 2 former mechanisms is active in this model. In the context of hindlimb ischemia, neovascularization occurs via Nox2-mediated activation of MMP9. ROS can have a direct effect on MMP activation via the cysteine switch or by altering the interaction between TIMPs and MMPs, as well as by increasing the expression of certain MMPs. We previously showed that Poldip2 increases Nox4 activity in vascular smooth muscle cells and that it inhibits the secretion of collagen in an ROS-dependent manner. Because Nox4 has been associated with angiogenesis,
these data suggest that the reduction in MMP activity seen in Poldip2+/− mice (Figure 5) is a consequence of reduced Nox4-derived H$_2$O$_2$ (Figure 6A). This would preserve the extracellular matrix and impair ischemia-induced neovascularization by limiting the infiltration of inflammatory cells as well as the liberation of circulating growth factors from extracellular matrix that would maintain the new vascular network.

It is likely, however, that Poldip2 has additional Nox4-independent effects on cellular processes that contribute to collateral formation. Several reports indicate that Nox4 has a protective function under distinct pathological conditions (eg, obesity, pulmonary arterial hypertension, kidney fibrosis, and myocardial infarction).14,46–49 Conversely, other articles suggest that Nox4 induces apoptosis, especially in cancer cells, endothelial cells, and cardiac myocytes.45,48,50,51 Nox4 exerts these effects by inactivating the protein tyrosine phosphatase-1B and enhancing vascular endothelial growth factor receptor-2 and mTOR (mammalian target of rapamycin) signaling,
as well as by regulating endothelial nitric oxide synthase expression, phosphorylation of members of the Bcl-2 family, and mitochondrial oxidative stress. In contrast, although Poldip2 regulates cytoskeletal dynamics and matrix deposition in a Nox4 and ROS-dependent manner, it has additional functions potentially unrelated to Nox4. For example, Poldip2 interacts with DNA polymerase δ and proliferating cell nuclear antigen, suggesting an important role in the processes of DNA replication and repair. In addition, as a mitochondrial protein, it has been shown that Poldip2 might regulate viral DNA replication. Klaile et al showed that Poldip2 is a binding partner for carboxyemobryonic antigen-related cell adhesion molecule 1 and serves to regulate its trafficking from the cell periphery to the nucleus. Finally, a recent publication from our group showed that Poldip2 affects mouse embryonic fibroblast proliferation by regulating Cdk1 (cyclin-dependent kinase) and cyclinA. Although the Nox4 dependence of these roles of Poldip2 has not been studied in detail, at least some are likely to be Nox4 independent because deletion of Poldip2 is embryonically lethal, but deletion of Nox4 is not. Because of the potential role of Poldip2 in so many fundamental cellular processes, it will be important to explore in detail how this novel protein influences cardiovascular physiology and pathophysiology.

Conclusions

In summary, we have shown that heterozygous deletion of Poldip2 results in reduced H2O2 production during hindlimb ischemia, which is associated with reduced MMP2 and MMP9 activity, decreased formation of arterioles but increased connectivity between larger vessels, increased vascular rarefaction and apoptosis, and ultimately decreased blood flow and impaired functional recovery. In addition, MMP activity, but not expression, as well as endothelial cell proliferation, is significantly decreased compared with WT mice. Given the complex cellular and molecular interactions that contribute to neovascularization, as well as the multifunctional nature of Poldip2, much work remains to be done to dissect the potential molecular pathways regulated by this intriguing protein.

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Disclosures

None.

