Thioredoxin-Interacting Protein Is a Biomechanical Regulator of Src Activity
Key Role in Endothelial Cell Stress Fiber Formation

Oded N. Spindel, Ryan M. Burke, Chen Yan, Bradford C. Berk

**Rationale:** Fluid shear stress differentially regulates endothelial cell stress fiber formation with decreased stress fibers in areas of disturbed flow compared with steady flow areas. Importantly, stress fibers are critical for several endothelial cell functions including cell shape, mechanosignal transduction, and endothelial cell–cell junction integrity. A key mediator of steady flow–induced stress fiber formation is Src that regulates downstream signaling mediators such as phosphorylation of cortactin, activity of focal adhesion kinase, and small GTPases.

**Objective:** Previously, we showed that thioredoxin-interacting protein (TXNIP, also VDUP1 [vitamin D upregulated protein 1] and TBP-2 [thioredoxin binding protein 2]) was regulated by fluid shear stress; TXNIP expression was increased in disturbed flow compared with steady flow areas. Although TXNIP was originally characterized for its role in redox and metabolic cellular functions, recent reports show important scaffold functions related to its α-arrestin structure. Based on these findings, we hypothesized that TXNIP acts as a biomechanical sensor that regulates Src kinase activity and stress fiber formation.

**Methods and Results:** Using en face immunohistochemistry of the aorta and cultured endothelial cells, we show inverse relationship between TXNIP expression and Src activity. Specifically, steady flow increased Src activity and stress fiber formation, whereas it decreased TXNIP expression. In contrast, disturbed flow had opposite effects. We studied the role of TXNIP in regulating Src homology phosphatase-2 plasma membrane localization and vascular endothelial cadherin binding because Src homology phosphatase-2 indirectly regulates dephosphorylation of Src tyrosine 527 that inhibits Src activity. Using immunohistochemistry and immunoprecipitation, we found that TXNIP prevented Src homology phosphatase-2–vascular endothelial cadherin interaction.

**Conclusions:** In summary, these data characterize a fluid shear stress–mediated mechanism for stress fiber formation that involves a TXNIP-dependent vascular endothelial cadherin–Src homology phosphatase-2–Src pathway. (Circ Res. 2014;114:1125-1132.)

**Key Words:** disturbed flow ■ endothelial cells ■ laminar flow ■ stress fibers ■ TXNIP
endothelial growth factor (VEGF) receptor 2, Tie1, and platelet-derived growth factor receptor. Consequently, Src family kinases are activated, promoting phosphorylation of PM scaffolds such as platelet EC adhesion molecule 1 and vascular endothelial cadherin (VE-cadherin). These pathways are important for multiple endothelial functions, such as vasodilation, inflammation, angiogenesis, and migration. Many of these events require formation and remodeling of stress fibers that are specialized actin structures in ECs.

In particular, VE-cadherin is a transmembrane receptor that acts as a FSS-sensing molecule to regulate endothelial stress fiber formation by 2 mechanisms: first, by increased phosphorylation and transduction of extracellular signals into the cytoplasm; and second, by acting as a docking site for adapter proteins and phosphatases. VE-cadherin interacts with Src homology phosphatase-2 (SHP2) and Src to regulate EC stress fiber formation. For example, in response to VEGF, the endothelial receptor VEGF receptor 2 is activated, leading to consequent steps: recruitment of SHP2 to the VE-cadherin complex followed by activation of Src kinase protein and changes in stress fiber dynamics.

We recently showed that thioredoxin-interacting protein (TXNIP) is highly regulated by changes in blood flow. In d-flow regions, TXNIP is highly induced and promotes endothelial inflammation and apoptosis, whereas in s-flow regions, TXNIP expression is suppressed. Furthermore, TXNIP is an α-arrestin protein, which has an important function in cellular signaling, acting as a scaffold protein, to regulate formation of signaling complexes and subcellular-specific signaling events. Specifically, TXNIP is important for activation of VEGF receptor 2 at the PM and downstream signaling, which is important for Src activation. Based on our recent TXNIP structure–function analyses, we propose that in response to FSS, TXNIP regulates Src activation via regulation of VE-cadherin–SHP2–Src complex, resulting in altered EC stress fibers.

