The Scaffolding Protein EBP50 Promotes Vascular Smooth Muscle Cell Proliferation and Neointima Formation by Regulating Skp2 and p21cip1

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Objective—The Ezrin-radixin–moesin–binding phosphoprotein 50 (EBP50) is a scaffolding protein known to regulate ion homeostasis in the kidney and intestine. Previous work showed that EBP50 expression increases after balloon injury in rat carotids. This study was designed to determine the role of EBP50 on vascular smooth muscle cells (VSMC) proliferation and the development of neointimal hyperplasia.

Methods and Results—Wire injury was performed in wild type (WT) and EBP50 knockout (KO) mice. Two weeks after injury, neointima formation was 80% lower in KO than in WT mice. Proliferation of KO VSMC was significantly lower than WT cells and overexpression of EBP50 increased VSMC proliferation. Akt activity and expression of S-phase kinase protein2 decreased in KO cells resulting in the stabilization of the cyclin-dependent kinase inhibitor, p21cip1. Consequently, KO cells were arrested in G0/G1 phase. Consistent with these observations, p21cip1 was detected in injured femoral arteries of KO but not WT mice. No differences in apoptosis between WT and KO were observed.

Conclusion—EBP50 is critical for neointima formation and induces VSMC proliferation by decreasing S-phase kinase protein2 stability, thereby accelerating the degradation of the cell cycle inhibitor p21cip1. (Arterioscler Thromb Vasc Biol. 2012;32:33-41.)

Key Words: EBP50 ■ Skp2 ■ p21cip1 ■ proliferation ■ smooth muscle ■ NHERF1

Neointimal hyperplasia is a common complication of atherosclerosis and surgical vascular interventions, particularly angioplasty. Although it is clear that neointima formation following arterial injury originates from several cellular processes, including the initial inflammatory response, increased matrix production and migration of vascular smooth muscle cells (VSMC), the proliferation of VSMC is a major contributing factor. Consequently, several studies examined how manipulation of the expression of molecules involved in cell cycle progression affects neointimal hyperplasia. Of particular interest in this respect are the cyclin-dependent kinase inhibitors p21cip1 and p27kip1.

Studies in animal models clearly demonstrated that increased expression of p21cip1 in vessels decreases the progression and severity of neointima formation following injury. p21cip1 is undetectable in uninjured human and porcine arteries but its expression increases following injury and inversely correlates with VSMC proliferation.2 Adenoviral delivery of p21cip1 inhibits neointimal thickening in rats and pigs.2,3 Moreover, p21cip1 mediates the antiproliferative effect of nitric oxide on VSMC.4 However, 2 studies show that reduction of p21cip1 does not increase neointimal hyperplasia in animal models.5,6 Similar to p21cip1, p27kip1 exerts important effects on disease progression. Overexpression of p27kip1 in pig arteries reduced intima formation by 50%.7 Increased expression of p27kip1 attenuates neointima formation,7 and expression of a dominant negative form of Skp2 inhibits intimal hyperplasia by increasing p27kip1.8 The upregulation of p27kip1 accounts, at least in part, for the remarkable inhibition observed on adenoviral delivery of a parathyroid hormone–related protein (PTHrP) devoid of the nuclear localization signal.9

The ezrin–radixin–moesin–binding phosphoprotein 50 (EBP50), also known as NHERF1, is a cytoplasmic adaptor protein involved in the regulation of a variety of membrane receptors, channels, and transporters.10 EBP50 contains 2 tandem PDZ motifs that mediate protein-protein interactions.11 n addition, EBP50 possesses a C-terminal merlin–ezrin–radixin–moesin binding motif that tethers the protein to the cytoskeleton.11 EBP50 is highly expressed in the kidney and the small intestine, where it exerts important regulatory functions on electrolyte transporters. The role of EBP50 on vascular function is largely unknown. We showed that in rat carotid arteries EBP50 expression is restricted to the endothelium and the adventitia with little (but detectable)
expression in VSMC. However, EBP50 expression increases during neointima formation following arterial injury and its expression inhibits the antiproliferative effect of the parathyroid hormone type 1 receptor, suggesting that this adaptor protein may contribute to neointimal hyperplasia.

The studies reported here establish important effects of EBP50 in the response of VSMC to injury. Specifically, EBP50 is critical for neointima formation and regulates VSMC proliferation by decreasing the stability of the cell cycle inhibitor p21

Materials and Methods

Detailed methodologies for femoral artery injury, cell culture and transfection, proliferation and fluorescence-activated cell sorting assays, immunohistochemical staining and microscopy analysis, Western blot analysis, RT-PCR, and statistical methods are described in the Supplemental Methods, available online at http://atvb.ahajournals.org.

Experimental Animals and Surgeries

Animal studies were performed on 10-week–old wild type (WT) C57BL/6 mice and EBP50 knockout (KO) littermates. Injury was performed with a 0.014" guide wire passed within the femoral artery 3 times. The right femoral artery was used as a control uninjured artery. Femoral arteries were removed 2 weeks after surgery. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Immunofluorescence and Immunohistochemistry

Femoral arteries (both control and injured) were excised from WT and KO mice and stained with hematoxylin and eosin. In WT mice exuberant neointima formed in 2 weeks (top right panel). Reduced neointimal hyperplasia was detected in KO mice (bottom right panel). Of note, the proliferative response in the adventitia is evident in both mouse strains (right panels). Sections of femoral arteries 2 weeks after injury were fixed and immunostained for EBP50 (in red). Shown in green are autofluorescent elastic fibers. Nuclei were visualized with DAPI (in blue).

Primary VSMC Culture and Transfection

Primary VSMC were isolated from abdominal aortic explants and cultured in Dulbecco’s modified eagle media containing 10% fetal bovine serum (FBS) in 5% CO2 at 37°C. All experiments were performed with cells between passages 3 and 15. Cells were transfected with siRNA for EBP50 and p21

Results

Role of EBP50 on Neointima Formation

The observation that EBP50 expression increases after endoluminal injury suggests that it contributes to neointimal hyperplasia. To determine directly if this is the case, wire injury was performed in 10-week–old WT and KO male littermate mice. As shown in Figure 1A and 1B, exuberant neointima formed in WT mice (top panels) 2 weeks after injury. In contrast, neointima formation was significantly reduced in KO mice (lower panels). The histomorphometric
parameters of the injured vessels are shown in Figure 1D. The neointimal area in WT mice was significantly larger than in KO mice, whereas the areas of the media were not different. Consequently, KO mice displayed an 80% reduction in neointima/media ratio compared to WT mice (2.54 ± 0.58 in WT mice versus 0.53 ± 0.25 in KO mice, \( P = 0.02, N = 5 \)) (Figure 1C).