References

Peripheral arterial obstructive disease leading to limb ischemia is a major challenge in cardiovascular research. Here, we demonstrate that polymerase δ-interacting protein 2 regulates several cellular functions integral to ischemia-induced collateral vessel formation, including matrix metalloproteinase activity, angiogenesis, endothelial proliferation, and vascular regression. Fully understanding how polymerase δ-interacting protein 2 promotes neovascularization may ultimately allow for the development of new therapeutics to compensate for obstructive vascular lesions and consequently to preserve tissue function in patients with peripheral arterial obstructive disease.
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**Figure S1: Effective downregulation of Poldip2 by siRNA.** HUVEC were transfected with siControl or siPoldip2. Downregulation of Poldip2 protein expression was assessed by Western Blot using an antibody against Poldip2. Tubulin was used to normalize each sample. (n = 3 per genotype). Bars are means ± SEM. * P<0.05 vs. siControl.
Figure SII: Poldip2 downregulation does not affect expression of MMPs and TIMPs. mRNA levels of MMP2, TIMP2, MMP9 and TIMP1 were measured in ischemic and nonischemic muscle at indicated time points. 18S rRNA was used to normalize each sample and results are shown as the ratio between the matrix metalloproteinase enzyme (MMP) and its corresponding tissue inhibitor of metalloproteinase (TIMP). (n = 3-5 per genotype). Bars are means ± SEM.
| **Supplemental Table 1**: Primer sequences and reaction conditions used for quantitative RT-PCR (Ta annealing temperature) |
|------------------|------------------|-------------|-------------|-------------|
| **Forward (5’to3’)** | **Reverse (5’to3’)** | [Mg] (mM) | [primers] (nM) | Ta (°C) |
| ARG               | CTGACCTATGTGTCAATTGG | CATCTGGGAACTTTCCCTTTTC | 4 | 300 | 51 |
| B2M               | GCCCTGTATGCTATCCAGAA | GAAAGACACGTCCTTGGCTGA | 4 | 100 | 55 |
| iNOS              | TGAATCCCTCTCGATCTTG | CATGTAACCAAACCACTTTGAAG | 4 | 450 | 50 |
| IL-1β             | GGATGATGATGATAACCTGC | CATGGGAAATATACCTTGTGGG | 4 | 450 | 50 |
| IL-6              | GTCTATACACTTCAACAAGTC | TGCATCATCGTTGTTCAAC | 4 | 450 | 50 |
| IL-10             | ACCTGTTAGAAGTGTGCCAGCAGGA | CATGCAAGTTGTGAGATCGATCAAA | 4 | 450 | 50 |
| MMP2              | ACAGGACATTGTCTTTTGTG | TACACAGCACTTCATTTTTC | 4 | 450 | 51 |
| MMP9              | CTTCCAGTACCAAGCACAAG | ACCTGTTCACCTCATTTTG | 4 | 450 | 51 |
| MRC               | AAATGATGAGCTGTGGATTG | CCATCTTGCTTTTCATAAC | 4 | 300 | 51 |
| Poldip2           | AGTTCCGAGGACGAGGGGTGTTG | GAATGCTGGGGATCCGGACATCAAGA | 3 | 250 | 65 |
| TIMP1             | CTAGAGACACACACACAGATAC | CCCATGAATTTAGCTTCCTATG | 4 | 450 | 51 |
| TIMP2             | GGATTCAGTATGAGATCAAGC | GCCCTTCCTCAGAATTAGATAC | 4 | 450 | 51 |
Poldip2 promotes postischemic neovascularization of the mouse hindlimb

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Materials and Methods

Poldip2 gene trap mice

Mice (C57BL/6) with a gene trap insertion in the first intron of Poldip2 (chromosome 11, NCBI Gene ID: 67811) were produced at the Texas A&M Institute for Genomic Medicine (College Station, TX). Because homozygous deletion of Poldip2 causes perinatal lethality,1 male heterozygous mice were used for this study. Mice were genotyped using a standard 3-primer PCR method.1 All animal protocols were approved by the Institutional Animal Care and Use Committee of the Emory University School of Medicine. For all experiments, the observer was blinded to the genotype up until the analysis of the data.

Hindlimb ischemia surgery

At 9 to 13 weeks of age, animals were anesthetized using isoflurane (oxygen delivered at 0.5 L/min with 3% isoflurane for induction and 2.0% isoflurane for maintenance). A unilateral incision was made over the left medial thigh, and the superficial femoral artery and vein were ligated proximal to the caudally branching deep femoral artery and proximal to the branching of the tibial arteries. The portion of the artery and vein between ligations was excised.

