Thioredoxin-Interacting Protein Mediates Sustained VEGFR2 Signaling in Endothelial Cells Required for Angiogenesis

Young Park, Shin-Young Park, Xi Shi, Jinjiang Pang, Chen Yan, Bradford C. Berk

Objective—Thioredoxin-interacting protein (TXNIP) is an α-arrestin protein whose function is important for the regulation of vascular endothelial growth factor receptor 2 (VEGFR2) signaling and endothelial cell survival. Because VEGFR2 is critical for angiogenesis, we explored the role of TXNIP in VEGF-induced angiogenesis.

Approach and Results—TXNIP knockdown inhibited VEGF-induced endothelial cell tube formation and proliferation in cultured human umbilical vein endothelial cell. To elucidate the mechanism by which TXNIP altered VEGFR2 signaling in human umbilical vein endothelial cell, we studied phosphorylation of VEGFR2, PLCγ1, endothelial NO synthase, and Akt. TXNIP knockdown significantly decreased phosphorylation of VEGFR2 and PLCγ1 at times >5 minutes, but phosphorylation was unchanged at 2 minutes, as was Akt and endothelial NO synthase phosphorylation. Cell-surface biotinylation assay showed that TXNIP knockdown significantly attenuated VEGFR2 internalization. These results suggested that TXNIP was required for sustained VEGFR2 signaling, which is mediated largely by internalized VEGFR2. Rab5 knockdown to inhibit the trafficking and fusion of early endosomes significantly blocked VEGF-induced VEGFR2 internalization and phosphorylation of VEGFR2 and PLCγ1. Immunofluorescence and coimmunoprecipitation showed that TXNIP was part of a complex that included Rab5 and VEGFR2. Finally, TXNIP knockdown prevented the association of VEGFR2 and Rab5.

Conclusions—Our results show that TXNIP is essential for VEGFR2 internalization in Rab5 positive endosomes, which is required for endothelial cell growth and angiogenesis. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: angiogenesis ■ Rab5 ■ signaling ■ thioredoxin-interacting protein ■ VEGFR2

Angiogenesis is the formation of new blood vessels from the existing vascular network that includes endothelial cell (EC) proliferation, tube formation, and migration, involving several growth factors. Among them, vascular endothelial growth factor (VEGF) is a major proangiogenic factor that regulates EC function by binding to the VEGF receptor 2 (VEGFR2). In particular, the phosphorylation of VEGFR2 is a key event for internalization of the activated receptor and signaling. Regulation of internalization and localization of VEGFR2 promotes numerous downstream signal transduction pathways that lead to proliferation, migration, permeability, and differentiation of vascular EC. Therefore, internalization of VEGFR2 is considered an important mechanism through which cells regulate signal transduction and EC functions. Until recently, most studies on VEGFR2 internalization have focused on receptor ubiquitylation with degradation. However, the mechanisms by which internalization of VEGFR2 mediates angiogenesis are not fully understood.

Thioredoxin-interacting protein (TXNIP) is an α-arrestin family member and acts as a scaffold protein with several protein–protein interacting domains. TXNIP regulates inflammation in EC by binding to and inhibiting thioredoxin (TRX) in a redox-dependent fashion. We previously showed that the TXNIP–TRX1 complex translocated to the plasma membrane in response to physiological concentrations of hydrogen peroxide (H2O2) or tumor necrosis factor. Membrane TXNIP plays an essential role in VEGFR2 phosphorylation, EC survival, and EC migration. However, the mechanisms by which TXNIP regulates downstream VEGFR2 signaling and angiogenesis have not been elucidated.

In the present study, we found that TXNIP was required for VEGF-mediated angiogenesis as shown by decreased EC tube formation and proliferation. TXNIP was also required for VEGF signaling via regulation of VEGFR2 internalization in Rab5-positive endosomes.