To better characterize the cellular composition of the vessels, control and injured femoral arteries from WT and KO mice were immunostained for CD31 (to visualize endothelial cells) and \( \alpha \)-smooth muscle actin (to visualize smooth muscle cells). No major differences were observed in uninjured vessels from WT and KO mice (Figure 1B, left panels). Two weeks after angioplasty, the neointima in WT and KO vessels was predominantly composed of cells expressing \( \alpha \)-smooth muscle actin (Figure 1B in green) and a complete endothelial lining was observed (Figure 1B in red). Cells in the adventitia did not express \( \alpha \)-smooth muscle actin, compatible with the predominant presence of fibroblasts. The expression of EBP50 in injured vessels was determined by immunofluorescence (Figure 1E and F). Consistent with previous observations in rat carotid arteries, EBP50 expression in the intima of injured femoral arteries was significantly higher than the media (ratio intima/media 3.2 ± 0.3, \( P < 0.004, N = 4 \)). A similar increase was determined in the adventitia (ratio adventitia/media 4.3 ± 1.3, \( P < 0.03, N = 4 \)).

**Figure 2.** Ezrin–radixin–moesin–binding phosphoprotein 50 (EBP50) induces vascular smooth muscle cells (VSMC) proliferation. A, EBP50 expression in WT and KO VSMC was determined by immunoblot (top panel). Beta-actin was used as a loading control (lower panel). B, Proliferation of wild type (WT) and knockout (KO) VSMC maintained in 10% fetal bovine serum determined by \([3H]\)-thymidine incorporation. *, \( P < 0.002 \). C, Proliferation of WT and KO VSMC maintained in 10% fetal bovine serum determined by cell counting. Ten thousand cells were plated on day 0 and counted at the indicated times. D, Proliferation of WT VSMC transfected with control siRNA (si-C) or siRNA for EBP50 (si-EBP50) determined by \([3H]\)-thymidine incorporation. *, \( P < 0.002 \). Insert shows the expression of EBP50 in si-C and si-EBP50 treated cells. E, Proliferation of WT VSMC transfected with control plasmid (pcDNA3) or EBP50 determined by \([3H]\)-thymidine incorporation. *, \( P < 0.0001 \). Insert shows the expression of EBP50 in control and EBP50-transfected cells.

**EBP50 Affects VSMC Proliferation**

The preceding observations raise the possibility that EBP50 contributes to neointimal hyperplasia by increasing VSMC proliferation, a key cellular event leading to neointima formation. VSMC proliferation in vivo following injury was determined by immunodetection of Ki67 (Figure 1G, 1H, and 1I). The percentage of Ki67-positive cells in the intima was significantly reduced in vessels from KO mice compared to WT vessels (Figure 1H). The number of Ki67-positive cells in the adventitia of WT and KO arteries was not significantly different (Figure 1I). To further characterize the effect of EBP50 on VSMC proliferation, we used primary VSMC isolated from WT and KO littersmates. Western blot analysis verified that EBP50 was expressed in WT cells but not in KO cells (Figure 2A). Thymidine incorporation assays and cell counting demonstrated that proliferation of KO cells was significantly lower than that of WT cells (Figure 2B and C). Similarly, the proliferation of WT cells was significantly reduced by inhibition of EBP50 expression with siRNA (Figure 2D). Conversely, increasing expression of EBP50 (by electroporation) increased WT VSMC proliferation (Figure 2E).

To determine which cell cycle phase is affected by EBP50, the cell cycle distribution of WT and KO cells grown in 10% FBS was determined by fluorescence-activated cell sorting analysis. As shown in Figure 3A and Supplemental Figure 1A, WT cells display a cell cycle distribution with 56% G0/G1, 25% S, and 19% G2/M. In contrast, KO cells display a distribution of 79% G0/G1 (\( P < 0.04 \) versus WT), 10% S (\( P < 0.02 \) versus WT), and 11% G2/M (\( P < 0.05 \) versus WT). These experiments indicate that the absence of EBP50 causes cell cycle arrest in G0/G1, with consequent inhibition of cell proliferation.

**EBP50 Regulates p21cip1 Expression**

Next, the expression of the critical regulators of the G1-S transition was determined in WT and KO cells. Western blot analysis indicated equal expression of cyclin E and cyclin D1 (Supplemental Figure 1B). Similarly, the expression of cdk4 was unchanged in WT and KO cells. A small, not statistically significant, reduction in cdk2 and cyclin D3 expression was observed in KO cells (Supplemental Figure 1B). In striking contrast, the expression of the cyclin-dependent kinase inhibitor p21cip1 was 8-fold higher in KO cells compared to WT (Figure 3B and C). Identical observations were made in independent preparations from 2 other mice (Supplemental Figure 1C). Quantitative RT-PCR showed that the mRNA levels of p21cip1 were similar in WT and KO cells (Figure 3D), suggesting that EBP50 regulates p21cip1 expression posttranslationally. Consistent with this interpretation, the expression of p53, a well-known upstream regulator of p21cip1 transcription, was unchanged in KO cells (Figure 3B). In addition, the regulation of p21cip1 expression by EBP50 was fully recapitulated in Chinese Hamster Ovary cells following recombinant expression of myc-tagged p21cip1 and EBP50 under the control of CMV promoters (Supplemental...
both WT and KO cells on treatment with the proteasome inhibitor MG132. The increase in WT cells was significantly higher than in KO cells, suggesting an increased degradation rate of p21cip1 in WT cells (Supplemental Figure IIB and IIC). Similarly, the total levels of p27kip1 increased on treatment with MG132, but the effect was similar in WT and KO cells (Supplemental Figure IIC). To determine whether the expression of EBP50 correlates with p21cip1 degradation, p21cip1 was visualized in KO VSMC transfected with YFP-EBP50. p21cip1 was detected in the nucleus of more than 90% of KO VSMC. In contrast, only 50% of cells expressing YFP-EBP50 were also positive for p21cip1 (P=0.0001) (Supplemental Figure III). Collectively, these experiments show that p21cip1 levels are lower in cells expressing EBP50, and that EBP50 increases degradation of p21cip1. Indeed, the half-life of p21cip1 (determined in the presence of cyclohexamide) was reduced in WT cells compared to KO cells (45±8 minutes in WT cells versus 82±16 minutes in KO cells) (Figure 4A and Supplemental Figure IV).