Measurements of perfusion by LASER Doppler perfusion imaging (LDPI)

LDPI with a LASER of 810 nm (MoorLDI, Moor Instruments, Wilmington, DE) was used to evaluate perfusion in the ischemic and nonischemic legs after surgery. The distance between the source of the laser and the leg was 21 cm, the scan speed was 4 ms/pixel, and the resolution was 256 × 256 pixels. Mean perfusion was estimated in the foot and in the ischemic portion of the leg, extending from the thigh to the ankle. The nonischemic legs and feet were used as controls. The results for mean perfusion were reported as ratios of the values in the ischemic limb (IL) to those in the contralateral nonischemic limb (NIL) for each animal.2

Measurement of morphological parameters by micro-CT

After the animals were euthanized, the thoracic cavity was opened and the inferior vena cava was severed. The vasculature was flushed with 0.9% normal saline containing 4mg/ml papaverine at a pressure of ≈100 mmHg via a needle inserted into the left
ventricle. Excess papaverine was flushed with saline and the vasculature fixed with 10% neutral buffered formalin. Formalin was flushed from the vessels using saline. The vasculature was injected with undiluted Microfil (MV-122, Flow Tech; Carver, MA). Samples were stored at 4°C overnight for contrast agent polymerization.

Mouse legs (not including the feet) were dissected from the specimens and soaked in 10% neutral buffered formalin to ensure complete tissue fixation. Tissues were subsequently treated for 48 h in Cal Ex II (Fisher Scientific; Pittsburgh, PA) and then stored in 10% neutral buffered formalin. The hindlimb vasculature was imaged with a micro-CT imaging system (µCT 40, Scanco Medical; Bassersdorf, Switzerland). The scanner was set to a voltage of 55 kVp and a current of 145 µA. Resolution was set to medium, and the limbs were scanned at a 30-µm voxel size. A threshold of 110 was chosen based on visual interpretation of thresholded two-dimensional tomograms. Surgery and control legs were evaluated individually to quantify the three-dimensional histomorphometric values vessel volume, connectivity, density, thickness distribution, and spacing. The results were reported as ratios of IL to NIL for each animal to account for potential changes in vessel volume after injection of microfil.

Running test

Animals were individually housed in cages with a single activity wheel (Lafayette Instrument, Lafayette, IN) to which they had free access. The distance run in the wheels was recorded continuously and reported as a daily total.

Quantification of vasculature by I-isolectin B4 and smooth muscle alpha-actin staining

Mice were euthanized at postoperative days 7, 14 and 21 and tissues were perfused with saline and fixed with 10% buffered formalin. Paraffin-embedded hindlimbs were cut in 5 µm sections and stained with FITC fluorescein-labeled GSL I-isolectin B4 (Vector Laboratories, cat# FL-1201) or alpha-smooth muscle actin (Sigma Aldrich, cat# A5691). Images were acquired using the x20 objective on a Zeiss Axioskop microscope equipped with an AxioCam camera. The number of vessels from four animals per group and six fields of view per hindlimb was counted. The results for density of microvessels were reported as ratios of the IL to NIL.

Macrophage detection by immunofluorescence

Sections from paraffin-embedded hindlimbs were prepared as above. Antigen retrieval was performed in citrate buffer, pH 6.0 (Invitrogen), before incubation with Mac-3 antibody (1:200 in 3% BSA, BD Pharmingen, San Diego, CA, cat#553322), followed by
incubation with anti-mouse secondary antibody (1:400 in 3% BSA; Vector Labs) and incubation with Streptavidin QDot 655 (1:200 in 3% BSA; Invitrogen, Grand Island, NY, cat#Q101123MP). Images were acquired using a Zeiss Axioskop microscope equipped with an AxioCam camera. Total fluorescence intensity was quantified with ImageJ 1.46g software and expressed as ratios of the IL to NIL.

**cDNA preparation and real-time quantitative PCR**

Immediately after CO₂ euthanasia, tissues were rapidly harvested and stored in RNA later solution (Ambion, Austin, TX) at 10°C overnight. Muscles were homogenized with a motorized rotor/stator device and total RNA was purified with the RNeasy Fibrous Tissue kit (Qiagen, Chatsworth, CA, cat# 74704), including digestion with DNase I. Following reverse transcription (RT) with random primers and Superscript II enzyme (Invitrogen), cDNA was purified with the QIAquick kit (Qiagen).

Quantitative PCR was carried out with a LightCycler instrument (Roche Applied Science, Indianapolis, IN) in glass capillaries, using PlatinumTaq DNA polymerase (Invitrogen) and SYBR green (Invitrogen) dye. Primer sequences are indicated in Supplemental Table 1. Data analysis was performed using the mak3 module of the qpcR software library³ in the R environment.⁴

**Measurement of H₂O₂ production**

H₂O₂ production from muscle tissue was measured using the Amplex Red assay kit (Invitrogen, cat# A22188) per the manufacturer’s instructions. Briefly, gastrocnemius muscle was harvested and incubated with Amplex Red (50 µM) and horseradish peroxidase (0.1 U/ml) for 60 min at 37°C in Krebs-HEPES buffer protected from light. Fluorescence was measured (excitation 530 nm, detection 590 nm), and normalized to milligrams of wet tissue.