**Methods**

**Animals**

All animal experiments were conducted in accordance with experimental protocols that were approved by the University Committee on Animal Resources of the University of Rochester. Animals were maintained under pathogen-free conditions at the Aab Cardiovascular Research Institute of the University of Rochester.

**En Face Staining**

Immunofluorescence staining of mouse aortic EC was performed using 24-week-old control or TXNIP-knockout mice. Mice were anesthetized with ketamine/xylocaine cocktail (0.130.0088 mg/g body weight), the jugular vein was cut, and the arterial tree was perfused with saline containing 40 USP U/United States Pharmacopeia unit/mL heparin from left ventricle for 5 minutes, followed by perfusion of prechilled 4% paraformaldehyde in PBS (pH 7.4) for 10 minutes. Subsequently, the whole aorta was dissected from iliac bifurcation to the heart, cut open longitudinally, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and blocked with 10% normal goat serum in Tris-buffered saline containing 2.5% Tween-20 for 1 hour at room temperature. Next, aortas were incubated with primary antibody in blocking buffer overnight at 4°C. After rinsing with washing solution (Tris-buffered saline containing 2.5% Tween-20) 3x, fluorescence-conjugated secondary antibodies were applied for 1 hour at room temperature. Finally, after another 3 rinses in the washing solution, aortas were mounted in the ProLong antifade reagent (Invitrogen, Eugene, OR). Aortas were examined by a laser-scanning confocal microscope (FX-1000 mounted on IX81, Olympus) with UPlanSApo ×20 or UPlanFL N ×40 lens.

**Cell Culture, Western Blot, and Small Interfering RNA Transfection**

Human umbilical vein EC (HUVEC) or bovine aortic EC were grown and transfected with plasmid/small interfering RNA (siRNA) as detailed in the online-only Data Supplement and previously described.

**In Vitro Flow Experiments**

To perform biochemical studies, we used a cone-and-plate flow apparatus, and HUVEC cultured in 60-mm dishes were exposed to s-flow at 12 dyn/cm² by using smooth cones (120 rpm) or exposed to d-flow at 2 dyn/cm² by using grooved cones (5 rpm) for 24 hours. The shear stress imposed on the surface of the plate was calculated by the formula $\sigma = \mu \omega \theta$, where $\omega$ is the rotation speed, $\mu$ is the fluid viscosity, and $\theta$ is the angle of the cone.

**Immunofluorescence**

Treated HUVEC in 35-mm dishes were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 15 minutes, permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, and blocked with 10% normal goat serum in PBS containing 0.5% Tween-20 for 1 hour at room temperature. Then cells were stained with primary antibodies overnight at 4°C in blocking solution. Cells were then rinsed with 0.5% Tween-20 in PBS 3x and incubated with the fluorescence-conjugated secondary antibodies for 1 hour at room temperature. After another 3 rinses with the washing solution, images were acquired using an inverted epifluorescence microscope (IX50, Olympus) equipped with a charge-coupled device camera (Spot; Diagnostic Instruments, Inc) with Cropland water x40 (numerical aperture, 0.8) or x60 (numerical aperture, 0.9) objective lens or laser-scanning confocal microscope (FX-1000 mounted on IX81, Olympus) with UPlanSApo ×20 or UPlanFL N ×40 lens.

**Statistical Analysis**

Group differences were analyzed using the standard Student $t$ test. All values are expressed as mean±SE. $P<0.05$ was considered statistically significant.