**EBP50 Regulates Skp2 Expression**

The reduced degradation of p21cip1 in EBP50 KO cells suggests that EBP50 may regulate the activity of the Skp1/Cullin-1/F-box (SCF) E3-ligase responsible for p21cip1 degradation. Of particular interest is the F-box protein Skp2 that directs recognition of p21cip1 by the SCF complex. The expression of Skp2 was reduced in primary VSMC from KO mice (Figure 4B). Similar reduction in Skp2, accompanied by a corresponding increase in p21cip1, was observed in WT cells in which EBP50 expression was inhibited by 2 distinct lentivirus expressing shRNA against EBP50 (Figure 4C, left panel). Conversely, expression of YFP-EBP50 in VSMC increased Skp2 and reduced p21cip1 (Figure 4C, right panel). The relation between EBP50 and Skp2 expression was established in distinct VSMC preparations in which EBP50 expression was reduced to various degrees by shRNA. As shown in Figure 4D, a direct and significant correlation was found between the expression of EBP50 and that of Skp2 in primary VSMC. The stability of Skp2 (determined in the presence of cyclohexamide) was reduced in KO cells compared to WT cells (Figure 4E). In contrast, mRNA for Skp2 was not different in WT and KO VSMC (Figure 4F), indicating that EBP50 predominantly regulates Skp2 expression posttranslationally. To determine if the expression level of Skp2 is critical to VSMC proliferation, KO VSMC were transiently transfected with Flag-Skp2. Immunofluorescence analysis showed the clear localization of Flag-Skp2 in the nucleus (Supplemental Figure V). In these cells, proliferation (determined by costaining for BrDU) was significantly increased (Figure 4G).

Collectively, these experiments show that EBP50 regulates Skp2 levels and, consequently, p21cip1 stability. However, we found no difference in p27kip1 expression in WT and KO cells (Figure 3B). This suggests the possibility that p27kip1 degradation is less sensitive to modest variations in Skp2 expression than p21cip1. Indeed, overexpression of Skp2 in KO VSMC efficiently decreased both p21cip1 and p27kip1 (Supplemental Figure VI).

**Figure 3. Effect of ezrin-radixin-moesin–binding phosphoprotein 50 (EBP50) on vascular smooth muscle cell (VSMC) cycle and p21cip1 expression.** A, Cell cycle distribution analyzed by FACS in wild type (WT, gray bars) and knockout (KO, white bars) VSMC. The percentage ±SE of the population of cells in G1, S, and G2/M phases are derived from 5 independent experiments. Probability values for WT vs KO are indicated. B, Immunoblots for p21cip1, p27kip1, and p53 in WT and KO VSMC. Beta-actin was used as loading control. Blots are representative of at least 5 independent experiments. C, Quantification of the expression of p21cip1 in WT (gray bars) and KO (white bars) VSMC. The ratio of p21cip1 expression over β-actin was determined in 5 independent experiments. *P<0.04. D, Quantitative RT-PCR for p21cip1 and p27kip1 using mRNA from WT (gray bar) and KO (white bar) VSMC. Results are presented as fold over WT ±SE of triplicate determinations. Values were normalized relative to GAPDH. E, Inhibition of p21cip1 expression by siRNA increases VSMC proliferation. Immunoblot for p21cip1 in KO VSMC transfected with either control siRNA (si-C) or p21cip1 siRNA (si-p21cip1) as indicated. Beta-actin is used as loading control. Proliferation of KO VSMC transfected with either control siRNA (si-C) or p21cip1 siRNA (si-p21cip1) and maintained in 10% fetal bovine serum determined by [3H]-thymidine incorporation. Data are presented as percentage of si-C treated cells ±SE of triplicate determinations. Similar results were obtained in 2 additional experiments. *P<0.003.

Figure 3D). The expression of p27kip1, another inhibitor of the G1-S transition, was equal in WT and KO cells (Figure 3B and 3D), indicating that the regulation of p21cip1 by EBP50, and the consequent effects on cell cycle progression, are specific. To determine if the expression of p21cip1 is critical for the different proliferation of WT and KO VSMC, its expression in KO cells was inhibited by siRNA. This treatment resulted in an 80% reduction in p21cip1 protein (Figure 3E). Decreasing p21cip1 expression significantly accelerated KO cell proliferation (Figure 3E), indicating that the expression level of this cell cycle inhibitor is sufficient to regulate SMC growth.14

Immunofluorescence analysis of KO VSMC demonstrated high expression and clear nuclear localization of p21cip1 (Supplemental Figure IIA). In contrast, p21cip1 expression was significantly reduced in WT VSMC (Supplemental Figure IIA). Total levels of p21cip1 increased in
EBP50 Regulates Akt Activity

Akt is a major transducer of mitogenic signals in a variety of cells. Akt is activated by arterial injury and contributes to neointima formation. Because EBP50 regulates growth factors signaling and Akt activity in a cell-specific manner, we determine Akt activity in WT and KO VSMC. As shown in Figure 5A, serum stimulation of previously starved WT cells induced robust phosphorylation of Akt after 5 and 30 minutes. In contrast, the response of KO cells to the same stimulus was blunted. Similar reduction in Akt activation was determined in KO cells on stimulation with EGF (Figure 5B). Moreover, as reported previously, EBP50 coimmunoprecipitated with Akt (Figure 5C and Supplemental Figure VIII), an interaction that potentiates Akt activation. The expression and cellular localization of phosphatase and tensin homolog (PTEN), a regulator of Akt, was not different in WT and KO VSMC (Supplemental Figure IX). Collectively, these experiments show that EBP50 potentiates Akt activity in VSMC by regulating growth factor receptor signaling and by directly interacting with Akt.