Materials and Methods

Materials and Methods are available in the online-only Supplement.
Results
TXNIP Is Required for VEGF-Induced Tube Formation and Proliferation in EC
To investigate the role of TXNIP in angiogenesis, we analyzed EC tube formation in vitro. In control siRNA-treated cells, VEGF treatment increased tube length by 5.4±1.1-fold (Figure 1A, 1B, and 1E) and tube numbers by 5.8±1.5-fold (Figure 1F) compared with PBS-treated cells. Tube length and tube numbers were significantly reduced by TXNIP knockdown (1.3±0.6-fold and 2.2±0.8-fold, respectively; Figure 1C–1F, \( P<0.05 \)). Because EC proliferation is a key step for angiogenesis, we evaluated the role of TXNIP on VEGF-induced proliferation in human umbilical vein endothelial cell (HUVEC). As shown in Figure 1G, VEGF increased the number of cells (1.6±0.2-fold), which was significantly decreased by TXNIP knockdown (1.2±0.3-fold; \( P<0.05 \)). To confirm a role for TXNIP in cell proliferation, we performed a BrdU incorporation assay. In control siRNA-treated cells, VEGF treatment increased cell proliferation by 3.9±0.8-fold compared with PBS-treated cells, which was significantly reduced by TXNIP knockdown (1.5±0.3-fold; \( P<0.05 \); Figure 1H). These data suggest that TXNIP is required for VEGF-induced EC tube formation and proliferation.

TXNIP Defines 2 VEGF–VEGFR2 Signal Pathways that Differ Temporally
To investigate the functional role of TXNIP in VEGF-mediated signaling events in EC, we measured phosphorylation of VEGFR2 and PLC\(_\gamma\)-1 using HUVEC transfected with TXNIP siRNA or control siRNA. TXNIP siRNA efficiently reduced endogenous TXNIP expression, compared with control siRNA (Figure 2A). In control siRNA-treated cells, VEGF-stimulated phosphorylation of VEGFR2 (Figure A–C, Lower panels are quantification analysis. \( *P<0.05 \) vs control siRNA + VEGF (mean±SD; \( n=3 \)).
2A and 2B) and PLCγ1 (Figure 2A and 2C) as early as 2 minutes, and the phosphorylation was maintained for 15 minutes. Interestingly, TXNIP knockdown did not alter the phosphorylation of VEGFR2 and PLCγ1 at 2 minutes but significantly attenuated phosphorylation from 5 to 15 minutes of VEGF stimulation. Another rapidly activated VEGF pathway is phosphorylation of Akt-endothelial NO synthase (eNOS). To investigate the role of TXNIP in this pathway, HUVECs were transfected with control siRNA or TXNIP siRNA. On VEGF stimulation, both Akt and eNOS were rapidly phosphorylated at 1 and 2 minutes with no significant difference after TXNIP knockdown (Figure IA in the online-only Data Supplement). These data show that TXNIP is not required for VEGF2 regulation of eNOS and Akt, defining a novel role for TXNIP in EC.

### Protein Tyrosine Phosphatases Do Not Mediate TXNIP-Dependent VEGF2 Phosphorylation

Protein tyrosine phosphatases (PTPs), such as PTP1B and Src homology 2 domain-containing PTP (SHP)-1, inhibit VEGFR2 phosphorylation and EC functions. Therefore, we hypothesized that TXNIP regulates VEGFR2 phosphorylation through inhibiting PTP1B or SHP1. Specific inhibition of PTP1B or SHP1 by drugs or siRNA knockdown increased VEGF-stimulated VEGFR2 phosphorylation. However, the TXNIP effect on VEGFR2 phosphorylation was not altered by PTP1B knockdown (Figure IIA in the online-only Data Supplement) or SHP1 knockdown (Figure IIB in the online-only Data Supplement). These data suggest that inhibiting of PTP1B or SHP1 by TXNIP is not the mechanism of TXNIP-mediated regulation of VEGFR2 phosphorylation.