**Results**

TXNIP Regulates Src Phosphorylation In Vivo

To evaluate the role of TXNIP in Src phosphorylation in vivo, we measured the phosphorylation state of tyrosine 416 (Y416) and tyrosine 527 (Y527) using en face staining of EC lining the aorta. Under basal conditions, Src Y527 is phosphorylated (phospho-tyrosine 527 [pY527]), which maintains low kinase
activity. In response to many stimuli, Src pY527 is dephosphorylated, which induces a conformational change in the activation loop. Full kinase activation then occurs by phosphorylation of Src Y416 (phospho-tyrosine 416 [pY416]).\textsuperscript{34} To demonstrate an endothelial role of TXNIP in differential phosphorylation states of Y416 and Y527 of Src, we used TXNIP-knockout mice. In the aorta exposed to s-flow, control animals exhibited low levels of phospho-Y416 (pY416) and high levels of pY527 (Figure 1A–1C, 1H–1J). In contrast, the aorta of knockout animals showed a dramatic increase in Src pY416 and decrease in pY527 (Figure 1D–1G, 1K–1N). To confirm these findings, we also scanned sites where the spinal arteries branch from the aorta; these sites are characterized by both s- and d-flow regions. There was a clear increase in Src pY416 and decrease in pY527 as shown in Online Figure I. Total Src expression was not significantly different between these animals (Online Figure II and III), suggesting that the effect observed was because of TXNIP’s effect on a Src regulatory mechanism.

**Src Phosphorylation State of Tyrosine 416 and 527 Is Differentially Regulated by TXNIP In Vitro**

To confirm this observation in vitro, we measured Src pY416 and pY527 in cultured HUVEC exposed to s-flow or d-flow. Shown in Figure 2, TXNIP expression is reduced under s-flow conditions, whereas it is dramatically increased under d-flow conditions. In addition, Y416 is highly phosphorylated under s-flow conditions and dephosphorylated under d-flow conditions (Figure 2). Finally, Y527 phosphorylation level was low under s-flow conditions but highly phosphorylated under d-flow conditions (Figure 2). This observation was also confirmed by an immunofluorescence experiment using HUVEC exposed to s-flow or d-flow (Online Figure IV).

In addition, we also measured pY416 and pY527 in HUVEC in which TXNIP was depleted using siRNA. HUVEC transfected with control siRNA had low levels of pY416 and high levels of pY527, whereas TXNIP siRNA–transfected cells had a 2.5-fold increase in pY416 and a 0.7-fold decrease in pY527 (Online Figure V). Furthermore, we confirmed this differential phosphorylation state of Y416 and Y527 using an immunofluorescence approach to measure pY416 and pY527 in cultured HUVEC in which TXNIP was depleted using siRNA. In HUVEC transfected with control siRNA, low levels of pY416 were observed, whereas in TXNIP siRNA–transfected cells, a 2.5-fold increase in pY416 was observed (Online Figure VIA–VIF and VIM). Increased pY416 was at the PM and diffusely in the cytosol. The results obtained for pY527 were opposite for those observed with pY416, as expected. In control siRNA–transfected cells, high levels of pY527 were observed, whereas in TXNIP siRNA–transfected cells, there was a significant 60% decrease in pY527 (Figure VIG–VIH and VIM). To confirm these results, we also used confocal microscopy as shown in Online Figure VII. All together, our in vivo and in vitro data strongly suggest that TXNIP acts as an endogenous inhibitor of Src activation in EC.