EBP50 Promotes Neointima Formation

The reduction of Skp2 expression in EBP50 KO cells prompted us to determine if, in addition to the reduction in cell proliferation, other phenotypes observed on deletion of Skp2 were also evident in EBP50 knockout. The body weight of EBP50 KO mice was reduced compared to WT littermates up to seven weeks of age, after which the mice were of similar size (Supplemental Figure VIIA). Skp2 null cells are enlarged compared to normal cells. Consistent with these observations, EBP50 KO VSMC were larger than WT cells (Supplemental Figure VIIIB).

Figure 4. Ezrin-radixin–moesin–binding phosphoprotein 50 (EBP50) regulates Skp2 and p21cip1 stability. A, Primary vascular smooth muscle cell (VSMC) from wild type (WT) (upper panels) and knockout (KO) (lower panels) mice were treated with cyclohexamide (10 μg/mL) or MG132 (2 μM/L) for the indicated times. Equal amounts of proteins were analyzed by immunoblotting for p21cip1 and β-actin (as loading control). Data are presented as mean (± SE) of the p21cip1 intensity (normalized with β-actin) relative to time zero. *P<0.05, N=3. B, The expression of Skp2 was determined by immunoblotting in WT and KO cells as indicated. C, WT VSMC were infected with control adenovirus expressing GFP or two distinct shRNA against EBP50 (shEBP50 #1 and #2) (left panel) or transfected with YFP-EBP50 (right panel). The expression of EBP50, Skp2, and p21cip1 was determined by immunoblotting. D, Correlation between EBP50 and Skp2 expression in 6 independent VSMC preparations. E, Primary VSMC from WT and KO mice treated as described in A were analyzed by immunoblotting for Skp2 and β-actin (as loading control). Graph shows the quantitation of three independent experiments. Data are presented as mean (± SE) of the Skp2 intensity (normalized with β-actin) relative to time zero. *P<0.008, N=4. F, Quantitative RT-PCR for Skp2 using mRNA from WT (gray bar) and KO (white bar) VSMC. Results are presented as fold over WT±SE of triplicate determinations. Values were normalized relative to GAPDH. G, Primary VSMC from KO mice were electroporated with control vector (pcDNA3) or Flag-Skp2 and cultured in the presence of BrDU for 16 hours. Cells were fixed and stained for BrDU and Flag. The percentage of BrDU positive cells was determined from 3 independent experiments. *P=0.04, N=3.
The preceding observations raise an intriguing question. In most mammalian cells, Akt regulates Skp2 expression both transcriptionally and by increasing its stability.\textsuperscript{27,28} The latter effect has been attributed to the direct phosphorylation of Skp2 by Akt.\textsuperscript{27,28} However, Akt does not phosphorylate mouse Skp2.\textsuperscript{27} Thus, the mechanisms by which Akt regulates Skp2 expression in mouse cells, and the possible involvement of EBP50, remain unclear. Consistent with other reports,\textsuperscript{14,27,28} inhibition of Akt with either LY294002 or AKTI reduced Skp2 expression in VSMC maintained in serum-supplemented media (Figure 5F), confirming the key role of Akt on Skp2 transcription. Furthermore, immunoprecipitation assays show that EBP50 constitutively interacts with Skp2 and this interaction is increased by expression of a constitutively active form of Akt (Myr-Akt) (Figure 5G). This observation suggests a possible mechanism for the observed stabilization of Skp2 in EBP50 expressing mouse cells.

**EBP50 Regulates p21cip1 Expression In Vivo**

The preceding observations in primary VSMC clearly indicate that in the absence of EBP50 the stability of p21cip1 is increased resulting in inhibition of cell proliferation. To determine if similar regulation occurs in vivo, the expression of p21cip1 was determined in the femoral arteries of WT and KO mice. p21cip1 was undetectable in control uninjured vessels (Figure 6 left panels). Similarly, little expression of p21cip1 was observed in injured vessels of WT mice. In contrast, p21cip1 was present both in the neointima and in the adventitia of femoral arteries of KO mice 2 weeks after injury (Figure 6, right panels). Consistent with the findings in cells, analysis of p21cip1 expression in WT and KO femoral arteries revealed similar expression in both control and injured vessels (Supplemental Figure XI).

VSMC growth is the balance between proliferation and apoptosis, and p21cip1 plays important roles on both cellular events. To determine if the different expression of p21cip1 in WT and KO mice affects VSMC survival, apoptosis in injured vessels was determined by TUNEL staining. The number of apoptotic cells in the intima and media of WT and KO vessels 2 weeks after injury was very low (approximately 0.25% of total cells) and not different between mice (Supplemental Figure XII). To assess possible differences in apoptosis on cellular stress, TUNEL assays were performed in WT VSMC and in cells in which EBP50 expression was inhibited by shRNA following 24 hours of serum starvation. Again, no differences in TUNEL staining were observed between control and shRNA-treated cells (Supplemental Figure XI).}

Figure 5. Ezrin–radixin–moesin–binding phosphoprotein 50 (EBP50) ablation decreases Akt signaling and Skp2 expression. **A**, Wild type (WT) and knockout (KO) vascular smooth muscle cells (VSMC) were serum-starved for 16 hours followed by the addition of 10% fetal bovine serum (FBS) for the indicate times. Phosphorylated and total Akt were determined by immunoblotting. Blots are representative of 4 independent experiments. **B**, WT and KO VSMC were serum-starved for 16 hours followed by the addition 10 ng/mL EGF for the indicate times. Phosphorylated and total Akt were determined by immunoblotting. Graph shows the quantitation of 3 independent experiments. *<0.05. **C**, Primary VSMC were transiently transfected with Myc-Akt and Flag-EBP50, as indicated. Cells were lysed, and immunoprecipitation experiments were performed with anti-Flag antibody followed by SDS-PAGE and immunoblotting with anti-Flag or anti-Myc antibodies. **D**, Proliferation (determined by 3H-thymidine incorporation) of WT and KO VSMC treated without or with 10 μmol/L LY-294002 (LY) or 5 μmol/L AKTI for 16 hours and maintained in 10% FBS. Data are expressed as mean±SE for triplicate determinations. Shown is also a Western blot analysis of phosphorylated and total Akt in similarly treated cells. **E**, Primary VSMC from KO mice were electroporated with YFP-EBP50, treated without or with 10 μmol/L LY-294002 (LY) or 5 μmol/L AKTI in the presence of BrDU for 16 hours. Cells were fixed and stained for BrDU. The percentage of BrDU positive nontransfected and YFP-EBP50-expressing cells was determined from 3 independent experiments. *P=0.03 vs EBP50, #P<0.02 vs vehicle, &P<0.01 vs vehicle; N=3. **F**, VSMC maintained in 10% FBS were treated without or with 10 μmol/L LY-294002 (LY) or 5 μmol/L AKTI. Skp2 expression was determined by immunoblotting. Beta-actin was used as loading control. **G**, Chinese Hamster Ovary cells were transiently transfected with constitutively active HA tagged Myr-Akt and Flag-EBP50, as indicated. Cells were lysed, and immunoprecipitation experiments were performed with anti-Flag antibody followed by SDS-PAGE and immunoblotting with anti-Flag, anti-HA, or anti-Skp2 antibodies.
The major finding in this report is the remarkable effect that the scaffolding protein EBP50 has in the development of neointimal hyperplasia following arterial injury. Two key and novel observations are presented. First, genetic ablation of EBP50 in mice results in almost complete inhibition of restenosis following endothelial denudation. Second, EBP50 exerts important effects on the cell cycle of VSMC, in particular by regulating the expression of Skp2 and the stability of the cell cycle inhibitor p21cip1.