### TXNIP Is Required for VEGF-Induced VEGF2 Internalization

We hypothesized that TXNIP may regulate VEGF2 internalization for 2 reasons. First, receptor internalization is directly linked to VEGF2 phosphorylation and downstream signaling. Second, TXNIP depletion did not affect VEGF2 phosphorylation at 2 minutes, whereas sustained VEGF2 signaling was inhibited. To study VEGF2 internalization, cell-surface VEGF2 in HUVEC was labeled with a membrane-impermeable biotin derivative, and the cells were then exposed to VEGF at 37°C to allow endocytosis. As controls, cells were also biotin labeled and stimulated with VEGF for 15 minutes at 4°C to prevent VEGF2 internalization. After VEGF exposure, the remaining cell-surface biotin was cleaved with glutathione, and internalized proteins were collected using streptavidin-conjugated beads. The amount of internalized VEGF2 was determined by Western blotting. There was a significant increase of internalized VEGF2 in control siRNA-treated cells exposed to VEGF (16.4±4.1 and 27.2±5.9-fold at 5 and 15 minutes, Figure 3A and 3B). In contrast, internalized VEGF2 was decreased in TXNIP knockdown cells after VEGF stimulation (6.1±2.1 and 7.1±3.3-fold at 5 and 15 minutes, Figure 3A and 3B). These results indicate that TXNIP regulates VEGFR2 internalization.

Rab5 Is Involved in TXNIP-Mediated VEGF2 Internalization and Signaling

After VEGF stimulation, VEGF2 internalization involves a Rab5 GTPase-dependent endocytotic pathway. Furthermore, endocytosis of activated VEGF2 is required for multiple signal pathways in EC. Rab5 is necessary for the early steps of endocytosis, such as budding and docking/fusion activities. Manipulation of Rab5 function leads to early endosome-specific modulation of VEGF2 signaling. Thus, we determined whether VEGF2 internalization in endosomes is critical for the sustained VEGF2 phosphorylation using Rab5 siRNA to specifically block the internalization system. We found that Rab5 siRNA effectively blocked VEGF-stimulated VEGF2 internalization (Figure 4A and 4B). Importantly, we demonstrated that blocking VEGF2 internalization by Rab5 siRNA attenuated sustained phosphorylation of VEGF2 and PLCγ1 but did not affect the early 2 minutes phosphorylation (Figure 4C–4E). In addition, Rab5 depletion did not affect phosphorylation of Akt and eNOS in response to VEGF (Figure 4B in the online-only Data Supplement), indicating the endosome independent of VEGF-stimulated phosphorylation of Akt and eNOS. To prove VEGF2 internalization is functionally required for angiogenesis, we examined tube formation. As shown in Figure 4F and 4G, VEGF-induced tube length was significantly reduced by Rab5 knockdown compared with control siRNA (1.3±0.5-fold versus 4.5±1.2-fold, *P<0.05), indicating the requirement for VEGF2 internalization to induce angiogenesis.

![Figure 3](https://example.com/fig3.png)