**TXNIP Prevents SHP2 Phosphatase Interaction With VE-Cadherin**

Next, we hypothesized that TXNIP prevents SHP2 to be recruited to VE-cadherin. Previously, it was shown that SHP2 dephosphorylates C-terminal Src kinase (CSK), which is an important inhibitor of Src via phosphorylation of the inhibitory Y527.\textsuperscript{25} Inhibition of CSK allows relief of the inhibitory effect of pY527 and activation of Src. SHP2 activity is regulated in EC through an interaction with VE-cadherin that acts as a docking site for a complex of proteins including Src, CSK, and SHP2.\textsuperscript{25} Because TXNIP is an α-arrestin protein and contains several protein–protein binding motifs (eg, PPxY [proline, proline, any amino acid, tyrosine] motif or ITIM [immunoreceptor tyrosine-based inhibition] motif), we hypothesized that TXNIP prevents SHP2 from interacting with VE-cadherin. To test this hypothesis, we measured VE-cadherin–SHP2 interaction (Figure 3) in cells transfected with TXNIP or control siRNA. In addition, we cotransfected these cells with 2 different plasmids: TXNIP wild type (TX-WT) or TXNIP mutant Y378A (Y378A). Tyrosine 378 (Y378) is part of an identified PPxY motif within the arrestin domain of TXNIP. Based on an extensive mutagenesis analysis, we discovered that Y378A fails to interact with SHP2 (Figure 3A). To test the hypothesis that TXNIP regulates SHP2 ability to interact with VE-cadherin, we introduced 2 green fluorescent protein (GFP)–tagged plasmids TX-WT-GFP or Y378A-GFP and measured VE-cadherin–SHP2 interaction (Figure 3B). In control TX-WT-GFP–transfected cells, low levels of VE-cadherin–SHP2 interaction were observed as expected. In
contrast, Y378A-GFP–transfected cells showed a 2-fold increased interaction between VE-cadherin and SHP2. These data demonstrate that TXNIP binding to SHP2 is regulated by the PPxY domain (AA375-378).

To demonstrate the functional mechanism by which TXNIP–SHP2 binding regulates Src activation, we performed a rescue experiment. We cotransfected cells with control or TXNIP siRNA, and then expressed TX-WT-GFP or Y378A-GFP plasmids, to rescue the activation of Src. When TX-WT-GFP was expressed, Src pY416 was low, but when Y378A-GFP was expressed, a 3.5-fold increase of Src pY416 was observed. When Y378A-GFP was expressed, Src pY416 was increased significantly similar to transfection with control siRNA as shown in Figure 3C. These data suggest that Y378A outcompetes endogenous TX-WT.

The in vivo data (Figure 1 and Online Figure I) suggest that s-flow promotes the VE-cadherin–SHP2 interaction based on the high levels of Src pY416. To show further the role of TXNIP in regulating VE-cadherin–SHP2 interaction, we exposed HUVEC to s- or d-flow conditions. There was a significant 3.5-fold increase in VE-cadherin–SHP2 interaction when s-flow was applied to HUVEC compared with d-flow samples (Online Figure VIII; *P<0.05). To provide further evidence that TXNIP regulates SHP2 function, we measured SHP2 localization in control siRNA– or TXNIP siRNA–transfected cells by confocal microscopy (Online Figure VIII). In control siRNA cells, SHP2 was found mainly in perinuclear and nuclear regions (Online Figure VIII). In contrast, in TXNIP siRNA–transfected cells, SHP2 was excluded from the nucleus and was more evenly distributed throughout the cell (Online Figure VIII).

Finally, to prove this mechanism, we measured CSK activation (serine 364 phosphorylation) under s-flow or d-flow conditions. Under s-flow conditions, CSK activation was low, consistent with low TXNIP expression and high Src activity. In contrast, under d-flow conditions, CSK activation was high, consistent with high TXNIP levels and low Src activity (Figure 4). To confirm this observation, we also transfected HUVEC with control or TXNIP siRNA. In control siRNA–transfected cells, a basal level of active CSK was observed, whereas in TXNIP siRNA–transfected cells, a 60% reduction in active CSK was observed (Online Figure IX).

These data confirm our hypothesis that TXNIP regulates Src activation through indirect regulation of CSK inhibition by SHP2. Specifically, TXNIP regulates SHP2 subcellular recruitment to VE-cadherin to dephosphorylate and inhibit CSK.