EBP50 is widely expressed and its function in the regulation of electrolyte transport has been well-characterized in the kidney and intestine.29,30 The original observation that EBP50 inhibition of neointimal hyperplasia while reducing p21cip1 expression.6 Collectively, these observations indicate that although p21cip1 is competent to reduce VSMC proliferation and inhibit neointima formation, its absence does not necessarily promote these events, suggesting the existence of compensatory effects from other cell-cycle controlling proteins (such as p27kip1). Interestingly, the regulation of p21cip1 by EBP50 occurs entirely posttranslationally. This is supported by several lines of evidence. The mRNA levels of p21cip1 and the expression of p53, the major regulator of p21cip1 transcription,31 are identical in WT and KO cells. The half-life of p21cip1 was increased in KO cells compared to WT cells. Inhibition of the proteasome by MG132 increased total p21cip1 levels in both WT and KO cells, but to a greater extent in WT cells, indicating an increased degradation of p21cip1 in these cells. Finally, experiments in single cells indicate that EBP50 expression inversely correlates with p21cip1 expression.

These observations indicate that EBP50 facilitates p21cip1 degradation. This process is dependent on the action of the E3 ubiquitin ligase complex consisting of Cullin-1, Skp1, and RBX1, as well as Skp2, which regulates substrate recognition.15,16 We show that ablation of EBP50 induces a marked decrease in Skp2 and, consequently, stabilization of p21cip1 in cultured cells and in vivo. Conversely, EBP50 overexpression increases Skp2 and decreases p21cip1 levels in VSMC. Consistent with these findings, adenoviral delivery of a dominant negative form of Skp2 reduced VSMC proliferation and neointima formation in rats.8 Several studies demonstrated that the PI3K/Akt pathway is a major regulator of Skp2 expression.14,27,28,32 Indeed, complete inhibition of Akt abrogated Skp2 expression in primary VSMC. We did not detect differences in Skp2 mRNA between WT and KO cells—possibly indicating that the level of Akt signaling in KO cells is sufficient to fully induce gene expression. However, the stability of Skp2 in WT cells was increased. Interestingly, direct phosphorylation of Skp2 by Akt, leading to cytoplasmic retention and stabilization of Skp2,27,28 does not occur in mice because Ser72, the site of phosphorylation, is a Gly in the murine Skp2. It should be noted that the importance of phosphorylation of Ser72 for the cellular localization of Skp2 is still controversial.33 Our observations indicate that a different mechanism mediates Akt-dependent regulation of Skp2. The interaction between EBP50 and Skp2 and the potentiation of this interaction by a constitutively active Akt provide a possible explanation for the regulation of Skp2 stability by Akt in mouse cells. Further studies are required to establish the molecular events underlying the effect of Akt on Skp2 stability in murine systems.

EBP50 deficiency attenuates Akt activity in primary VSMC. This regulation occurs at several levels. First, EBP50 interacts constitutively with Akt and increases Akt activation.26 Second, EBP50 increases the function of the receptors for epidermal growth factor and platelet-derived growth factor, 2 major activators of Akt signaling in VSMC.21,22 EBP50 has been shown to regulate membrane localization, and therefore activity, of the PI3K inhibitor PTEN in glioblastoma cells.23 However, we detected no differences in PTEN expression and localization in VSMC from WT and KO mice. It is interesting to observe that the effect of EBP50...
on proliferation is remarkably tissue- and cell-specific. EBP50 is overexpressed in glioblastomas, hepatocellular, and breast carcinomas. Yet, the direction of the effect of EBP50 on cell growth is variable. Although in breast cancer cells and in embryonic fibroblasts EBP50 inhibits cell proliferation, in biliary epithelial cells, as in VSMC, EBP50 stimulates cell growth. EBP50 regulates a variety of molecules involved in cell growth, including growth factor and protein-coupled receptors, β-catenin and PTEN. Therefore, whether EBP50 stimulates or inhibits proliferation depends on the relative importance of these signaling pathways. We show here that the PI3K/Akt/Skp2/p21cip1 is the major pathway mediating the proliferative effect of EBP50 on VSMC. In contrast, the effect of EBP50 on adventitial cells, predominantly fibroblasts, was not different between WT and KO mice.

EBP50 does not alter the regulation of p27kip1 in VSMC, consistent with observations made in clonal rat A10 cells. This finding is somewhat puzzling because, like p21cip1, p27kip1 is subject to regulation by Akt and ubiquitin ligase complexes. We observed that p27kip1 was actively degraded in both WT and KO cells. This suggests the possibility that p27kip1 degradation is less sensitive to variations in Akt signaling and Skp2 expression than p21cip1. It is important to note that in the primary cultures used in these studies neither Akt signaling nor Skp2 expression were entirely abrogated or overactivated. In fact, overexpression of Skp2 in WT VSMC reduced both p21cip1 and p27kip1. In addition, p21cip1 and p27kip1 are targeted by more than 1 E3 ligase and by different kinases. It is also possible that EBP50 regulates the ability of Skp2 to specifically recognize and discriminate substrates for degradation. Determining the exact molecular mechanisms underlying the effect of EBP50 on substrate recognition by Skp2 requires further investigation.