**Figure 3.** Thioredoxin-interacting protein (TXNIP) regulates vascular endothelial growth factor receptor 2 (VEGF2) internalization. **A.** Human umbilical vein endothelial cells were transfected with control siRNA or TXNIP siRNA for 48 hours, and the cells were surface labeled with biotin and stimulated with VEGF (10 ng/mL) for the indicated times. After stripping remaining cell-surface biotin, cell lysates were immunoprecipitated with streptavidin beads to bind the internalized biotinylated proteins. Samples were immunoblotted for VEGF2 to determine internalized VEGF2 (top). Total cell lysates were also immunoblotted with VEGF2 antibodies to detect total (surface + internalized) VEGF2 levels (bottom). **B.** Internalized VEGF2 was quantified relative to the total amount of VEGF2 using Image J. *P<0.05 vs control siRNA +VEGF (mean±SD; n=3).
We next determined whether TXNIP localizes to Rab5-positive endosomes. First, we performed immunofluorescence on cultured HUVEC to show the colocalization of TXNIP and Rab5 in response to VEGF. Colocalization of TXNIP and Rab5 at membrane and cytosolic region significantly increased after 5 minutes of VEGF stimulation (Figure 5A and Figure III in the online-only Data Supplement). The peak in colocalization of TXNIP and Rab5 occurred at 15 minutes (8.2±2.0-fold; \( P < 0.05 \); Figure 5A). To confirm that TXNIP interacts with early endosomes, we used the early endosomal marker 1, a well-known Rab5 effector, that functions as a tethering protein in early endosome fusion.23 In response to VEGF, TXNIP colocalized with early endosomal marker 1 at membrane and cytosol (peak at 5–15– minutes; Figure IV in the online-only Data Supplement). In addition, we showed that TXNIP and VEGFR2 traffic together after 5 minutes of VEGF treatment at membrane and cytosol, but very little at 2 minutes and 30 minutes (Figure V in the online-only Data Supplement). Consistent with these results, coimmunoprecipitation studies showed that VEGF stimulates the association of TXNIP with Rab5 and VEGFR2 complex after 5 minutes, but not at 2 minutes (Figure 5B). This suggests that TXNIP and VEGFR2 traffic together to endosomes after 5 minutes of VEGF stimulation (Figure 5B). Further, TXNIP siRNA blocked the association of VEGFR2 with Rab5 in response to VEGF (1.3±0.3 and 1.1±0.2-fold at 5 and 15 minutes) compared with control siRNA (7.8±1.9 and 8.2±2.0-fold at 5 and 15 minutes; Figure 5C), indicating a critical role of TXNIP in the Rab5 and VEGFR2 complex formation. In summary, the formation of VEGFR2 and Rab5 complex mediated via TXNIP is critical for VEGFR2 internalization and sustained VEGFR2 signaling.

**Discussion**

The major findings of this study are that TXNIP is essential for VEGFR2 internalization in Rab5-positive endosomes, which is required for EC growth and angiogenesis. On the basis of our data, we propose the following model for the role of TXNIP in VEGF–VEGFR2 signaling in EC (Figure 6). The binding of VEGF induces autophosphorylation of Tyr1175 in the cytoplasmic domain of VEGFR2 receptor, which is required for VEGFR2 activation and angiogenesis. The earliest signaling events that occur within 2 minutes, such as phosphorylation of VEGFR2, PLC\( \gamma \), Akt, and eNOS (Figure 2), do not require TXNIP (Figure IA in the online-only Data Supplement). In contrast, sustained signaling, such as persistent VEGFR2 and PLC\( \gamma \)1 phosphorylation, requires TXNIP. A key finding was that TXNIP-mediated sustained signaling occurred in internalized VEGFR2 localized to Rab5-positive endocytotic vesicles (Figures 4

![Figure 4](image-url)
and 5). TXNIP-dependent signaling in EC was found to have significant physiological effects as shown by findings that TXNIP was required for VEGF-mediated tube formation and proliferation.

TXNIP is a member of the α-arrestin family that functions as an intracellular scaffold, which participates in cellular signaling by formation of signaling complexes and localization of signaling components in the cell. TXNIP-dependent signaling in EC seems to be dependent on TXNIP as shown in the present study. We showed previously that TXNIP was required for VEGFR2 activation and EC survival in response to low concentrations of H2O2 and tumor necrosis factor-α. These data support our concept that TXNIP plays a critical role in regulating VEGFR2 signaling and angiogenesis in EC.

A novel finding of the present study is that TXNIP seems to be required for the earliest stage of VEGFR2 internalization. Receptor tyrosine kinases are regulated by endocytosis through the internalization of plasma membrane receptors. For example, the internalization of epidermal growth factor receptor is mediated by clathrin-mediated endocytosis and is essential for sustained epidermal growth factor receptor signaling. Internalized receptor is delivered from the plasma membrane to early endosomes in endocytic vesicles, and canonically defined Rab5 is an early endosome marker. We showed that VEGFR2 internalization in endosomes is critical for sustained VEGFR2 signaling and angiogenesis in EC.