TXNIP Acts as a Negative Regulator of Src and Stress Fibers Formation In Vitro

Because Src activity is important for F-actin stress fiber homeostasis,13 we determined the role of TXNIP in stress fiber formation. We transfected HUVEC with control or TXNIP siRNA to evaluate Src activation state (pY416) and F-actin fibers (rhodamine–phalloidin fluorescence). Cells transfected with control siRNA had a low amount of stress fibers under baseline conditions that was consistent with low Src activity (Figure 5A–5C). In contrast, after transfection of TXNIP siRNA, stress fibers formed spontaneously that correlated with increased Src activation at the periphery of the cells and in cytosolic structures (Figure 5D–5F). Active Src

![Figure 2](http://circres.ahajournals.org/)

Figure 2. Western blot analysis demonstrates thioredoxin-interacting protein (TXNIP) expression. Src pY416 and pY527 are regulated by flow. A, Total cell lysates of human umbilical vein endothelial cell exposed to steady flow (s-flow) or disturbed flow (d-flow) were immunoblotted for pY416, pY527, Src, and TXNIP. B, C, Quantification of the data using ImageJ (*P<0.05 vs s-flow; n=4).

![Figure 3](http://circres.ahajournals.org/)

Figure 3. Vascular endothelial (VE)-cadherin–Src homology phosphatase-2 (SHP2) interaction is regulated by thioredoxin-interacting protein (TXNIP). A and B, Western blot analysis of immunoprecipitation samples from bovine aortic endothelial cell (BAEC) transfected with TX-WT-GFP or Y378A-GFP plasmids. C, Western blot analysis of total cell lysates samples from BAEC transfected with control or TXNIP siRNA and TX-WT-GFP or Y378A-GFP mutant TXNIP. Quantification of the data using ImageJ (n=3).

![Figure 4](http://circres.ahajournals.org/)

Figure 4. C-terminal Src kinase (CSK) activity is regulated by flow. A, Western blot analysis of human umbilical vein endothelial cell exposed to steady flow (s-flow) or disturbed flow (d-flow) and immunostained for active CSK, thioredoxin-interacting protein (TXNIP), and actin. B, Quantification of CSK activation in response to flow (*P<0.05 vs s-flow; n=3).
localized with stress fibers, suggesting a link between reduced TXNIP expression and Src regulation of F-actin stress fibers (Figure 5F).

**Flow Regulates TXNIP Expression and F-Actin Stress Fiber In Vitro and In Vivo**

To study the physiological relevance of these observations, we analyzed the relationship among s-flow or d-flow, TXNIP expression, and F-actin stress fiber formation. First, we performed en face staining for TXNIP in s-flow and d-flow regions of the aortic arch of WT animals (Figure 6A–6F). Consistent with previous studies, TXNIP expression was significantly suppressed in regions of s-flow (greater curvature; Figure 6A–6C) compared with d-flow regions (lesser curvature; Figure 6D–6F). Interestingly, TXNIP expression in s-flow and d-flow regions inversely correlated with formation of F-actin stress fibers. These data suggest an important inhibitory effect of TXNIP on EC stress fiber formation (Figure 6G–6I). Measuring the correlation between TXNIP expression and F-actin formation showed a clear inverse correlation (Online Figure X).

To confirm a role for TXNIP in the regulation of stress fibers, HUVEC were exposed to no flow, s-flow, or d-flow conditions and TXNIP and F-actin levels were measured using immunofluorescence. Under no flow conditions, there were few stress fibers, and TXNIP was localized to the nucleus as indicated by colocalization of TXNIP and 4',6-diamidino-2-phenylindole staining (Figure 7A–7C). After s-flow stimulation (12 dyn/cm², 24-hour), EC aligned in the direction of flow and were characterized by increased stress fibers, and TXNIP expression was decreased (Figure 7D–7F). In contrast, EC that were exposed to d-flow (2 dyn/cm², 24-hour) demonstrated no cell alignment, low stress fibers, and significantly increased TXNIP expression in both nucleus and cytosol (Figure 7G–7I; quantification of these data presented in Online Figure XI). These data confirm the inhibitory role TXNIP plays in stress fiber formation under different flow patterns.