In summary, we describe the identification of the scaffolding protein EBP50 as a key regulator of vascular remodeling. EBP50 is required for neointima formation following endoluminal arterial injury in mice and, by controlling Skp2 expression and the stability of p21cip1, exerts important actions on cell cycle progression of VSMC. These findings reinforce the emerging importance of scaffolding and adaptor proteins as major regulators of physiological and pathological events.

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Disclosures
Nathalie M. Fiaschi-Taesch is coinventor on a United States patent application no. 20050261183. Nathalie M. Fiaschi-Taesch has a financial interest in Vasculostatin LLC. All other authors have nothing to disclose.

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The scaffolding protein EBP50 promotes VSMC proliferation and neointima formation by regulating Skp2 and p21\(^{cipl}\).

SUPPLEMENTAL MATERIAL

Supplemental Methods

*Experimental animals and surgeries.* Animal studies were performed in 10 weeks old wild type (WT) C57BL/6 mice and EBP50 knockout (KO) littermates. Mice were anesthetized using Ketamine (45 mg/kg) and Xylazine (5 mg/kg) i.p., shaved locally and washed with betadine. The left femoral artery was exposed and lidocaine was topically applied to induce vasodilatation. A guidant hi-torque guide wire 0.014" was inserted into the femoral artery and passed within the artery 3 times. The right femoral artery was used as a control uninjured artery. Sterile packaged autoclips were used to close the wound and the area was wiped with betadine. Femoral arteries were removed 2 weeks post surgery. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

*Immunofluorescence and immunohistochemistry.* Femoral arteries (both control and injured) were excised from WT and KO mice, fixed with 4% paraformaldehyde and embedded in OCT Tissue-Tek (Sakura Finetek). Sections (10 \(\mu\)m) were analyzed by immunohistochemistry. For CD31 and \(\alpha\)-actin staining, sections were treated with 10% goat serum/ 0.5% TritonX-100 and incubated sequentially with rat anti-mouse CD31 (BD Pharmingen), Alexa546-conjugated anti-rat secondary antibody (Invitrogen) and FITC-conjugated anti-smooth muscle \(\alpha\)-actin antibody (Sigma) for 1 h at RT. For EBP50 and Ki67 (cellular marker for proliferation) staining, sections were incubated in boiled Tris-EDTA buffer (10 \(\mu\)M Trizma base, 1.25 \(\mu\)M EDTA, 0.05% Tween 20, pH 9.0) for 10 min, blocked in 4% NGS, 1% BSA, 0.5% Triton X-100 for 20 min at room temperature and incubated with anti-EBP50 antibody (Thermo Pierce, 1:250 dilution) or Ki67 antibody (Labvision, 1:100 dilution) overnight. As a secondary antibody, anti-rabbit Alexa546-conjugated IgG (Invitrogen, 1:250 dilution for EBP50 and 1:200 for Ki67) was used.

For p21\(^{cipl}\) and p27\(^{kip1}\) staining, sections were boiled in citric acid buffer for 10 min and incubated with 0.3% H\(_2\)O\(_2\) in 80% methanol for 30 min. Anti-p21\(^{cipl}\) and anti-p27\(^{kip1}\) antibodies (Santa Cruz, 1:100 dilution) were added in 1% goat serum and 1% BSA after blocking in 5% normal goat serum and 1% BSA in PBS for 30 min. As a secondary antibody, anti-mouse IgG HRP-conjugated (Cell signaling, 1:250...
dilution) was used. After washing with PBS, sections were incubated with ABC reagent for 30 min and visualized with DAB kit (VECTOR).

Expression of p21<sup>cip1</sup> and PTEN in VSMC was determined as follows. Cells on glass coverslips were fixed with 2% paraformaldehyde and incubated with blocking buffer containing 5% goat serum and 0.2% Nonidet P-40 (NP-40), 0.01% sodium dodecyl sulfate (SDS) in PBS. A rabbit anti-p21<sup>cip1</sup> antibody (Santa Cruz) or anti-PTEN antibody (Santa Cruz) was applied at a dilution of 1:500 in the same buffer overnight at 4 °C. Coverslips were washed with PBS, incubated with Alexa546-conjugated anti-rabbit secondary antibody (1:1000, Molecular Probes) and 4',6-diamidino-2-phenylindole (DAPI, 0.1 μg/ml; Sigma) for 3 h and washed again. Coverslips were mounted for immunofluorescence microscopy and analyzed with an Olympus Fluoview confocal laser-scanning microscope with a x63 oil immersion objective. To quantify expression, images were acquired in five regions of the coverslip using an identical microscope setting. The focus was adjusted to obtain maximal intensity of the DAPI signal. Membrane expression of PTEN was imaged using total internal reflection fluorescence (TIRF) microscope (Nikon Ti-TIRF) equipped with 60x Oil TIRF objective (NA=1.49). Apoptosis in vessels and cultured cells was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling [TUNEL] assays using the “Click-it TUNEL Alexa Fluor594 Imaging Assay” (Invitrogen), according to the manufacturer’s instructions. The fluorescence intensity of each image was determined with Image J software (National Institutes of Health).

**Histomorphometric analysis.** Frozen femoral artery sections (10 μm thick) from WT and KO mice were selected at 100 μm intervals and stained using an Elastic Stain Kit (Sigma). H&E staining of arteries sections were performed by the University of Pittsburgh Research Histological Services. Images were captured using a Leica DM5000B light microscope using a 40x objective. Images were analyzed with the Image J software (National Institutes of Health).