Figure 5. Thioredoxin-interacting protein (TXNIP) is critical for Rab5 and vascular endothelial growth factor receptor 2 (VEGFR2) complex in response to VEGF. Human umbilical vein endothelial cells (HUVECs) were untreated or treated with 10 ng/mL VEGF for indicated time (A and B). A, Quantification of TXNIP and Rab5 binding (punctate spots) were performed by image Pro. *P<0.05 vs unstimulated control (mean±SD; n=6). B, Immunoblot analysis of anti-TXNIP immunoprecipitates from the cell lysates with anti-VEGFR2 or anti-Rab5. Immunoblot analysis of total cell lysates from IP samples was performed with anti-VEGFR2, TXNIP, or Rab5. C, HUVECs were transfected with control siRNA or TXNIP siRNA. After 48 hours, the cells were treated with VEGF (10 ng/mL) for indicated times. Cell lysates were used to immunoprecipitate Rab5 and immunoblotting for VEGFR2. Immunoblot analysis of total cell lysates from IP samples was performed with anti-VEGFR2, TXNIP, or Rab5. IB indicates ; IgG, ; and TCL, .

Figure 6. Model for thioredoxin-interacting protein (TXNIP)-mediated vascular endothelial growth factor receptor 2 (VEGFR2) signaling in endothelial cell (EC) angiogenesis. VEGF triggers autophosphorylation of VEGFR2 (Y1175), which is internalized inside endocytotic vesicles and leads to PLCγ1 phosphorylation in TXNIP dependent. TXNIP sustains VEGFR2 signaling that is required for EC tube formation and proliferation in angiogenesis. On the contrary, transient VEGFR2 signaling (p-AKT/p-endothelial NO synthase [p-eNOS] pathway) is independent of TXNIP.
data demonstrate that VEGFR2 internalization is critical for sustained VEGFR2 signaling.

Our findings have potential clinical implications. We identified TXNIP as a critical component in sustained VEGFR2 signaling, such as VEGF-induced tube formation, proliferation, and angiogenesis. Inhibiting VEGFR2 signaling with antibodies and small molecules has demonstrated efficacy in diseases, such as cancer and proliferative retinopathy, by limiting angiogenesis.13–18 However, these therapies are associated with side-effects, such as hypertension, attributable to inhibiting VEGFR2 signaling that includes generation of nitric oxide. The present findings suggest that the TXNIP-dependent sustaining VEGFR2 signal pathway is separate from the VEGFR2 pathway that activates eNOS. If this true in vivo, inhibiting TXNIP function in EC specifically might limit side-effects dependent on nitric oxide. Also, because TXNIP is α-arrested and deleting TXNIP mimics the effect of inhibiting Rab5 activity, it is likely that other receptor tyrosine kinases will be regulated by TXNIP. Future studies will be required to elucidate the specific domains of TXNIP and interacting proteins that are responsible for sustained VEGFR2 signaling required for angiogenesis.

Acknowledgments
We thank Amy Mohan and Alison J. Hobbins for technical assistance. We thank Dr Mark Sowden for critical reading and editorial assistance.

Sources of Funding
The study was supported by National Institutes of Health grant R01 HL106158 to B.C.B.

Disclosures
None.

References
Regulation of receptor internalization is considered as an important mechanism that regulates downstream cell signaling pathways and cell functions. In particular, internalization of vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2) is essential for VEGF downstream signaling that leads to endothelial cell proliferation, migration, and survival. However, the mechanisms by which internalization of VEGFR2 contribute to VEGFR2 signaling and endothelial cell angiogenesis are not clear. The novel findings of this study are that thioredoxin-interacting protein is required for sustained VEGFR2 activation, endothelial cell proliferation, and tube formation. Specifically, thioredoxin-interacting protein regulates VEGF signaling via regulation of VEGFR2 internalization in Rab5 endocytotic vesicles. These findings provide new insight into the role of thioredoxin-interacting protein as a critical regulator for endothelial cell angiogenesis by regulating endosome-dependent VEGFR2 signaling and VEGFR2 internalization.
Thioredoxin-Interacting Protein Mediates Sustained VEGFR2 Signaling in Endothelial Cells Required for Angiogenesis
Young Park, Shin-Young Park, Xi Shi, Jinjiang Pang, Chen Yan and Bradford C. Berk