**Discussion**

The major findings of this study are that TXNIP is a biomechanical regulator of Src activity that controls EC stress fibers formation. Based on both in vitro and in vivo studies, we propose that TXNIP regulates a SHP2–CSK–Src signaling cascade.
Under d-flow conditions, TXNIP expression is high and CSK is active, leading to increased Src Y527 phosphorylation and low Src activity. In contrast, s-flow reduces TXNIP-SHP2 interaction that leads to dephosphorylation of CSK, decreased Src Y527 phosphorylation, and activation of Src. Importantly, active Src was observed mainly in linear structures that appeared similar to stress fibers, as indicated with white arrows in Figure 1. In EC, Src activation correlated with increased F-actin stress fibers formation both in vivo and in vitro. Physiologically, this model explains the presence of stress fibers in regions of s-flow and absence of stress fibers in regions of d-flow, as originally described by Kim et al.36 Furthermore, stress fiber formation is likely to be important in diverse EC functions, such as migration, permeability and apoptosis, cell shape change and alignment, mechnano-signal transduction, EC–cell junction, and EC structural and functional integrity.37–44

The present study further supports the role of TXNIP as an arrestin-like scaffold protein that coordinates cell-signaling mechanisms that occur in specific subcellular locations. Recently, we performed a sequence analysis of TXNIP and described several binding motifs that can facilitate TXNIP interaction with multiple proteins under different biological conditions.32 Particularly important are the 4 SH3 and 2 PPxY motifs. The current article demonstrating the critical role for Y378 in the C-terminal PPxY is the first description of a functional TXNIP motif that interacts with SHP2. Future studies will describe the binding partners that mediate TXNIP localization to specific subcellular locations, such as nucleus, cytosol, and PM.28,45,46 For example, we and others showed previously that PARP1 (poly-ADP-ribose polymerase 1) and thioredoxin regulate TXNIP localization.47 Recently, we also showed that in response to VEGF receptor 2 activation, TXNIP translocates from the nucleus and interacts with Rab5 to regulate endocytosis and angiogenesis.48

Our data show that TXNIP is a key regulator of Src activity, which is important in physiological and pathological cell functions. For example, under physiological conditions, when EC are exposed to s-flow, TXNIP expression is low. Therefore, Src activity is high because Src pY527 is low and pY416 is high, resulting in EC stress fiber formation. Other examples that the relationship between TXNIP expression and Src activity include both tumorogenesis and metabolism. Decreased expression of TXNIP was shown to result in spontaneous tumorigenesis in multiple tissue types, which is related to its role as growth arrest protein.49 This is consistent with data that Src is a well-known oncogene, which is highly active in tumor cells.50 In addition, tumor metastasis requires stress fiber rearrangement and loss of cell interaction with matrix, processes facilitated by decreased TXNIP expression.51 In terms of metabolism, Src and TXNIP have been shown to play important roles in glucose metabolism, mitochondrial function, and cell death.47,52–57 In summary, this is the first demonstration that a specific TXNIP subcellular motif (C-terminal PPxY) mediates a critical cell function, specifically the interaction with SHP2 and regulation of Src activity. Furthermore, these findings suggest that TXNIP may play a broader role in regulating Src activity in multiple cell types, particularly in response to oncogenic and inflammatory stimuli.

Sources of Funding

This work was supported by National Institutes of Health HL 106158 to B.C. Berk.

Disclosures

None.

References


---

**Novelty and Significance**

**What Is Known?**
- Thioredoxin-interacting protein (TXNIP), a scaffold protein of the α-arrestin family, regulates target protein activity through multiple protein–protein interacting domains.
- TXNIP is highly regulated by multiple mechanisms, such as glucose, redox state, and biomechanical forces, including shear stress and stretch.
- Src kinase activity regulates stress fibers formation via multiple targets, such as paxillin or focal adhesion kinase.