**Primary VSMC culture and transfections.** Primary VSMC were isolated from abdomen aortic explants and cultured in DMEM containing 10% FBS in 5% CO<sub>2</sub> at 37°C. All experiments were performed with cells between passage 3 and 15. Small interfering RNA (siRNA) for EBP50 and p21<sup>cip1</sup> were generated by Dharmacon (Thermo Scientific) as follows. EBP50: 5'-GAAGGAGAGCAGCCGUGAAddTdT3' (sense) and 5'-UUCACGCCGUCUCUCCUUdCdTdT-3' (antisense). p21<sup>cip1</sup>: 5'-GACAAGAGGCCCAGUACU UdTdT3' (sense) and 5'-AAGUACUGGGCCCUUGUCdCdTdT-3' (antisense). Accell Non-targeting siRNA (Dharmacon, Thermo Scientific) was used as a control siRNA. Cells were transfected with siRNAs (0.1 μM) using DharmaFECT Duo (3 μl/well of 12 well plates) transfection reagent (Dharmacon, Thermo Scientific) in DMEM with 1% FBS in the absence of antibiotic according to the manufacturer’s instructions. Cells were used for the experiment 72 h after transfection.
YFP-tagged and Flag-tagged EBP50, Flag-tagged Skp2 and Myc-Akt (4 μg) were introduced in primary VSMC (1x10^6) by electroporation using an AMAXA electroporator and the Basic Nucleofect kit for primary smooth muscle cells (Lonza). EBP50, constitutively active Myr-Akt (a generous gift from Dr. Daniel Altschuler, University of Pittsburgh School of Medicine) and Myc-p21cip1 (a generous gift from Dr. Richard Steinman, University of Pittsburgh School of Medicine) was transfected in CHO cells using Fugene6. Lentiviral shRNA vectors (pLKO.1-puro) specific to mouse EBP50 (NM_012030) and GFP-expression control lentivirus (pLKO.1-puro-TurboGFP) were prepared by the Lentiviral Facility at the University of Pittsburgh Cancer Institute. The target sequences of the EBP50 shRNAs are: shEBP50 #1 GAGTTCTTCAAGAAGTGCAAA and shEBP50 #2 GACCGAATTGTGGAGGTCAAT. Aliquots of virus (titer ~10^6), plus 8 μg/mL of polybrene, were used to infect exponentially growing cells (1 x 10^5/mL). Fresh medium was supplemented at 24 hours after the infection. Cells were used 72 hours after infection.

Western blot analysis. Cells were lysated in urea lysis buffer (4M urea, 62.5mM TrisCL, 2% SDS, 1mM EDTA) containing a protease inhibitor cocktail. The cell lysates were resolved on 12% SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane, which was then subjected to two sequential incubations with appropriate primary antibodies (1:500 dilution for EBP50, cyclin E, p21cip1, cyclin D1, cyclin D3, CDK2, CDK4 and 1:1000 dilution for SKP2 (all from from Santa Cruz); 1:1000 dilution for p27kip1, pAKT, AKT, Myc tag antibodies (from Cell Signaling); 1:5000 dilution for β-actin (from Sigma)) and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (1:2000 dilution, Cell signaling). Immunoreactivity was detected by incubation with Immune-Star ECL (Bio-Rad). Quantitation of band intensity was performed with the Image J software (National Institutes of Health).

Quantitative Real-Time PCR. Total RNA from VSMC was obtained by using the RNA easy micro kit (Qiagen, Valencia, CA) following the instructions of the manufacturer. One μg of total RNA was reverse transcribed in the following reaction buffer (Promega, Madison, WI): ImProm-II reverse transcriptase (1 μl), 1X reaction buffer, RNase inhibitor (20 U), dNTP (10 mM each) and Oligo(dT)15 primer (1 μM) at 42 °C for 1 h. As a negative control, water was used instead of reverse transcriptase. cDNA was used to amplify p21cip1 and GAPDH using specific primer sets (p21cip1, forward 5’-ACGTTGGAACCTTTGACCTCG-3’ and reverse 5’-GAGTGCAAGACACGGACAAG-3’; p27kip1, forward 5’-GATACGAATGGCAGGAGGTG-3’ and reverse 5’-TCTGACGAGTCCAGGCTTGGT-3’; Skp2, forward 5’-CCAACACCTCTCGCTCAG-3’ and reverse 5’-CCAGGTCTCTCTCTTGCTGCTCC -3’; GAPDH, forward 5’-CTCATGACCACAGTCCATGC-3’ and reverse 5’-
ATGTAGGCCATGAGGTCCAC-3`). Quantitative gene expression analysis was performed on an ABI PRISM 7700 (Applied Biosystems, Warrington, UK) using SYBR Green technology. In 48-wells optical plates, 10 µl of SYBR Green master mix (Applied Biosystems, Warrington, UK) was added to 2 µl of cDNA and 300 nM forward and reverse primers in water. Following 10 min at 95 ºC, 40 cycles of 15 s at 95 ºC, 30 s at 60 ºC and 60 s at 72 ºC were applied. At the end of the run, samples were heated to 95 ºC to check melting curve. The absence of genomic DNA contamination in the RNA preparations was confirmed by using total RNA samples that had not been subjected to reverse transcription. GAPDH was used as the standard housekeeping gene. Relative gene expression was normalized by GAPDH expression.

Proliferation Assays. Cells on 24-well plates were transfected as indicated and cultured until 70-80% confluent. Cells were incubated with 1 µCi/ml [³H]thymidine in the culture media for 18 hours at 37 ºC, rinsed with PBS and exposed to 10% trichloroacetic acid (TCA) for 10 min. TCA was removed and the cell monolayers dissolved in 1 N NaOH for the determination of radioactivity. For BrdU incorporation experiments, cells were grown on coverslips and incubated in the presence of 100 µM BrdU for 16 h. After fixation with 4% paraformaldehyde, cells were permeabilized in 0.5% Triton X-100/PBS for 30 min and incubated with anti-BrdU antibody (1:100, Biodesign International) in blocking solution (10% promega RQ1 DNaseI, 1% BSA, 1% FBS in PBS) at 37ºC for 1 hour. Flag-Skp2 was visualized with anti-Flag antibody (Sigma, 1:1000) in the same buffer. Cells were rinsed in PBS, incubated with secondary antibody anti-sheep Alexa 594 (1:1000, Molecular Probes) or anti-rabbit Alexa 488 (1:1000, Molecular Probes) in PBS containing 1% BSA, 1% FBS.

For cell cycle profile, VSMC were grown to subconfluence in DMEM containing 10% FBS. Cells collected from 6 cm dishes were rinsed twice with PBS and fixed with 70% ethanol at 4 ºC overnight. Fixed cells were washed with PBS, pelleted, and resuspended in the staining PBS solution containing 50 µg/mL propidium iodide, 100 U/mL RNAse A, and 1 g/L glucose. The cell cycle profile and forward scatter were determined using a Becton Dickinson FACS Caliber, and data were analyzed using the ModFit LT 2.0 (Verity Software House, Inc.).