Arterioscler Thromb Vasc Biol. published online February 7, 2013;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2013/02/07/ATVBAHA.112.300386

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/02/07/ATVBAHA.112.300386.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Data

Supplemental Figure. I: Effect of TXNIP knockdown in VEGF-induced activation of Akt and eNOS.

(A) HUVEC were transfected with control siRNA, or TXNIP siRNA. After 48 hrs, the cells were treated with VEGF (10 ng/ml) for the indicated times. Cell lysates were immunoblotted with p-Akt (S473), Akt, p-eNOS (S1177), eNOS, and TXNIP. (B) HUVEC were transfected with control siRNA, or Rab5 siRNA. After 48 hrs, the cells were treated with VEGF (10 ng/ml) for the indicated times. Cell lysates were immunoblotted with p-Akt (S473), Akt, p-eNOS (S1177), eNOS, and Rab5.

Supplemental Figure. II: Inhibition of protein tyrosine phosphatases (PTPs) had no effect on TXNIP mediated VEGFR2 Y1175 phosphorylation.

(A) HUVEC were transfected with control siRNA, TXNIP siRNA, or PTP1B siRNA. After 48 hrs, the cells were treated with VEGF (10 ng/ml) for the indicated times. Cell lysates were immunoblotted with p-VEGFR2 (Y1175), TXNIP, and PTP1B. (B) HUVEC were transfected with control siRNA, or TXNIP siRNA for 48 hrs and then pretreated with or without SHP1 inhibitor (10 nM for 1 hr) before VEGF (10 ng/ml) stimulation. Cell lysates were immunoblotted with p-VEGFR2 (Y1175), VEGFR2, TXNIP, and α-tubulin.

Supplemental Figure. III: Co-localization of Rab5 and TXNIP in response to VEGF in HUVEC.

HUVEC were treated with VEGF (10 ng/ml) for the indicated times and fixed cells were
incubated with antibodies to TXNIP (Red) and Rab5 (Green). TXNIP interacts with rab5 at the membrane and cytosol (arrowheads) and at later time points there was only perinuclear (short arrows). Scale Bars, 20 μm.

**Supplemental Figure. IV: Co-localization of EEA1 and TXNIP in response to VEGF in HUVEC.**

HUVEC were treated with VEGF (10 ng/ml) for the indicated times and fixed cells were incubated with antibodies to TXNIP (Red) and EEA1 (Green). TXNIP interacts with EEA1 at the membrane and cytosol (arrowheads) and at later time points there was only perinuclear (short arrows). Scale Bars, 20 μm.

**Supplemental Figure. V: Co-localization of VEGFR2 and TXNIP in response to VEGF in HUVEC.**

HUVEC were treated with VEGF (10 ng/ml) for the indicated times and fixed cells were incubated with antibodies to TXNIP (Red) and VEGFR2 (Green). TXNIP interacts with VEGFR2 at the membrane and cytosol (arrowheads) and at later time points there was only perinuclear (short arrows). Scale Bars, 20 μm.
### Supplemental Figure I

#### A

<table>
<thead>
<tr>
<th>VEGF</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TXNIP siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- p-Akt (S473)
- Akt
- p-eNOS (S1177)
- eNOS
- TXNIP

#### B

<table>
<thead>
<tr>
<th>VEGF</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rab5 siRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- p-Akt (S473)
- Akt
- p-eNOS (S1177)
- eNOS
- Rab5
Supplemental Figure II