**What Is New Information Does This Article Contribute?**
- Steady flow (s-flow) increases Src activity and stress fiber formation while decreasing TXNIP expression, whereas disturbed flow (d-flow) has the opposite effect.
- Our observation that TXNIP plays a role in stress fibers formation expands the role of TXNIP as a biomechanical sensor.
- Stress fiber formation under conditions of fluid shear stress is mediated by the TXNIP–Src homology phosphatase-2–C-terminal Src kinase–vascular endothelial cadherin–Src signaling pathway.

Fluid shear stress differentially regulates endothelial cell function; most notably, atherosclerosis is greater in areas of d-flow compared with s-flow. Conversely, stress fiber formation is greater in s-flow than d-flow areas. Importantly, stress fibers are critical for several endothelial cell functions including cell shape, mechano-signal transduction, and cell–cell junction integrity. A key mediator of s-flow–induced stress fiber formation is Src, although the exact mechanisms that control Src remain unclear. A new signal mediator that links biomechanical forces with redox state in endothelial cell has been identified as the TXNIP, which is highly induced by d-flow. Using en face immunohistochemistry of the aorta and cultured endothelial cell, we show that s-flow increased Src activity and stress fiber formation whereas it decreased TXNIP expression. In contrast, d-flow had opposite effects. We found that TXNIP regulated Src by binding to Src homology phosphatase-2 and inhibiting the Src negative regulator C-terminal Src kinase. These data show that TXNIP plays a role in stress fibers formation and expands its role as a biomechanical mediator. Furthermore, these findings suggest that TXNIP is a novel regulator of Src. Because TXNIP is highly regulated by multiple factors (eg, glucose, circadian rhythm, redox), these results provide new insights into Src regulation in many pathophysiological conditions.
Thioredoxin-Interacting Protein Is a Biomechanical Regulator of Src Activity: Key Role in Endothelial Cell Stress Fiber Formation

Oded N. Spindel, Ryan M. Burke, Chen Yan and Bradford C. Berk

Circ Res. 2014;114:1125-1132; originally published online February 10, 2014;
doi: 10.1161/CIRCRESAHA.114.301315

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/114/7/1125

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/02/10/CIRCRESAHA.114.301315.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research 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 Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Material

Detailed Methods

Antibodies and Reagents

Antibodies and reagents were purchased as follows: mouse anti Actin, mouse anti VE-cadherin and goat anti VE-cadherin antibodies (Santa Cruz); Src pY416, Src pY527, SHP2 (Cell Signaling); TXNIP (Invitrogen); Rat anti VE-cadherin (BD Biosciences Pharmingen); Phalloidin (Sigma-Aldrich); CSK pSer 364 (Abcam); Green fluorescent protein (Clontech)

Cell Culture and siRNA Transfection

Human umbilical vein endothelial cell (HUVEC) or bovine aortic endothelial cell (BAEC) were seeded onto gelatin-coated dishes maintained in Medium 200 or Medium 199 respectively (Cascade Biologics, Portland Or) with low serum growth supplement and 5% FBS as previously described\(^1\). Cells were used at passages 2 to 6. Cells were transiently transfected with siRNA (TXNIP targeted or control) or plasmids as described in the text. Transfection was performed using Opti-MEM I Reduced Serum Media (Invitrogen) and Lipofectamine 2000. Experiments were performed 24 hours post-transfection.

Western Blot and Immunoprecipitation

Cells were washed twice in ice-cold PBS and harvested in lysis buffer (20 mmol/L Tris pH7.5, 150mmol/L NaCl, 1 mmol/L EDTA, 1 µmol/L EGTA, 1% Triton X-100, 2.5 µmol/L sodium pyrophosphate, 1 µmol/L β-glycerolphosphate, 100 mmol/L NaVO4, 1 mol/L NaF and protease inhibitor cocktail). Immune complex samples were separated by SDS-PAGE, transferred to nitrocellulose, and incubated with appropriate primary antibodies (overnight; 4°C). After washing and incubation with secondary antibodies (LiCor Biosciences, Lincoln NE), immunoreactive proteins were visualized with the Odyssey LiCor Infrared Imaging System. Densitometry of blots was performed using Image J software (version 1.36b, National Institutes of Health).