Statistical Analysis. Results from each experiment were averaged and expressed as mean ± S.E. Results were analyzed by ANOVA with Tukey’s test or Student’s t-test. Statistical calculations were performed with the GraphPad InStat3 software (GraphPad Software Inc., San Diego CA). P-values were considered statistically significant when lower than 0.05.
References


Supplemental Figure I. Effect of EBP50 on vascular smooth muscle cell cycle.

A. Cell cycle profile of WT (left panel) and KO (right panel) VSMC.

B. Immunoblots for key G1-S transition molecules (cyclin E/ckd2 and cyclin D/ckd4, as indicated) in WT and KO VSMC. β-actin was used as loading control. Blots are representative of 2-4 independent experiments.

C. Immunoblot for p21cip1 in two different primary VSMC preparations from WT and KO mice.

D. p21cip1 and EBP50 expression in CHO cells. Cells were transfected with the plasmids for Myc-p21cip1 and EBP50 as indicated. Total DNA in the transfections was equalized with empty plasmid (pcDNA3). Equal amount of proteins were analyzed. Blots are representative of two independent experiments.
Supplemental Figure II. EBP50 and p21cip1 expression in VSMC. A. Primary VSMC from WT (upper panels) and KO (lower panels) mice were grown on coverslips in the presence of 10% FBS. Cells were fixed and immunostained for p21cip1 (in red). Nuclei were visualized with DAPI (in blue). Higher expression and nuclear localization of p21cip1 is evident in KO cells. B. Left Panel. Primary VSMC from WT (left panels) and KO (right panels) mice were grown on coverslips in the presence of 10% FBS. Cells were treated in the absence (upper panels) or the presence (lower panels) of the proteasome inhibitor MG132 (2 μM for 6 hours) fixed and immunostained for p21cip1 (in red). Nuclei were visualized with DAPI (in blue). Right Panel. Quantification of p21cip1 expression in primary WT (black bars) and KO
(white bars) VSMC. Graph shows the average fluorescence intensity ± standard error of p21cip1 determined from sections. P values relative to untreated WT cells are shown above each bar. C. Primary VSMC from WT and KO mice were treated with MG132 (2 μM) for 6 h. Equal amounts of proteins were analyzed by immunoblotting for p21cip1, p27kip1 and β-actin (as loading control). Graph shows the quantitation of three independent experiments. Data are presented as mean (± standard error) of the p21cip1 or p27kip1 intensity (normalized with β-actin) relative to untreated cells. *, p=0.01.
Supplemental Figure III. EBP50 and p21<sup>cip1</sup> expression in VSMC. Primary VSMC from KO mice were electroporated with YFP-EBP50 (in green), fixed and stained for p21<sup>cip1</sup> (in red). Nuclei were visualized with DAPI (in blue). The arrow indicates a cell expressing p21<sup>cip1</sup> but not EBP50. The arrowhead indicates a cell expressing EBP50 but not p21<sup>cip1</sup>. C. The percentage of p21<sup>cip1</sup> positive non-transfected and YFP-EBP50-expressing cells was determined from three independent experiments. *, p=0.0001.
Supplemental Figure IV. EBP50 regulates p21<sup>cip1</sup> stability. Primary VSMC from WT and KO mice maintained in 10% FBS were treated with cyclohexamide (10 µg/ml) or MG132 (2 µM) for the indicated times. Cell lysates were analyzed by immunoblotting for p21<sup>cip1</sup> and β-actin (as loading control).
Supplemental Figure V. Primary VSMC from KO mice were electroporated with Flag-Skp2, cultured for 16 h in the presence of BrDU, fixed and stained for Flag (in green) and BrDU (in red). Nuclei were visualized with DAPI (in blue). Nuclei positive for DAPI, Skp2 and BrDU (indicated by the arrows) appear in white in the merged image.
Supplemental Figure VI. Primary KO VSMC were electroporated with Skp2 and cultured for 2 days. Equal amounts of proteins were analyzed by immunoblotting for Skp2, p21\textsuperscript{cip1} and p27\textsuperscript{kip1}, as indicated. β-actin was used as loading control.
Supplemental Figure VII. A. Weight of WT and KO mice at the indicated ages. P values are indicated. N=5. B. Area (in arbitrary units) of WT and KO VSMC. *, p<0.006, N=4.
Supplemental Figure VIII. Primary VSMC were lysed, and immunoprecipitation experiments were performed with mouse anti-Akt or mouse IgG antibodies followed by SDS-PAGE and immunoblotting with rabbit anti-Akt or anti-EBP50 antibodies.
Supplemental Figure IX. PTEN expression. A. PTEN expression in primary VSMC from WT and KO mice was analyzed by immunoblotting. β-actin was used as loading control. B and C. Primary VSMC from KO mice were fixed and stained for PTEN (in green). Nuclei were visualized with DAPI (in blue). Cells were analyzed by confocal microscopy (B) and TIRF microscopy (C). Graph shows the fluorescence intensity of PTEN in WT and KO cells from TIRF images.
Supplementary Figure X. Primary WT VSMC were electroporated with YFP-EBP50 (in green), cultured for 16 h in the presence of BrDU, fixed and stained for BrDU (in red). Nuclei were visualized with DAPI (in blue).
Supplemental Figure XI. Expression of p27kip1 in mouse femoral arteries. Sections from uninjured femoral artery (left panels) or 2 weeks after wire injury (middle panels) from WT (upper panels) and KO (lower panels) mice were stained for p27kip1 (in brown). N indicates the neointima and M the media. The expression of p27kip1 is evident in femoral arteries from both mouse strains in uninjured vessels and two weeks after injury.
Supplemental Figure XII. Apoptosis in WT and KO vessels and VSMC. A. *Top Panel.* TUNEL staining (in red) of sections from injured femoral arteries of WT (left panel) and KO (right panel) mice. Nuclei are stained in blue and the elastic lamina autofluorescence is in green. TUNEL positive cells are indicated by yellow arrows. M indicates media and I the intima. *Bottom Panel.* Quantitation of the percentage of TUNEL positive cells over total cells in the intima and media of WT (gray bars) and KO (white bars) mice. N=4. B. TUNEL staining of WT VSMC infected with control GFP or shRNA for EBP50. Cells were serum starved for 24 h. N=3.