A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VEGF (min) 0</th>
<th>VEGF (min) 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con siRNA</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TXNIP siRNA</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>PTP1B siRNA</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VEGF (min) 0</th>
<th>VEGF (min) 2</th>
<th>VEGF (min) 5</th>
<th>VEGF (min) 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con siRNA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>TXNIP siRNA</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>SHP1 inhibitor</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Legend:**
- **p-VEGFR2 (Y1175):** Phosphorylation of VEGFR2 at Y1175
- **TXNIP:** TXNIP protein
- **PTP1B:** PTP1B protein
- **VEGF (min):** VEGF (min) protein
- **α-tubulin:** α-tubulin protein

**Note:** The table and diagrams illustrate the effects of different treatments on the expression of proteins of interest.
Supplemental Figure III

Control

VEGF 2min

VEGF 5min

VEGF 15min

VEGF 30min
Supplemental Figure V

Control

VEGF 2min

VEGF 5min

VEGF 15min

VEGF 30min

TXNIP  VEGFR2  Merge  Merge 3X
Materials and Methods

Reagents
Recombinant mouse VEGF\textsuperscript{164}, Rabbit anti-TXNIP (VDUP1) and mouse anti-\(\alpha\)-tubulin were purchased from Invitrogen. Rabbit anti-Flk-1 (for detecting VEGFR2), mouse anti-PLC\(\gamma\)1, Akt, Actin, and PTP1B were purchased from Santa Cruz Biotechnology. Mouse anti- Rab5, p-eNOS (Ser1177), eNOS, EEA1 and growth factor reduced Matrigel were purchased from BD Biosciences. Rabbit anti-p-VEGFR2 (Tyr1175), p-PLC\(\gamma\)1 (Tyr783) and p-Akt (Ser473) were purchased from Cell Signaling Technology. Streptavidin agarose beads and EZ-Link\textsuperscript{®} Sulfo-NHS-SS-Biotin were purchased from Thermo Scientific. Glutathione was purchased from Sigma. The SHP1 inhibitor (PTP inhibitor 1) was purchased from Calbiochem (CAS 2491-38-5), with chemical name \(\alpha\)-Bromo-4-hydroxyacetophenone. This chemical inhibits SHP-1 (\(\Delta\)SH2), the catalytic domain of the SH2 domain-containing phosphatase SHP-1 (\(K_i = 43 \, \mu\)M), and PTP1B (\(K_i = 42 \, \mu\)M).

Cell Culture and siRNA transfection
Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical veins and seeded onto gelatin-coated dishes maintained in Medium 200 (Cascade Biologics) with 5% fetal bovine serum (FBS), 100 \(\mu\)g/ml streptomycin, 100 IU/ml penicillin and low-serum growth supplement (LSGS; Invitrogen). To knockdown TXNIP (# J-010814-05, Dharmacon), Rab5 (# J-004009-05-0005, Dharmacon) or PTP1B (# L-003529-00-0005, Dharmacon) in HUVEC, a mixture of the relevant siRNA (50 nM) was transfected using Lipofectamine 2000 (Invitrogen) in Opti-MEM media (Invitrogen) into each well and incubated for 2 hrs followed by full culture medium change. 48 hrs later, experiments were performed on siRNA transfected HUVEC.
Western Blot

HUVEC were harvested and lysed in ice-cold 1x lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined by Bradford protein assay (Bio-Rad). Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto nitrocellulose membranes, and were subsequently blocked in 5% (v/v)-skim milk in PBST (Phosphate-buffered saline containing 0.1% Tween 20) for 1 hr. Then the blots were incubated overnight at 4 °C with appropriate antibodies. Then after being washed 3 times with PBST, membranes were incubated with horseradish peroxidase-coupled goat anti-rabbit or anti-mouse IgG (1:5000 dilution; Amersham) for 1 hr. Visualization of signals were by chemiluminescence (ECL Reagent, Amersham) or using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Densitometric analysis of films was performed using Image J software (version 1.36b, National Institutes of Health).