Reference

**Supplemental Figure I**

*En face* staining demonstrates differential regulation of Src pY416 and pY527 in KO animals compared to control. Aortas of control (A-C; H-J) or KO (D-F; K-M) were immuno-stained for VE-cadherin (A, D, H, K), Src pY416 (B, E), Src pY527 (I, L) or presented as merged images (C, F, J, M).
Supplemental Figure II

*En face* staining demonstrates equal amounts of Src in control and KO animals.
Supplemental Figure III

Negative controls for *En face* staining demonstrate low green channel background in various regions of the aorta: s-flow, d-flow and spinal artery exit. Aortas were stained for VE-cadherin (A, D, G), IgG control (B, E, H), or no antibody and no IgG (J, K) or presented as merged images (C, F, I, L). No significant background signals were observed.
Immunofluorescence assay demonstrate different cellular distribution and phosphorylation state of Src in response to flow. HUVEC exposed to s-flow (A-C & G-I) or d-flow (D-F & J-L) were immuno-stained for pY416 (A, D), pY527 (G, J), VE-cadherin (B, E, H, K) or merged (C, F, I, L). (N=4).

Supplemental Figure IV
Supplemental Figure V

Western blot analysis demonstrates Src pY416 is regulated by TXNIP expression. HUVEC transfected with control siRNA or TXNIP siRNA (A) were left untreated and immuno-blotted for pY416, pY527, Src and TXNIP. (B, C) Quantification of the data using Image J (* P<0.05 vs. control siRNA; n=3).
Supplemental Figure VI

Immunofluorescence assay demonstrate different cellular distribution and phosphorylation state of Src after TXNIP depletion. HUVEC transfected with control siRNA (A-C & G-I) or TXNIP siRNA (D-F & J-L) were left untreated and immuno-stained for pY416 (A, D), pY527 (G, J), VE-cadherin (B, E, H, K) or merged (C, F, I, L). (M, N) Quantification of the data using Image J show pSrc intensity (* P<0.05 vs. control siRNA; n=4).
Supplemental Figure VII

Confocal microscopy demonstrates different cellular distribution and phosphorylation state of Src after TXNIP depletion. HUVEC transfected with control siRNA (A-C & H-J) or TXNIP siRNA (D-F & K-M) were left untreated and immuno-stained for pY416 (A, D), pY527 (H, K), VE-cadherin (B, E, I, L) or merged (C, F, J, M).
Supplemental Figure VIII

(A) Quantification of VE-cadherin-SHP2 interaction under s- or d-flow conditions (* P<0.05 vs. control siRNA; n=3). (B) Colocalization quantification using ImageJ of VE-cadherin-SHP2 shown in panels C (* P<0.05 vs. control siRNA; n=3). (C) SHP2 subcellular localization after transfection of Control or TXNIP siRNA. White arrowheads demonstrate areas of SHP2 redistribution and colocalization.
Supplemental Figure IX

Western blot analysis demonstrates CSK pSer364 is regulated by TXNIP expression. HUVEC transfected with control siRNA or TXNIP siRNA (A) were left untreated and immuno-blotted for CSK pSer364. (B) Quantification of the data using Image J (* P<0.05 vs. control siRNA; n=3).
Supplemental Figure X

Quantification of *in vivo* stress fiber formation and TXNIP expression experiments demonstrate differential regulation of TXNIP and stress fibers in s-flow and d-flow regions (*P*<0.05 vs. s-flow, n=5).
Supplemental Figure XI

Quantification of *in vitro* stress fiber formation experiments demonstrate differential regulation of TXNIP and stress fibers in response to no flow, s-flow and d-flow conditions (* P<0.05 vs. control siRNA, n=5).