**Zymography and gelatinase/collagenase activity**

Briefly, mice were perfused with saline and muscles were dissected and placed at -80°C until use. Each tissue was homogenized on ice with 150 µl lysis buffer (100 mM Tris, 200 mM NaCl, 100 mM CaCl₂, 0.1% Triton X-100, pH 7.6), the lysates were spun at 10,000 x g for 5 min at 4°C and the supernatants were collected. Protein concentration was assessed using the Bradford assay method. Gelatin zymography was performed as described by Von den Hoff et al.⁵ Thirty µg of lysates were loaded in each lane of a premade 10% gelatin zymogram gel (Invitrogen, cat# EC61752). After electrophoresis, the gel was incubated with Zymography Renaturating Buffer (Invitrogen, cat# LC2670)
for 1 hr at room temperature followed by incubation with Zymogram Development Buffer (Invitrogen, cat# LC2671) overnight at 37 °C with slight rocking. Next, the gel was stained with SimplyBlue™ Safe Stain (Invitrogen, cat# LC6060) overnight and destained with water for 6 hr.

The gelatinase activity from muscle was measured with EnzChek Gelatinase/Collagenase Assay kit (Invitrogen, cat# E-12055) per the manufacturer’s instructions. Sample preparation was identical to gelatin zymography described above. Supernatants were incubated with DQ gelatin fluorescein conjugate at 37°C for 4 hours and the fluorescence monitored with a microplate reader (excitation 485 nm, detection 530 nm).

**Apoptosis assay**

Mice were euthanized at postoperative day 21 and tissues were perfused with saline and fixed with 10% buffered formalin. Paraffin-embedded hindlimbs were cut in 5 µm sections and stained with In Situ Cell Death Detection Kit TMR red (Roche cat# 12156792910). Images were acquired using the x20 objective on a Zeiss Axioskop microscope equipped with an AxioCam camera. The number of positive cells from three animals per group and six fields of view per hindlimb was counted.

**Proliferation assay**

HUVEC (Human Umbilical Vein Endothelial Cells) at passages 7-12 were seed at 10^4 cells per well in 6 well culture plates in EGM™ complete medium (Lonza #CC-3124). Twenty-four hours later HUVECs were transfected with siAllStar Negative control (Qiagen cat# 1027281) or siPoldip2 (sense 5'-CGUGAGGUUUGAUCAGUAAd(TT)-3', antisense 5'-UUACUGAUCAAACCUCACGd(TG)-3') using Lipofectamine RNAiMAX Reagent (Invitrogen cat# 13778) in OptiMEM reduced serum medium (Life Technologies cat# 31985070). Cells were trypsinized and counted every 24h for 4 days using a Scepter 2.0 counter (Millipore cat# PHCC20060).

**In vivo Angiogenesis model**

SIS (porcine small intestinal submucosa) (Cook Biotech Inn cat# P112628) was implanted subcutaneously into the mice, and vessel ingrowth was assessed at day 21 after implantation using histology. Endothelial cells were stained with FITC fluorescein-labeled GSL I-isolectin B4 (Vector Laboratories, cat# FL-1201) as described above in the section “Quantification of Vasculature”.

**Western Blot**
Samples were lysed in Hunter's buffer (25 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1 mM Na-orthovanadate, 1% Na deoxycholate, 1% Triton X-100, 0.1% SDS, 10% Glycerol, and protease inhibitors). Lysates were then sonicated and cleared at 10 000 rpm for 10 minutes. Proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes (Amersham cat#RPN68D), blocked, and incubated with appropriate primary antibodies. Proteins were detected by ECL (Amersham, cat#RPN2106). Band intensity was quantified by densitometry using ImageJ 1.38 software.

Statistical analysis

Results are expressed as mean±SEM from at least 3 independent experiments. Statistical significance was assessed using ANOVA, followed by Tukey’s multiple comparison post hoc test. In some cases, a Student t-test was used to assess significance between WT and Poldip2⁺/⁻ groups when no other variables were considered. A value of P<0.05 was considered statistically significant.

References