Immunoprecipitation

Cells were washed two times with cold PBS and lysed in immunoprecipitation lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.25% deoxycholate, 1% NP-40, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitors (Sigma, MO USA). Cleared lysates (13,000g; 10 min; 4 °C) were incubated with specific antibodies at 4 °C for O/N, followed by incubation with immobilized protein G-agarose (Santacruz, USA) at 4 °C for 2 hrs. Precipitates were washed three times with IP wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.25% deoxycholate, 1 mM EDTA, and protease inhibitors), followed by resuspension in 2X Laemli’s SDS-sample buffer (200 mM Tris, pH 6.8, 8 M urea, 5% SDS, 0.1 mM
EDTA, 0.03% bromophenol blue, 1.5% dithiothreitol). Denatured-immunocomplexes were then subjected to Western blot.

**Tube formation assay for in vitro angiogenesis**

For in vitro angiogenesis assay, HUVECs were transfected with control siRNA or TXNIP siRNA (50 nM). After 48 hrs, the cells were seeded on 96-well plates at 5x10^4 cells/well coated with growthfactor-reduced Matrigel and maintained in 1% FBS growth media contains VEGF (20ng/ml) for 18 hrs at 37 °C. The tube formation was visualized by a light microscope (Olympus CK40) at different time points and imaged with digital camera (Olympus DP11).

**BrdU Incorporation assay**

HUVEC were seeded in 24-well plates in 500μl culture medium per well for overnight, and incubated in 1% FBS for 24 hrs and then incubated with or without VEGF (20 ng/ml) in 1% FBS for 48 hrs. Proliferation was determined using BrdU Incorporation assay (Roche, Cat. No. 11 647 229 001) following the manufacturer’s protocol.

**Analysis of VEGFR2 Internalization by Cell-Surface Biotinylation**

Cells were grown to confluence on 10 cm collagen-coated dishes and starved for 1 hr in media without FBS. After rinsing with cold PBS, cells were incubated with 5 ml/dish EZ-Link sulfo-NHS-SS-Biotin (0.5 mg/ml) in PBS at 4 °C for 30 min, quenched with cold PBS + 50 nM glycine, and rinsed with cold media with 1% BSA. Cells were then stimulated with VEGF-A (20 ng/ml) at 37 °C. At specified time points, cells were rinsed with cold PBS and incubated for 20 min on ice with glutathione (45 mM) in 75 mM NaCl, 75 mM NaOH, 1 mM EDTA, 1% BSA. Glutathione was quenched with cold PBS with iodoacetamide (5 mg/ml).
Cells were rinsed with cold PBS and protein lysates prepared using Protein lysis buffer. Lysate (500µg) was immunoprecipitated with 50µl Streptavidin agarose beads at 4 °C overnight, then rinsed with 1ml IP wash buffer and resuspended in 50µl 2X Lameli’s SDS-sample buffer. Samples were analyzed by SDS-PAGE followed by blotting with anti-Flk-1 (VEGFR2).

**Immunofluorescence staining and microscopy**

Treated HUVEC were washed with PBS and fixed with 4% formaldehyde in PBS followed by washing with PBS three times at room temperature. Cells were then permeabilized with 0.1% Triton-X100 containing PBS for 10 min followed by three times washing with PBS, and blocked with 10% normal goat serum in PBS containing 0.5% Tween 20 for 1 hr at room temperature. Cells were incubated with rabbit anti-TXNIP (1:200 dilution), mouse anti-Rab5 (1:200 dilution), mouse anti-EEA1 (1:200 dilution) and mouse anti-VEGFR2 (1:200 dilution) overnight at 4 °C in the blocking solution. After two times washing with PBS, fluorescence-conjugated secondary antibodies (1:2000 dilution, Alexa Fluor 546 and 488, respectively) were incubated and followed by three times washing with PBS. Images captured by a fluorescence microscope (Olympus BX51, Software: SPOT Imaging software advanced).

**Statistics**

Group differences were analyzed using the standard two-tailed Student’s t-test. All values are expressed as mean ± SD from three to six independent experiments.